

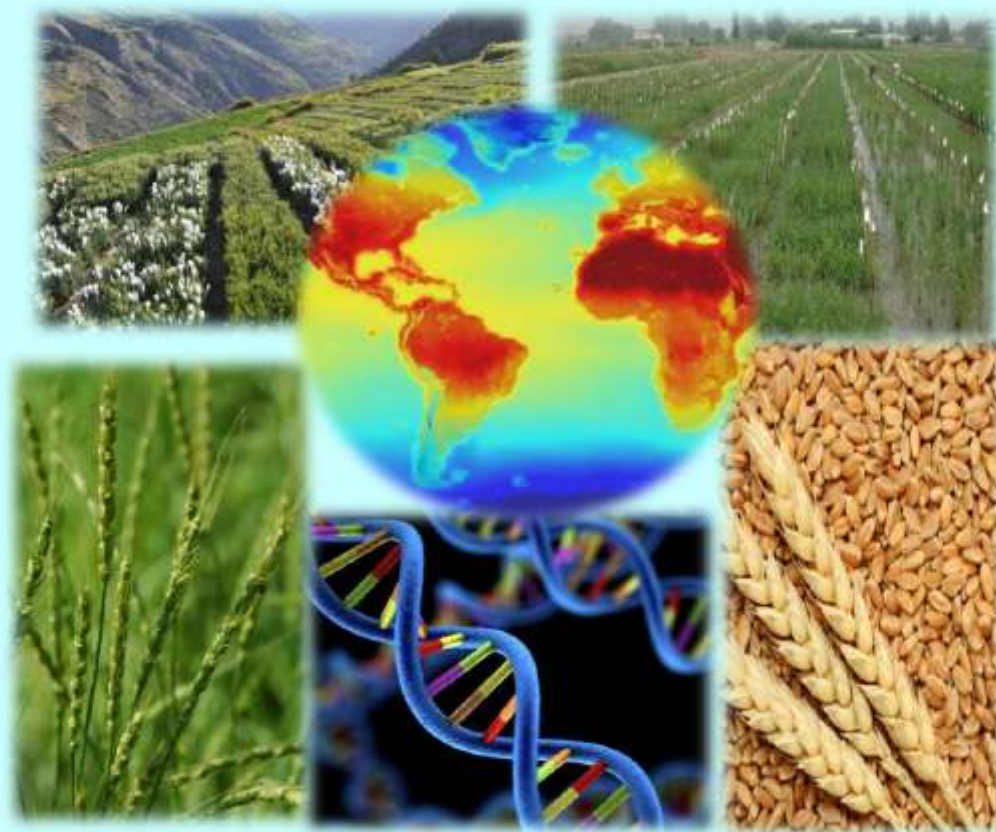


Biotic and Abiotic Stress Tolerance in Plants under Changing Climatic Conditions



Instruction Manual

06 August to 26 August 2019



S.K. Dhillon, D. Bhatia, Gurjit K. Gill, Pritpal Singh and G.S. Mangat

**CENTRE FOR ADVANCED FACULTY TRAINING IN GENETICS AND
PLANT BREEDING**

**DEPARTMENT OF PLANT BREEDING AND GENETICS
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2019**

PREFACE

Since inception of Centre for Advanced Faculty Training (CAFT) in Genetics and Plant Breeding by ICAR, New Delhi in 1995 at Punjab Agricultural University, the centre has organized 33 training programmes and trained scientists of various ICAR institutes and SAUs. The objective of the training programmes is to share the knowledge and experience of the researchers of the PAU and other institutes with breeders/ researchers/ teachers as trainees so as to update their knowledge in the field of crop improvement.



The current training programme “Biotic and abiotic stress tolerance in plants under changing climatic conditions” was planned to equip young scientist with various methodologies, strategies and tools to face the challenges posed by changing climatic conditions to crop plants. The emphasis was given on designing new plant architecture of field crops aimed at producing more productive and climate resilient crop varieties. The advances and prospects of advanced genotyping and genomic technologies in crop improvement programmes were discussed. Special emphasis was laid on familiarizing breeders with modern tools of molecular biology and bioinformatics and their utilization in different crop plants including cereals, oilseeds, pulses, vegetables and fruit crops. Twenty one teachers/scientists working on crop improvement in different SAUs and ICAR institutes have been imparted training under this programme. Experts from the Departments of Plant Breeding and Genetics, School of Agricultural Biotechnology, Vegetable Science and Fruit Science were invited to deliver lectures. Guest lectures of scientist from ICAR institutes were also organised for the benefit of trainees.

To provide practical training in modern technology, 14practicals were organised on topics viz., screening for diseases and insects under artificial conditions, basic molecular assays, *Insilico* genome analysis, Gene/QTL mapping etc. In addition, field visits to various crop experimental fields were also conducted.

This e-manual comprehend the text on most of the topics delivered including theory and practicals. I wish this material will be of immense utility to our trainees for reference in future. This manual is also available at <http://web.pau.edu/caftpbg/>

A handwritten signature in blue ink, appearing to read 'Gurinder'.

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Training Course (6th Aug to 26th Aug 2019)

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DEVELOPING CLIMATE-RESILIENT CROPS

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Abstract

Crop production is central to human survival, and production of increased quantities of food is a necessity to sustain current and future global populations. Agricultural crops provide nearly 70%–80% of calories and 60%–70% of all proteins consumed by human beings. Of about 50,000 edible plant species, less than 50 are used, of which only 15 supply 90% of the world's food, and just 3—wheat, rice, and maize—supply 60% of the total, making our agricultural system very vulnerable and non-resilient (Kang and Banga 2013). There are also growing pressures of demands for nonfood usage, for example, biofuels. A major cause of the unprecedented increases in food prices in 2008 was biofuels that were produced from grains (e.g., maize) and used to substitute bioenergy for fossil fuel energy to mitigate climate change. Only a significantly enhanced biological adaptation potential of crops can help improve the current agricultural production levels and meet future food needs. Even present production systems that produce less and have variable yields are acutely vulnerable, less efficient, and less resilient to shocks than they normally could be.

Adaptation of agriculture to climate change is any response that improves an outcome in terms of stable and enhanced productivity. Strengthening the adaptive capacities of farmers include diversification of production systems besides more efficient use of natural resources in both irrigated and dryland ecologies. Like in the past, plant breeding is expected to play a major role in adapting crops to climate change. Present situation requires that superior crop performance must be achieved under adverse environmental conditions. The growing global demand for food and competition for resources among different economic sectors compel future agricultural systems to be defined relative to targeted agronomics, farming systems, crops, and cultivars best suited to predicted environments.

Crop Biodiversity: adaptation, preservation, and utilization

A resilient agricultural system is integral to ecosystem services, such as food, feed, and livelihoods (Altieri, 1999; Tilman et al., 2002). With greater climate variability, a range of crop and ecosystem responses may alter nutrient cycling, soil moisture, as well as distribution of pests and diseases. All of these can adversely impact food production and food security (Fuhrer, 2003; Jones and Thornton, 2003). Extinction of species may cause ecosystems to move from a sustainable equilibrium to inherently turbulent state, thereby constraining their ability to provide ecosystem services (Folke et al., 2004). For example, as a result of coevolution, the life cycle stages of pollinator insects are in perfect consonance with crop flowering phenologies of target crops. Any uncoupling of insect life cycle stage and crop flowering phenology caused by alterations in annual temperature cycle can strongly impact cross-pollination. Under natural ecosystems,

this can have devastating effects on adaptive potential of pollinator insect species as well as on target host species.

Crop wild relatives (CWRs) constitute reservoirs of genetic diversity accumulated during millions of years of evolution. CWRs are key resources for adaptation to climate change, as they provide researchers with genes and traits for developing plants resistant to biotic and abiotic stresses (Lane and Jarvis, 2007; Maxted et al., 2008). The problem, however, is that CWRs themselves are now under threat of extinction because of climate change. Thomas et al. (2004) has predicted that 15%–37% of CWRs were in danger of extinction. Under elevated CO₂ levels, CWRs produce relatively less fruit and seed than domesticated crops (Jablonski et al., 2002), increasing their risk of extinction. For genetic evolution to occur, genetic diversity and gradual onset of change are prerequisites. Contrarily, wild populations are now required to adapt fast to a variety of environmental stresses, despite themselves suffering decline and shifts as a consequence of environmental change. Although pesticide resistance and heavy metal tolerance (MacNair, 1997) and a few other developmental traits (Bradshaw and Holzapfel, 2006; Gienapp et al., 2008) are known to evolve rapidly, not all species or populations show rapid adaptive genetic responses, mostly because of the absence of underlying mutations for resistance. The development of resistance has been, in most cases, based on the presence of specific alleles, even prior to the actual onset of stress.

Developing genetic responses to climate change is incumbent on effective evaluation and exploitation of the existing genetic variability. Use of molecular markers to predict adaptive variability is rather ineffective because little correlation exists between molecular genetic diversity and quantitative genetic variation. Also, heterozygosity at these markers shows no correlation with level of inbreeding. Availability of complete genomic sequences may help tackle the issues of inbreeding depression, structure and extent of adaptive variation, the level of local adaptation, and genotype-by-environment interactions. Genome sequencing of many CWRs is under way in several plant families, including Gramineae and Brassicaceae. These are likely to provide a conceptual toolbox for genomic research in conservation biology and highlight possibilities for the mechanistic study of functional variation, adaptation, and inbreeding (Angeloni et al., 2012). While breeding programmes have significantly enhanced productivity, they have also narrowed genetic diversity. Besides wild relatives, farmers' fields and bio-reserves also represent constantly evolving gene pool that may already reflect species responses to changing climate. The gene banks of the world, including CGIAR, hold large numbers of genetically diverse plant collections—improved crop varieties and traditional landraces and wild crop species. This gene pool should be adequately cataloged and made available for adapting farming systems to future climates. There is a need to plant sufficiently large “evolutionary” breeding populations of different crops at multiple sites across the globe. Such programs can capitalize on recombination and natural selection to evolve and adapt. Breeders can use such breeding populations as germplasm conduits of continuously evolving genetic variation. Data mining through metaanalysis of past and present multilocation trials may also help in analyzing climate sensitivity and tolerance in existing germplasm. As

germplasm accessions are sensitive to climatic variables, such as temperature and photoperiod, georeferencing of data will help elucidate the sensitivity of accessions to temperature and photoperiod.

Improving plant tolerance to heat, Drought, and submergence

The agricultural sector alone uses 70%–80% of the available freshwater supplies of the world; this figure may go up as temperatures rise further (Brookes and Barfoot, 2008). In addition, almost 10.12 million hectares of land is practically lost each year because of salinity caused by unsustainable irrigation techniques (Ruane et al., 2008). This would translate into 30% land loss within the next 20 years or so and up to 50% loss by 2050. Anthropogenic increases in greenhouse gas and aerosol concentrations have made a detectable contribution to the observed drying trend as reflected by the PDSI. Managing water scarcity is a major concern. Unsustainable water demand is driven in part by underpricing of water, together with subsidies for groundwater pumping to raise crops, such as rice and sugarcane, in ecologically fragile areas of the world. Climate change will further exacerbate water scarcity through increased evapotranspiration, decreased groundwater recharge, loss of glacial water storage, and saline water intrusion. Another negative impact of climate change on agriculture is the increased exposure of crop plants to heat stress. The impact of high temperatures on final yield is generally dependent on the crop growth stage. Wollenweber et al. (2003) found that the plants experienced warming periods as independent events and that critical temperature of 35.8°C for a short period around anthesis had severe yield-reducing effects. However, high temperatures during the vegetative stage may not have significant effects on growth and development. Changes in short-term temperature extremes can be critical, especially if they coincide with key stages of plant development. Only a few days of extreme temperature at the flowering stage of most crops can reduce yield drastically (Wheeler et al., 2000). Temperatures as low as 25°C can reduce grain-filling period in wheat, after which a 1°C temperature rise shortens the reproductive phase by 6% and shortens the grain-filling duration by almost 5%; grain yield and harvest index are also reduced proportionately. Heat stress impacts key crop metabolic and developmental processes, such as pollination, grain filling, and approach to maturity, which are adversely affected after temperature-tolerance thresholds are exceeded.

The past decade has witnessed an increase in studies related to detection of quantitative trait loci (QTL) for drought-related traits, and the first encouraging results in QTL cloning have been reported. The QTL cloning has enabled better understanding and effective manipulation of the traits influencing drought tolerance (Tuberosa and Salvi, 2006). With the availability of whole genome sequences of plants, physical maps, and genetic and functional genomics tools, integrated approaches using molecular breeding and genetic engineering offer new opportunities for improving stress resistance (Manavalan et al., 2009). Although genetic variation exists for the plants' ability to cope with temperatures that are significantly higher than current adaptation regimes of most crops, it may still be necessary to identify genes that control adaptation to much higher levels of abiotic stresses. This may include extensive gene expression assays to identify genes in both cultivated and CWRs that are important for survival in different climatic

conditions. The dehydration-responsive element-binding proteins (DREBs) or C-repeat-binding proteins (CBFs) are among the first families of transcriptional regulators that are transcriptionally upregulated by water deficit or low temperature (Morran et al., 2011). The expression of individual DREBs/CBFs may be regulated by drought, salt, and cold or by drought and cold only. Most published evaluations of transgenic plants for drought tolerance considered survival rather than final productivity or grain yield. The drought protection offered by overexpression of TaDREB2 or TaDREB3 is typical of the DREBs/CBFs and is largely related to survival and recovery from severe drought stress. Molecular control mechanisms for abiotic stress tolerance are based on activation and regulation of specific stress-related genes. Hundreds of genes and their products respond to these stresses at transcriptional and translational levels (Umezawa et al., 2006). Alternative strategies for engineering drought tolerance also include increasing the depth or vigor of the root system to facilitate water acquisition and increase water-use efficiency (WUE). Although adaptation to stress under natural conditions has some ecological advantages, the metabolic and energy costs may far exceed benefit to agriculture (Apse and Blumwald, 2002). Transgenic plants can be engineered on the basis of different stress mechanisms: metabolism, regulatory controls, ion transport, antioxidants and detoxification, late embryogenesis abundant proteins, heat-shock processes, and heat-shock proteins (Wang et al., 2001, 2003).

A promising path to improve high-temperature tolerance has been indicated by a “photorespiratory bypass” engineered in Arabidopsis chloroplasts by the introduction of Escherichia coli glycolate catabolic pathway that substantially suppresses photorespiratory flux (Maurino and Peterhansel, 2010). This photorespiratory bypass in turn would be expected to increase the temperature optimum of net photosynthesis in transformed plants. The specific aim must be to identify genes that control the plants’ ability to tolerate stress and to develop biotechnology-oriented selection and breeding procedures (functional analysis, marker probes, and transformation with specific genes). Further improvement and adaptation of current agricultural practices may also be needed (Wang et al., 2003). The use of biotechnology to improve crops genetically has not yet been widely accepted, but the pressures on the world’s agricultural systems may hasten its acceptance, with all the appropriate biosafety precautions in place.

Specific tolerance or resistance: pests, Diseases, and nutrient stress

Rising temperature and atmospheric CO₂ are also indirectly affecting crops through their effects on pests and diseases. These interactions are complex, and their full impact on crop yield is yet to be fully appreciated. Impacts of warming or drought on resistance of crops to specific diseases may be through the increased pathogenicity of organisms or by mutations induced by environmental stresses (Gregory et al., 2009). The influence of climate change on plant pathogens and their consequent diseases has been reviewed extensively (Chakraborty, 2005). Different individual parameters associated with climate change, such as warming, increased levels of CO₂, decreased rainfall, and erratic pattern of rainfall, have been studied for their influence on different aspects of pathogens and diseases across various crops (Chakraborty, 2005). These include pathogen life cycle (Chakraborty and Datta, 2003), expression of host

resistance, disease epidemiology and severity of disease epidemics (Butterworth et al., 2010), and pathogen inoculum production (e.g., Chakraborty et al., 2000b). Conventional breeding initiatives have contributed significantly to crop adaptations through the development of strains that are resistant to biotic stresses, such as insects, fungi, bacteria, and viruses (Bianchi et al., 2006). Transgenic Bt crops are proving to be valuable tools for integrated pest management programs by giving farmers new pest control choices. Also, GM cassava, potatoes, bananas, and other crops carrying resistance to fungi, bacteria, and viruses are in the pipeline; some have already been commercialized while others are undergoing field trials (Clive, 2011). Studies carried out between 2002 and 2005 determined that biotic stress-tolerant GM crops accounted for increases in average yield of 11%–12% for canola and maize compared with conventional crops (Brookes and Barfoot, 2009). The significantly increased pest

population pressure, their rapid evolution, and their ability to move, combined with possible impact of climate change on the expression of current resistance gene(s), will require a coordinated breeding response to source genes from wild relatives of crops and microorganisms. Microorganisms might be a better source of resistance gene(s) because of their faster rate of evolution as compared with plants. Transgenic/cisgenic breeding approaches may be used increasingly to combat stresses that require genetic responses for multitude of stresses at the same time.

Enhanced CO₂ Fertilization and nutrient- and water-use efficiency of Crop plants

Between 1958 and 2008, atmospheric concentration of CO₂ increased from 316 to 385 mg/kg, and continued increases in CO₂ concentration are expected to significantly affect long-term climate change, including variations in agricultural yields (Kirkham, 2011). Increased atmospheric CO₂, coupled with water and temperature constraints, is likely to impact WUE and nutrient-use efficiency of crops, pastures, and forests; nutrient recommendations for a changed climate are also not likely to operate on the same premise as current recommendations. Changed metabolic responses of plants resulting from environmental stresses may have to be factored in. Understanding of metabolic processes associated with such changes is expected to help with formulation of adaptation strategies under both rainfed and irrigated agroecosystems. Improved nutrient-use efficiency in plants can assist with mitigation through reduced carbon intensity of agricultural operations. Because nutrient uptake and utilization are genetically controlled, a major requirement would be to identify genes/alleles that regulate nutrient-use efficiency. Projections show that by including increased CO₂ fertilization, all but the very driest regions would show increases in productivity. Global-scale comparisons of the impacts of CO₂ fertilization with those of changes in mean climate reveal that the strength of CO₂ fertilization effects is a critical factor for projecting global-scale crop yields (Nelson et al., 2009). North America and Europe may benefit from increased CO₂ fertilization effects, at least in the short run. Other regions, such as Africa and India, are nevertheless projected to experience up to 5% losses in food grain production by 2050, even with strong CO₂ fertilization. These losses increase up to 30% if the effects of CO₂ fertilization are omitted. In fact, without CO₂ fertilization, all regions are projected to experience a loss in productivity by 2050

because of climate change. However, existing global-scale studies (Nelson et al., 2009) are based only on a limited sample of available climate model projections. Higher atmospheric concentrations of CO₂ are normally thought to increase leaf and canopy photosynthesis, especially in C₃ plants, with little alteration in dark respiration. Additional CO₂ may increase biomass without significantly altering dry matter partitioning, but it will reduce transpiration of most plants and improve WUE (Field et al., 1995). However, their overall impacts on crop productivity and water use will vary as nutrient acquisition is closely associated with overall biomass and strongly influenced by root surface area. Modest, crop-specific benefits in productivity may be possible only where nutrient availability can be optimized and where climate change increases temperatures to a species-specific optimum and changes precipitation patterns to reduce water stress (drought or flooding) days. The C₃ species may also accrue a direct benefit from CO₂ fertilization under such conditions. Plant availability of nutrients in the soil also depends on soil chemical properties, nutrient availability, and location of nutrient ions relative to the root surface and the length or distance the nutrient must “travel” in the soil to reach the root surface (Jungk, 2002). Soil moisture and temperature are primary determinants of nutrient availability and root growth and development. It is expected that process outcomes will be dependent on changed climate. There are also suggestions that climate change impacts on nutrient-use efficiency will be primarily accomplished through direct effects on root surface area (Itoh and Barber, 1983). In the absence of roots, steady-state solution-phase concentrations of nutrient ions are controlled by adsorption–precipitation and desorption–dissolution. Crops may, in general, become more water-use efficient at elevated CO₂ concentrations, as stomata do not need to stay open for longer periods to enable plants to receive the required CO₂. As a consequence, there may be only marginal yield appreciations. Despite the potential positive effects on yield, elevated CO₂ may be detrimental to yield quality of certain crops. For example, elevated CO₂ is detrimental to wheat flour quality through reductions in protein content (Sinclair et al., 2000). Elevated atmospheric CO₂ may even reduce plant growth when combined with other likely consequences of climate change, namely, elevated temperatures, increased precipitation, or increased nitrogen deposits in the soil. The interactive effects of soil moisture and nutrient availability are two key edaphic factors that determine crop yield (Ziska and Bunce, 2007).

During their long evolutionary history, plants have evolved different mechanisms of adaptation, for example, changes in ion transporter expression and activity, increase in root growth to explore more soil volume (Jordan-Meille and Pellerin, 2008), or root zone acidification of the surrounding soil for mineral nutrient mobilization (Ryan et al., 2001). Despite these adaptations, the mechanisms involved in sensing and signaling low mineral nutrient status are still not fully understood, although some progress has been made (Wang and Wu, 2010). QTL have been detected for growth-related traits, ion content, biochemical traits, and their responses to reduced supplies of potassium or phosphate in *Arabidopsis* (Prinzenberg et al., 2010). Improved nutrient uptake through transgenic manipulation of transports has yet to be realized. Low input cultures may be used to identify traits and genotypes associated with high nutrient-use efficiency. These

traits may include greater root biomass, higher root surface area, longer/denser root hairs, more adventitious roots, smaller root diameter and shallower basal roots in surface soils, more dispersed laterals, and enhanced exudation (Lynch, 2007). Breeding for root traits has just begun, but current availability of rapid root zone screening techniques may facilitate rapid screening of global germplasm resources.

Revisiting Crops and Cropping systems, reintroduction of hardy Crop species to Commercial Farming

Post-Green Revolution, significantly higher productivity, abundant and cheap supplies of nitrogenous fertilizers, and subsidized irrigation facilities helped wheat and rice occupy niches that were previously occupied by environmentally hardy crops, such as sorghum, pearl millet, proso millet, legumes, and oilseeds. Cropping systems were developed around these highly input-intensive crops at the cost of environment and long-term sustainability. Rice cultivation in northwest India is a prime example where rice, with a high water requirement, replaced more temperature- and drought-hardy millets, legumes, and oilseeds. Similar instances are available across the globe where sustainability was disregarded or sacrificed in favor of productivity and food security. The major gross virtual water exporters (i.e., export of agricultural commodities), which together account for more than half of the global virtual water export, include the United States (314 Gm³/year), China (143 Gm³/year), India (125 Gm³/year), Brazil (112 Gm³/year), Argentina (98 Gm³/year), Canada (91 Gm³/year), Australia (89 Gm³/year), Indonesia (72 Gm³/year), France (65 Gm³/year), and Germany (64 Gm³/year) (Hoekstra and Mekonnen, 2012). Of these, the United States, Pakistan, India, Australia, Uzbekistan, China, and Turkey are the largest blue virtual water exporters, accounting for 49% of the global blue virtual water export. All of these countries are partially under water stress.

Crop improvement efforts can only hasten the evolutionary and adaptive processes. Cook et al. (1998) studied long-term (>100 years) effects on high concentration of CO₂ on *Nardus stricta* plants growing near a natural CO₂ spring (concentration of CO₂ ≈ 790 μmol/mol) by comparing with plants growing nearby in an ambient CO₂ environment (≈360 μmol/mol). The plants selected in and adapted to high concentration CO₂ exhibited earlier senescence and reductions in photosynthetic capacity (≈25%), RuBisCo content (≈26%), RuBisCo activity (≈40%), RuBisCo activation state (≈23%), chlorophyll content (≈33%), and leaf area index (≈22%) compared with plants growing away from the spring. It is now important to revisit crops and cropping systems across the globe with the aim of achieving WUE at both regional and global levels. There is also a need to develop farming system– specific varieties that fit in a farming system context and are freely accessible to farmers. There is also a need for large-scale reintroduction of hardy crop species, such as sorghum and millets, to commercial farming in water-scarce countries in Asia, Latin America, and Africa. Of special interest are the adaptation techniques, such as no-till agriculture and bed planting. By leaving at least 30% of residue on the soil surface, no-till agriculture reduces loss of CO₂ from agricultural systems and also plays a role in reducing water loss through evaporation, increases soil stability, and creates a cooler soil microclimate.

Conservation agricultural practices that help prevent soil erosion may also sequester soil carbon and enhance methane consumption. Powlson et al. (2011) have suggested that climate change benefit of increased soil organic carbon from enhanced crop growth must be balanced against greenhouse gas emissions from production and use of nitrogenous fertilizers required for higher growth trajectories. In modern agricultural practices, GM Roundup Ready™ (herbicide-resistant) soybean technology has accounted for up to 95% of no-till area in the United States and Argentina and led to sequestration of 63,859 million tons of CO₂ (Kleter et al., 2008). Transgenic canola (oilseed rape) and soybean have been modified to be resistant to specific herbicides (Bonny, 2008). These GM crops should allow farmers to adopt no-till farming practices. Such climate change mitigation practices enhance soil quality and retain carbon. WUE needs to be achieved at both regional and global levels. Crops differ widely in their WUE; some crops have low (need >2000 m³/ton), moderate (need >750 m³/ton), and high efficiency (need <500 m³/ton).

As indicated earlier, farming system-specific varieties that fit in a farming system and are freely accessible to farmers are needed. Hardy crop species, such as sorghum and millets, need to be reintroduced for commercial farming in water-scarce countries in Asia, Latin America, and Africa. In Australia, a collection of greater lotus (*Lotus uliginosus*) breeding lines is being adapted to low latitudes and possibly with improved heat tolerance, which could push greater lotus (with its ability to tolerate waterlogging/acidity) further north and out of its current niche in New South Wales. Similarly, birdsfoot trefoil (*Lotus corniculatus*) is being evaluated as a new perennial legume adapted to low-fertility acidic conditions. There is known genotypic diversity in birdsfoot trefoil to extend its usage through breeding into high altitude and low rainfall dryland grazing and cropping environments. Similarly, wild and related species of *Medicago sativa* (lucerne) are being evaluated because of their significantly higher tolerance to grazing, ability to spread by rhizomes, and, in some cases, greater tolerance to salinity and waterlogging. These plants will potentially provide a perennial legume for areas where lucerne will not persist because of overgrazing or uncontrolled grazing and/or salinity or drought. Other species of perennial legumes of very early maturity that would be good at adapting to hotter/drier conditions are Yelbeni yellow serradella, Toreador hybrid medic (a hybrid between strand and disc medic), and Trigonella balansae, which is about to be released. All of these species flower between 70 and 85 days. In addition, burgundy bean, a summer perennial legume, can flower and set seed in as little as 60 days under moisture stress, but under better conditions, it will not flower till 90 days. Such efforts need to be repeated with additional species across the globe.

Gene Discovery, germplasm enhancement, and genome-Based approaches to Improve Breeding efficiency

Development of new, more climate-resilient crop varieties, as always, will be critical to agricultural adaptation. Because varietal development is a long-term endeavor, region-by-region prediction of future climatic conditions and trait-by-trait cataloging of global crop genetic resources are extremely important. Crop breeders need to pursue

specific targets rather than moving targets. Use of genome-wide association studies, integrating genome sequencing, single nucleotide polymorphism (SNP) analysis, epigenetic analysis, and transcriptomics may help identify gene sources for developing plants adapted to emerging climate change stresses. Such efforts may also include massive genome sequencing, particularly in CWRs. Such sequencing data should allow selection of specific SNPs as candidates for adaptation to environmental stresses. Identification of genes and alleles for improving nutrient-uptake and nutrient-use efficiencies is also critical for reducing carbon footprint of agriculture. Novel alleles are likely to be available in CWRs and landraces, where the local environment has affected selection for tolerance to environmental extremities. New knowledge and resources should be freely available to breeders. By screening populations with a large number of SNP markers, especially using SNP chips, signals of selection can be identified. Variation in such markers will provide insights into functional rather than neutral genetic variation. Next-generation sequencing should facilitate the study of gene expression. Also, the epigenetic and transcriptome analyses will need to be used to achieve a far more detailed gene mapping than currently possible. Because such studies map heat-sensitive and nutrient utilization traits in the genome. There is a need to reanalyze the domestication history of crops. Wild-to-domesticated complexes are excellent experimental systems to investigate evolutionary issues. Both the progenitors and their descendants are known. This allows for the integration of evolutionary and developmental genetics and for a closer look at those differences at the molecular level that are responsible for the phenotypic differences between wild and domesticated types. This knowledge can assist with gene discovery and also possibly aid in finding for new domesticates.

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COMBATING CLIMATE CHANGE: AN AGRICULTURAL PERSPECTIVE

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Emissions of greenhouse gases (GHGs), particularly carbon dioxide (CO₂), methane (CH₄), oxides of nitrogen (nitrous oxide, N₂O, and nitric oxide, NO), and halocarbons, are enforcing global climate change. The atmospheric concentrations of CO₂, CH₄ and N₂O have increased to levels unprecedented in at least the last 800,000 years (IPCC, 2013). CO₂ is the major anthropogenic GHG accounting for 76% (38 ± 3.8 Gt CO₂eq yr⁻¹) of total anthropogenic emissions. Methane and N₂O account for 16% (7.8 ± 1.6 Gt CO₂eq yr⁻¹) and 6 % (3.1 ± 1.9 Gt CO₂ eq yr⁻¹) of the anthropogenic emissions, respectively. Remaining 2% (1.0 ± 0.2 Gt CO₂eq yr⁻¹) of the emissions came from fluorinated gases. The increased concentration of GHGs causes global warming, depletes the concentration of ozone in the stratosphere, and also contributes to acid deposition. Though CO₂ has the highest atmospheric concentration among GHGs, N₂O and CH₄ have several times higher global warming potentials (GWP) than CO₂ (Table 1). Most of the anthropogenic emissions come from the combustion of fossil fuel and from industrial processes but there is substantial contribution from agriculture and land-use changes. Agriculture accounts for estimated emissions of 5.1–6.1 Gt CO₂-eq/year, including 3.3 Gt CO₂-eq/year as CH₄ and 2.8 Gt CO₂-eq as N₂O. During 1970 to 2010, emission of GHGs in the agriculture sector increased by 35%, from 4.2 Gt CO₂ eq yr⁻¹ to 5.7 Gt CO₂ eq yr⁻¹ (IPCC, 2014). The emissions from agriculture sector represent 10%–12% of the total anthropogenic emissions of GHGs. Of the total non-CO₂ emissions from agriculture, N₂O emission from soils and CH₄ emission from enteric fermentation account for 38% and 32%, respectively. Biomass burning, rice agriculture, and manure management account for 12%, 11%, and 7% of the emissions, respectively. Compared to 1990, agricultural emissions of CH₄ and N₂O have increased by nearly 17%, representing a mean emissions increase of about 60 Mt CO₂-eq/year. It is estimated that CO₂ emissions from agricultural soils constitute less than 1% of global anthropogenic CO₂ emissions. The increase in agricultural land, coupled with the greater demand for food, especially meat, is expected to enhance annual GHG emissions from agriculture. Land misuse and soil mismanagement could exacerbate the emission of GHGs from soil to the atmosphere. Soil contributes to the greenhouse effect and also acts as a sink of GHGs. It is intricately linked to the atmospheric–climate system through carbon, nitrogen, and hydrologic cycles. In this lecture an assessment of soil's contributions to the emission of GHGs and its potential role in mitigation and adaptation to climate change is discussed.

Table 1 Characteristic of three principal greenhouse gases (*Source: IPCC, 2013*)

Characteristic	CO ₂	CH ₄	N ₂ O
Pre-industrial concentration (1750)	278 ppm	722 ppb	271 ppb
Concentration in 2011	391 ppm	1803 ppb	324.2 ppb
Rate of concentration change per year (2002-2011)	2 ± 0.1 ppm	6 ppb	0.73 ± 0.03 ppb
Increase in concentration since 1750 (%)	40	150	20
Atmospheric life time (years)	~100-300	12.4 ± 14	121 ± 10
100-yr Global warming potential	1	28	265
Radiative forcing (W m ⁻²) in 2011	1.82 ± 0.19	0.48 ± 0.05	0.17 ± 0.03

Agriculture as a source of GHGs

The GHG inventory for India shows that the contribution of agriculture including enteric fermentation and manure management, to country's total emissions is 18 percent of which about 21 percent is related to nitrous oxide (N₂O) emissions from agricultural soils and 18 percent to rice production. Agriculture sector contributes to GHG emissions through energy use in the production and use of farm machinery, synthetic fertilizers, and crop protection chemicals such as herbicides, insecticides and fungicides and by direct use of fuel in farm operations. Cultivation or ploughing of soil resulting in the loss of soil organic matter (SOM), is another source of carbon (C) emission (Benbi 2018). Quantification of the carbon footprint of intensive crop production systems in the state of Punjab revealed the overriding importance of groundwater irrigation and fertilizer use. Intensification of agriculture resulted in ~2.5 fold increase in food grain production and 3-fold increase in emission of GHGs during 1980 to 2015 (Benbi, 2018). The major soil-borne GHGs include carbon dioxide (CO₂), methane (CH₄) and nitrous oxide (N₂O). Soils constitute the largest pool of actively cycling C in terrestrial ecosystems and stock about 2000 Gt C (to a depth of 1m) in various organic forms. The total quantity of CO₂-C exchanged annually between the land and atmosphere as gross primary productivity is estimated at ~120 Gt C yr⁻¹ and about half of it is released by plant respiration giving a net primary productivity of ~60 Gt C yr⁻¹. Heterotrophic soil respiration and fire return ~60 Gt C yr⁻¹ to the atmosphere. Soil disturbance associated with tillage enhances emission of CO₂ through decomposition of soil organic matter (SOM). Tillage breaks soil aggregates, increases oxygen supply and exposes the surface area of organic material promoting decomposition of organic matter.

Methane is produced in soil during microbial decomposition of organic matter under anaerobic conditions. Rice fields submerged under water are the potential source of CH₄ production. Continuous submergence, higher organic C content and use of organic manure in puddled soil enhance CH₄ emission. Burning of crop residues also contributes to the global methane budget. The enteric fermentation in ruminants is another major source of CH₄ emission. Nitrous oxide is produced in soils through the processes of nitrification and denitrification. Nitrous oxide is a gaseous intermediate in the reaction sequence of denitrification and a by-product of nitrification that leaks from microbial cells into the soil and ultimately into the atmosphere. One of the main factors governing this reaction is the availability of inorganic N in soil through addition of fertilizers, manure, crop residues, sewage sludge or mineralization of N in soil organic matter.

Agricultural management and land-use for combating climate change

Climate change will have considerable impact on agriculture through direct and indirect effects on crops, soils, livestock and pests. Development of technologies for adaptation and mitigation and their adoption by the farmers are essential for combating climate change. Agriculture offers opportunities for mitigation from supply as well as demand-side. The supply-side opportunities include sustainable intensification, improving N fertilizer production and management, reducing emissions from enteric fermentation, reducing methane emissions from rice paddies and improving manure management. The demand-side opportunities include C sequestration, reduction in food wastage and change in dietary habits. Methane emission from rice fields could be reduced by intermittent flooding, which involves flooding a rice field and then allowing the irrigation water to drain for 2-3 days, followed by re-flooding and draining in cycles throughout the season. Direct-seeding of rice (DSR) and system of rice intensification (SRI) do not require continuous soil submergence, and therefore reduce or eliminate CH₄ emission from rice fields. Improved rice straw management, composting, turning into biochar are the other options for GHG mitigation in rice. Options for reducing N₂O emission from soil include enhancing N-use efficiency and reducing loss of applied N, plant breeding and genetic modifications to increase N uptake, integrated use of manure and fertilizer to reduce reliance on chemical fertilizer, application of N based on soil-test and use of slow-release fertilizers or nitrification inhibitors.

Carbon sequestration in agricultural soils

The strategies to lower CO₂ emissions include reducing energy use, developing low or no-carbon fuel, and sequestering CO₂ through natural and engineering techniques. The latter techniques are expensive and have biogeochemical and technical limitations. Therefore, C sequestration in soil and vegetation is considered a feasible option for mitigating atmospheric abundance of CO₂. Globally, soils have lost an estimated 55-78 Gt C because of land-use changes; therefore soils have considerable capacity to sequester C and mitigate atmospheric C load (Nieder and Benbi, 2008). However, the rate and magnitude of soil C sequestration differs with factors such as soil quality,

antecedent C level, climatic conditions, land-use, and management. In the last three decades, several studies have enumerated the effect of agricultural management on organic matter turnover and soil's feed-back to global climate change. A number of management practices have been evaluated and recommended for enhanced soil C sequestration (Table 2). Management practices or technologies that increase C input to soil and reduce C loss or both lead to net carbon sequestration (Nieder and Benbi, 2008). Increased C input in agro-ecosystems can be achieved through selection of high biomass producing crops, residue recycling or residue retention by lessened tillage intensity, application of organic materials (e.g. animal manure, compost, sludge, green manure etc.), adoption of agroforestry systems, intensification of agriculture through improved nutrient and water management practices, reducing summer or winter fallow, changing from monoculture to rotation cropping, and switching from annual crops to perennial vegetation. Soil organic carbon (SOC) loss could be decreased by adopting conservation agriculture and minimizing soil disturbance, checking erosion through reduced tillage intensity, and using low quality organic inputs.

Table 2. Strategies for C sequestration in agricultural soils

Increase input	Decrease output
Increasing crop productivity	Erosion control
Diversified crop rotations	Reduced or no tillage
Higher return of crop residues	Mulch farming
Increasing use of organic manures	Reduced bare fallow
Green manuring	Input of low quality organic material
Intensive cropping	
Elimination of fallow	
Agroforestry systems	
Improved irrigation	
Greater root biomass	
Depth placement of carbon	
Switching from annual crops to perennial vegetation	

Intensive agriculture with improved nutrient and water management can increase water and nutrient use efficiency and crop biomass production, thereby increasing plant-mediated C input to soil and reducing rates of organic matter decomposition. In Punjab,

intensification of agriculture with rice-wheat system improved SOC by 38 per cent over a 25-year period (Benbi and Brar, 2009). The enhanced C sequestration resulted from increased productivity of rice and wheat. Besides improving crop yields, intensification of agriculture saves emission that could occur because of bringing more land under cultivation for meeting the food requirement of growing population. Application of recommended amounts of fertilizers leads to C sequestration by increasing crop productivity and nutrient use-efficiency. Balanced application of N, P and K fertilizers across several sites in India improved SOC concentration by 6 to 100 per cent and C sequestration by 20-600 kg C ha⁻¹ y⁻¹ depending on soil, crop and climatic conditions (Fig. 1; Benbi, 2015). Integration of inorganic fertilizers with organic amendments further enhances soil C sequestration. Integrated use of organic sources, such as farmyard manure (FYM) and inorganic fertilizers can enhance C sequestration by 100-1200 kg C ha⁻¹ yr⁻¹ under various soil, crop and climatic conditions in India (Benbi, 2013). Integrated nutrient management involving addition of organic manures/composts along with inorganic fertilizers results in improved soil aggregation (Benbi et al., 1998) and greater C sequestration especially in macro-aggregates (Benbi and Senapati, 2010; Sodhi et al., 2009). Generally, manures and composts provide greater C stabilization with longer residence time than those with low C/N ratio such as green manures (Sodhi et al. 2009; Benbi and Khosa, 2014). With an aim to improve stabilization of the added C in soil, application of biomass-derived black C or biochar, with very long turnover time has been suggested. A study of the decomposition of rice residue-derived by-products showed that rice straw and rice-husk exhibited smaller mean residence time (MRT) than did biochar and rice straw compost suggesting that their incorporation may lead to only short-term C accrual in soil (Benbi and Yadav, 2015). On the contrary, biochar and rice straw compost with high MRT (1100-2000 days) will stay longer in soil leading to long-term C sequestration.

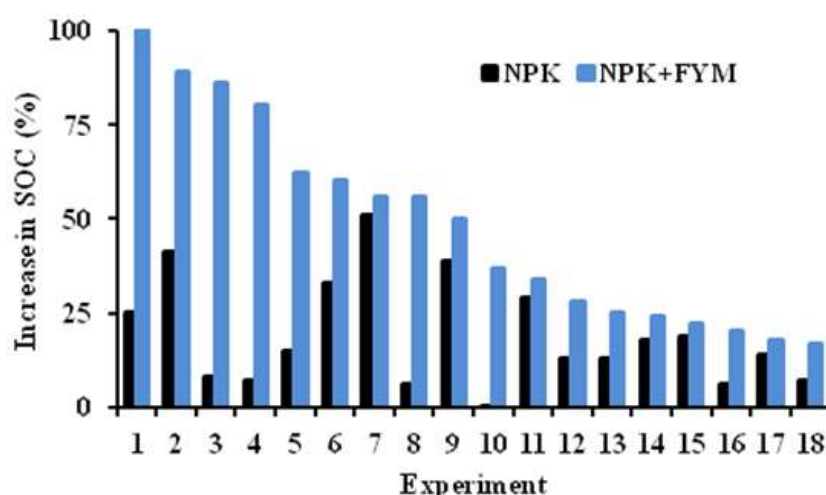


Fig. 1. Response of soil organic carbon to NPK and NPK+FYM application at different sites in India

Conservation agriculture has been suggested as a strategy to minimize C emission and enhance C sequestration. Physical disturbance associated with intensive soil tillage increases the turnover of soil aggregates and accelerates the decomposition of aggregate associated C. No-till (NT) practices increase aggregate stability and promote the formation of recalcitrant C within aggregates. Croplands under no-till systems have been shown to increase soil C compared with more intensive tillage operations. Retention of crop residue with NT is expected to contribute to soil C sequestration, but the effect is determined by soil texture. In wheat-based cropping systems in India, conversion from CT to NT resulted in net C sequestration rates of 219–359 kg C ha⁻¹ y⁻¹ with relatively high rate in rice-wheat (244 to 359 kg C ha⁻¹ y⁻¹) than in maize–wheat and cotton–wheat systems (219–231 kg C ha⁻¹ y⁻¹) (Grace et al., 2012). The potential for C sequestration in wheat-based systems of India on conversion to NT is estimated at 50.7 Tg C over 20 years. Crop residues returned to the soil provide substrate to soil organisms, which help in SOM turnover. In India, an estimated 500–550 Tg of crop residues are produced annually; after accounting for multiple competitive uses, about 141 Tg are surplus most of which are burnt *in situ*. Addition of surplus crop residues to soils can result in C sequestration of 6.3 Tg C annually. In north Indian state of Punjab, about 22 Tg of rice residue is produced out of which 80% is burnt *in situ*. The incorporation of rice residue in soil instead of burning can lead to C accrual of 0.80 Tg C per year over an area of 2.28 m ha in Punjab, which is equivalent to about 350 kg C ha⁻¹ (Benbi, 2015). Still higher rates of C sequestration, upto 745 kg C ha⁻¹, can be achieved if both animal manure and rice residue are applied annually in the rice-wheat cropping sequence (Benbi et al., 2012b). Removal or burning of crop residues besides causing environmental pollution can adversely impact soil quality and production system sustainability.

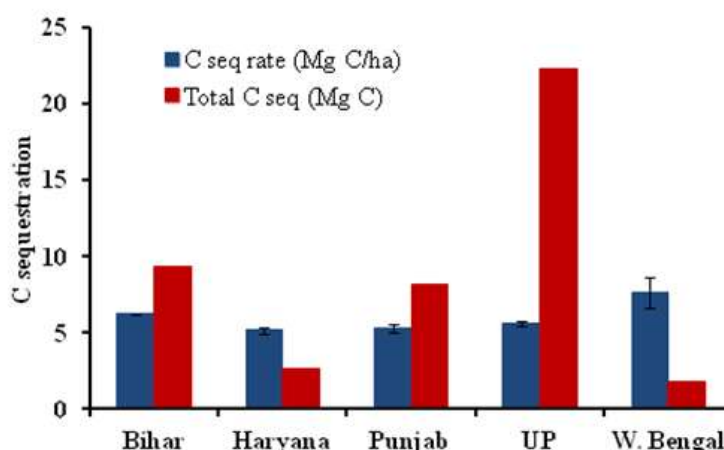


Fig. 2 Effect of reduced tillage on C sequestration in wheat-based cropping systems in selected states of India

Soil organic C stocks are strongly influenced by land-use, for example conversion of native ecosystems to agricultural production usually results in loss of SOC. On the contrary, conversion of cropland to forest or grassland can lead to build-

up of SOC. Average global C sequestration rate, when changing from agriculture to forest or grassland is estimated at 335 kg C ha⁻¹ yr⁻¹ (Post and Kwon, 2000). Agroforestry is considered an important option for C sequestration; poplar-based agroforestry systems in northern India are reported to have 88% higher SOC stocks than the rice-wheat system, but the accrued C mainly occurs in labile forms (Benbi et al., 2012a; Benbi et al., 2015). Among different cropping systems, C sequestration could be higher under submerged rice soils compared to aerobic soils due to incomplete decomposition of organic materials, and decreased humification of organic matter under flooded conditions. Results from landscape studies in China show that paddy ecosystems have the ability to accumulate organic C faster than other agro-ecosystems (Wu, 2011).

Obviously, there are several management practices that can mitigate agricultural emission of GHGs but the most appropriate practices are site-specific. IPCC (2014) identified the following mitigation options in agriculture: (1) plant management: improved variety, rotation, cropping system; (2) nutrient management: type of fertilizer, urease/nitrification inhibitor; (3) tillage/residue management: reduced tillage, residue retention; (4) water management: improved water application, drainage; (5) land-use change: agro-forestry, bio-energy crops, and (6) biochar application. Available best management practices will require evaluation and adaptation with reference to soil type, land-use, climatic conditions and economic feasibility.

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EPIGENOMICS IN ENHANCING STRESS TOLERANCE IN PLANTS IN THE ERA OF CLIMATE CHANGE

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In order to deal with the challenge of crop improvement in the era of climate change, it is essential that we adopt the approaches in which plant responds to environmental changes in terms of producing novel phenotypes. Epigenomics is one such approach. First I explain some important terms in epigenetics and compare them with their corresponding terms in genetics.

Genetics and Epigenetics

Genetics is the study of genes, heredity, and genetic variation in living organisms. We explain genetic variation on the basis of changes in the DNA sequences. Epigenetics is the study of cellular and physiological trait variations that are not caused by changes in the DNA sequence; it is essentially the study of external or environmental factors that turn genes on and off and affect how cells read genes. Epigenetic marks such as DNA methylation or histone modifications are key players in epigenetics (Figure 1).

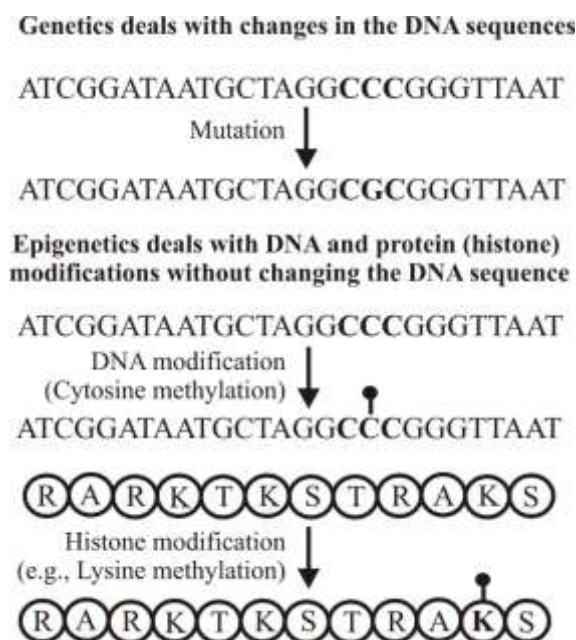


Figure 1. Difference between genetics and epigenetics. (Miglani, G.S. and R. Singh. 2019. Epigenome editing in crop improvement. Chapter 15. In: *Quantitative Genetics, Genomics and Plant Breeding*. Second edition. Ed. Kang, M.S. CAB International. Oxford, U.K.)

Genome and Epigenome

Genome is the genetic material present in a single set of chromosomes. In all eukaryotes, we have nuclear and mitochondrial genomes. Plants in addition have

chloroplast (plastid) genome. Epigenome is complete set of epigenetic modifications on the genetic material of a cell. The epigenome is a multitude of chemical compounds that can tell the genome what to do. DNA holds the instructions for building the proteins that carry out a variety of functions in a cell. The epigenome is made up of chemical compounds and proteins that can attach to DNA and direct such actions as turning genes on or off, controlling the production of proteins in particular cells. Some of the epigenome compounds, come from natural sources like food and others from man-made sources like medicines or pesticides. The epigenome marks the genome in two main ways, both of which play a role in turning genes on or off. When epigenomic compounds attach to DNA and modify its function, they are said to have "marked" the genome. These marks do not change the sequence of the DNA. Rather, they change the way cells use the DNA's instructions. The marks are sometimes passed on from cell to cell as cells divide. They also can be passed down from one generation to the next. There are two types of epigenomic marks. The first type of mark, called DNA methylation, directly affects the DNA in a genome. In this process, proteins attach chemical tags called methyl groups to the bases of the DNA molecule in specific places. The methyl groups turn genes on or off by affecting interactions between the DNA and other proteins. In this way, cells can remember which genes are on or off. The second kind of mark, called histone modification, affects DNA indirectly. DNA in cells is wrapped around histone proteins, which form spool-like structures that enable DNA's very long molecules to be wound up neatly into chromosomes inside the cell nucleus. Proteins can attach a variety of chemical tags to histones. Other proteins in cells can detect these tags and determine whether that region of DNA should be used or ignored in that cell.

Genomics and Epigenomics

Genomics is study of structure and function of every bit of genetic material present in an organism. Epigenomics is the study of the complete set of epigenetic modifications on the genetic material of a cell, known as the epigenome. The field is analogous to genomics and proteomics, which are the study of the genome and proteome of a cell. Epigenomics seeks to identify and characterize epigenetic modifications on a global level. Epigenomics relies heavily on bioinformatics. Epigenomics is the systematic analysis of the global state of gene expression not attributable to mutational changes in the underlying DNA genome. An organism has multiple, cell type-specific, epigenomes comprising epigenetic marks such as DNA methylation, histone modification and specifically positioned nucleosomes. RNA-directed DNA methylation is also known.

Mutations and Epimutations

Mutations involve changes in the sequence of nucleotides. Changes in nucleotides include deletion, duplication, inversion, translocation, and transposition of one or more nucleotides. Epigenetic modifications result epimutations modifications on a cell's DNA or histones that affect gene expression without altering the DNA sequence. Epigenetic modifications are reversible changes. Epigenetic modifications play an important role

in gene expression and regulation, and are involved in numerous cellular processes such as in differentiation/development (Zhu *et al.* 2013) and tumorigenesis.

Just as mutations can occur in forward and reverse direction, epimutations are also known to be forward or reverse. Forward epimutations occur due to De novo methylation whereas reverse epimutations are caused by demethylation. Ethionine and 5-azacytidine are two agents that cause reverse epimutations by undermethylation. Ethionine is a methionine analogue. It inhibits most methyltransferases and causes undermethylation of the DNA. 5-Azacytidine is cytosine analogue with a nitrogen atom at position 5. It cannot accept methyl group and thus causes undermethylation.

Gene and Epigene

Geneticists have genes to work with. On the contrary, epigeneticists do not have an epigene. Thus epigene is a hypothetical term.

EPIGENETIC MODIFICATIONS

Differences in the presence or types of histones (variants) and modifications of DNA (methylation) or histones (methylation, acetylation, etc) and small RNAs that are often associated with epigenetic variation but can be influenced by genetic variation or development/ environment.

DNA modifications

DNA modifications and histone modifications are the two types of epigenetic modifications. In eukaryotes, DNA modification usually means cytosine methylation at carbon 5, which normally occurs in CpG dinucleotides. CpG are part of recognition sequences of a large number of restriction enzymes. DNA methyltransferase methylates DNA. This enzyme does not recognise sequence having no cytosine methylated. Enzyme recognises half-methylated DNA after replication. The set of nucleic acid methylation modifications in an organism's genome or in a particular cell is termed as methylome. DNA methylome is a map showing DNA methylation patterns. While cytosine methylation is a stable modification of the genomic DNA that can be inherited, it also dynamically changes during the lifespan of certain cells and tissues of an organism and it is susceptible to diet and other environmental influences. Indeed, it is essential for the correct onset of differentiation processes and for defining tissue-specific transcriptional profiles, and can be dysregulated in disease states.

Some examples of phenomenon that occur due to DNA methylation are: host restriction and modification, chromosome imprinting, X chromosome inactivation, paramutation, monozygotic twins, epigenetic recombinant inbred lines (epiRILs), small RNAs, heterosis, tissue culture variation, T-DNA integration, programmed cell death of CD of tapetum Cells, DNA methylation and PCR efficiency.

DNA methylation types. In prokaryotes a methyl group can be added to both cytosines and adenine in palindrome target sequences while in eukaryotes DNA methylation is restricted to cytosines. The most common sequence context where this epigenetic mark

is found is the CpG (or CG) di-nucleotide. CpG islands (CGI), are usually depleted of 5meC. CGI are often within upstream gene regulatory regions and their presence, altered methylation status, and the overall CpG content of the promoter regions are highly predictive of the transcriptional potential of the downstream gene. In several eukaryotic organisms 5meC can also be found in other sequence contexts. In general, this type of modification is named non-CG methylation. In particular 5meC in the CHG and CHH sequence context were found; H being A, C or T. mCHH are highly enriched in *A. thaliana* transposons. In some organisms different enzymes and pathways are responsible for these alternative types of methylation.

Recently, the presence of an additional DNA methylation modification, 5-hydroxymethylcytosine (5hmC), was found in mouse Purkinje neurons and the brain. This base can be detected using thin-layer chromatography, while standard restriction endonuclease and bisulfite conversion based methods appear not to be able to reveal it. TET proteins can oxidize 5meC to 5hmC, which is poorly recognized by DNMT1 and can be converted to cytosine, providing a possible pathway for passive de-methylation.

Histone modifications

Histones are chemically-modified by covalent linking of extra moieties. Histone modification includes post-translational addition of acetylation, methylation, phosphorylation, ubiquitination, SUMOylation, ADP-ribosylation, deamination and proline isomerization. Many different amino acids (e.g., lysine, arginine) are modified post-translationally. Histone methylome is a map showing histone methylation patterns.

There are two consequences of histone modifications – establishment of global chromatin environment and orchestration of DNA-based processes. Establishment of global chromatin environment help partition the genome into distinct domains such as euchromatin, where DNA is kept “accessible” for transcription, and heterochromatin, where chromatin is “inaccessible” for transcription. Heterochromatic/euchromatic boundaries are maintained by the presence of methylation at H3K4 and H3K9. Orchestration of DNA-based Processes play role in DNA-based functions, such as replication, transcription, repair, chromosome condensation require the ordered recruitment of the machinery to unravel DNA, manipulate it and then put it back to the correct chromatin state. The term “histone code” has been loosely used to describe the role of modifications to enable DNA functions. There is a cross-talk between histone modifications. The positive or negative influence of one histone modification over another has functional consequences.

Why are epigenetic modifications important?

A human being has trillions of cells, specialized for different functions in different types of cells. Each of these cells carries essentially the same genome in its nucleus. The differences among cells are determined by how and when different sets of genes are turned on or off in various kinds of cells. Specialized cells in the eye turn on genes that make proteins that can detect light, while specialized cells in red blood cells make

proteins that carry oxygen from the air to the rest of the body. The epigenome controls many of these changes to the genome.

Phenomenon involving DNA and Histone Methylation Together

Both DNA and histone methylation are involved in gene imprinting, formation of complex regulatory network, and formation of functional domains in eukaryotic chromosomes. DNA methylation, histone deacetylation and histone methylation in H3K9 (9th lysine of H3) and H3K27 are associated with gene repression, and DNA demethylation. Epigenetic modifications play important roles in various aspects of the plant life cycle such as genome integrity, transgene silencing, nucleosome arrangement, nucleolar dominance, paramutation, flowering, and parent of origin-specific gene expression

EPIGENOME

The genome-wide distribution of chromatin modifications or DNA methylation patterns that may include non-heritable changes or genetically influenced patterns.

Is the epigenome inherited? The genome is passed from parents to their offspring and from cells, when they divide, to their next generation. Much of the epigenome is reset when parents pass their genomes to their offspring; however, under some circumstances, some of the chemical tags on the DNA and histones of eggs and sperm may be passed on to the next generation. When cells divide, often much of the epigenome is passed on to the next generation of cells, helping the cells remain specialized.

What is imprinting? Diploid eukaryotic genome contains two copies of every gene—one copy inherited from the mother and one from the father. For a small number of genes, only the copy from the mother gets switched on; for others, only the copy from the father is turned on. This pattern is called imprinting. The epigenome distinguishes between the two copies of an imprinted gene and determines which is switched on. Some diseases are caused by abnormal imprinting. They include Beckwith-Wiedemann syndrome, a disorder associated with body overgrowth and increased risk of cancer; Prader-Willi syndrome, associated with poor muscle tone and constant hunger, leading to obesity; and Angelman syndrome, which leads to intellectual disability, as well motion difficulties.

Can the epigenome change? Although all cells in the body contain essentially the same genome and it does not change during a person's lifetime, the DNA marked by chemical tags on the DNA and histones gets rearranged when cells become specialized. The epigenome can also change throughout a person's lifetime.

What makes the epigenome change? Lifestyle and environmental factors (such as smoking, diet and infectious disease) can expose a person to pressures that prompt chemical responses. These responses, in turn, often lead to changes in the epigenome, some of which can be damaging. However, the ability of the epigenome to adjust to the

pressures of life appears to be required for normal human health. Some human diseases are caused by malfunctions in the proteins that "read" and "write" epigenomic marks.

How do changes in the epigenome contribute to cancer? Cancers are caused by changes in the genome, the epigenome, or both. Changes in the epigenome can switch on or off genes involved in cell growth or the immune response. These changes can lead to uncontrolled growth, a hallmark of cancer, or to a failure of the immune system to destroy tumors. To compile a complete list of possible epigenomic changes that can lead to cancer, researchers in The Cancer Genome Atlas (TCGA) Network, which is supported by the National Institutes of Health (NIH), are comparing the genomes and epigenomes of normal cells with those of cancer cells. Among other things, they are looking for changes in the DNA sequence and changes in the number of methyl groups on the DNA. Understanding all the changes that turn a normal cell into a cancer cell will speed efforts to develop new and better ways of diagnosing, treating and preventing cancer.

EPIGENETIC VARIATIONS CAN CAUSE PHYSICAL VARIATION

In eukaryotes, the genome is compacted into chromatin, and the chromatin structure plays an important role in gene expression: Gene expression can be controlled by changes in the structure of chromatin without changing the DNA sequence, and this phenomenon is termed “epigenetic” control. Epigenetic change can cause phenotypic variation, and thus epigenetic change can be considered as an important factor in understanding phenotypic change. Phenotypic plasticity is defined as capacity of a cell to change its state in response to stimuli. DNA methylation and histone modification are well known epigenetic modifications (Fujimoto 2012). Specific mechanisms were proposed in which information was superimposed on DNA sequences. These phenomena were considered exceptions to Mendelian genetics since the outcomes of genetic segregation often appeared to contest Mendel’s predicted laws of inheritance (Kinoshita and Jacobsen 2012).

Epigenetic variation has the potential to contribute to the natural variation of plant traits. Epigenome analysis aids in explaining how natural epigenetic variation causes phenotypic differences in plants. Stable inheritance of complex traits such as flowering time has been observed in these epi-recombinant inbred line (epi-RIL) populations, providing important evidence that epigenetic variation can contribute to complex traits. Bringing together two divergent genomes and epigenomes in the same nucleus has the potential to alter gene expression and the phenotype of an organism (Greaves *et al.* 2012). This is evident in plant crop systems where hybrids commonly show increased vigor or heterosis. Recent studies have found the epigenome to be a possible contributor to heterosis, especially in intraspecies hybrids, where genetic diversity is limited. In the F₁ hybrids, interaction occurs between the epigenomes contributed by the two parental sets of chromosomes leading to altered gene expression (Greaves *et al.* 2012).

Epigenetics has a role in heterosis. Groszmann *et al.* (2011) has reported that changes in short interfering RNA and DNA methylation levels appear to be related to heterosis, and sRNAs are known to be involved in establishing DNA methylation through RNA-directed DNA methylation.

EPIGENOMIC RESEARCH

It is planned to 'genomicize' epigenomic research and pave the way for breakthroughs in the prevention, diagnosis and treatment of human disease (The American Association for Cancer Research Human Epigenome Task Force and The European Union, Network of Excellence. Scientific Advisory Board 2008). The coding and structural information in the base sequence of DNA is organized in chromatin to form multiple epigenomes. DNA cytosine methylation and covalent modifications of the tails of histones and histone variants contribute information to nucleosome remodeling machines that render genes and non-coding RNAs susceptible to transcription. Transcription factors also play a major part in the competence and organization of the epigenome. The AHEAD project will map epigenetic marks in a defined set of epigenomes.

First mathematical models for different nucleosome states affecting gene expression were introduced in 1980s. Later, this idea was almost forgotten, until the experimental evidence has indicated a possible role of covalent histone modifications as an epigenetic code (Strahl and Allis 2000). In the next several years, high-throughput data have indeed uncovered the abundance of epigenetic modifications and their relation to chromatin functioning which has motivated new theoretical models for the appearance, maintaining and changing these patterns (Sedighi and Sengupta 2007; Dodd *et al.* 2007). These models are usually formulated in the frame of one-dimensional lattice approaches (Teif and Rippe 2010).

EPIGENOMIC MAPS

In epigenomics, researchers try to chart the locations and understand the functions of all the chemical tags that mark the genome. Such charts are called epigenomic maps. Epigenomic maps may someday enable doctors to determine an individual's health status and tailor a patient's response to therapies. Epigenomics, thus, has become a vital part of efforts to better understand the human body and to improve human health.

As part of the ENCODE (ENCyclopedia Of DNA Elements) project-which aims to catalog the working parts of the genome – the National Human Genome Research Institute is funding researchers to make epigenomic maps of various cell types. NIH-supported investigators have developed a number of epigenomic maps from several human organs and tissues. These NIH projects are part of an international effort to understand how epigenomics could lead to better prevention, diagnosis and treatment of disease.

DNA Methylation Assays

Techniques for characterizing primary DNA sequences could not be directly applied to methylation assays. For example, when DNA was amplified in PCR or bacterial cloning techniques, the methylation pattern was not copied and thus the information

lost. The DNA hybridization technique used in DNA assays, in which radioactive probes were used to map and identify DNA sequences, could not be used to distinguish between methylated and non-methylated DNA (Laird 2010; Eads 2000). Methods of analyzing DNA methylation patterns are divided into two broad categories – nongenome-wide approaches and genome-wide approaches (Fujimoto 2012).

The earliest methylation detection assays used methylation modification sensitive restriction endonucleases. Genomic DNA was digested with both methylation-sensitive and insensitive restriction enzymes recognizing the same restriction site. The idea being that whenever the site was methylated, only the methylation insensitive enzyme could cleave at that position. By comparing restriction fragment sizes generated from the methylation-sensitive enzyme to those of the methylation-insensitive enzyme, it was possible to determine the methylation pattern of the region. This analysis step was done by amplifying the restriction fragments via PCR, separating them through gel electrophoresis and analyzing them via Southern blot with probes for the region of interest (Eads *et al.* 2000; Laird 2010)).

Exemplifying methods based on methylation sensitive RE, the HpaII tiny fragment enrichment by ligation-mediated PCR assay (HELP) can be used to determine genome-wide patterns based on the combined activity of HpaII and MspI restriction enzymes (RE) (Khulanet *et al.* 2006). The main disadvantages of this approach are in the resolution of the data and the bias due to the non-uniform distribution of RE cutting sites.

This technique was used to compare the DNA methylation modification patterns in the human adult and hemoglobin gene loci. Different regions of the gene (gamma delta beta globin) were known to be expressed at different stages of development. Consistent with a role of DNA methylation in gene repression, regions that were associated with high levels of DNA methylation were not actively expressed (Van der Ploeg *et al.* 1980).

This method was limited not suitable for studies on the global methylation pattern, or ‘methyloome’. Even within specific loci it was not fully representative of the true methylation pattern as only those restriction sites with corresponding methylation sensitive and insensitive restriction assays could provide useful information. Further complications could arise when incomplete digestion of DNA by restriction enzymes generated false negative results (Laird 2010). Several methods have been developed which enable capture of genome-wide profiling of DNA methylation (Laird 2010; Pelizzola and Ecker 2011).

Non-CG methylation

Non-CG methylation was distributed non-randomly in the genome and was particularly enriched in the gene bodies, with increasing levels corresponding to more highly transcribed genes. Non-CG methylation was also particularly enriched in genes related with processing of mRNA and in genes having higher pre-mRNA levels. These

findings point to the potential involvement of DNA methylation with the splicing machinery.

Methylation of regulatory elements

Cells in different states of differentiation use different DNA methylation mechanisms to mark these important regulatory features.

Differential methylation

While non-CG methylation was absent in differentiated cells, widespread differences were found in the distribution and levels of CG methylation. CG DNA methylation is usually considered a repressive mark. Indeed, methylation in the context of promoters is inversely related with the transcriptional level of the downstream gene. However, CG methylation in gene bodies is positively correlated with the gene transcriptional level, indicating that the meaning of this epigenetic mark is rather complex and context dependent. Genome-wide studies showed that gene-body non-CG methylation is also clearly positively correlated with gene expression. There is evidence in *A. thaliana* for association between gene body methylation and transcriptional elongation, suggesting a scenario where transcription contributes to maintaining or enhancing DNA methylation levels.

Histone Modification Assays

Posttranslational covalent modifications of histone tails, which include methylation, acetylation, phosphorylation, and ADP-ribosylation, are thought to control gene expression by regulating the strength of DNA-histone interactions determining the accessibility of DNA to transcriptional regulators. Historically, histone modifications have been identified by chromatin immunoprecipitation (ChIP) which, in brief, involves cross-linking proteins to DNA, followed by immunoprecipitation of a protein of interest with a specific antibody, and characterization of the bound DNA by hybridization or PCR amplification.

Li *et al.* (2008) provided insights into the rice epigenomes and their effect on gene expression and plant development. High-resolution maps of DNA methylation and H3K4 di- and trimethylation of two entire chromosomes and two fully sequenced centromeres were obtained from rice (*Oryza sativa*) shoots and cultured cells. This analysis reveals combinatorial interactions between these epigenetic modifications and chromatin structure and gene expression. Cytologically densely stained heterochromatin had less H3K4me2 and H3K4me3 and more methylated DNA than the less densely stained euchromatin, whereas centromeres had a unique epigenetic composition. Most transposable elements had highly methylated DNA but no H3K4 methylation, whereas more than half of protein-coding genes had both methylated DNA and di- and/or trimethylated H3K4. Methylation of DNA but not H3K4 was correlated with suppressed transcription. By contrast, when both DNA and H3K4 were methylated, transcription was only slightly reduced. Transcriptional activity was positively correlated with the ratio of H3K4me3/ H3K4me2: genes with predominantly

H3K4me3 were actively transcribed, whereas genes with predominantly H3K4me2 were transcribed at moderate levels. More protein-coding genes contained all three modifications, and more transposons contained DNA methylation in shoots than cultured cells. Differential epigenetic modifications correlated to tissue-specific expression between shoots and cultured cells.

Methods for profiling RNAs

It is often of interest to generate gene expression profiles in epigenomic studies, such that the relationship and correlated changes between epigenomic states and transcriptional activities can be identified in a genome-wide manner. Detailed characterization of the cellular RNA pools has also uncovered novel intergenic transcripts, noncoding RNAs (ncRNAs), alternative splicing isoforms and antisense transcripts, thereby providing new opportunities for discovery-based research. In addition to established microarray-based methods, high-throughput RNA sequencing (RNA-Seq) is being used in an increasing number of studies. Two major types of libraries can be constructed for RNA-Seq: cDNA libraries and strand-specific RNA libraries (Schmitz and Zhang 2011). The advantage of cDNA library sequencing is the cost of library preparation and the ability to construct a library with limited starting material, and it is now possible to profile the transcriptome of a single cell. The advantage of strand-specific RNA-Seq (also known as directional RNA-Seq) is the ability to determine the strandedness of the RNA, which allows the identification of novel ncRNAs and antisense transcripts.

Plant cellular RNA pools also include several types of small RNAs (sRNAs), including small interfering RNAs (siRNAs), microRNAs (miRNAs), trans-acting small interfering RNAs (tasiRNAs) and natural antisense small interfering RNAs (nat-siRNAs). These sRNAs are highly abundant and play important roles in many biological processes, such as regulating gene expression, heterochromatic silencing, and antiviral defense. A major goal in sRNA studies is the detailed characterization of the sRNA populations accumulated in specific cells or tissues. The source and targets of sRNAs can then be identified to address questions regarding the mechanisms and functions of the sRNA pathways. There are currently two primary methods for isolation of sRNAs. First, size selection from total cellular RNA is the most popular method and has been extensively utilized to catalog sRNAs in different tissue types, mutants and accessions or subspecies. Second, immunoprecipitation of sRNA-binding proteins has been employed, but it requires a few additional features, including a protein that strongly associates with sRNAs (such as ARGONAUTE proteins) and the ability to immunoprecipitate the protein of interest. Following ligation to RNA adaptors and reverse transcription, millions of sRNAs sequences can be revealed by RNA-Seq. In addition to the sequences and relative abundances, sequencing results also provide length and information about strand of origin, which can be used to infer the proteins that might be involved in generating and processing sRNA precursors.

Different strategies used for high-throughput sequencing the epigenome (genome-wide DNA methylation, histone modification, and small RNAs analysis) are shown in Figure 2 (He *et al.* 2011).

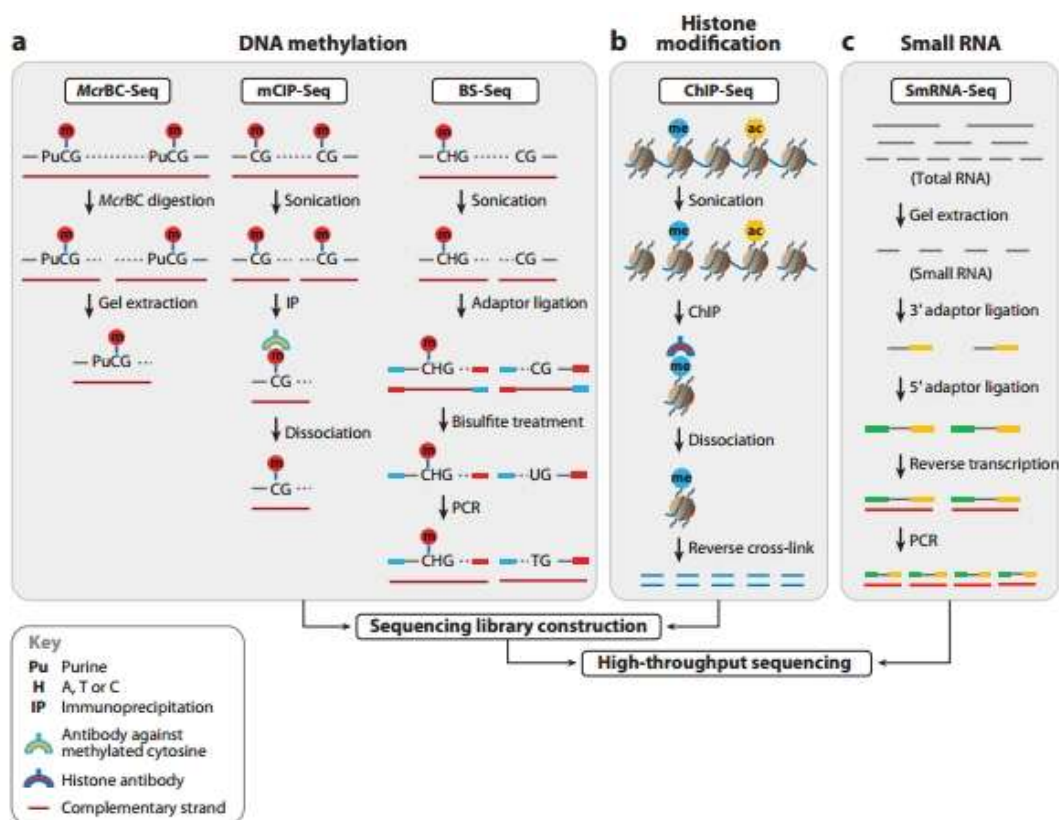


Figure 2. Strategies for high-throughput sequencing the epigenome. (a) DNA methylome profiling. Methylated DNA is enriched by gel purification of MCrBC-digested genomic DNA in the MCrBC-Seq approach, or by immunoprecipitation of sonicated genomic DNA with an antibody against methylated cytosine in the mCIP-Seq approach. In the BS-Seq approach, specific adaptors are used in PCR to turn single-stranded DNA obtained from bisulfite treatment into double-stranded DNA. The final DNA fragments are used to generate high-throughput-sequencing libraries according to the manufacturer's instructions. (b) Genome-wide profiling of histone modifications. DNA fragments associated with modified histones are purified using ChIP assay and are used to generate sequencing libraries. (c) Small RNA (smRNA) transcriptome profiling. The purified smRNAs are ligated sequentially with 3 and 5 adaptors. The PCR products are subjected directly to high-throughput sequencing (He *et al.* 2011)

COMPARTMENTALIZATION IN PLANT GENOMES WITH RESPECT TO EPIGENOMIC MODIFICATIONS OF PLANT EPIGENOMES

The collective results from published epigenomic studies suggest that plant genomes are highly compartmentalized, with different components of the genome associated with distinct combinations of epigenomic modifications (Schmitz and Zhang 2011). That is, transposable elements and other repetitive sequences are highly DNA-methylated in all three sequence contexts and are also targeted by the siRNA and

histone lysine 9 dimethylation (H3K9me2) pathways. Developmentally repressed genes are enriched in H3K27me3 and bound by LikeHeterochromatin Protein 1 (LHP1), whereas actively transcribed genes are enriched in a different set of histone modifications including acetylation, H3K4me, H3K36me and H3K9me3, and many moderately to highly transcribed genes also contain heavy gene-body DNA methylation but only in the CG context. In other words, constitutively silenced, developmentally repressed and actively transcribed regions are associated with largely nonoverlapping sets of modifications, indicating that these modifications likely play important roles in modulating transcriptional activities. Consistent with this notion, nonrepetitive intergenic regions that are transcriptionally inactive are generally devoid of these modifications. In addition to transcription, tantalizing new results have also implicated H3K27me1 in the activities of origins of DNA replication and associated DNA methylation with nucleosome positioning and histone variants. It is conceivable that some of the pathways described above may also function in other molecular events that are affected by the local chromatin environment, such as recombination or DNA repair. The application of epigenomic approaches in combination with other methods, particularly those that allow cell-type-specific studies, will undoubtedly lead to novel and exciting discoveries in the near future.

METHYLOMES AND EVOLUTION

The role of DNA methylation in different genomic contexts can be remarkably different, and alternative mechanisms are available for establishing 5^{me}C patterns in different genomic regions. The density of possible methylation sites, as well as the distribution of 5^{me}C, is not uniform in the genome. In particular, control of methylation levels in promoters, gene bodies, regulatory features, and transposable and repetitive elements appears to be critical.

Recently, base-resolution sequencing of several eukaryotic methylomes has provided initial insights into the evolutionary history of DNA methylation and its distribution in these key genomic regions. Currently, the complete methylomes for 23 organisms are available (10 animals, 8 plants and 5 fungi; Figure 3) (Pelizzola and Ecker 2011). In general the DNA methylation landscape can be either continuous along the genome, or constituted by a series of heavily methylated DNA domains interspersed with domains that are methylation free. When considering the data available for a limited set of loci in an even wider set of organisms, the current perspective is that the evolutionary history of DNA methylation in gene bodies and transposons may be independent. These genomic regions are present in both plants and vertebrates; however, transposon methylation is only conserved in fungi while gene body methylation only occurs in invertebrates. Gene body methylation seems to be a property inherited from ancient genomes, while transposon methylation appears to be related to the degree of sexual outcrossing. In general, for those organisms where the methylation of certain genomic elements was present, their methylation pattern is quite conserved. However, the presence of non-CG methylation is less common, but when present, it is always at a lower level than mCG.

DNA METHYLATION LANDSCAPES IN PLANTS

The first high-resolution genome-wide map of DNA methylation in plants was obtained using the mCIP-chip method. Since then, many other studies using various approaches profiled diverse DNA methylation landscapes in different plant tissues and species. The results from these studies, although differing slightly depending on the strategies used in profiling, strongly support the concept that DNA methylation plays a key role in controlling chromatin structure.

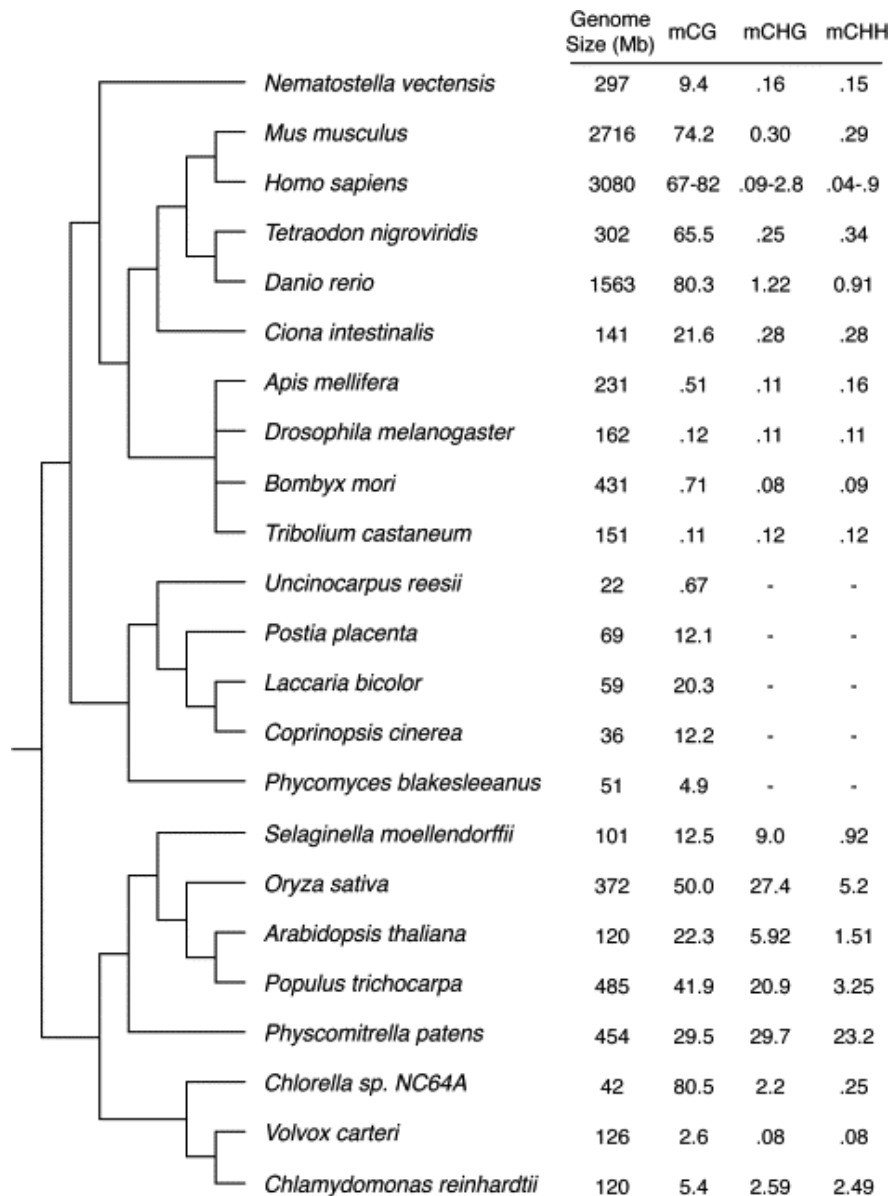


Figure 3. Methylation levels in 23 eukaryotic organisms. The organisms are organized according to their evolutionary distance. The genome size is indicated together with the percentage of methylated sites within three sequence contexts: CpG, CHG and CHH (H being A, C or T) (Pelizzola and Ecker 2011)

In general, DNA methylation was found to be highly enriched in pericentromeric heterochromatin regions, which are associated with transposable elements (TEs) and other repetitive sequences (He *et al.* 2011). This suggests that one of the most important functions of DNA methylation lies in suppressing the activity of transposons and maintaining genome stability. Although initially unexpected, DNA methylation was also found to extend to the euchromatic regions. A large number of protein-coding genes are methylated within transcribed regions, in contrast to the low frequency of DNA methylation within promoter regions. Further examination of the position of genic methylation in nontransposon genes (non-TE genes) revealed a biased distribution of DNA methylation, with a slight increase in the 3'-half, and a depletion in the immediate 5' and 3' flanking regions of genes. What is consistent in all studies is that throughout the genome there is significant DNA methylation of transposons or transposon-related genes (TE genes), pseudogenes, and other repetitive sequences. Furthermore, DNA methylome profiling at single-base resolution in plants revealed that whereas CG, CHG, and CHH methylation are enriched in transposons and repetitive sequences, genic methylation of non-TE genes was found to consist almost exclusively of CG methylation.

Further investigations of the positions of these histone modifications relative to genes revealed that whereas H3K4me1 and H3K9me2 were distributed across the entire transcribed region, H3K4me2, H3K4me3, H3K9ac, H3K9me3, H3K27ac, H3K27me3, and H3K36me3 were enriched at the 5'-end of the gene body (He *et al.* 2011). H3K4me1 and H3K4me2 are not correlated with transcript abundance. The role of these two modifications in the regulation of gene expression depends on their interaction with other epigenetic marks such as H3K4me3 and H3K27me3.

Epigenomic Reprogramming during Gametogenesis and Seed Development in Plants

In flowering plants, gametogenesis is initiated with the differentiation and meiosis of diploid microspore and megaspore mother cells, followed by sequential mitotic division to produce male and female gametophytes. Male gametophytes (pollen grains) contain two smaller haploid generative cells (sperm cells) enclosed entirely within the cytoplasm of a larger vegetative cell. The vegetative cell delivers the sperm cells to the female gametophyte by forming a pollen tube. The female gametophyte (embryo sac) contains seven cells and eight nuclei, including a haploid egg cell and a homodiploid central cell. Seed development is initiated by a double fertilization event in which one sperm cell fertilizes the haploid egg cell to generate a diploid embryo, whereas another sperm cell fertilizes the diploid central cell to generate triploid endosperm. The endosperm is essential for plant development and growth because it nourishes the embryo and seedling during their early developmental stages. During gametogenesis and seed development in plants, it is very important to keep transposons inactive so as to maintain genome stability in gamete and embryo and to ensure the accuracy of genetic information during the life cycle. The accessory cells and tissues such as the vegetative cell, central cell, and endosperm do not contribute genetically to the next

generation. Recently, genome-wide epigenetic analyses revealed extensive epigenomic reprogramming during the development of pollen and endosperm as well as a potential epigenetic contribution of the supporting cells or tissues to repress transposon expression and maintain genome stability in sperm cell and embryo through an siRNA-mediated silencing pathway (He *et al.* 2011).

The recent availability of a large amount of epigenome data from diverse species allows us to analyze the interactions and relationships between genetic and epigenetic variations and their significance in determining phenotypic plasticity in plant species from a genome-wide viewpoint (Figure 4).

An interesting feature of the maize epigenomic landscape that was found is that the genic DNA methylation pattern is similar to that of rice in that it shows a peak around ATG sites but differs substantially from that of *Arabidopsis*, which shows higher DNA methylation in transcribed regions. In addition, it was found that whereas the histone modifications associated with gene activation tend to occur together, the two gene-repressive histone marks under study, H3K27me3 and DNA methylation, tend to exclude each other at the same locus.

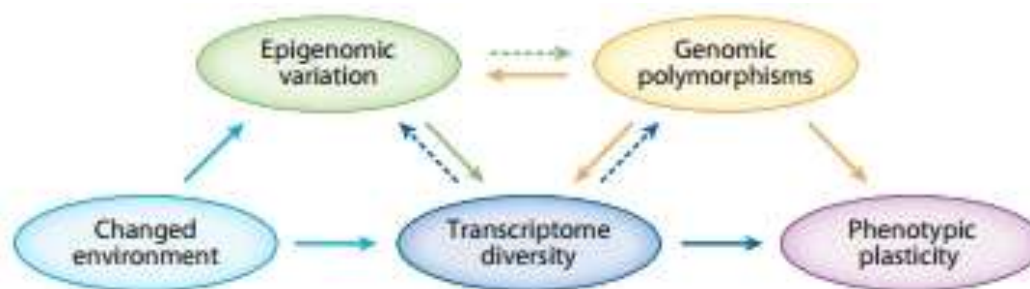


Figure 4. The interactions of variations in genome, epigenome, and transcriptome and their relationships with environmental sensing and phenotype determination. Dashed arrows indicate the possible relationships in given situations, such as the epigenomic and genomic variation resulting from transposon reactivation and translocation (He *et al.* 2011)

Deep Sequencing Maps

Massively parallel signature sequencing (MPSS), sequencing by synthesis (SBS) method, 454 Life Sciences' SBS pyrosequencing method, Applied Biosystems' SOLiD sequencing by ligation system, Helicos Biosciences' single-molecule synthesis platform, and several other single-molecule synthesis platforms are often referred to as “deep sequencing” because of their ability to generate huge numbers of sequencing reads per experiment or instrument run (Eckardt 2009).

These sequencing-based approaches have some distinct advantages over microarray-based approaches for genome-wide transcriptomics (the study of gene expression) and epigenomics (the study of chromatin organization and dynamics), such as avoiding complex intermediate cloning and microarray construction steps and the ability to generate a massive amount of sequence quickly. Using these approaches, gene

expression is assayed by directly sequencing cDNA molecules obtained from an mRNA sample and simply counting the number of molecules corresponding to each gene to assess transcript abundance.

Wang *et al.* (2009) report genomic landscapes of representative epigenetic modifications and their relationships to mRNA and small RNA (smRNA) transcriptomes in maize shoots and roots. The epigenetic patterns differed dramatically between genes and transposable elements, and two repressive marks (H3K27me3 and DNA methylation) were usually mutually exclusive. They found an organ-specific distribution of canonical microRNAs (miRNAs) and endogenous small interfering RNAs (siRNAs), indicative of their tissue-specific biogenesis. Furthermore, we observed that a decreasing level of mop1 led to a concomitant decrease of 24-nucleotide siRNAs relative to 21-nucleotide miRNAs in a tissue-specific manner. A group of 22-nucleotide siRNAs may originate from long-hairpin double-stranded RNAs and preferentially target gene-coding regions. Additionally, a class of miRNA-like smRNAs, whose putative precursors can form short hairpins, potentially targets genes in trans. In summary, these data provide a critical analysis of the maize epigenome and its relationships to mRNA and smRNA transcriptomes. This study largely confirms epigenomic patterns that have been established in other species (*Arabidopsis* and other organisms), such as, for example, H3K4me3, H3K9ac, and H3K36me3 being associated with transcriptionally active genes, and H3K27me3 and DNA methylation predominantly being found in transcriptionally inactive genes and repetitive elements (Figure 5).

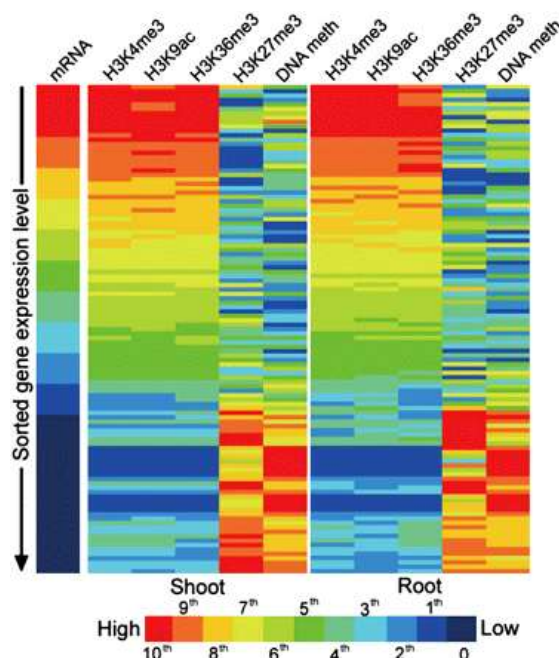


Figure 5. Heat maps of epigenetic modification levels on ~60,000 genes sorted by their expression measured by deep sequencing. Gene expression levels and modifications levels were transformed to 100 percentiles, and each bar represents the averaged level of nearly 600 genes within each percentile (Eckardt 2009)

AN EPIGENETIC APPROACH TO CROP BREEDING

As part of the large research consortium EPIGEN, Professor Serena Varotto from the University of Padua is working on maize to determine how stress-induced epigenetic variation can help to improve the stress tolerance and yields of crops. Epigenetic variation is when the phenotypic traits of an individual vary without altering the primary sequence of its DNA. This can occur through changes in the expression of particular genes via processes such as DNA methylation and chromatin remodelling, and by influencing the activity of RNA structures which regulate levels of gene expression. Epigenetic changes in gene expression enable an individual to respond to changes in the environment and adjust the synthesis of proteins accordingly. It has become apparent that while many of the epigenetic modifications to the genome are reset during the process of meiosis, some epigenetic information can be transmitted between generations, so that the phenotypic traits of offspring are affected without altering the primary structure of the DNA. Thus offspring can inherit tolerance to a particular environmental condition before they have been exposed.

The EPIGEN project has the broad aim of understanding how epigenetic mechanisms regulate biological processes, determine phenotypic variation and contribute to the onset and progression of diseases. Made up of a group of more than 40 Italian researchers, the project is divided into eight subprojects, one of which is coordinated by Professor Serena Varotto from the University of Padua. This project uses the model crop species maize to study the natural and induced epigenetic variation that occurs as a result of environmental stress, and in particular to consider transposed regions of the genome that are epigenetically responsive. Furthermore, the analysis will aim to distinguish between the obligatory and pure components of epigenetic variation – the former being due to structural variation in the DNA sequence, and the latter as a result of variation that is independent of genetic variation and therefore more significant for environmental adaptation.

Extremes of genetic variation

To achieve this, the team has made use of appropriate genetic materials representing the two extremes of genetic variation. To create the low genetic variation material the researchers used the progeny of a single plant belonging to an inbred maize line. The plants of the progeny were separately propagated in a common environment for six generations and consequently were expected to have very low genetic variation. In parallel, the highly genetically variable material used the same progeny but both parent and progeny plants were exposed to a common environmental stress before selfing. Varotto explains: “Using this approach, it is hoped that we will be able to establish how, and to what extent, epigenetic information is dynamically restructured following genomic recombinations, such as those that occur during meiosis”.

Epigenetics for evolution

Epigenetic changes that occur independent of changes to the genotype have important implications for evolution. Over the last century, improvements to crop yields have been achieved by genetic breeding to improve tolerance to abiotic and biotic

stresses and to improve basic yield under non-stressful conditions. The 21st Century will bring with it increasingly severe fluctuations in climatic conditions; causing more frequent drought, desertification, salt stress and reduction in soil quality. Thus there is a pressing need to enhance the ability of crops to grow in adverse environmental conditions.

The researchers treated genetically variable and genetically homogenous maize lines for 10 days with different stresses – drought conditions, salt treatment and drought plus salt treatment – in addition to a non-stressed control. Plants were then allowed a period of recovery. Leaf samples were taken at the end of treatment, then after four and seven days of recovery, and genetically analysed to determine the levels of RNA transcripts, the state of chromatin modifications and the population of small RNAs. Following this, the plants were self-pollinated to produce genetically identical progeny that could be analysed to see to what extent the memory of the stress was epigenetically preserved between the generations. If the inheritable component of epigenetic variation can be understood, this knowledge can be used to develop high yielding and stress-resistant crop varieties via breeding programmes.

Initial results

Initial sequencing of the stressed plants has identified a ‘stress-specific transcriptome’ which demonstrates the modulation of many stress-related genes through alterations to their expression levels, as well as the action of transposable elements and long noncoding RNAs. Looking forward in the project, the identified genes of interest are in the process of being characterised to determine their function and epigenetic component. The project will enable the translation of knowledge from basic research into novel strategies and methodologies to improve plant yield production and stability in adverse environmental conditions.

Epigenetics and Response to the Environment

The concept of environmental responses as epigenetic is often used to describe a range of findings from heritable phenotypic consequences to the observation of chromatin changes after environmental treatments. Stable phenotypic consequences after an environmental treatment may reflect physiological or morphological changes triggered by the initial environmental cues that are maintained without epigenetic contributions. However, the observation of chromatin changes does not necessarily imply epigenetics, because these changes may be transient effects that require the ongoing presence of the stimulus. For example, drought conditions may result in altered leaf wax deposition, and this morphological change could influence phenotypic responses to subsequent environmental conditions but would not reflect cellular inheritance of the memory of the stress. Conceptually, mitotically transmissible memory that programs responses to environmental cues may provide part of the mechanism that plants use to alter gene expression in response to the environment. There are some clear examples of epigenetic mitotic memory of environmental conditions, such as vernalization (see below), but there are many more examples in which dynamic changes in chromatin modifications

provide a portion of the mechanism for transient gene expression changes in response to the environment.

Another potential example of epigenetic contributions to environmental response is the priming or training response to environmental conditions. Priming refers to changes in phenotype or gene expression after repeated exposures to an environmental stress. A phenotypic response or gene expression change will be trained by initial exposures to stress and will exhibit more pronounced or rapid responses to subsequent treatments of the same environmental condition.

USE OF EPIGENOMICS IN IMPROVEMENT OF *BRASSICA*

An Australian scientist, G.J. King has demonstrated potentials of use of epigenomics in crop improvement by using *Brassica oleracea*. Some of his important contributions in this area of research are described here:

Epiallelic Variation

Treatment with the demethylating agent 5-azacytidine induced a range of developmental changes at high frequency in *Brassica oleracea* (King 1995). The spectrum of morphological variation was similar to that previously observed in *Brassica*, either as somaclonal variation as a result of tissue culture, or as a result of mutation with nitroso-N-methylurea. Where floral development permitted, inheritance of developmental variants was demonstrated to the M₂ generation.

Fruit Ripening

A major component in the regulatory network controlling fruit ripening is likely to be the gene at the tomato Colorless non-ripening (*Cnr*) locus (Manning *et al.* 2006). The *Cnr* mutation results in colorless fruits with a substantial loss of cell-to-cell adhesion. The nature of the mutation and the identity of the *Cnr* gene were previously unknown. Using positional cloning and virus-induced gene silencing, here we demonstrate that an SBP-box (SQUAMOSA promoter binding protein-like) gene resides at the *Cnr* locus. Furthermore, the *Cnr* phenotype results from a spontaneous epigenetic change in the SBP-box promoter. The discovery that *Cnr* is an epimutation was unexpected, as very few spontaneous epimutations have been described in plants. This study demonstrates that an SBP-box gene is critical for normal ripening and highlights the likely importance of epialleles in plant development and the generation of natural variation. Thus epigenetic variation has been shown to influence tomato ripening (Seymour *et al.* 2008). These discoveries are likely to have a major impact on strategies for crop improvement in fruit bearing species.

Exploring and Exploiting Epigenetic Variation

DNA methylation has role as an epigenetic mark that contributes to epiallelic diversity and modulation of gene regulation. King *et al.* (2010) outline the prevalence and distribution of epigenetic marks in relation to eukaryote developmental processes, and in particular identify where this may be relevant to crop traits both in terms of specific

developmental stages and in relation to physiological responses to environmental change. Recent whole genome surveys have identified specific characteristics of the distribution of DNA methylation within plant genomes. Together with greater understanding of the mode of action of different maintenance and *de novo* methyltransferases, this provides an opportunity to modulate DNA methylation status at specific loci as an intervention strategy in crop genetic improvement.

Epigenetic QTL Mapping

Long *et al.* (2011) analyzed the stability and distribution of epialleles within the complex genome of the oilseed crop plant *Brassica napus*. For methylation sensitive AFLP (MSAP) and retrotransposon (RT) epimarkers, they found a high degree of stability, with 90% of mapped markers retaining their allelic pattern in contrasting environments and developmental stages. Moreover, for two distinct parental lines 97% of epialleles were transmitted through five meioses and segregated in a mapping population. For the first time they have established the genetic position for 17 of the 19 centromeres within this amphidiploid species. Epiloci and genetic loci were distributed within distinct clusters, indicating differential detection of recombination events. This enabled them to identify additional significant QTL associated with seven important agronomic traits in the centromeric regions of five linkage groups.

Generation of Novel Epialleles

Epigenetic marks superimposed on the DNA sequence of eukaryote chromosomes provide agility and plasticity in terms of modulating gene expression, ontology, and response to the environment. Modulating the methylation status of cytosine can generate epialleles, which have been detected and characterised at specific loci in several plant systems, and have the potential to generate novel and relatively stable phenotypes. There have been no systematic attempts to explore and utilise epiallelic variation, and so extend the range of phenotypes available for selection in crop improvement. Amoah *et al.* (2012) developed an approach for generating novel epialleles by perturbation of the DNA methylation status. 5-Azacytidine (5-AzaC) provides selective targeting of ^{5m}CG, which in plants is associated with exonic DNA. Targeted chemical intervention using 5-AzaC has advantages over transgenic or mutant modulation of methyltransferases, allowing stochastic generation of epialleles across the genome.

Plant Plasticity

Plasticity in plants is assisting in homeostasis in response to variable environmental conditions. Plant plasticity is facilitated by epigenetic processes that modulate chromatin through dynamic changes in DNA methylation, histone variants, small RNAs and transposable elements (Bloomfield *et al.* 2014). Plant plasticity needs to be understood in the context of epigenetic regulation of developmental phases and transitions and map these onto the key stages of crop establishment, growth, floral initiation, pollination, seed set and maturation of harvestable product. We need to take

a fresh look at the crosstalk between regulatory systems that confer plasticity in the context of crop development. Insights into genotype \times environment ($G \times E$) interaction should underpin development of new crop management strategies, both in terms of information-led agronomy and in recognizing the role of epigenetic variation in crop breeding.

Genotype \times Environment Interactions

King (2015) proposes that within plant nuclei, general and localized ionic homeostasis plays an important role in maintaining chromatin conformation, whilst maintaining complex genomic regulation that involves specific patterns of epigenetic marks. More generally, reversible changes in DNA methylation appear to be consistent with the ability of nuclear chromatin to manage variation in external ionic and temperature environment. Understand in greater detail the internal environment of plant nuclei will generate a deeper understanding of the molecular mechanisms underlying genotype \times environment interactions that may be beneficial for long-term improvement of crop performance in less predictable climates.

EPIGENOME EDITING

Epigenome editing is a type of engineering in which the epigenome is modified at specific sites using engineered molecules targeted to those sites (as opposed to whole-genome modifications). “Editing” epigenomic features in this manner allows researchers to determine the exact biological role of an epigenetic modification at the site in question. The engineered proteins used for epigenome editing are composed of a DNA binding domain that target specific sequences and an effector domain that modifies epigenomic features (Figure 6). Currently, two major groups of DNA binding proteins have been predominantly used for epigenome editing: Transcription Activator-Like Effectors (TALEs) (Figure 7) and nuclease deficient Cas9 fusions (CRISPR). Commonly used effector proteins TET1 induces demethylation of cytosine at CpG sites. This protein has been used to activate genes that are repressed by CpG methylation and to determine the role of individual CpG methylation sites. LSD1 induces the demethylation of H3K4me1/2, which also causes an indirect effect of deacetylation on H3K27. This effector can be used on histones in enhancer regions, which can change the expression of neighbouring genes. The interaction allows the chromatin modifier to act on the desired location. This means that the modification can be performed in an inducible and reversible manner, which reduces long-term secondary effects that would be caused by constitutive epigenetic modification.



Figure 6. Basic structure of an epigenome editing tool. (Miglani, G.S. and R. Singh. 2019. Epigenome editing in crop improvement. Chapter 15. In: *Quantitative Genetics, Genomics and Plant Breeding*. Second edition. Ed. Kang, M.S. CAB International. Oxford, U.K.)

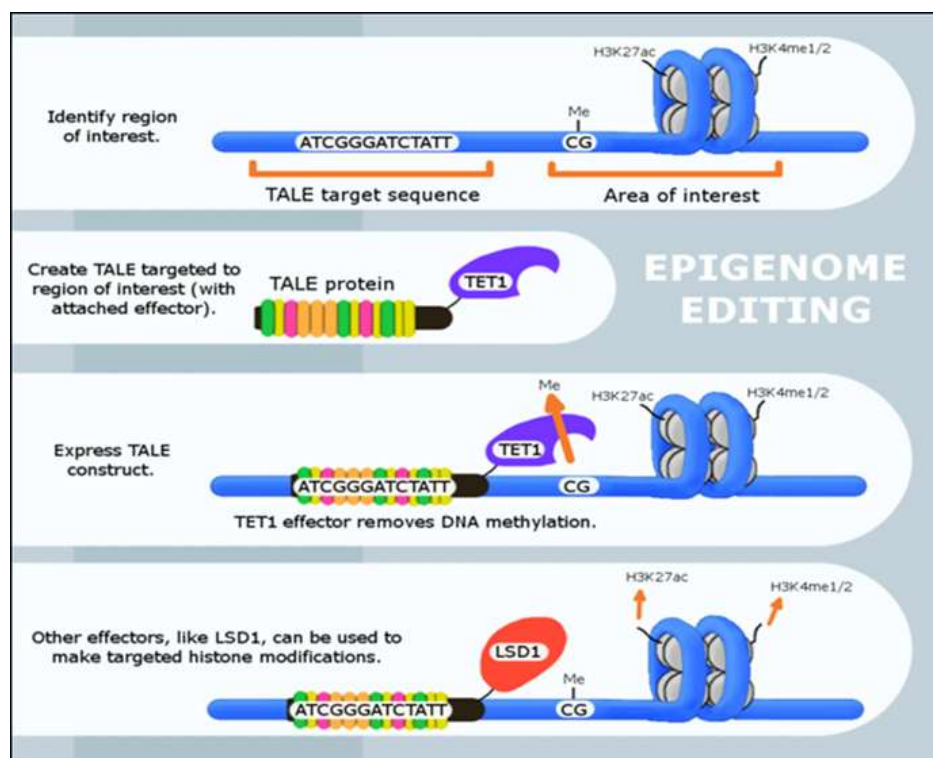


Figure 7. Epigenome Editing (https://en.wikipedia.org/wiki/Epigenome_editing)

APPLICATIONS OF EPIGENOME EDITING

There are various applications of epigenome editing (Figure 8): modulation of gene expression, study of regulatory elements, modulation of environmental traits, and cell type studies. Some of these applications are described here.

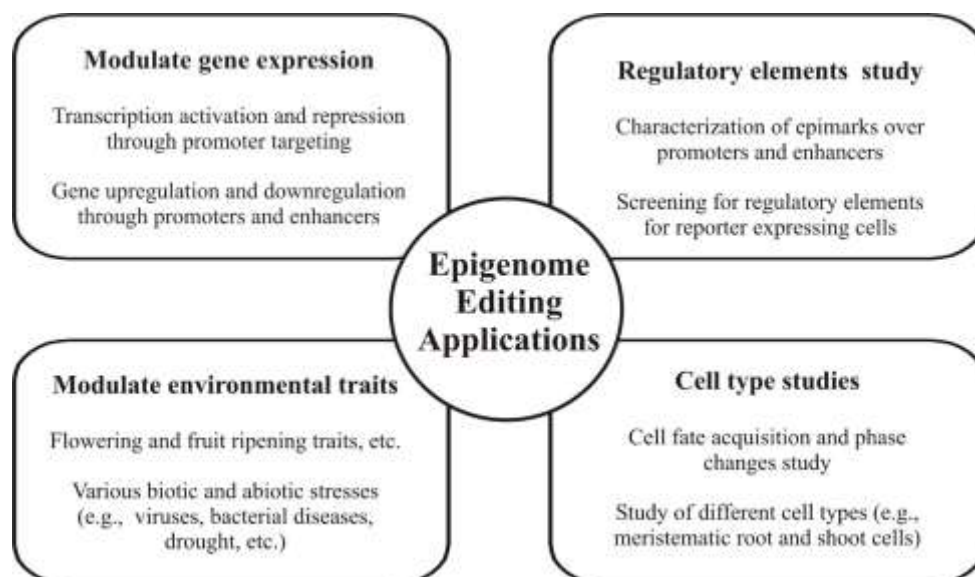


Figure 8. Applications of epigenome editing. (Miglani, G.S. and R. Singh. 2019. Epigenome editing in crop improvement. Chapter 15. In: *Quantitative Genetics, Genomics and Plant Breeding*. Second edition. Ed. Kang, M.S. CAB International. Oxford, U.K.)

1. Studying enhancer function and activity

Editing of gene enhancer regions in the genome through targeted epigenetic modification has been demonstrated by Mendenhall *et al.* (2013). This study utilized a TALE-LSD1 effector fusion protein in order to target enhancers of genes, to induce enhancer silencing in order to deduce enhancer activity and gene control. Targeting specific enhancers followed by locus specific RT-qPCR allows for the genes affected by the silenced enhancer to be determined. Alternatively, inducing enhancer silencing in regions upstream of genes allows for gene expression to be altered. RT-qPCR can then be utilized to study effects of this on gene expression. This allows for enhancer function and activity to be studied in detail

2. Determining the function of specific methylation sites

It is important to understand the role specific methylation sites play regulating in gene expression. To study this, Maeder *et al.* (2013) used a TALE-TET1 fusion protein to demethylate a single CpG methylation site. Although this approach requires many controls to ensure specific binding to target loci, a properly performed study using this approach can determine the biological function of a specific CpG methylation site.

3. Determining the role of epigenetic modifications directly

Epigenetic editing using an inducible mechanism offers a wide array of potential use to study epigenetic effects in various states. Konermann *et al.* (2013) employed an optogenetic two-hybrid system which integrated the sequence specific TALE DNA-binding domain with a light-sensitive cryptochrome 2 protein (CIB1). Once expressed in the cells, the system was able to inducibly edit histone modifications and determine their function in a specific context.

Besides editing genome sequence, genome editing systems have been used in altering the transcription of specific gene(s) without altering the underlying sequence. This modification for transcriptional alterations involves exclusion or inactivation of nuclease activity followed by use of the targeting system fused to a modular effector domain and is known as epigenome editing.

It should be noted that the effect of epigenetic marks is context dependent and thus the context of this table is the average consequence of depositing these marks in a promoter or enhancer. These systems have been used to target integrated/synthetic as well as endogenous loci, a distinction that is reviewed by Kescu (2014). The function of ATF was the primary purpose for DNA sequence specificity of designer ZFs and TALEs with the goal of precise transcriptional activation, also known as transactivation. Transactivation effector domains are based on viral elements. The original VP16 domain comes from Herpes Simplex Viral Protein 16 and consists of amino acids 437–447 (DALDDFDLDMML). VP16 was later engineered into VP64 domain, which is a fusion containing four tandem copies of VP16 connected by glycine-serine linkers (DALDDFDLDMML)-GS-(DALDDFDLDMML)-GS-(DALDDFDLDMML)-GS-(DALDDFDLDMML)

It is the most widely used transactivation domain. Interestingly, one effect of using the VP64 transactivation domain is that it recruits p300, which causes activating H3K27Ac to increase at the targeted locus over time and represents an example of transcription driving transcription. Transcriptional repression, on the other hand, utilizes repression domains and is typically achieved by variants of a 45-aa segment from Krüppel-associated boxes (KRAB) or repressive epigenomic modifications (Kuscu *et al.* 2014). The KRAB repressor domain appears to be the most potent natural repressor in the genome and used by half of zinc fingers, which make up half of the genome's transcription factors. Interestingly, the KRAB repressor domain recruits histone modifying domains and results in a decrease of activating H3K4me3 and increase of repressive H3K9me3 and H3K27me2 but does not alter DNA methylation (Lister *et al.* 2013). However, these modifications may not reflect the immediate effect of transcriptional repression and could be a later consequence. A bacterial DNA methyltransferase (M.SssI) is also capable of repression and recruiting a heterochromatin protein, H3K9me3, and H3K27me2. The following are examples of how some of these effector domains have been used with DNA binding platforms to modulate gene expression. Another approach involves further enhancing the sgRNA to create a system known as CRISPR-Display (Mali *et al.* 2013). CRISPR-Display allows for functional RNA domains (~4.8 kb) to be inserted into the sgRNA loops at multiple positions, including the same loop as sgRNA 2.0 as well as 5' and 3' positions. This approach uses functional motifs like the protein-binding cassettes of earlier sgRNA 2.0 approaches but also enables long non-coding RNA to be inserted into the dCas9/sgRNA complex. Ultimately, the CRISPR-Display system enables precision ectopic targeting of RNA and ribonucleoprotein to loci of interest in order to fully characterize the functionality of the RNA (Shen *et al.* 2014).

Combinatorial Biotechnology

(Epi)genome editing systems allow not only for fusing a genome editing system to an effector but they can also add additional genetic changes and facilitating methodologies. For example, Konermann *et al.* (2015) used transcriptional control or histone modifying (acetylation and methylation) effector domains along with a light inducible (optogenetic) element.

The optogenetic induction system involves light-sensitive cryptochrome 2 (CRY2) and CIB1, its binding partner. These two proteins only heterodimerize upon exposure to blue light, a process that is rapid and reversible, and can be applied to study neurons in mammalian brains. They can then be separately fused to an epigenome editing system, with one attached to the targeting system and the other to the effector. This can be utilized in epigenome editing by allowing for the editing to be induced when and where it is desired in a rapid and reversible manner. The optogenetic approach has also been combined with CRISPR/Cas9 transactivation systems. Alternate inducible transactivation systems use steroids that has been created using TALEs. Finally, cell imaging can also be achieved using an EGFP effector domain that can visualize pericentric, centric, and telomeric repeats. This allows for the visualization of repetitive

sequences using a single sgRNA or an array of sgRNAs for non-repetitive sequences to enable visualization and tracking through cellular processes.

This technique was demonstrated by imaging telomere dynamics and the dynamic sub-nuclear localization of a single gene through mitosis. Another visualization system has been developed that allows for multicolor analysis. It was initially successful in TALEs and was recently adopted for dCas9 orthologs with three spectral systems. The spectral systems were used to target telomeres, several target loci, and also determine the intranuclear distance between loci on different chromosomes, which allowed for the assessment of DNA compaction in live cells. Furthermore, epigenome editing systems have been used for enrichment and purification of proteins interacting with target loci. They have the potential to allow for an examination of all the proteins and histone PTMs associated with a single genomic locus, the epiproteome (Zhang *et al.* 2012). These techniques couple chromatin immunoprecipitation and the target specificity of genome editing systems (without the catalytic activity) by using an effector domain to allow for enrichment that can then be coupled to analysis by mass spectrometry. One variant of this approach is known as engineered DNA-binding molecule-mediated chromatin immunoprecipitation (enChIP). It has been developed using TALEs (Cong *et al.* 2012) and CRISPR/Cas9 to study telomeres.

Epigenome editing is a auspicious approach for durable gene regulation, with many applications in basic research including the investigation of the regulatory functions and logic of chromatin modifications, stimulating tissue regeneration, compensating for genetic defects, activating silenced tumor suppressors, controlling stem cell differentiation, performing genetic screens, and creating synthetic gene circuits and cellular reprogramming. From a clinical point of view, targeted regulation of disease-related genes offers novel therapeutic avenues for many diseases (Konermann *et al.* 2015). However, progress made in this field opens questions in epigenetic regulation and its stability, methods to increase the specificity of epigenome editing, and improved delivery methods for targeted EpiEffectors. Future work will reveal if the approach of epigenome editing fulfills its great promise in basic research and clinical applications (Ran *et al.* 2013).

CASE STUDY

Improved drought stress tolerance in *Arabidopsis* by CRISPR/dCas9 fusion with a histone acetyltransferase

The awareness of climate change and global warming emphasizes the need to implement some efficient and sustainable solutions. In several countries, a current important issue is the maintenance of crop production during drought. Drought varies spatially and temporally as well as in strength. Accordingly, plants have diversified their responses and have evolved to exhibit multiple morphological and physiological behaviours. These behaviours consist of different degrees of drought escape, avoidance and tolerance. Exploiting genetic traits that enhance the drought stress response while

maintaining high yields remains of critical interest for crop management (Paixão et al. 2019).

Comprehensive molecular analyses have deciphered the cellular pathways that orchestrate the drought stress response. Abscissic acid (ABA) is a phytohormone that plays a role as a key regulator of the drought stress response in plants by regulating gene expression and controlling stomatal closure to prevent water losses by transpiration. The basic leucine zipper (bZIP) transcription factors, termed ABA-responsive element binding proteins/ABRE binding factors (AREB/ABFs), are important determinants in ABA signalling. Over-expression of *AREB1* (also named *ABF2*) exhibited enhanced drought stress tolerance in *Arabidopsis*, rice and soybean, while *AREB1* loss of function causes drought stress sensitivity. Indeed, *AREB1* regulates a large set of genes downstream of the ABA signalling pathway in response to drought stress and participates in osmotic stress protection, ABA biosynthesis and antioxidant signalling. Thus, *AREB1* represents an attractive candidate gene for improving the drought stress response.

Histone acetyltransferase (HAT) catalyzes the acetylation of core histones through the addition of an acetyl group to the lysine residue on the terminal tail of the histones. Histone acetylation triggers DNA relaxation and leads to exposure of DNA to the transcriptional machinery. Thus, HAT activity is correlated with gene expression activation (Figure 9). The use of dCas9 in fusion with HAT (dCas9-HAT), combined with the directed targeting of sgRNAs, appears promising for positively regulating the activity of a targeted promoter. dCas9 epigenome editing of specific drought stress response genes therefore emerges as an encouraging strategy for improving stress tolerance in plants (Paixão et al. 2019).

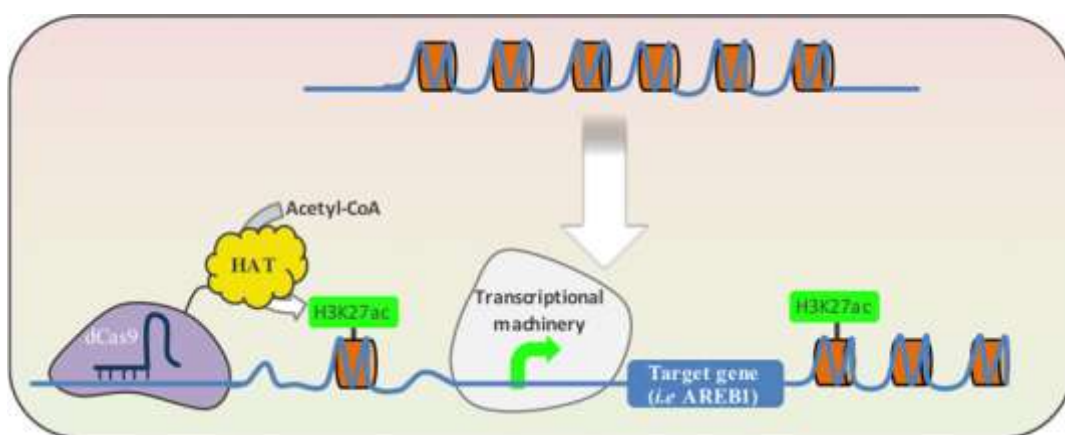


Figure 9. Schematic illustration of a model for dCas9^{HAT} function in transcriptional activation of a target gene. Upper: the histone compaction (in orange) induces DNA condensation and limits transcription. Below: the dCas9HAT in complex with a single guide RNA (in dark blue) binds DNA on a target locus. The histone acetyltransferase (HAT) triggers histone acetylation on the lysine 27 (H3K27ac) and induces local DNA relaxation. The DNA relaxation strengthens the interaction of the transcriptional machinery and /or transcriptional enhancers with the target locus. (Paixão et al. 2019)

EPIGENOMICS IN CROP IMPROVEMENT IN ERA OF CLIMATE CHANGE

Abiotic stress drastically limits agricultural crop productivity worldwide. Climate change threatens the sustainable agriculture with its rapid and unpredictable effects, making it difficult for agriculturists and farmers to respond to the challenges cropping up from environmental stresses. This challenge comes at a time when plant sciences are witnessing remarkable progress in understanding the fundamental processes of plant growth and development. Plant researchers have identified genes controlling different aspects of plant growth and development, but many challenges still exist in creating an apt infrastructure, access to bioinformatics and good crop results (Ahmad et al. 2014).

There is considerable excitement about the potential for epigenetic information to contribute to heritable variation in many species. Our understanding of the molecular mechanisms of epigenetic inheritance is rapidly growing, and it is now possible to profile the epigenome at high resolution. Epigenetic information plays a role in developmental gene regulation, response to the environment, and in natural variation of gene expression levels. Because of these central roles, there is the potential for epigenetics to play a role in crop improvement strategies including the selection for favorable epigenetic states, creation of novel epialleles, and regulation of transgene expression (Springer 2013).

Impacts of climate change

The challenges of abiotic stress on plant growth and development are evident among the emerging ecological impacts of climate change. Climate change is predicted to affect agricultural production the most, primarily at low latitudes populated by developing countries, with adverse effects of increasing carbon dioxide and high temperature, challenging researchers toward devising adaptation strategies (Pereira 2016). Plants are rooted in the environment they grow in, and have to adapt to the changing conditions brought about by the multitude of environmental factors, with extreme levels eliciting abiotic stress.

Climate change and its physiological impacts on productivity

Climate change is characterized by higher temperatures, elevated atmospheric CO₂ concentrations, extreme climatic hazards, and less water availability for agriculture (Debaekeet *al.* 2017). Physiological impacts of climate change on crop productivity include CO₂ fertilization effect, drought effects, high temperature, interactions between temperature, water and CO₂, and multiple stress approach.

Climate change-linked stress tolerance in plants

Current and anticipated impacts of climate change and atmospheric gases on water availability and weather extremes affect the production of crops (<https://www.volkswagenstiftung.de/en/events/calendar-of-events/other-events/climate-change-linked-stress-tolerance-in-plants-keystone-symposia>). Innovative research on crops, related wild species and model plant species is enabling the identification of stress signaling, metabolic and developmental strategies that limit yield loss in zones with increased climate stress and variability. The use of functional genomics and epigenomics to dissect abiotic stress sensing and signaling networks and the downstream adjustments in metabolism and development continue to provide solutions

for crop improvement. These advances are accelerated by tools such as genome editing, epigenome editing and use of synthetic biology to fine-tune key pathways.

Adaptation of Plant to Climate Change

Biologists are studying whether genes could be switched on and off in plants depending on climatic conditions. What if plants could switch their genes on and off, depending on which traits were important in a certain climate? That's what a team of researchers at the Salk Institute for Biological Studies investigated in a series of studies on *Arabidopsis thaliana*, a common mustard weed (Stecker2013).

The potential of switching genes on and off falls under the relatively new field of epigenetics (the prefix "epi" means "on top of" in Greek). It is the study of a pattern of chemical markers that regulate genes that sit over the DNA sequence. Genetic mutations are caused by changes in the pattern of nucleotide bases – the building blocks of DNA. Epigenomic modifications do not affect these building blocks and therefore don't leave a permanent change in DNA. Epigenetic modifications are, in theory, reversible. This means plants could temporarily activate or deactivate genes. Epigenetic modifications might be reversed in response to stress or a change in the environment.

The sum total of the biochemical modifications in nuclear DNA, post-translational modifications in histone proteins and variations in the biogenesis of non-coding RNAs in a cell is known as an epigenome. These changes are often responsible for variation in the expression of the gene without any change in the underlying nucleotide sequence. The changes might also cause variation in chromatin structure resulting in the changes in function/activity of the genome. The epigenomic changes are dynamic with respect to the endogenous and/or environmental stimuli, which affect phenotypic plasticity of the organism. Both the epigenetic changes and variation in gene expression might return to the pre-stress state soon after the withdrawal of the stress (Kumar 2018). A part of the epigenetic changes may be retained, which may play a role in acclimatization and adaptation as well as in the evolutionary process. Probable exploitation of epigenome-engineering for improved stress tolerance in plants has become essential for better utilization of the genetic resources.

Epigenetic regulation in the adaptation and stress responses

Enormous progress has been made in understanding the role of epigenetic regulation response to environmental stimuli, especially in response to stresses. We now understand how new stable phenotypes can be generated quickly in response to environmental modifications. In some cases, it has also been shown that epigenetic modifications can be stably transmitted to the next generations. The vast majority of studies have been carried out with model plants, particularly with *Arabidopsis*, and very little is known on how native plants in their natural habitat react to changes in their environment. Climate change has been affecting, sometimes drastically, the conditions of numerous ecosystems around the world, forcing populations of native species to adapt quickly. A part of the adaptation can be explained by the preexisting genetic variation in the populations, recent studies have shown that new stable phenotypes can be generated through epigenetic modifications in few generations, contributing to the stability and survival of the plants in their natural habitat. Thus

epigenetic variation can help natural populations to cope to with change in their environments (Thiebaut et al. 2019).

Epigenetic marks are of three types: DNA methylation, histone modifications and small RNAs. In many cases small RNAs can trigger DNA methylation and chromatin modification using RNA-directed DNA Methylation (RdDM) pathway. DNA methylation is also occurring in gene-coding regions affecting gene expression. In *Arabidopsis*, one-third of methylated genes occur in transcribed regions, and 5% of genes showed methylation in promoter regions, suggesting that many of these are epigenetically regulated by DNA methylation. Stress can also result in changes in DNA methylation. DNA methylation content can also be regulated in response to abiotic stress. Experiments in maize and *Arabidopsis* showed that cold stress might induce modification of the DNA methylation status. Vernalization treatments result in reduction of levels of DNA methylation and induced the initiation of flowering. Some stress-induced modifications are reversed to the basal level; however, some of these modifications may be stable and heritable, being named the epigenetic “stress memory”. The knowledge of these stress memories can increase our understanding the processes of plant adaptation to stresses (Thiebaut et al. 2019).

An important question is: what is the contribution of epigenetic modification to phenotypic variation in native plants in their environment? Recent data have suggested that epigenetic variation can contribute to natural populations to cope to with changes in their environments (Figure 10).

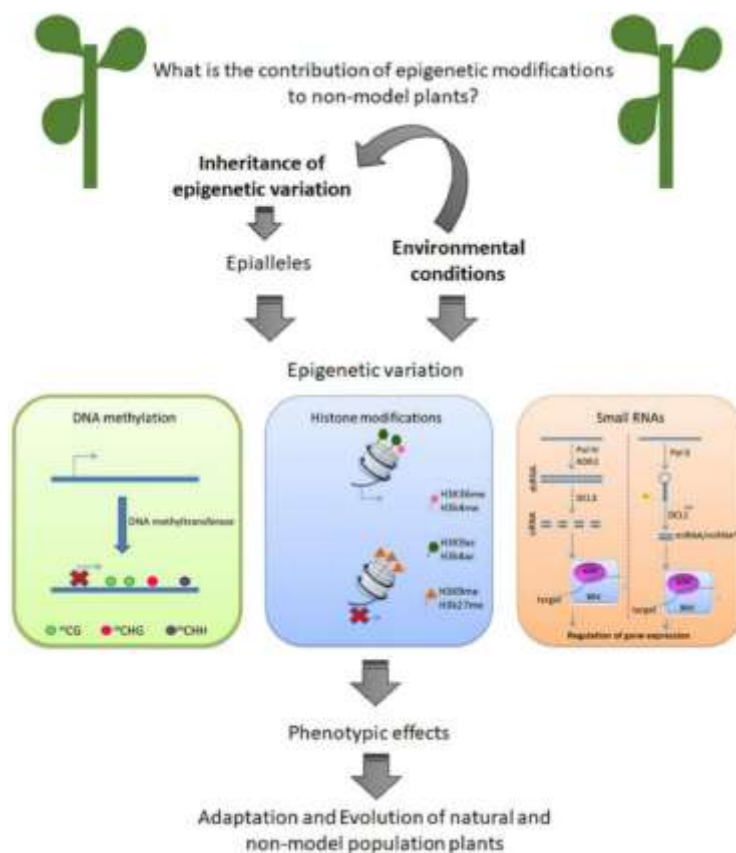


Figure 10. Epigenetic variation can contribute to adaptation and evolution of non-model plants. (Thiebaut et al. 2019)

Inheritance of epigenetic variation

Transgenerational epigenetic inheritance requires that epigenetic marks can be transmitted to the progeny. Epigenetic marks might be transmitted through mitosis and sometimes also meiosis. The variation in methylation of the same gene between different plants is denominated epialleles. Epialleles differ in the number or distribution of methylated nucleotides at specific gene sequences. Different epialleles can result in different phenotypes which are heritable in a new generation. Some epigenetic marks may result in heritable phenotypic variation whereas others do not. DNA methylation is an epigenetic mark easier to pass through generations. In plants some germline cells are descended from somatic cells and they carry epigenetic marks, which can contribute with the heritability of epigenetic marks. The majority of studies on epigenetic inheritance focus on DNA methylation. Heritable epigenetic marks may result in heritable phenotypic variation, influencing fitness, and so be subject to natural selection (Thiebaut et al. 2019).

Plants can sense the environmental conditions during vegetative growth and this could result in epigenetic modifications in a cell lineage that can generate a germline. Studies using the model plant *Arabidopsis* have showed that stress-induced transgenerational responses depend on changes in DNA methylation. Based on this observation, it is possible that phenotypic effects caused by epialleles are inherited across generations and influenced by environmental conditions also in native plants. Therefore, heritable epialleles will influence plant evolution through their effects on both phenotypic trait distributions and fitness (Thiebaut et al. 2019). In addition, many plants are propagated asexually through clonal reproduction, where meiotic epigenetic reset does not occur. The epigenetic information among clonal generations is more effective than in sexual reproduction.

Cytosine methylation in the improvement of *P. pinea* fitness under different environmental conditions

A study addressing phenotypic variation of native *Pinus pinea* plants showed a remarkable degree of phenotypic plasticity, despite having low levels of genetic variation. However, analysis of different vegetatively propagated trees showed a high degree of DNA methylation, suggesting the role of cytosine methylation in the improvement of *P. pinea* fitness under different environmental conditions. A study with the oil palm *Elaeisguineensis* revealed the impact of DNA methylation in an important characteristic of the fruit. Approximately, 75% of hypomethylated loci were transposons and repeats, while less frequent hypermethylated loci included genic sequences regions. Methylation near the Karma transposon predicts normal fruit and hypomethylation predicts homeotic transformation, parthenocarpy and marked loss of yield.

Adaptation Through Epigenetics Might Happen Much Faster

Genetic adaptation occurs through evolution, which can take hundreds of years. Adaptation through epigenetics might happen much faster. A *A. thaliana* plant that can reproduce a new generation every eight weeks, what looked like epigenetic modifications after 30 generations, or about five years.

Creating Genetic Diversity In The Lab

Epigenetics could also help promote diversity in species that have been steadily losing it. In the domestication process for agriculture, humans domesticated a rather small portion of wild plants to bring out certain traits, like high-yielding plants or tasty fruit. This was a genetic bottleneck and led to limited diversity. Breeding techniques over the last several centuries have led to the loss of genes that could be helpful to plant geneticists looking for drought-tolerant, heat-withstanding varieties that could be valuable for agricultural climate change adaptation.

Epigenetic Diversity

Epigenetic research could save scientists from going into the wild to collect seeds to reclaim lost genetic material. Scientists are creating epigenetic variation to replace genetic diversity in the lab. Using epimutations, the range of phenotypes found can be great. Phenotype is the physical manifestation of a gene. There is potential for creating new diversity, epigenetic diversity.

Epigenomic Studies in relation to Stress Tolerance in Plants in the Era of Climate Change

Molecular mechanisms of stress memory in plants remain to be investigated (Kumar 2018). Insights into the molecular conservation of stress memory in crop species are scarce. Understanding the mechanisms of stress priming/memory might enable manipulation for tailored responses of crop plants to respond more efficiently to the challenges presented by the climate change. Thus, there is great potential for generation of environment-mediated heritable epigenetic variations, which actually drive/influence the evolution process in living organisms. Epigenome editing can be used to decipher the role of epimarks. This would allow functional interrogation of epigenetic marks and their usage towards stable improvement in the agriculturally important traits. Various biochemical, physiological, genetic and epigenetic mechanisms associated with defense responses of plant under abiotic (e.g., salt) stress are: (1) Stress sensing; (2) Signal transduction through various inducers (e.g., reactive oxygen species, nitric oxide etc.); (3) Induction of transcription factor genes; (4) Expression of stress-responsive genes; (5) Activation/repression of epigenetic (DNA methylation/demethylation, histone modifications and non-coding RNA (ncRNA) biogenesis) factors involved in the regulation of stress-associated gene expression; (6) Transcriptional and translational reprogramming to combat the stress; (7) biochemical and (8) cellular responses leading to the (9) enhanced stress tolerance. ABA: abscisic acid; CAT: catalase; GR: glutathione reductase; H₂O₂: hydrogen peroxide; IAA: indol acetic acid; mRNA: messenger RNA; NAC-TF: NAC transcription factor; NO: nitric oxide; POX: peroxidase; ROS: reactive oxygen species; SOD: superoxide dismutase; SOS: salt overly sensitive; SSRG: salt stress responsive gene (Figure 11).

Challenges

The research on understanding role of epigenetics for crop improvement is still preliminary. The time period for many experiments hasn't been long enough to observe the stability of epigenetic modifications. The study of epigenetics has only taken hold

in the last decade and skeptics of the science do exist. It has yet to be proven as a useful approach to plant breeding.

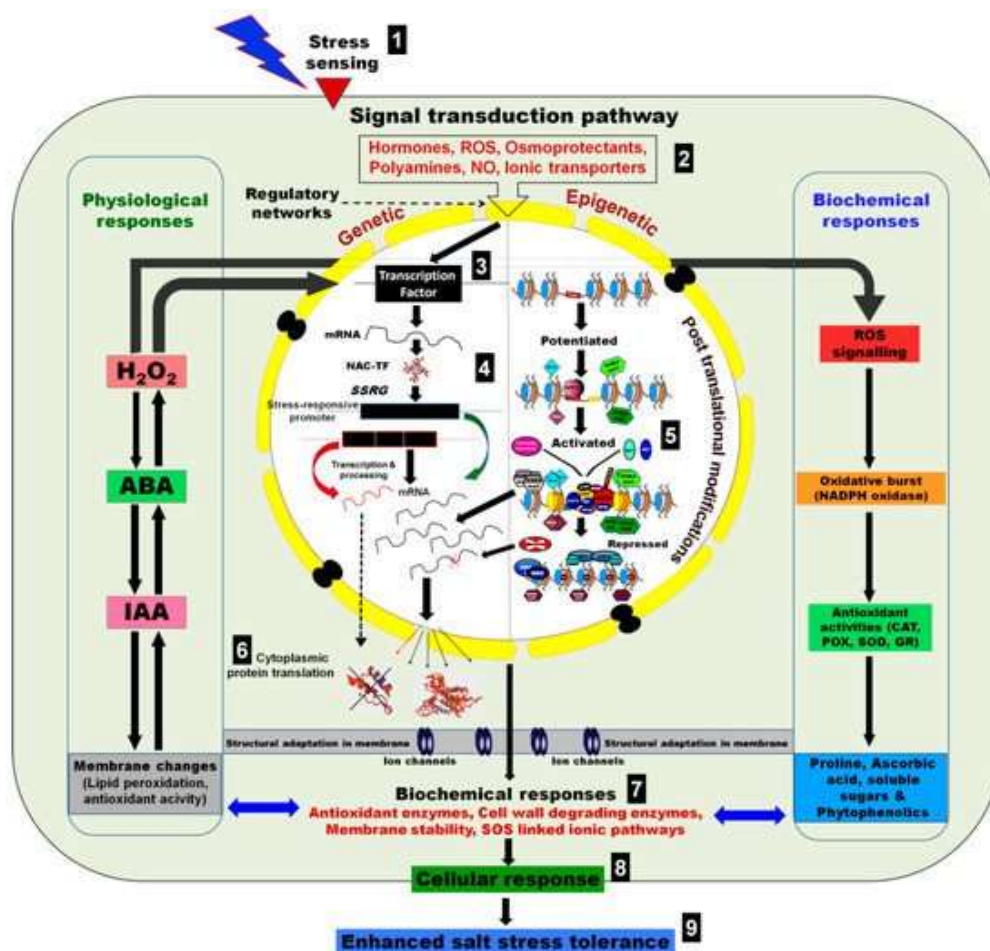


Figure 11. Various biochemical, physiological, genetic and epigenetic mechanisms associated with defense responses of plant under abiotic (e.g., salt) stress. (Kumar 2018)

The challenges before us in plant biology and crop improvement are to integrate the systems level information on abiotic stress response pathways, identify stress protective networks, and engineer environmentally stable crops that yield more, with less water and dwindling natural resources, to feed the growing world population (Pereira 2016).

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FUNGAL DISEASE CONTROL IN CROP PLANTS THROUGH TRANSGENESIS

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Introduction

Plants produce antifungal proteins in response to invasion by fungal pathogens. These proteins were first noticed in tobacco leaves showing hypersensitive reaction to tobacco mosaic virus and were therefore designated as pathogenesis-related proteins. In general, these proteins hinder pathogens growth, differentiation and multiplication, despite this significant yield losses occur in crop plants (Agrios 2005). Some fungal diseases have been effectively controlled by application of chemical fungicides but their effects are often non-specific i.e. beneficial organisms as well as pathogens are targeted together with adverse health, safety and environmental risks. The incorporation of genetic resistance in plants has been successfully achieved using plant breeding methods leading to transfer of genes through crosses between resistant and susceptible parents, followed by screening and selection under disease conducive conditions (Rommens and Kishore 2000).

The modern day agriculturally important crop cultivars are bred to have in-built genetic resistance against a number of diseases; however, evolution of virulent phytopathogens may overcome the success of resistant cultivars (Cornelissen and Schram 2000). The plant breeding methodologies are laborious, applicable only within sexually compatible species and also lead to transfer of undesirable traits together with the resistance genes. The most important developments for fungal disease resistance in last two decades have been through the use of molecular biology and genetic engineering techniques that have led to i) cloning and characterization of several resistance genes whose products are involved in recognizing invading pathogens; ii) dissection of a number of signalling pathways that trigger in response to pathogen infection ; iii) identification of antifungal compounds synthesized by plants to combat fungal infections; iv) development of genetic transformation approaches enabling integration of genes from any species to any plant genome leading to engineering of transgenic plants with enhanced resistance.

The most commonly used approach for conferring genetic resistance against a broad range of plant pathogenic fungi is through constitutive over-expression of genes encoding hydrolytic enzymes leading to improved resistance in transgenic crop plants against many fungal diseases (Punja 2006). The type and source of the transgene are critical factors in determining the levels of disease resistance e.g. genes encoding chitinase from plants provide insufficient resistance (Mauch et al. 1998) as compared to the expression of fungal chitinases, both in level and range of disease resistance to fungal pathogens (Awady-El et al. 2007). This is mainly attributed to the optimization

of lytic enzymes from microorganisms to break down fungal cell walls (Park et al. 1996).

Fungal cell wall

The fungal cell wall primarily comprises of polysaccharides (80-90%), proteins (3-20%), lipids i.e. in lesser amounts (Latgé 2007), and the composition of fungal wall often varies distinctly between the species e.g. in higher fungi such as Ascomycetes, Basidiomycetes, Deuteromycetes the key macromolecular components of the cell walls are chitin, β -glucan and mannoproteins (glycoproteins), whereas chitin is preponderated by cellulose in the lower fungi e.g. Myxomycetes, Phycomycetes. In almost all fungi, the central core of cell wall is a branched β -1-3, β -1-6 glucan which is linked to chitin by a β -1,4 linkage. The interchain β -1-6 glucosidic linkages are three and four per cent of the total glucan linkages, in *Saccharomyces cerevisiae* and *Ascomycetes fumigates*, respectively (Perez and Ribas 2004). The chitin (chitosan in some fungi) and β -glucan microfibrils form the scaffolding that provides shape and strength to the cell wall (Ruiz-Herrera 1992). The chitin biopolymer is composed of β -1-4 linked N-acetylglucosamine (GlcNAc) units having a three-dimensional β -helical configuration stabilized by intramolecular hydrogen bonding (Kas 1997). Chitin does not occur in plants, vertebrates or prokaryotes (Havukkala 1991), whereas in many plant pathogenic fungi it comprises of 22-44 per cent cell wall material of fungi and is essential for maintaining the structural integrity of hyphae (Peberdy 1990). The glucans, primarily β -1-3, β -1-6, α -1-3, β -1-4 within the fungal cell wall have a key role as a structural polymer for maintaining rigidity and conferring protection (Wessels and Siestma 1981).

Over-expression of hydrolytic enzymes in plants through transgenesis

i) Through plant genes

The improved tolerance of transgenic plants towards fungal pathogens through the expression of heterologous genes is summarized in Table 1. Transgenic tobacco plants engineered using bean chitinase gene under the control of CaMV 35S promoter induced 20-40 fold higher chitinase activity coupled with improved resistance to pathogenic fungi *Rhizoctonia solani* leading to delayed disease development (Broglie et al. 1991). The over-expression of rice chitinase gene in grapevine, rice and peanut led to increased resistance against *Uncinula necator* (Yamamoto et al. 2000), *R. solani* (Datta et al. 2001) and *Cercospora arachidicola* (Rohini and Rao 2001), respectively. Grey mold resistant transgenic chrysanthemum was developed by inserting a chitinase gene (*RCC2*) from rice (Takatsu et al. 1999) and transgenic grapevine, cucumber, trifoliate orange expressing the same gene showed improved resistance against *U. necator*, *Botrytis cinerea* and fungal pathogens, respectively (Tabei et al. 1998, 1999; Yamamoto et al. 2000; Kishimoto et al. 2002; Mitani et al. 2006). Leaf spot resistant groundnut was developed by inserting a tobacco chitinase gene (*Chi-V*) (Rohini and Rao 2001). The sheath blight resistant rice was produced by incorporating rice chitinase gene *RC7* (Datta et al. 2001) and *chi11* (Kumar et al. 2003). Takakura et al. (2000)

developed fungal resistant rice by incorporating *OsChia* chitinase gene isolated from rice pistils. Transgenic soybean having resistance to sheath blight was produced by expressing the bean chitinase (*chi*) gene (Li et al. 2004). The same gene was used to develop cotton having resistance to *Verticillium dahliae* (Tohidfar et al. 2005). The introduction of rice chitinase gene (*chi11*) in barley led to development of fungal-resistant barley (Tobias et al. 2007). *Sclerotium rolfsii* resistant taro was developed by the expression of *ricchi11* rice chitinase gene (He et al. 2008, 2010). Leaf blast resistant finger millet was developed by incorporating *chi11* rice chitinase gene (Ignacimuthu and Ceasar 2012). In addition, inhibition of fungal disease symptoms by over-expressing chitinase genes from plant sources has been demonstrated in several transgenic crops e.g. canola (Terakawa et al., 1997), rice (Nishizawa et al. 1999; Baisakh et al. 2001; Kim et al. 2003; Sridevi et al. 2003; Maneewan et al. 2005; Asghar et al. 2007; Sheidai et al. 2009), chrysanthemum (Takatsu et al. 1999; Sen et al. 2011), wheat (Lifang et al. 2001; Shin et al. 2008), groundnut (Sharma et al. 2006; Iqbal et al. 2012; Prasad et al. 2012), grapevine (Nirala et al. 2010), strawberry (Chalavi et al. 2003), pigeon pea (Kumar et al. 2004), cotton (Ganesan et al. 2009; Tohidfar et al. 2009, 2012), potato (Hussain 2007; Ahmad et al. 2012), tomato (Abbas et al. 2009; Chen et al. 2009; Jabeen et al. 2009), safflower (Kumar et al. 2009), banana (Sreeramanan et al. 2009), sorghum (Aruselvi et al. 2010), litchi (Das and Rahman 2012), *Rosa damascence* (Pourhosseini et al. 2012), apple (Sharma et al. 2012).

ii) Through non-plant genes

The over-expression of antifungal genes in transgenic plants has revealed enhanced resistance to fungal diseases in several studies (Table 1). The best known example of improved resistance is transgenic tobacco and potato plants carrying *chit42* gene from *T. harzianum* that were either highly tolerant or completely resistant to *Alternaria alternata*, *A. solani*, *B. cinerea*, *R. solani* with no visible effect on plant growth and development (Lorito et al. 1998). The endochitinase encoding gene from *T. harzianum* led to development of *B. cinerea* resistant grapevine (Kikkert et al. 2000). The *ech42* gene of *T. harzianum*, encoding a powerful endochitinase was constitutively expressed in apple and the transgenic plants showed a high level of resistance to *Venturia inaequalis*, however reduced plant growth was observed (Bolar et al. 2000). The *ThEn42/chit42* gene provided increased resistance to broccoli from *A. brassicicola* attack (Mora and Earle, 2001). Increased resistance towards Fusarium infection was demonstrated in barley expressing *T. atroviride* endochitinase *ech42* (McIntyre et al. 2004). Likewise, resistance against *Colletotrichum* in strawberry was reported through heterologous expression of *chit42* and in melon and tomato plants containing *chit42* and a β -1,6 glucanase transgenes. Thus, protection of plants appears to result exclusively from the increased antifungal enzymatic activities. Enhanced resistance in transgenic rice was demonstrated against *R. solani* by expressing *chit42* gene from *T. virens* (Shah et al. 2009). Significant resistance to *A. alternata* and *R. solani* was reported in transgenic cotton plants expressing the endochitinase gene *Tv-ech1* from *T. virens* (Emani et al. 2003). Transgenic tobacco having resistance to *R. solani* was

developed by introduction of *chit33* and *chit42* genes from *T. harzianum* (De las Mercedes et al. 2006). Transgenic carrot expressing the endochitinase *chit36* gene from *T. harzianum* drastically enhanced tolerance to *A. radicina* and *B. cinerea* (Baranski et al. 2008). Transgenic lemon having resistance to *Phoma tracheiphila* and *B. cinerea* was developed by introducing *chit42* gene from *T. harzianum* (Gentile et al. 2007; Distefano et al. 2008). The *Th-chit* gene from *T. harzianum* induced resistance against *A. alternata* in transgenic tobacco (Saiprasad et al. 2008). The *T. atroviride chit42* gene was used to develop transgenic potato having resistance to *R. solani* (Esfahani et al. 2010). Transgenic tomato and tobacco were developed by introducing *ech42* gene from *T. virens* for resistance against fungal diseases (Shah et al. 2010). The β -1,3-glucanase gene (*bgn13.1*) from *T. virens* was used to generate transgenic potato that provided resistance against *R. solani* (Esfahani et al. 2010) and same gene was used to develop *S. sclerotiorum* resistant rape (Kheiri et al. 2014).

A synergistic increase in antifungal activity leading to enhanced resistance against *B. cinerea*, *A. alternata*, *A. solani* and *R. solani* was reported in transgenic tobacco and potato plants carrying *ThEn-42* and *chit42* genes from *T. harzianum* (Lorito et al. 1998). Increased resistance to *V. inaequalis* in apple has been correlated with co-expression of endo-(*ech42*) and exo-(*nag70*) chitinases from *T. atroviride* (Bolar et al. 2000; Schafer et al. 2011). Likewise, the expression of multiple antifungal transgenes in rice encoding β -1,3-glucanase (*gluc78*) and two chitinases (*ech42* and *nag70*) of *T. atroviride* resulted in resistance to *R. solani* and *Magnaporthe grisea* (Mei et al. 2004). Dana et al. (2006) reported generation of intrinsic defense responses and improved salt stress tolerance in tobacco plants over-expressing the *chit33* and *chit42* chitinase genes from *T. harzianum*, suggesting that chitinases might act as plant fungi by releasing chitoooligosaccharides as inducers of defense responses.

Conclusions

The successful degradation of fungal cell wall may require harmonious action of more than one enzyme e.g. in fungal cell wall the chitin is protected by β -glucan and is not accessible to chitinases (Cherif and Benhamou 1990), thus the chitinase action might be synergistic with hydrolytic action of glucanases. Therefore, several processes such as the production of cell wall degrading enzymes, antifungal metabolites and genes related to signal transductions responding to multiple signals occur consecutively. Implying the foundation of effective biocontrol relies on knowledge about domains and targeting mechanisms of genes engaged in biocontrol. It is proposed, that through transgenesis the mycoparasitic ability can be enhanced by designing expression cassettes comprising of components such as i) cellulose-binding modules enabling tight binding of chitinases to insoluble chitin substrates (Limón et al. 2004) ii) chitosanases encoding domains responsible for hydrolyzing chitosan, a partially deacetylated form of chitin (Kubicek et al. 2011) iii) β -1,6-glucan-binding domains as β -1,6-glucanase has been identified in the area of interaction between *Trichoderma* spp. and pathogen (Montero et al. 2007). The overexpression cassettes can be functionally characterized by generating recombinant

Trichoderma strains and evaluating their performance through confrontation assays. These interventions will ensure improved constitutive gene expression of the encoding enzymes leading to biosynthesis of secondary metabolites and synergistic action of lytic enzymes for targeted cell wall lysis.

Table 1 Application of antifungal genes in generating fungal resistant transgenic plants

Gene	Host plant	Fungal pathogen/disease resistance/trait	Reference
Plant sources			
Rice chitinase (<i>RCC2</i>)	Cucumber	<i>Botrytis cinerea</i> /Gray mold	Tabei et al. (1998; 1999)
Rice chitinase (<i>Cht-2</i> , <i>Cht-3</i>)	Rice	<i>Magnaporthe grisea</i>	Nishizawa et al. (1999)
Rice chitinase (<i>RCC2</i>)	Chrysanthemum	<i>B. cinerea</i> /Gray mold	Takatsu et al. (1999)
Rice chitinase (<i>Chi11</i>)	Rice	<i>Rhizoctonia solani</i>	Datta et al. (2001)
Rice pistils chitinase (<i>OsChia</i>)	Rice	Fungal resistance	Takakura et al. (2000)
Rice chitinase (<i>RCC2</i>)	Grapevine	<i>Uncinula necator</i>	Yamamoto et al. (2000)
Rice chitinase (<i>chi1</i>)	Rice	Sheath blight	Baisakh et al. (2001)
Rice chitinase (<i>RC7</i>)	Rice	<i>R. solani</i>	Datta et al. (2001)
Rice chitinase (<i>RCH8</i>)	Wheat	Scab	Lifang et al. (2001)
Tobacco chitinase (<i>Chi-V</i>)	Groundnut	<i>C. arachidicola</i> & Leaf spot	Rohini and Rao (2001)
Rice chitinase	Groundnut	<i>R. solani</i>	Rohini and Rao (2001)
Rice chitinase (<i>RCC2</i>)	Cucumber	<i>B. cinerea</i> /Grey mold	Kishimoto et al. (2002)
Tomato chitinase	Strawberry	<i>Verticillium dahliae</i>	Chalavi et al. (2003)
Rice chitinase (<i>RCH10</i>)	Rice	Sheath blight	Kim et al. (2003)
Rice chitinase (<i>chi11</i>)	Rice	<i>R. solani</i>	Kumar et al. (2003)
Rice chitinase (<i>Chi11</i>)	Rice	<i>R. solani</i>	Sridevi et al. (2003)
Rice chitinase (<i>Rchit</i>)	Pigeonpea	<i>Fusarium oxysporum</i>	Kumar et al. (2004)
Bean chitinase (<i>Chi</i>)	Soyabean	Sheath blight	Li et al. (2004)
Rice chitinase (<i>Chi 11</i>)	Rice	Fungal disease	Maneewan et al. (2005)
Bean chitinase (<i>Chi</i>)	Cotton	<i>V. dahliae</i>	Tohidfar et al. (2005)

Gene	Host plant	Fungal pathogen/disease resistance/trait	Reference
Rice chitinase	Groundnut	<i>Aspergillus flavus</i>	Sharma et al. (2006)
Rice chitinase (<i>RCC2</i>)	Trifoliate orange	Fungal disease	Mitani et al. (2006)
Rice chitinase (<i>RCC2</i>)	Basmati rice	<i>Uncinula necator</i>	Asghar et al. (2007)
Rice chitinase (<i>RCG3</i>)	Potato	Fungal disease	Hussain (2007)
Rice chitinase (<i>Chi11</i>)	Barley	Fungal disease	Tobias et al. (2007)
Rice chitinase (<i>Chi 11</i>)	Tomato	<i>F. oxysporum</i>	Abbas et al. (2009)
Rice chitinase (<i>Chi11</i>)	Taro	<i>Sclerotium rolfsii</i>	He et al. (2008)
Barley chitinase	Wheat	<i>F. graminearum</i>	Shin et al. (2008)
Rice chitinase (<i>ChiII</i>)	Cotton	<i>F. oxysporum</i> & <i>Alternaria macrospora</i>	Ganesan et al. (2009)
Rice chitinase (<i>CHT-3</i>)	Tomato	Fungal disease	Jabeen et al. (2009)
Rice chitinase	Safflower	<i>Alternaria</i> /Leaf spot	Kumar et al. (2009)
Rice chitinase (<i>RICCH-1</i> , <i>RICCH-2</i> , <i>RICCH-3</i>)	Rice	Sheath blight	Sheidai et al. (2009)
Rice chitinase (<i>RCC2</i>)	Banana	Fungal disease	Sreeramanan et al. (2009)
Rice chitinase (<i>chi11</i>)	Cotton	<i>F. oxysporum</i> & <i>A. macrospora</i>	Tohidfar et al. (2009)
Rice chitinase	Sorghum	Fungal disease	Arulselvi et al. (2010)
Rice chitinase (<i>ricchi 11</i>)	Taro	<i>S. rolfsii</i>	He et al. (2010)
Rice class1 chitinase (<i>RCC2</i>)	Grapevine	<i>Uncinula necator</i>	Nirala et al. (2010)
Rice chitinase (<i>chiII</i>)	Chrysanthemum	Fungal disease	Sen et al. (2011)
Rice chitinase (<i>RCG3</i>)	Potato	Fungal disease	Ahmad et al. (2012)
Rice chitinase	Litchi	Leaf spot	Das and Rahman (2012)
Rice chitinase (<i>chi11</i>)	Finger millet	Leaf blast	Ignacimuthu and Ceasar (2012)
Rice chitinase-3	Groundnut	Leaf spot	Iqbal et al. (2012)
Rice chitinase	Rose	Powdery mildew	Pourhosseini et al. (2012)
Rice chitinase (<i>chi11</i>)	Apple	Fungal disease	Sharma et al. (2012)

Gene	Host plant	Fungal pathogen/disease resistance/trait	Reference
Rice chitinase (<i>Rchit</i>)	Groundnut	Foliar fungal pathogens	Prasad et al. (2012)
Bean endochitinase	Cotton	<i>V. dahlia</i>	Tohidfar et al. (2012)
<i>Rhizopus oligosporus</i> chitinase (<i>Chi1</i>)	Tobacco	<i>S. sclerotiorum</i> & <i>B. cinerea</i>	Terakawa et al. (1997)
Non-plant sources			
<i>Trichoderma harzianum</i> chitinase(<i>chit42</i>)	Tobacco, potato	Foliar & soil borne fungal pathogen	Lorito et al. (1998)
<i>T. harzianum</i> endochitinase	Grape	<i>B. cinerea</i>	Kikkert et al. (2000)
<i>T. harzianum</i> chitinase (<i>ech42</i>)	Apple	<i>Venturia inaequalis</i>	Bolar et al. (2000)
<i>T. harzianum</i> chitinase (<i>ThEn42</i>)	Broccoli	<i>A. brassicicola</i>	Mora and Earle (2001)
<i>T. virens</i> endochitinase (<i>Tv-ech1</i>)	Cotton	<i>R. solani</i> & <i>A. alternata</i>	Emani et al. (2003)
<i>T. atroviride</i> (<i>ech42</i>)	Barley	Fusarium infection	McIntyre et al. (2004)
<i>T. harzianum</i> chitinase	Alfaalfa	<i>Phoma medicaginis</i> & <i>C. trifolii</i>	Tesfaye et al. (2005)
<i>T. harzianum</i> chitinase	Lemon	<i>Phoma tracheiphila</i> & <i>B. cinerea</i>	Gentile et al. (2007)
<i>T. harzianum</i> chitinase (<i>chit36</i>)	Carrot	<i>A. radicina</i> & <i>B. cinerea</i>	Baranski et al. (2008)
<i>T. harzianum</i> chitinase (<i>chit42</i>)	Lemon	<i>B. cinerea</i>	Distefano et al. (2008)
<i>T. harzianum</i> chitinase (<i>Th-chit</i>)	Tobacco	<i>A. alternata</i>	Saiprasad et al. (2008)
<i>T. virens</i> endochitinase (<i>Tv-ech1</i>)	Cotton	<i>R. solani</i>	Kumar et al. (2009)
<i>T. virens</i> chitinase (<i>Chit42</i>)	Rice	<i>R. solani</i>	Shah et al. (2009)
<i>T. atroviride</i> chitinase (<i>chit42</i>)	Potato	<i>R. solani</i>	Esfahani et al. (2010)
<i>T. virens</i> chitinase (<i>ech42</i>)	Tomato & tobacco	Fungal disease	Shah et al. (2010)
<i>T. virens</i> TvGST	Tobacco	Cadmium tolerance	Dixit et al. (2011)
<i>T. harzianum</i> Thke11	<i>A. thaliana</i>	Salt & osmotic stress	Hermosa et al. (2011)

Gene	Host plant	Fungal pathogen/disease resistance/trait	Reference
<i>T. harzianum</i> chitinase	Sorghum	<i>Colletotrichum sublineolum</i>	Kosambo-Ayoo et al. (2011)
<i>T. viride ech42</i>	Tobacco	<i>Sclerotium rolfsii</i> & <i>R. bataticola</i>	Kumari et al. (2011)
<i>T. harzianum</i> chitinase	Wheat	<i>Erysiphe graminis</i>	Rana et al. (2012)
<i>T. virens</i> \square -1,3-glucanase (<i>bgn13.1</i>)	Potato	<i>R. solani</i>	Esfahani et al. (2010)
<i>T. virens</i> \square -1,3-glucanase (<i>bgn13.1</i>)	Rape	<i>S. sclerotiorum</i>	Kheiri et al. (2014)
<i>T. harzianum</i> (<i>ThEn-42</i> and <i>chit42</i>)	Potato & tobacco	<i>B. cinerea</i> <i>A. alternata</i> , <i>A. solani</i> , <i>R. solani</i>	Lorito et al. (1998)
<i>T. atroviride</i> (<i>ech42</i> , <i>nag70</i>)	Apple	<i>V. inaequalis</i>	Bolar et al. (2000)
<i>T. atroviride</i> chitinase (<i>ech42</i> , <i>nag70</i>), \square -1,3-glucanase (<i>gluc78</i>)	Rice	<i>M. grisea</i>	Mei et al. (2004)
<i>T. harzianum</i> chitinase (<i>chit33</i> , <i>chit42</i>)	Tobacco	<i>Pseudomonas syringae</i> & <i>R. solani</i>	Dana et al. (2006)
<i>T. harzianum</i> chitinase (<i>chit33</i> , <i>chit42</i>)	Tobacco	<i>R. solani</i>	De las Mercedes et al. (2006)
<i>Metarhizium anisopliae</i> <i>Chit1</i> /endochitinase <i>CHIT42</i>	Tobacco	<i>R. solani</i>	Kern et al. (2010)
<i>T. atroviride</i> chitinase (<i>ech 42</i> , <i>nag 70</i>)	Apple	Fungal pathogens	Schafer et al. (2011)

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GENE-FOR-GENE HYPOTHESIS AND GENETIC BASIS OF DISEASE RESISTANCE

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Plants are used by a number of other organisms including viruses, bacteria, fungi, insects etc., as primary source of food. When these organisms feed on crop plants, they lead to adverse effects on plant. Any abnormal condition in the plant produced by an organism is called a disease. Diseases reduce biomass and hence Yield. Diseases may lead to: killing of plants (e.g. vascular wilts, various soil borne fungi), general stunting caused by metabolic disruption, nutrient drain or root damage (e.g. many viruses), killing of branches (e.g. some fungal diebacks), damage to leaf tissues (e.g. many rusts, mildews, blights, leaf spots), damage to reproductive organs including fruits and seeds. Often the loss due to diseases may range from a few to 20 or 30 %, and in case of severe infection, the total crop may be lost. When spread over a large area, diseases may lead to famines. To reduce the extent of disease caused crop losses, farmers use chemical sprays which leads to increase in cost of cultivation and other environment and toxicity related problems. Breeding disease resistant varieties is the most economic and environment friendly way to prevent losses due to diseases and ensure stable production.

Biffen (1905) was the first to demonstrate that resistance to stripe rust (*Puccinia striiformis*) in wheat is a heritable trait when he showed that the resistance to stripe rust was governed by a single recessive gene. However, there was considerable confusion as even with genetically stable wheat lines, reaction to rust differed between locations and years. Confusion about variability could be cleared after discovery and characterization of defined pathogen races. Differential reaction of a variety with a resistance gene towards particular races of pathogen suggested there was genetic variability in pathogen which specifically interacted with monogenic resistances in plant.

Barrus (1911) observed three isolates of the bean anthracnose fungus (*Colletotrichum lindemuthianum*) had different pathogenic properties. Stakman and Piemeisel (1917) found variation among different isolates of black rust (*Puccinia graminis* f. sp. *tritici*) of wheat and proposed the well-known concept of physiological races. This concept has since been used to specify pathogenic variation. Ausemus et al (1946) described three dominant monogenic resistances against the wheat leaf rust and five dominant genes against stem rust.

The Gene-for-Gene System

In studies on genetics of disease resistance before 1940, the variation was either considered in the host or pathogen only. Flor (1942, 1946, 1947) was the first to study both the inheritance of pathogenicity in pathogen and the inheritance of reaction in the host. In studying the inheritance of resistance in flax (*Linum usitatissimum* L.) and

virulence in the flax rust fungus (*Melampsora lini*), Flor (1955 and 1956) found that there is a gene-for-gene correspondence between resistance and virulence. Flor made a large number of crosses of flax rust races. He then identified how pathogenicity on flax was inherited. He used all the flax rust progeny he obtained to inoculate a large number of available flax lines, and scored the reaction of the plant to the pathogen (immune, resistance, semi-resistant, susceptible). Flor also made a large number of crosses of the host, each of which was resistant to none, one, or several races of the flax rust pathogen. He crossed two races of flax, cross the resulting progeny with itself, and analyze the second generation. In the second generation, the recessive genes segregate. Flor inoculated all the second generation flax progeny to study the inheritance of resistance.

Flor found that both resistance in plants as well as avirulence in the pathogens were inherited. He also noted that even if the plant had a resistance gene (R), it was only resistant if the pathogen infecting the plant had an avirulence (Avr) gene. Flor also found that in almost all the cases he looked at, resistance was dominant over susceptibility and avirulence was dominant over virulence. Flor formulated what is now called the gene-for-gene hypothesis. For every gene in the plant that confers resistance, there is a corresponding gene in the pathogen that confers avirulence.

<u>Parasite</u>	<u>Host</u>					
	rr	R-				
A-	<table><tr><td>+</td><td>-</td></tr><tr><td>Susceptible</td><td>Resistant</td></tr></table>	+	-	Susceptible	Resistant	
+	-					
Susceptible	Resistant					
aa	<table><tr><td>+</td><td>+</td></tr><tr><td>Susceptible</td><td>Susceptible</td></tr></table>	+	+	Susceptible	Susceptible	
+	+					
Susceptible	Susceptible					

Fig.1. Disease reaction in the common gene-for-gene pattern of interaction between resistance in the host and virulence in a pathogen. (+) indicates successful parasitism and (-) indicates resistant reaction and little or no disease incidence on host

The basic pattern of the gene-for-gene relationship is often referred to as the quadratic check (Fig 1). A resistant (incompatible) reaction occurs when the host has a gene for resistance that is not matched by a corresponding gene for virulence in the pathogen. Flor's (1955 and 1956) extensive analysis of the genetics of flax rust interactions showed that for each major gene conditioning resistance in the host, there is a specific gene conditioning virulence in the pathogen. In this type of gene-for-gene relationship, there is no major gene for resistance that cannot be overcome with a corresponding gene for virulence in the pathogen.

Reciprocal check for two loci of resistance (R1 and r2) in two cultivars. Cv1 and cv2 and corresponding two virulence loci in two pathogen races (A1 and A2). Combination of R1 and A1 or R2 and A2 results in plant resistance. This reciprocal check defines a gene for gene interaction

	Cv 1	R1r2	CV2	r1R2
Race 1 A1a2	—		+	
Race 2 a1A2	+		-	

	AVR1AVR2	AVR1avr2	avr1AVR2	avr1avr2
R1-R2-	Incompatible (Resistant)	Incompatible (Resistant)	Incompatible (Resistant)	Compatible (Susceptible)
R1-r2r2	Incompatible (Resistant)	Incompatible (Resistant)	Compatible (Susceptible)	Compatible (Susceptible)
r1r1R2-	Incompatible (Resistant)	Compatible (Susceptible)	Incompatible (Resistant)	Compatible (Susceptible)
r1r1r2r2	Compatible (Susceptible)	Compatible (Susceptible)	Compatible (Susceptible)	Compatible (Susceptible)

Salini-Kurti lecture 1

The significance of Flor's work was largely overlooked until Person (1959) published a theoretical analysis of gene-for-gene relationships in host parasite systems. He proposed that gene-for-gene interaction would occur widely in parasitic systems, and this has been verified. These interactions have been found not only in plant-fungal interactions but also in plant interactions with viruses, bacteria, nematodes and insects. Thompson and Burdon (1992) have listed over 40 associations between plants and pathogens that were either demonstrated or suggested to have such an interaction. Several characteristics are common to all plant-parasite systems where gene-to-gene relations have been demonstrated. First, resistance is usually dominant and controlled by single genes which may be linked, unlinked or in a multiple allelic series. Second, virulence is usually recessive and inherited by single independent genes. Third, a single combination for incompatibility is epistatic to all other interactions, and fourth, resistance and avirulence alleles function as conditional genes. Deviations have never been observed in the last two

characteristics. It is generally agreed that the specificity of gene-for-gene interactions stems from the specificity of interaction between the conditional resistance and avirulence. The gene-for-gene relationship has not only been observed between races of a formae specialis of the pathogen and host cultivars. Tosa (1989) obtained evidence in wheat for gene-for-gene relationship between formae specialis of *Erysiphe graminis* and genes of gramineous plants

Exceptions to the typical pattern of inheritance of resistance in gene-for-gene systems include: (i) recessive resistance genes, (ii) two host genes controlling resistance to a particular pathogen genotype (it is not always clear whether or not the two genes in the host are interacting with only one gene in the pathogen), (iii) modifiers which can enhance the effects of a major resistance gene (there is speculation that 'matched' resistance genes may provide some residual resistance and may act as modifiers), (iv) occasional suppressors of resistance genes (especially when genes from a distant relative are incorporated into the host). The major exception to gene-for-gene characteristics for resistance is resistance controlled by several genes that have small additive effects. This type of resistance is considered to be completely separate from major gene resistance. These exceptions to the typical gene-for-gene systems have been discussed in detail by Christ et al (1987).

MOLECULAR BASIS OF GENE FOR GENE HYPOTHESIS

<u>Pathogen</u>	<u>Plant host cell</u>	<u>Disease reaction</u>
A Avirulence gene	→ Resistance gene	→ Resistance
B Compatible pathogen	→ resistance gene	→ Susceptible
C Compatible pathogen	→ Susceptible host	→ Susceptible
D Incompatible pathogen	→ susceptible Host	→ susceptible

Molecular model of gene for gene interactions (Staskawicz et al 1995).

As shown above, resistance occurs only if there is a specific recognition between the resistance gene product and product of matching avirulence gene (A). In the absence of any recognition (B,C,D), no resistance reaction occurs and the pathogen can colonise. Product of resistance gene in this model would be a **receptor** that actively recognizes a direct/indirect product of avirulence gene. Only **receptor ligand** interaction results in specific recognition indicated by hypersensitive response and disease resistance.

During host invasion, pathogens release exogenous as well as endogenous elicitors (Vorwerk et al. 2006; Huckelhoven 2007). **Elicitors** are molecules produced by either the host or the pathogen that in turn induce a response by either the host or the pathogen. Fungal pathogen elicitors include enzymes which break down the cuticle or cell wall polysaccharides. Chitin, the major constituent of fungal cell walls is also known to be an elicitor which triggers a defense response in plants. The bacterial protein, flagellin, is also an elicitor of host defense. Cuticle and cell wall fragments are examples of endogenous elicitors of host defense. As these tissues are under physical or

enzymatic attack, released fragments are detected by R-proteins stationed in the cell membrane and a defense response is initiated. Within 15 minutes of pathogen recognition, host cells begin producing new proteins in reaction to the attack (Dangl and Jones 2001). This first response to invasion is known as a basal defense, and includes production of proteins that inhibit pathogen enzymes, structural and chemical remodeling of the host cell wall at penetration sites, and production of antimicrobial agents that will kill the intruder. A second, more extreme, line of defense is known as the **hypersensitive response (HR)**, or **programmed cell death (PCD)** (Greenberg and Yao 2004; Lam et al. 2001; Day and Graham 2007; Glazebrook 2005). The hypersensitive response is a strategy of “scorched earth” where the plant cell introduces toxic molecules into the surroundings; creating a localized environment that is incapable of sustaining life for both the pathogen and the cell itself. The HR is an efficient means of defense against many pathogens, but is ineffective against necrotrophic fungi

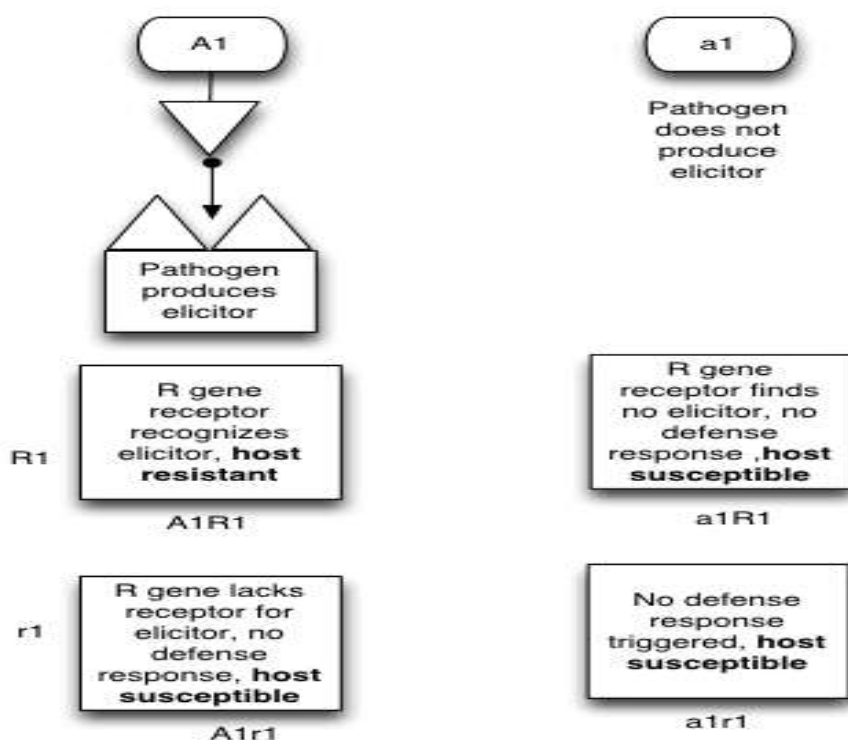


Fig.A more molecular model derived from the observation of the dominant character of both the avirulence gene and the resistance gene is shown in fig .

The product of the resistance gene in this model would be a receptor that actively recognizes a direct or indirect product of the avirulence gene. Only the receptor ligand interaction (fig2A) results in specific recognition indicated by the hypersensitive response and disease resistance.

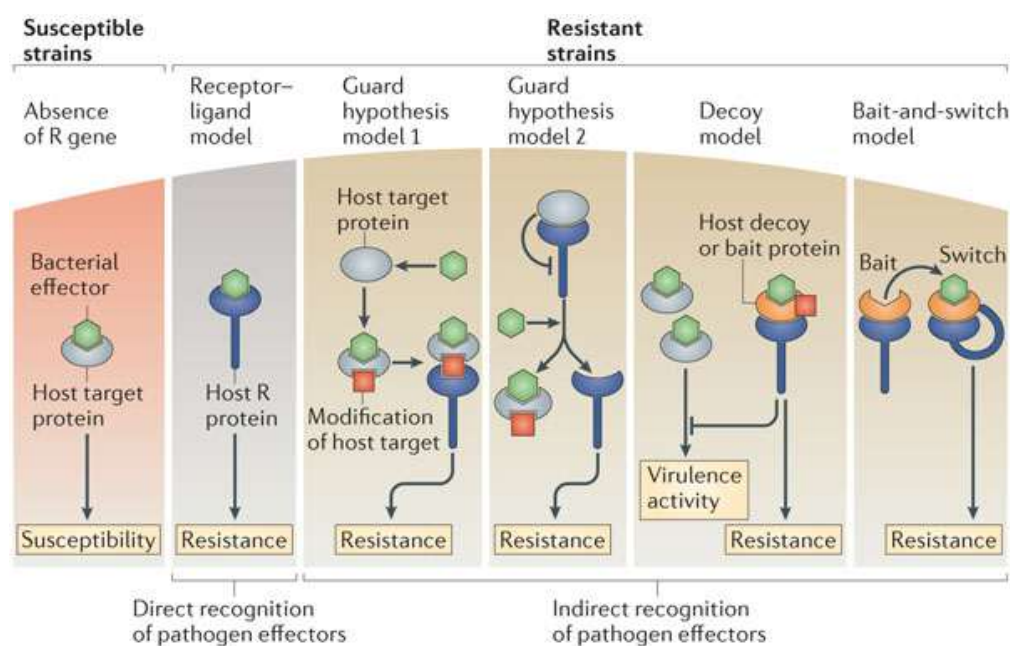
Different models of Host-pathogen interaction

Receptor–ligand model. Direct recognition occurs when a pathogen effector is directly recognized by a host receptor. Evidence of direct recognition of pathogen effectors in animals is currently lacking.

Guard hypothesis. In the guard model, certain important cellular targets are 'guarded' by a specific R protein, and an immune response is induced if they are perturbed. In model 1, engagement of the host target by the pathogen effector results in the modification of the target. This modification is recognized by the R protein to signal an immune response. In an alternative model (model 2), the R protein guard and the host target 'guardee' exist together in the healthy state and the immune response is repressed. During infection, the modification of the host target protein (guardee) by the pathogen effector liberates the R protein (guard), allowing the R protein to engage signalling pathways and induce a defence response.

Decoy model. This model suggests that during evolution, the host has acquired proteins that are decoys for the targets of pathogen effectors and that function as sentinels for the presence of these effectors. These decoys could arise through gene duplication events or could be splice variants of the normal target. However, decoys are generally thought to have no other primary function in the cell.

Bait-and-switch model. In this case, the pathogen effector is not sensed after binding its true target but rather is 'baited' by a decoy protein that engages an R protein. The function of the R protein switches to induce signalling once the pathogen effector is bound.



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Types of Resistance True resistance is classified into two major types (Nelson, 1978). The host either resists the establishment of a successful parasitic relationship by restricting the infection site and the infection process, or it resists subsequent colonization and reproduction of the parasite following successful infection. Put in an epidemiological frame work, the first type of resistance affects the disease onset by reducing the amount of effective initial inoculum. The other kind of resistance affects the apparent infection rate

and, thus, the amount of disease that finally develops on the plants. Vander Plank (1963) coined the terms 'Vertical Resistance' (VR) and 'Horizontal Resistance' (HR) for the former and the latter, respectively. According to him when a variety is more resistant to some races of the pathogen than to the others, the resistance is called 'Vertical' or 'Perpendicular'. When the resistance is evenly spread against all races of the pathogen, it is called 'Horizontal' or 'Lateral'. Resistance to infection i.e. vertical resistance has also been referred by such terms as hypersensitivity, race specific resistance, major gene resistance, qualitative resistance, non uniform resistance etc. Resistance to subsequent colonization and reproduction i.e. horizontal resistance has been characterized variably by the terms race nonspecific resistance, minor gene resistance, polygenic resistance, quantitative resistance, uniform resistance, partial resistance, durable resistance etc.

Horizontal or race non-specific resistance can be discerned according to Vander Plank (1963, 1968), from vertical or race specific resistance by a test in which a number of host genotypes are tested against a number of pathogen genotypes. If the total non-environmental variance in levels of resistance is due to main effects only (differences between cultivars and differences between isolates), the resistance and the pathogenicity (in broad sense) are horizontal in nature (Table 1). Vertical resistance and pathogenicity are characterized by the interaction between host and pathogen showing up as a variance component in this test due to interaction between cultivars and isolates (Table 2).

Therefore, as meant by Vander Plank, horizontal resistance is a type of resistance in which gene-for-gene relationship does not hold good. According to Parlevliet and Zadoks (1977), in Vander Plank's test in horizontal resistance, the interaction component is so small that is difficult to discern from a normal error variance. So called horizontal resistance can, therefore, be explained by a polygenic resistance, when the individual genes are vertical and operating in a gene-for-gene basis with virulence genes in the pathogen. Parlevliet and Zadoks (1977) formulated 'The Integrated concept of Disease Resistance' according to which the true resistance genes act in a gene-for-gene way with virulence genes in the pathogen, irrespective of whether resistance is controlled monogenically or polygenically. They formulated and compared two models for polygenic resistance and pathogenicity (i) the "addition model" in which the minor genes act in an additive way within the host, within the pathogen and between the host and pathogen genotypes, and the "interaction model", in which the resistance alleles act in an additive way within the host, as in the addition model, but interact on a gene-for-gene basis with the alleles for pathogenicity in the pathogen. Their analysis showed that variance in the interaction model is largely due to the two main effects, cultivars and isolates, and the variance is so small that it would be difficult to discern it from a normal error variance. Their models also suggested that populations with polygenic resistance based on gene-for-gene action have an increased level of resistance compared to populations following the addition model. Person et al (1983) criticized Parlevliet and Zadoks (1977) polygenic models and their conclusions because the addition model had a theoretical maximum of 200% disease severity even though the maximum possible is 100%.

Combined analysis of variance in two test of four races of *Phytophthora infestans* on three potato varieties. (from Vander Plank, 1968)

Source	Degrees of freedom	Mean square	P
Races	3	563.7	<0.001
Varieties	2	919.0	<0.001
Races x Varieties	6	23.5	
Tests	1	1230.0	
Error	132	19.6	

- The varieties differed significantly. The races differed significantly. But there was no significant interaction between varieties and races. This means that the varieties differed in resistance horizontally but not vertically, and the races differed in aggressiveness but not in virulence.

Table 2. Combined analysis of variance in three tests of three races of *Helminthosporium gramineum* in twelve barley varieties (from Vander Plank, 1968)

Source	Degrees of freedom	Mean squares	P
Races	2	11.08	
Varieties	11	113.05	<0.01
Races x varieties	22	107.86	<0.01
Tests	2	34.58	
Error	70	39.47	

The barley varieties differed significantly in resistance. The races of *H. gramineum* did not differ significantly, but there was significant differential interaction between races and varieties. The varieties differed in resistance both horizontally and vertically and the races differed in virulence but not in aggressiveness

BOOM AND BUST CYCLE: New variety carrying VR resistant to prevalent pathotypes are released for cultivation in a area. These become popular and occupy large growing area. This period of Boom (high popularity, large area) continues till a virulent pathotype develops or increases its frequency to cause an epidemic. As the new virulent pathotype becomes prevalent, popular variety loses popularity, area declines, the variety is busted by the virulent pathotype. Then the breeder develops a new variety with new VR genes

resistant to prevalent pathotype which again goes a boom and bust cycle. Selection pressure exerted on the pathogen by resistant variety is main cause of selection/origin of new pathotypes virulent on the existing popular variety. Mutation may lead to new race which may be virulent on the VR gene or virulent race which was in low frequency at the time of release becomes more frequent.

Breeders have successfully developed lines resistant to diseases by integrating R-genes into their cultivars for many years; but a durable, long lasting resistance in many cases has been difficult to achieve as pathogens quickly evolve and develop counter resistance genes that circumvent the host cultivars resistance. Breeders often spot this breakdown and hurriedly integrate a newly found effective R- gene into their populations. In time, the new R-gene loses its effectiveness and the boom- bust and induced co-evolution between crop and pathogen continues.

An example of this comes from the United States where in Indiana, 1955; a cultivar was released that held on R-gene conferring resistance to Hessian fly attack. Just six years later the Hessian fly population had developed a substantial amount of counter-resistance. By 1964, the Indiana breeders had introduced a second R-gene into their cultivars. This new R-gene provided resistance for eight years before counter-resistance developed. A third R-gene was introduced and released in cultivars in 1971; and again was overcome by the pathogen, this time in a period of ten years (Rausher 2001).

Vertifolia effect: Named after a potato cultivar of this name, in which the effect was very pronounced. Vertifolia effect is a loss of HR which occurs during breeding for VR. Later extended to include loss of HR that occurs during breeding under protection of pesticides. Level of HR can only be assessed by level of parasitism. If there is no parasitism because of a VR or a pesticide, level of HR cannot be assessed. Because individual plants with a high level of HR are rather rare in a mixed screening population, individuals with a low level of HR will be selected on the basis of other attributes. Loss is usually quite small in a single breeding cycle but, after many cycles can become very serious. Example of vertifolia effect is loss of HR to potato blight (*Phytophthora infestans*) that has continued ever since both the discovery of Bordeaux mixture in late 19th century, and discovery of VR in 20th century. A loss of HR to cotton pests has continued since discovery of DDT in the 1940s. Vertifolia effect is a very modern phenomenon. Its overall consequences are seen in the high levels of HR in heritage cultivars, compared to modern cultivars.

Due to use of strong VR genes by the breeders to safeguard against important diseases, there is strong selection pressure on the pathogen leading to development or selection of new virulent pathotypes. Thus, the life of any resistant variety with strong VR genes is limited. To prolong the life of resistant genes, the breeder can adopt following approaches for deployment of resistant genes.

i) Alternate approaches of gene deployment

Gene deployment is the guided distribution of genes in space and time (Parlevliet, J.E., 1993a) Gould (1983) divided the gene deployment strategies in to two broad categories

as 1) spatial (gene deployment at the farm level, at the field level and at the region level) and 2) temporal.

a) Spatial gene deployment

Spatial resistance gene deployment can also be done over a small geographic region such as within a single field. If a cultivar consists of individual plants that differ from each other in their resistance, the resultant heterogeneous population should reduce rate of epidemic development and prevent selection of a single compatible phenotype in the pathogen population. Gene deployment at the field level includes variety mixtures, species mixtures, multi lines and multi blends.

Species mixtures/ mixed cropping: The safest opinion for mixed cropping, which may indeed provide permanent protection for cultivars with durable resistance, is to grow species mixtures in which the important pathogens are incompatible with one or more of the host species used. Of the many examples available, mixtures of barley and oat cultivars are widely used in cereal producing countries, for example in Canada and Poland.

Multiline varieties: The term multiline variety was first defined by Jensen (20) as a blend of multiple pure lines, each of which is of a different genotype. Borlaug[27] used an equivalent term 'composite' and proposed as a new approach for resistance to stem rust of wheat (*Puccinia graminis tritici*). According to Borlaug's definition, these are mixtures of a number of phenotypically similar lines, which are genotypically different for resistance to a given disease. The term multiline in current usage designates a mixture of near-isogenic lines for resistance to a specific race of the pathogen (Wolfe 1993).

Multilines have been known for mid-America at least since 1898 (Browning and Frey 1969). Borlaug (1958) suggested a minimum of 8 to 10 lines, thereby requiring 8 to 10 backcrossing programmes simultaneously and screening for genes resistant to a specific race that makes it damn expensive. Marshal (1977) has given two approaches of multiline development. These are the clean crop approaches and the dirty crop approach. Dirty multilines are mixtures of lines each carrying a unique resistance gene and clean multilines are mixtures of lines each resistant to all prevalent races of the disease. Multiline varieties offer various advantages. For example, by using multilines we can exploit horizontal resistance, which is otherwise difficult to achieve because of the complexities associated with its genetic mechanism, multiline varieties proved to live longer than their corresponding pure stands because of stabilized selection. Another advantageous features of multilines over variety mixtures is that all the component lines are phenotypically similar the only difference being the gene for resistance.

Multiblend varieties/mass reservoirs: Multiblend varieties are generally simple mixtures of existing lines or varieties.

b) Gene deployment at the farm level (inter field diversification): A very large area of a single variety, or a number of varieties with similar resistance factors, would be vulnerable to any corresponding virulent isolate in the pathogen population. A practical

way to protect resistant factors, which the grower can understand and implement, is to diversify by growing a number of varieties, which differ in the form of their resistance to specific diseases (Priestley 1978). However, a crucial point is that for diversification to be successful, we need more precise information on the differences among cultivars in terms of the genetics and mechanisms of their durability (Wolfe 1993). If the specific resistance genes in each variety selected differ, a pathogen race, which possesses specific virulence genes for one variety, will not spread to the other varieties (Parry 1990). Diversification is used primarily in cereal varieties for reduction of mildew and yellow rust.

Gene deployment at the regional level: If the cultivars recommended in different regions of an epidemiological area carry consistently different resistance genes it would certainly reduce the rate at which complex races would develop (Parlevliet 1993).

Temporal gene deployment: Temporal gene deployment may include 1) sequential release of resistance genes where by each variety is used until populations reach the breakdown population level and is immediately replaced by another variety (Wilhoit 1992). The sequential release strategy has been employed for resistance to brown plant hopper (*Nilaparvata lugens*) at IRRI, 2) Variety rotations from season to season or recycling of resistance genes. Rotation of varieties with different resistances prevents selection of compatible isolates in populations of soil-born pathogens (Fry 1982). This approach was used to control stem rust of wheat in Australia between 1938 and 1950.

Multiple gene barriers/ multigenes/ gene pyramids: An alternative to multilines is to pyramid desirable genes to provide a wide array of horizontal resistance. To delay breakdown of resistance conferred by single genes, pyramiding of more than one resistance gene has been found to be effective (Gnanamanickam et al 1999). It is also known as gene stacking. Watson and Singh (1952) proposed the concept of multigene varieties. Resistance gene pyramids are expected to considerably extend the durability of resistance because of the low probability for the pathogen to assemble multiple, rare virulence genes by mutation and/ or recombination (Jorgensen 1993). In a way analogous to partial resistance breeding, the combining or pyramiding of resistance genes into individual cultivars has had considerable success in reducing the rate of evolution of pathogens particularly in situations where the pathogen does not reproduce sexually (Burdon 1993). However some difficulties arise like for instance, in order to arrange this, one has to know all the resistance genes involved and the breeders in the whole epidemiological area should stick rigorously to the agreements made over the use of the resistance genes. Moreover, bringing together two fully effective major genes in an agronomically successful cultivar is far from easy (Parlevliet 1993a). However, in the future it is possible this may also be achieved by combinations of resistance genes that would require combinations of virulence genes that may have detrimental effects on pathogen fitness (Burdon 1993). When sufficient RFLP markers have become available the application of a multiple gene barrier will become much easier as the tracing of the individual resistance gene becomes much easier (Parlevliet 1993a).

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UNDERSTANDING GENETIC ARCHITECTURE OF COMPLEX PLANT DISEASES USING NEXT GENERATION SEQUENCING

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Most of the complex diseases in plants are quantitative in nature and controlled by several genes of small effects, referred as quantitative resistance loci (QRL). Understanding genetic architecture of these QRLs can provide a platform to develop durable resistance in crop plants.

Two general categories of disease resistance

Two general categories of disease resistance have long been recognized in plants: qualitative and quantitative disease resistance. The qualitative resistance is conditioned by one or two genes with large effect and quantitative resistance controlled by several genes of small effect. Qualitative resistance are studied by dividing the number of plants segregating in a mapping population into distinct classes (Fig. 1). In quantitative resistance, the classes are overlapped with each other and follows a normal distribution. A variety of synonymous terms have been used in literature to refer to these two classes, including horizontal versus vertical resistance, complete versus incomplete resistance, major-gene versus minor-gene and narrow-spectrum versus broad-spectrum resistance. Although the phenomena of qualitative and quantitative resistance can be considered different, there is a great deal of grey area between the extremes, suggesting that it might be useful to re-examine the concepts in light of emerging evidence on mechanisms of resistance.

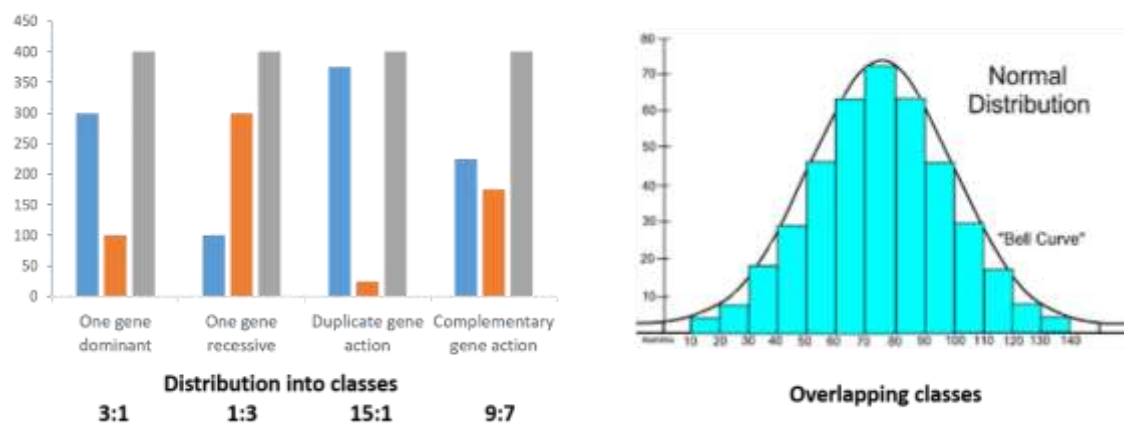


Fig 1: Frequency distributions observed for qualitative and quantitative resistance in a segregating population

The dwindling boundaries of qualitative resistance

Qualitative resistance usually works for biotrophic pathogens and follows gene for gene hypothesis given by Flor in 1942. The type of resistance is usually governed by plant

disease resistance genes (R-genes) which play a key role in recognizing proteins expressed by specific avirulence (*Avr*) genes of pathogens. R-genes typically provide high levels of resistance and are relatively easy to manipulate, both in basic research and applied breeding programs. These genes are important in many systems, but their utility varies among pathosystem and among genes within a pathosystem. The primary limitations of R-genes for crop protection are (i) a lack of durability in some systems (primarily with respect to pathogens that have high evolutionary potential (McDonald and Linde, 2002) and (ii) a lack of availability in others (primarily necrotrophic systems). The deployment and breakdown of R-genes for diseases has been a frustrating battle for plant breeders and pathologists and more durable resistance is needed (Ayliffe *et al.*, 2008). As an example, potato late blight, caused by *Phytophthora infestans*, lines carrying most R-genes have been rapidly overcome by virulent populations. In addition, the presence of R-genes shifts the trait distribution in such a way that underlying quantitative trait loci cannot be detected.

Genetic architecture of quantitative resistance

In the last two decades, several studies have been conducted to understand the genetic architecture of quantitative resistance and molecular mechanisms underlying QRLs. Based on these studies, several mechanisms with which these QRLs may work, are discussed in detail by Poland *et al* (2008). These are summarized as follows here:

QRLs may be genes underlying plant developmental traits

Quantitative resistance is conditioned by genes regulating morphological and developmental phenotypes. Plant diseases develop within the spatio-temporal context of plant development, so it sounds reasonable that some QRL are based on genes that control plant architecture or development. It has been well documented in many necrotrophic plant-pathogen systems that flowering time is strongly correlated with disease resistance, such that susceptibility is apparently enhanced after flowering (Collins *et al.*, 1999). Few studies conducted at PAU shows the correlation of sheath blight of rice with tiller angle and neck blast of rice with panicle exertion.

QRLs may be genes involved in basal defense

Flagellin and chitin are two widely conserved pathogen features that enable plants to recognize broad pathogen groups. Flagellin is the main constituent of bacterial flagella and chitin is the main component of fungal cell walls. In *Arabidopsis*, it has been demonstrated that a LRR receptor-like kinase gene (*Fls2*) involved in the perception of flagellin, has established a mechanistic basis of flagellin recognition and providing basal resistance in plants (Zipfel *et al.*, 2004). Mutations in *Fls2* quantitatively effect disease severity and bacterial colonization under natural conditions (Zipfel *et al.*, 2004). Fungal chitin also play a role in triggering basal resistance (Shibuya and Minami, 2001). The host receptor-like kinase, *CERK1* (also called *LysM RLK1*), has been identified as a component of chitin perception and defense signal transduction. Mutation in *CERK1* leads to reduced resistance to the normally incompatible

necrotrophic fungus *Alternaria brassicicola* (Miya *et al.*, 2007). These examples support the hypothesis that pattern recognition receptors acting in basal defense, can condition quantitative differences in the resistance phenotype.

QRLs may be the genes that produce phytoalexins to detoxify phytotoxins

Phytotoxins are the toxic compounds produced by pathogen that promote plant disease, whereas phytoalexins are the compounds, produced by plants to deploy against pathogen attack to detoxify the effect of phytotoxins. QRL might be the genes that produce phytoalexins. In *Arabidopsis*, biochemical studies for understanding *Arabidopsis-Botrytis* pathosystem, showed that levels of camalexin (a phytoalexin) were correlated with quantitative resistance, and that camalexin sensitivity of different pathogen isolates contributed to isolate specificity (Kliebenstein *et al.*, 2005). The study suggests that genes controlling phytoalexin levels underlie QRL. Additional evidence was found in rice, in which members of the glutathione *S* transferase (GST) gene family were found to co-localize with QRL (Wisser *et al.*, 2005) in bioinformatics analysis. Necrotrophic pathogens utilize numerous secondary metabolites to disrupt normal cell processes, trigger host cell death, and destroy host tissue. The well-studied pathogen *Botrytis cinerea* uses an arsenal of compounds including Botrydial, a phytotoxin to attack its hosts (Colmenares *et al.*, 2002). On the other hand, production of oxalic acid by host plant is a potent weapon for *B. cinerea*, causing a reduction in host oxidative burst and defense responses, as well as producing an acidic environment for further enzymatic degradation (van Kan, 2006). Thus, the mode of necrotrophic pathogen attack naturally implicates toxin production and mitigation in quantitative disease resistance.

QRL are involved in defense signal transduction

Plants have developed a complex system for the effective transmission of signals from initial pathogen perception to the activation of defense mechanisms. This involves the genes that regulate the production of phytohormones such as salicylic acid, jasmonic acid and ethylene. QRL might be genes that regulate signal transduction by production of these phytohormones. Often these signalling mutations increase resistance to a range of biotrophic pathogens but increase susceptibility to necrotrophs and could also contribute to the broad spectrum resistance of some QRL (Poland *et al.*, 2008).

QRLs: a unique set of unknown genes

QRL are a completely novel component of plant defense based on recent cloning of QRLs. In the wheat-leaf rust pathosystem, the resistance gene *Lr34* has been identified as a putative adenosine triphosphate binding cassette (ABC) transporter (Krattinger *et al.*, 2009). A second gene in wheat, *Yr26*, was isolated by map-based cloning and shown to be a Kinase- START gene (Fu *et al.*, 2009). Another QRL cloned is *pi21* from rice which confers resistance to rice blast. The recessive resistance of *pi21* results from the loss of a proline-rich gene (Fukuoka *et al.* 2009). None of these candidate genes have sequence similarity to any previously reported defense genes. With the

identification of additional QRL it is probable that new and novel classes of genes will be implicated in disease resistance.

An understanding of the developmental processes associated with pathogenesis, as well as the microscopic phenotypes associated with failure or attenuation of pathogenesis on resistant hosts, can provide important clues into the mechanisms of resistance. Current genomic advances situate QRL as an exciting field for systems biology research with the additional prospect of valuable application in crop improvement.

Evolution of NGS technologies

NGS platforms including Illumina HiSeq2500, ABI 5500xl SOLiD, Roche 454, Ion Torrent and PacBio RS and advances in bioinformatics tools has not only simplified the process of discovery of SNPs and INDELs (insertions/deletions), which comprise of most of variations in the genomes, but also provided the benefit of doing genotyping in a single and cost effective process (Chen *et al.*, 2013). Reduced representation techniques such as RRL (Reduced Representation Libraries), CRoPSTM (Complexity Reduction of Polymorphic Sequences), RAD-seq (Restriction Associated DNA Sequencing), GBS (Genotyping by Sequencing), ddRAD-seq (Double Digest Restriction Associated Sequencing), Rest-seq (Restriction DNA Sequencing) effectively subsample the genome of multiple individuals at homologous locations allowing for single nucleotide polymorphisms (SNPs) to be identified and typed for tens or hundreds of thousands of markers spread evenly throughout the genome in large numbers of individuals. This family of reduced representation genotyping approaches are generally called genotype-by-sequencing (GBS) or Restriction-site Associated DNA sequencing (RAD-seq). Genotyping by sequencing (GBS) is currently a powerful technique backed by power of NGS for genotyping large populations and more precise association of genotype and phenotype. GBS uses enzyme-based complexity reduction method coupled with DNA barcoded adapters to produce multiplex libraries of samples ready for NGS sequencing.

EVOLVING NATURE OF GBS

Marker discovery and assessing the polymorphism with number of genetic makers is the basic requirement for more precise recombination breakpoints in mapping studies, genotyping large number of individuals for marker assisted selection or for resolving phylogeography of wild populations (Davey *et al.*, 2011). Marker density not only enables the genome wide association studies in any organisms, but also answers number of biological questions with high accuracy. While whole genome sequencing was a costly affair, reduced representation sequencing holds promise for genome wide marker discovery and analysing the polymorphism by sequencing subset of genomic regions in number of individuals. RRLs and CRoPSTM were first two methods introduced for sampling and sequencing a small set of genome-wide regions without sequencing the entire genome. Both these methods used deep sequencing to sequence the entire genome at variable sites in the population. However RRL approach required

high quantity of DNA (50µg) and allows the detection of polymorphism in the pool of individuals, but not for each individual. Complexity Reduction of Polymorphic Sequences (CRoPSTM) was the first method to identify polymorphisms in each individual sample by incorporating short barcode identifier sequences also known as multiplex identifier sequences (MIDs) on the Roche Genome Sequencer platform into the ligated adaptors and using an adaptor containing a different barcode for each DNA sample. With introduction of MID, quantity of DNA in this approach was reduced to 300ng per sample. Low coverage sequencing protocols like RAD-seq, GBS, ddRADseq and Rest-seq were developed for further lowering the cost and time for genotyping the entire populations, but also modifying these methods making suitable to number of species and most of labs worldwide (Bhatia *et al.*, 2013). Well documented method for RAD-seq was first put forward by Baird *et al.*, (2008). It is one of a number of reduced representation methods that sample a shared set of sites across the genome in many individuals or pools, making population-scale sequencing possible at a fraction of the cost of whole genome sequencing (Davey *et al.*, 2011). Simple and modified protocols for RAD-seq were later developed as Whole Genome Resequencing (Huang *et al.*, 2009); as Multiplexed Shotgun Genotyping (Andolfatto *et al.*, 2011) and 2b-Restriction site-Associated DNA (Wang *et al.* 2012). The term GBS became more popular with two papers published by Elshire *et al.*, (2011) and Poland *et al.*, (2012a), who described simplified, robust and cost effective procedure using one restriction enzyme and two restriction enzymes respectively. Both RAD-seq and GBS target the genomic sequence flanking restriction enzyme sites to reduce the complexity of the genome. GBS requires less DNA (100-200ng per sample), avoids random shearing and size selection and is completed in only two steps on plates followed by PCR amplification of the pooled library (Elshire *et al.*, 2011, Poland *et al.*, 2012a). Double Digest Restriction Associated DNA Sequencing (ddRADseq) emphasizes on increasing the number of individuals sampled for the same resource investment by reducing the fraction of each genome sequenced with precise size selection, and also ensuring that same region is examined between all individuals (Peterson *et al.*, 2012). Reduction of marker densities can be important benefit in some cases like large size of genome or limited recombination event. ddRADseq introduced n*m individual multiplex using n*m indices, which is two indexes combinatorial approach. Restriction fragment Sequencing (RESTseq) was given by Stolle and Moritz (2013) for SNP genotyping even on small scale platforms by further reducing the complexity of genome and number of fragments than all other approaches. The method yields fragments potentially containing SNPs with an unbiased distribution across the genome. As a result SNP genotypes are obtained at much less sequencing effort reducing costs for chemicals and labour. The method is especially suitable for population genomic studies on small scale sequencing platforms like Ion Torrent PGM (Life Technologies), MiSeq (Illumina) and 454 GS Junior (Roche). Simple and straightforward flowchart for preparation of GBS library is shown in the Fig. 2.

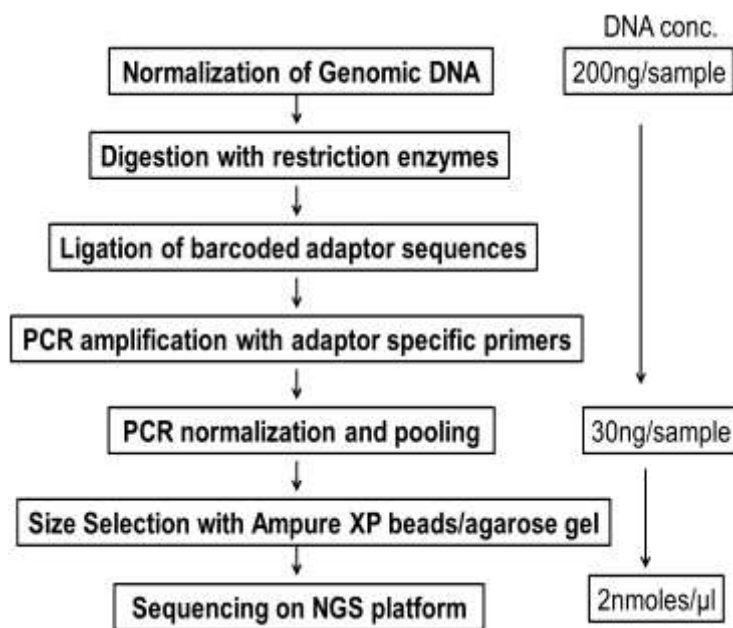


Fig.2: Flowchart of simple and straightforward steps for GBS protocol. Flowchart can be modified depending upon protocols suggested by GBS family approaches by various authors (adapted from Bhatia *et al.*, 2013).

Application of GBS

GBS can be an excellent approach for linkage/association based mapping with generation of more dense genetic maps leading to more accurate and high resolution mapping of genes/QTL. In rice, ‘green revolution gene’ *sd-1* was mapped to 100 kb region in RIL population demonstrating the worth of method for mapping with high resolution. GBS extended its virtues by using high density linkage maps generated in barley for precisely mapping previously characterized *Breviaristatum-e* (*ari-e*) locus and two small effect additional QTLs for plant height. RIL population (GPMx) derived from a cross between the two-rowed barley cultivar ‘Golden Promise’ and the six-rowed cultivar ‘Morex’ was genotyped with GBS two enzyme strategy. GBS mapping data and phenotypic measurements showed that *ari-e* maps to a small genetic interval on chromosome 5H and that alternative alleles at a region encompassing *Vrs1* on 2H along with a region on chromosome 3H also influence plant height (Liu *et al.*, 2014). An ultra-high-density linkage map based on high-quality SNPs generated from low-coverage sequences (~0.07 genome sequence) in a sorghum recombinant inbred line (RIL) population was used to map 57 major QTLs for eight agronomically important traits in two contrasting photoperiods. The high accuracy and quality of this map was evidenced by the finding that genes underlying two cloned QTLs, *Dw3* for plant height (chromosome 7) and *Ma1* for flowering time (chromosome 6), were localized to the correct genomic regions (Zou *et al.*, 2012). Genome-wide association studies (GWAS) require hundreds of thousands to millions of markers to generate sufficient information and coverage, and getting such resolution has been greatly enhanced by the emergence of NGS technologies. Association Mapping compared to the conventional linkage mapping, using the non-random associations of loci in haplotypes, is a powerful high-

resolution mapping tool for complex quantitative traits (Flint-Garcia *et al.*, 2003). GBS has been used to map sclerotinia stem rot (SSR) in a collection 130 diverse lines of soybean by association mapping (Bastien *et al.*, 2014). High-density genetic maps developed through GBS can be used to anchor and order physical maps and refine or correct unordered sequence contigs. In *D. simulans*, Andolfatto *et al.*, (2011) were able to assign 8 Mb to the linkage group, which comprised 30% of the unassembled *D. simulans* genome or about 6% of the total genome. This is a substantial improvement of an already well-characterized genome. Gao *et al.*, (2013) improved the genome sequences of paternal variety 93-11 and maternal cultivar PA64s of widespread Chinese hybrid Liang-You-Pei-Jiu by using GBS re-sequencing dataset. Genomic Selection (GS) provides the ability to select complex quantitative traits based on marker data alone and combines the benefit of high throughput technologies like GBS and developments in the statistical methods needed for data analysis. GS can greatly accelerate the breeding cycle while also using marker information to maintain genetic diversity and potentially prolong gain beyond what is possible with phenotypic selection (Lorenz *et al.*, 2011). The accuracy of genomic prediction using GBS is currently undergoing investigation in several important crops including maize and wheat. Poland *et al.*, (2012b) have demonstrated the suitability for GBS markers in developing GS models in the complex wheat genome.

The dense marker coverage obtained through GBS can enable the researcher not only to discover polymorphisms and then transfer these to a fixed assay, but also obtain genotypic information across the whole population of interest. This combined one step approach has made GBS a rapid and flexible platform for range of species. As sequencing output continues to increase, GBS will evolve from lower levels of complexity reduction to whole-genome resequencing to capture most of variants. Low cost of GBS will help most of labs in the developing countries to adopt this technology. Marker discovery, development of dense molecular maps will expedite the mapping of economically important genes/QTLs and thus crop improvement. Though challenges are there in maintaining and analysing huge datasets, but developments in this field looks promising in future with emerging field of bioinformatics.

Future directions

QRLs controlling quantitative resistance are an important weapon for host plant resistance. Recent advances in Next generation sequencing technologies provide an excellent platforms to understand the genetic architecture of QRL and deploying them into cultivars for broad spectrum resistance.

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ARTIFICIAL SCREENING OF BACTERIAL BLIGHT AND SHEATH BLIGHT OF RICE

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Introduction:

Bacterial blight: Rice (*Oryza sativa* L) is a major crop in Asia and top rice growing countries are situated in South and South-East Asia. Bacterial blight (BB) of rice caused by *Xanthomonas oryzae* pv. *oryzae* [(Ishiyama), Swing et al.1990], is widely distributed in particularly endemic to most parts of Asia (Mew et al. 1993). The occurrence and incidence increasing of the disease has also been reported in rice growing countries of Africa. The losses due to its outbreak can also reach up to 50% depending on stage, weather, location and the cultivar (Mew et al. 1993). The higher yield loss from 60 to 70% has been reported in Punjab state of Northern India when it occurred at *kresek* phase (Raina et al., 1981). For management of this disease, no any effective chemicals are available in the tropical monsoon climate of Asia (Ou, 1985).

Breeding for bacterial blight resistance with deployment of R genes in high yielding rice cultivars, is the only key approach, economical and eco-friendly strategy for the management of this disease. Globally different 43R genes (*Xa/xa*) conferring resistance against *Xoo* have identified so far (Cheema et al. 2008; Wang et al. 2009; Kim et al., 2015). However, the durability of resistance depends upon the prevalence of pathogen population in particular area. Pathogen *Xoo* is highly variable and showed diverse reaction to rice cultivars possessing genetic resistance to bacterial blight under field conditions (Fig.1). The pathogen population divided in many groups in different countries based on local rice cultivars/ near isogenic lines (Lore et al. 2011). The pathogen population became virulent to most of the single BB resistance gene even some of the *Xoo* strains showing virulence on pyramided genes such as *Xa4+xa5*, *Xa4+Xa21*, *xa5+Xa21* and *xa13 + Xa21* in India (Lore et al. 2016). The contrast interaction was also found of some recessive R genes with *Xoo* strains prevalent in across South and South-East Asia. Pyramding of two and more R genes has been done in mega rice varieties through marker assisted back cross breeding approach (Singh et al., 2001).

Pathogen Population:

The pathogen *Xoo* is highly variable and knowledge on pathogen population structure is an important for designing a scientific disease resistance breeding program. More than 30 races/pathotypes of the bacterium have been reported worldwide (Adhikari et al. 1999). However, the group of races/pathotypes depends on the specific area or country of origin. Ten groups of *Xoo* strains have been identified from eastern India. Seven pathotypes of *Xoo* have been reported in Punjab (Northern India) based on the

near isogenic lines (NILs) (Lore et al. 2011). Furthermore, three new races of *Xoo* have been reported which broken down the resistance of rice cultivar PAU 201 and PR120 including BB resistance genes *xa13*, *Xa21* and new gene *Xa38* in Northern India (Lore et al., 2013). These pathotypes showed virulence to almost all the single R gene viz. *Xa3*, *Xa4*, *xa5*, *Xa7*, *xa8*, *xa13* and *Xa21* and some of the pathotypes virulent on pyramided line viz. IRBB-50 (*Xa4+xa5*), IRBB52 (*Xa4+Xa21*), IRBB54 (*xa5+Xa21*) and IRBB55 (*xa13+Xa21*). A total of 22 pathotypes have been identified from 400 *Xoo* strains spread in diverse rice growing region of India (Laha et al., 2016).

Screening and Bacterial blight resistant varieties: Maintenance and purification of the pathogen/pathotypes is the key factor for screening of rice germplasm. Single colony purified culture approximately 10^9 cfu/ml has been used for screening and inoculation done by 'clip inoculation technique' (Kauffman *et al.*, 1973) at the maximum tillering stage of the crop (Fig.1). Disease assessment is done by using 0-9 scale (SES-IRRI) or based on lesion length (Resistant <5 cm, moderately resistance 5-10 cm, moderately susceptible 10-15 cm and susceptible >15 cm).

Bacterial blight resistance genes viz. *Xa4*, *xa5*, *xa13* and *Xa21* are widely used under the disease resistance breeding program in South and South-east Asia. The R gene *Xa4* has been widely used in breeding program for develops of resistance in high yielding rice cultivars of Asian. IR20 was the first high yielding bacterial blight resistant rice variety carrying *Xa4* gene, released during early 1970s (Mew et al., 1992). The high yielding cultivars with *Xa4* genes were grown in large scale areas in South and South-East Asian countries. These varieties were breakdown by the new emerging races of *Xoo* after 1980s (Mew et al., 1992).

Systematic work to identify resistance donor under artificial inoculation conditions was started in 1978 at PAU Rice Research Station, Kapurthala. Consequently, many effective donors were identified against bacterial blight to be further used in resistance breeding program (Raina *et al.*, 1979; Lore *et al.*, 2004) resulting in release of a number of bacterial blight resistant and high yielding varieties PR109, PR110, PR 111, PR 113, PR 114, PR 115, PR 116, PR 118, PAU 201, PR 120, PR 121, PR 122, PR 123, PR 124, PR 127, Punjab Basmati 3, Punjab Basmati 4 and Punjab Basmati 5 in Punjab (Table 1). Resistance of rice varieties was broken down by emerging new race of the pathogen. However, cultivars viz. PR 121, PR 122, PR 123, PR 124, Punjab Basmati 3, Punjab Basmati 4 and Punjab Basmati 5 carrying pyramided BB resistance genes *Xa4*, *xa13* and *Xa21* and showed broad spectrum resistance to all the ten pathotypes prevalent in Northern India (Table 1).

Using molecular marker technology, diverse bacterial blight resistance genes *xa5*, *xa13* and *Xa21* have been pyramided in PR106 and Pusa44 background at PAU, Ludhiana (Singh et al. 2001). The PR106 pyramided lines showed resistance to all the pathotypes of bacterial blight pathogen in Punjab. New Basmati rice cultivars Punjab Basmati 3, Punjab Basmati 4, Punjab Basmati 5 and non-Basmati rice cultivars PR 121,

PR 122, PR 123 and PR 124, has been developed using MAS breeding and released during the year 2013 to 2017.

Sheath blight: Sheath blight (ShBl) caused by *Rhizoctonia solani* Kuhn [Teleomorph *Thanatephorus cucumeris* (Frank) Donk] is a major disease of rice (*Oryza sativa* L.) globally and it causes important yield losses, under input intensive crop management and favorable environmental conditions (Savary et al. 2000). This disease appeared in low to moderate intensity on all the cultivars growing in the state but this severity has increased at higher level in India during past decade. Now the disease is ranked one of the major fungal diseases of rice in Punjab. The characteristic symptoms of this disease are lesions starting on the sheath at the base of the culms near the ground level and progressing upward. The pathogen has a very wide host range and major gene(s) which give complete resistance in rice against this disease have not been identified (Pinson et al. 2005; Srinivasacharya et al. 2011) but variation in quantitatively inherited resistance to *R. solani* in the field in the cultivated rice is known. To date, only partial resistance to rice sheath blight has been identified in the cultivars of *O. sativa* (Pinson et al. 2005; Srinivasacharya et al. 2011). In addition, some other *Oryza* species have been reported as source of resistance to this disease (Lore et al. 2011). The inoculation techniques such as placement of *Typha* spp. bits in whorl of each plant, the colonized rice straw or grains on sheath among the tillers or hills in the field were used by various workers (Ou 1985). Evaluation of sheath blight resistance is based in general on the field ratings of lesion height above the ground level when the plants are in the reproductive stage. However, some *Oryza* species have an open growth habit, not favorable for sheath blight infection in the field and may escape infection during screening in the field.

Host plant resistance represents a key approach for disease management. Screening for host plant resistance to rice sheath blight has been conducted for many years in different research centres, including IRRI, where more than 30,000 accessions have been tested (Mew et al. 2004). Over the past decade, screening of rice germplasm, wild species, as well as mapping of QTL associated with disease resistance, have been conducted, using an array of approaches. In spite of these efforts, no rice variety with high level of resistance is currently deployed in Asia (Srinivasacharya et al. 2011). Resistance to sheath blight is associated with quantitative, complex, traits (Pinson et al. 2005; Srinivasacharya et al. 2011). Only partial resistance against this disease has been reported. Efforts for donor identification and screening methodology development for host-plant resistance to sheath blight are in progress. Cultivar PR108 released in 1986 has tolerance to sheath blight and white backed plant hopper. Detached tiller technique for sheath blight screening has been standardized with collaborative efforts by PAU and IRRI (Willocket et al, 2011).

Screening methods for sheath blight resistance: There are four methods of screening for sheath blight resistance in rice. i) **Tiller detached method**, tillers are collected from plants at maximum tillering stage and inoculated using by single sclerotium. The sclerotium is placed inside the second fully expended leaf sheath (Fig 2a). The inoculated tillers are put in test tubes containing distilled water. These tillers are

sprayed with distilled water and covered with plastic for maintaining the humidity and put at 28 ± 2°C. The progression of lesion height (mm), lesion expansion, number of lesion and disease severity is recorded 7d after inoculation (dai). ii) **Sheath detached method**, sheaths are collected from plants at maximum tillering stage and inoculated using by single sclerotium inoculation method. The inoculated sheaths put in plastic tray and covered with plastic for maintaining moisture and put at 28 ± 2°C (Fig2b). The progression of lesion height (mm), lesion expansion, number of lesion and disease severity is recorded 4d, after inoculation. iii) in **Micro-chamber method**, seeds of different genotypes are sown in pots. 3-4 week old seedlings (3 seedlings / pot) are inoculated with mycelial disc and the seedlings covered with plastic bottle for maintaining the humidity. The progression of lesion height (mm), total length of culm, number of lesion type3, presence or absence of the lesion on leaf blade and number of dead leaf / seedling are recorded 7dai (Fig2c). iv) **Maize-meal sand medium** with 10 days growth of the pathogen is used @ 5 g per hill under field conditions. Disease assessment is done using 0-9 scale at 15 days after inoculation.

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Fig.1 Collection, maintenance, isolation and preservation of *Xanthomonas oryzae* pv. *oryzae*

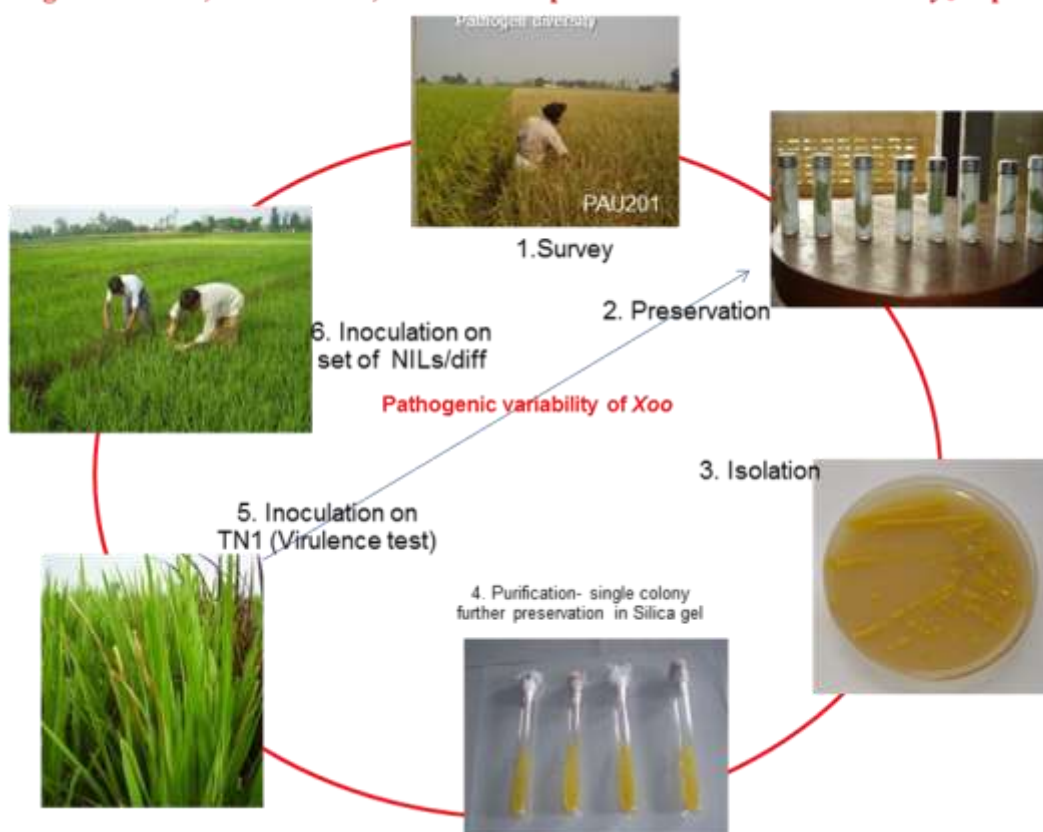


Fig 2. Different methods of screening for sheath blight resistance in rice. 2a. Tiller detached, 2b. Sheath detached, 2c. Micro-chamber

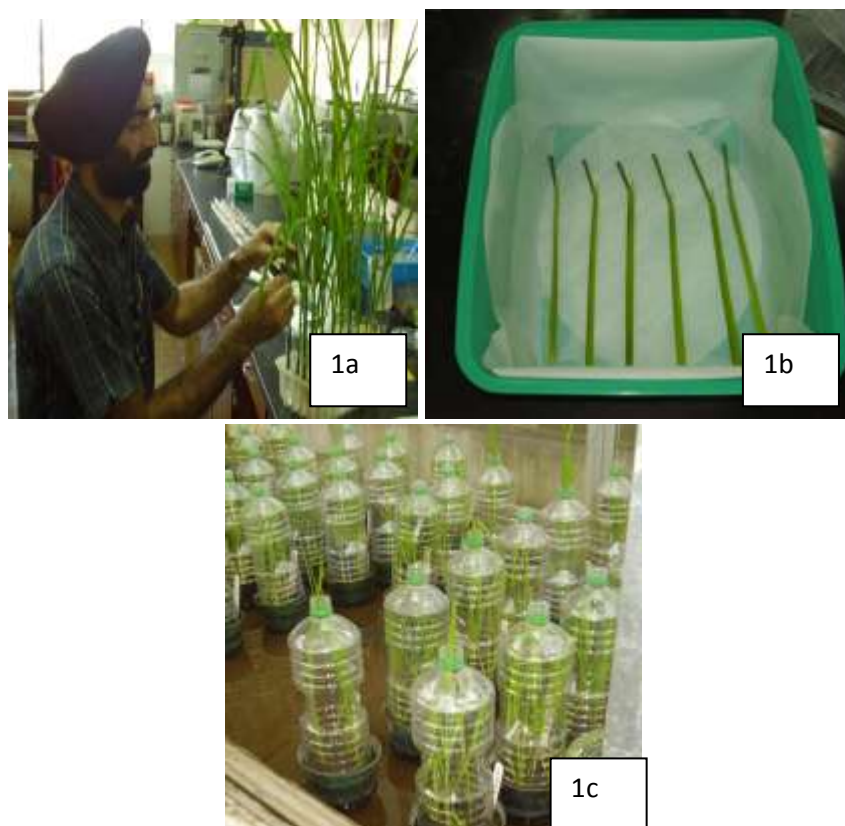


Table1. Release of BB resistant rice cultivars and their breakdown by emerging pathotypes

Variety	BB genes	Year	Yield (t/ha)	Grain type	Reaction to Pathotypes									
					PbX o-1	PbX o-2	PbX o-3	PbX o-4	PbX o-5	PbX o-6	PbX o-7	PbX o-8 Pi-902	PbX o-9 Tar 949	PbX o-10 Tar 950
PR109	Unknown	1986	6.5	LS	R	R	R	R	MR	R	R	S	S	S
PR110	Xa4 + ?	1992	6.5	LS	R	R	R	R	S	S	S	R	S	S
PR -111	Unknown	1994	6.8	LS	R	R	R	R	MR	R	R	R	S	MS
PR113	Unknown	1998	7.0	LB	R	R	R	R	R	R	R	R	MS	R
PR114	Xa4 + ?	1999	6.8	LS	R	R	R	R	S	S	S	R	S	S
PR115	Unknown	2000	6.3	LS	R	R	R	R	MR	R	MR	R	MS	R
PR116	Xa4 + ?	2000	7.0	LS	R	R	R	R	S	S	S	R	S	S
PR118	Xa4 + ?	2003	7.3	MS	R	R	R	R	S	S	S	R	S	S
PAU 201	Xa13	2007	7.5	LS	R	R	R	R	R	R	R	S	R	R
PR 120	Unknown	2008	7.1	LS	R	R	R	R	R	R	R	R	S	S
PR121	Xa4+xa13+Xa21	2013	7.6	LS	R	R	R	R	R	R	R	R	R	R
PR122	Xa4+xa13+Xa21	2013	7.9	LS	R	R	R	R	R	R	R	R	R	R
P. Bas 3	xa13+Xa21	2013	4.2	FLS	R	R	R	R	R	R	R	R	R	R
PR123	Xa4+xa13+Xa21	2014	7.4	LS	R	R	R	R	R	R	R	R	R	R
PR124	Xa4+xa13+Xa21	2015	7.6	LS	R	R	R	R	R	R	R	R	R	R
PR127	New gene (Og)	2018	7.7	LS	R	R	R	R	R	R	R	R	R	R

IN PLANTA TRANSFORMATION IN PIGEONPEA

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Pigeonpea [*Cajanus cajan* (L.) Millsp.], also called red gram or tur, belongs to family Fabaceae and has $2n = 22$. It is a major pulse crop of India, accounting for than 80 % of the world pigeonpea production (Popelka *et al.* 2004). This crop has a variety of uses, e.g. it is consumed as dhal, used as fodder, fuelwood, green manure and construction of roofs etc. Several biotic and abiotic stresses limit the potential yield of pigeonpea, amongst which *Helicoverpa armigera* (legume pod borer) and *Maruca vitrata* (spotted pod borer) are the most important polyphagous pests (Shanower *et al.* 1999) with annual yield losses of US\$ 400 million (Anonymous 2007). Conventional breeding methods to develop insect-resistant pigeonpea varieties have not been successful because of limited sources of resistance in crossable germplasm and incompatibility barrier with wild species (Bhosale and Nawale 1985). In this regard, genetic transformation of pigeonpea using *cry* genes from *Bacillus thuringiensis*, particularly acting against lepidopteran insect-pests provide improved insect resistance (Dita *et al.* 2006, Eapen 2008).

Among different genetic transformation methods, *Agrobacterium tumefaciens*-mediated transformation is widely used to generate transgenic crops, for which *in vitro* plant regeneration is a pre-requisite. Legumes, in general, are difficult to regenerate under *in vitro* conditions coupled with low regeneration frequency (Geetha *et al.* 1999, Lawrence and Koundal 2001). Although regeneration of shoots from different explants of pigeonpea through somatic embryogenesis and organogenesis has been reported (Krishna *et al.* 2010), however their applicability for genetic transformation is limited due to genotypic-specificity (Mohan and Krishnamurthy 1998) and long time taken for *in vitro* shoot differentiation, leading to somaclonal variation (Clough and Bent 1998). Rao *et al.* (2008) and Ramu *et al.* (2011) transformed pigeonpea following a simple *Agrobacterium* transformation method that did not involve *in vitro* shoot differentiation called as in planta transformation method. The method comprised of pricking pigeonpea seeds at the embryo axes, treating with *Agrobacterium* and sowing in soil for generation of T₀ plants. This method has now become a method of choice as it produces transgenic pigeonpea plants in a large number. A major problem with in planta transformation is the development of chimeric plants that has to be overcome through stringent selection (Mayavan *et al.* 2013, Subramanyam *et al.* 2013). Clough and Bent (1998) devised floral dip method of transformation by simply dipping developing floral tissues into a solution containing *Agrobacterium tumefaciens*, sucrose and a surfactant, that developed transgenic plants at a high frequency in *Arabidopsis*. They also reported that majority of the transformants produced through floral dip transformation method are non-chimeric.

Methodology for in planta pigeonpea transformation using embryo axes

Preparation of plant material

- Take mature seeds of pigeonpea

- Surface sterilize in 70 % ethanol (v/v) for 1 min followed by 0.1 % mercuric chloride (w/v) treatment for 7-10 min in a laminar air flow cabinet
- Wash with sterile distilled water thrice followed by overnight soaking in water with gentle shaking at 100 rpm on an orbital shaker
- De-coat the seeds and split them up
- Prick the exposed cotyledonary embryo axes with one cotyledon attached gently at the apical meristematic region once with a sterile needle and use for agroinfection

Preparation of *Agrobacterium* suspension

- Prepare *Agrobacterium* suspension by inoculating a colony of verified *Agrobacterium* strain carrying *cry* transgene in 10 ml liquid YEM medium (Vincent 1970) [Yeast extract (0.4 g/l), mannitol (10 g/l), NaCl (0.1 g/l), MgSO₄·7H₂O (0.2 g/l), K₂HPO₄·3H₂O (0.5 g/l); pH 7.0] supplemented with appropriate antibiotic
- Incubate the culture overnight at 28 °C with shaking at 120 rpm on an orbital shaker to obtain a suspension with optical density (OD) of 0.6 at 600 nm (Lin et al. 2009)
- Re-suspend the bacterial culture in 100 ml YEM medium, grow for 18 h and pellet the bacterial culture by centrifugation and suspend the pellet in half MS medium
- For *vir* gene induction, add acetosyringone to *Agrobacterium* suspension in medium 1 h before infection
- Immerse the prepared embryo axes in *Agrobacterium* suspension for 1 h, and then wash the embryo axes briefly with sterile water and blot dry on sterile filter paper
- The transformation is performed aseptically *in vitro* in a laminar air flow cabinet
- Subsequently, sow the agro-infected cotyledonary embryo axes in seedling trays (50 per tray) containing potting mixture (soil and farm yard manure in 3:1 ratio).
- Incubate the trays in transgenic glass house at 25 ± 2 °C under 14 h photoperiod
- Repeat the experiment
- Moisten the germinating T₀ seedlings daily with water and spray Urea (0.05 % w/v) at weekly intervals and FeSO₄ + ZnSO₄ (0.1 % w/v) mixture fortnightly
- Collect T₁ seed of each T₀ plant separately
- Screen T₁ plants through PCR
- Advance PCR positive plants to T₂ generation, and characterize them further through RT-PCR, Southern blotting, ELISA and insect bioassay

Methodology for in planta pigeonpea transformation using floral dip method

- ✚ Dip pigeonpea inflorescence containing unopened buds in a solution containing *Agrobacterium tumefaciens*, sucrose and a surfactant.
- ✚ Repeat for 4 consecutive days

- ✚ After each agroinfection, cover the plants for 1 day to retain humidity
- ✚ Collect the seeds from pods formed from treated flowers.
- ✚ Raise T₁ plants and analyze through PCR
- ✚ Raise T₂ plants and analyze them extensively.

Observation to be recorded

- Number of PCR-positive plants, i.e. number of putative transgenic plants showing the presence of *cry* gene
- Number of RT-PCR-positive plants, i.e. number of putative transgenic plants showing the expression of *cry* gene
- Gene copy number of *cry* in transgenic plants based on Southern blotting
- Cry protein content in transgenic plants based on ELISA
- Larval weight (mg) before and after feeding, larval mortality (%) after 24 h interval until pupation based on *in vitro* insect bioassay experiment

Conclusion

Pigeonpea is susceptible to a large number of insects leading to its low yield. There is lack of resistance in the cultivated gene pool of pigeonpea, thus transgenesis is considered as an alternative to overcome the problem. Poor *in vitro* regeneration and poor root induction leads to low transformation frequency, and are major concerns for transgenic development in pulses including pigeonpea. *In planta* transformation provides a good alternative to tissue culture based transformation method.

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SCREENING TECHNIQUES FOR DISEASE RESISTANCE IN PULSES

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Pulses can play an important role in crop diversification, which is vital for sustaining the long term productivity of cereal based cropping system in Punjab. In last decade, development of short duration varieties of mungbean with synchronous maturity, reduced photosensitivity, high yield and disease resistance offered a great scope for their introduction as a catch cum cash crop between rice-wheat/potato cropping systems. Further expansion is linked with replacement of cereals by other *rabi* and *kharif* pulses; chickpea, lentil, mungbean, urdbean and pigeonpea in few identified niche areas of Punjab. A number of high yielding varieties of these pulse crops have been developed at PAU. However, production stabilization can only be achieved through improved protection technologies. Screening of germplasm, segregating generations and advanced breeding lines of pulses against major diseases is the most important component of pulse pathology programme. Other research activities are focused on devising suitable chemical and cultural methods for management of important diseases of pulses.

Development of screening techniques

As far as Pulses are concerned, following diseases are important, which have been witnessed in varying epidemic form time to time.

Crop	Important diseases	Causal organism
Chickpea	Ascochyta blight	<i>Ascochyta rabiei</i> (Pass.) Labr.
	Fusarium wilt	<i>Fusarium oxysporum</i> f.sp. <i>ciceris</i> (Padwick) Matuo and Sauto (FOC)
	Botrytis grey mould	<i>Botrytis cinerea</i> .
Mungbean, urdbean, soybean	Yellow mosaic disease	Mungbean Yellow mosaic virus, Mungbean Yellow Mosaic India Virus
Pigeon pea	Phytophthora stem blight	<i>Phytophthora cajani</i>
Lentil	Rust	<i>Uromyces viciae-fabae</i> (Pers.) J. Schroöt

Chickpea

PAU centre pioneered in developing screening techniques; cut twig, field screening and cloth chamber for Ascochyta blight of chickpea. These techniques have

profound place at national and international forums. For Botrytis grey mould (BGM), cut twig and growth chamber methods have been developed. Cut twig method is very much useful for screening of wild germplasm of chickpea.

Multiplication of pathogens for inoculations

For AB screening, isolate 8, race 3968 (Singh, 1990) is used as it is the most virulent isolate. For BGM screening, isolate 24, race 510 (Singh and Bhan, 1986) are used. Mixture of isolates, representing broad spectrum of AB pathogen population should be used. These isolates are maintained on PDA (200 g potato, 20 g dextrose, 20 g agar, 1 L H₂O) slants and multiplied on Potato dextrose broth (500 g potato, 20 g dextrose, 1 L H₂O) at 22°C (AB) and 25°C (BGM) for inoculations.

Ascochyta blight

Field screening technique

In this screening technique, test lines are grown in 2 m rows, 40 cm apart. Susceptible varieties L550 and C214 are planted as indicator-cum-spreader rows after every 8 test rows. To establish uniform disease for AB screening, all plants of the test entries are spray-inoculated with conidial suspension of *A. rabiei* at 4×10^4 mL⁻¹ in the evening at around 17:00 h in the first week of February each year when the crop is at the flowering and pod initiation stage, i.e. 85 to 90 days after seeding. Water sprinkling is done with sprinkler system operated by 7 H power diesel engine from day following inoculation for 10 min at 2 h interval from 10:00 to 16:00 h to maintain > 85% RH for 21 days. Disease symptoms start to appear 10–11 days after inoculation. Observations are recorded on a 1–9 rating scale (Gurha et al., 2003) during the last week of March.



Fig 1: Field screening technique for AB resistance

Cloth chamber screening techniques

Seeds of test material are sown in polyethylene pots (15 x 10 cm) filled with sandy-loam soil. Highly susceptible varieties C 214 and L 550 are used as indicator-cum-infecter plants. Twenty-five polyethylene pots containing 20-25 days old seedlings are grouped along with a pot of check variety C 214/ L 550 placed in 8-10 cm deep pit. Water is added in and around the pots in the morning and evening before inoculation. Inoculation is done by spraying spore suspension of *Ascochyta rabiei* @ 4×10^4 spores mL⁻¹. A moist 'Dasuti' cloth chamber supported by iron frame is then placed

over these pots. The bottom of the cage is buried in the soil to check any free passage of air into these chambers. The chambers are kept moist for 48 hrs during incubation period by spraying water at 90 minute interval during day time to maintain the relative humidity (RH) above 85% and the temperature near 21-22°C. After the incubation period, chambers are removed, but spraying of water is continued for another 13 days. The disease symptoms start appearing 7 days after inoculation. Observations are recorded at 13 days after inoculation.

Cut twig screening technique

Material derived from wide hybridization is tested by cut twig method. The delicate twig about 4-6'' are cut from the main plant wrapped in cotton plug and immediately immersed in the test tube containing fresh water. These test tubes containing cut-twigs were placed in a test tube stand and inoculation was done by spraying spore suspension 1×10^4 spores ml^{-1} . Rest of the procedure for development of disease is same as described under cloth chamber screening technique, however, favorable conditions (RH and temp.) can also be provided by placing these plants in the field where disease is being produced by field screening technique. The disease symptom started appearing 7-10 days and the observations were recorded on 1-9 scale after 13 days of inoculation.

Important Symptoms of AB

The symptoms appear as circular light brown spots on leaves, petioles, flowers, pods, branches and stem. The fungus forms fruiting bodies, the pycnidia, in concentric rings. The lesions on stem are elongated, sunken dark brown often girdling the stem.

Rating scale for AB

Grade	Disease intensity	Reaction
1	No disease visible on any plant	Highly resistant
1.1-3	Lesions visible on less than 10% of the plants, no stem girdling	Resistant
3.1-5	Lesions visible on up to 25% plants, stem girdling on less than 10% plants but little damage	Moderately resistant
5.1-7	Lesions present on most of the plants, stem girdling on 50% of plants	Susceptible
7.1-9	Lesions profused on plants, stem girdling present in more than 50% plants	Highly susceptible

Botrytis gray mold

Growth chamber screening technique

The test lines are sown in polyethylene pots (15x10 cm) containing sandy-loam soil in a glass house. One-month old plants of the test lines along with susceptible check

G543/H208 are transferred to growth chambers, watered and inoculated with spore suspension containing 1×10^4 spore ml^{-1} . These plants are covered with moist chambers (polyethylene bags supported by iron cage) for 144 hrs. During incubation period 8 hrs dark and 16 hrs light period was provided. Observations on disease incidence are recorded on 1-9 point rating scale after 6 days of inoculation.

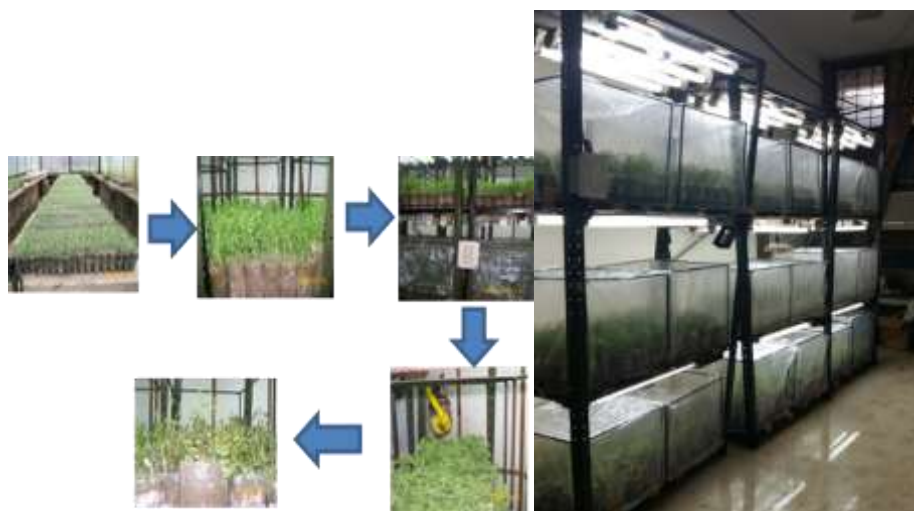


Fig 2: Growth chamber technique for BGM

Cut twig method

In the cut twig method, the tender shoots of the chickpea plants are cut in tray containing water, immediately wrapped in wet cotton plug and placed into a test tube (15 × 100 mm) containing fresh tap water. Three twigs are tested from each wild accession or derivative line. Twigs are inoculated by spraying spore suspension of *B. cinerea* (10,000 spores mL^{-1}) and covered with moist polythene covers. Incubation and rating is done as described for the growth chamber screening technique. Disease observations are recorded at 1–9 scale.



Fig 3: Cut twig method for BGM

Important Symptoms of BGM

Water soaked lesions one formed on leaves which then turn in to brownish black small round spots. The fungal growth is cottony white turning gray with age on stem and flowers/pods. Conidia borne in sporodochia are seen in humid conditions on pods and other tender apical parts.

Rating scale for BGM

Grade	Disease intensity
1	No infection on any part of the plant
2	Minute water-soaked lesions on emerging tender leaves, usually not seen
3	Minute water-soaked lesions on 1-5% emerging and upper most under leaves, usually seen after careful examination
4	Water-soaked lesions on 6-10% upper most tender leaves and tender shoots
5	Water-soaked lesions, soft rotting of 11-25% of tender leaves and shoots
6	Water-soaked lesions and soft rotting of 26-40% of top leaves and shoots
7	Soft –rotting and fungal growth on 41-55% of the leaves and branches
8	Soft-rotting, fungal growth on 56-70% of the leaves, branches and stems
9	Extensive soft rotting, fungal growth on above 70% of the leaves, branches and stems

Mungbean, urdbean and soybean

PAU, Ludhiana has been identified as hot spot location for yellow mosaic disease (MYMV) of mungbean, urdbean and soybean. Incidence of other foliar diseases (Cercospora leaf spot, bacterial leaf spot, anthracnose and web blight) is also observed on mungbean and urdbean in *kharif* season.

Yellow mosaic disease

Modified double infector row technique:

The test entries along with checks are sown in *kharif* season. Two rows (three meter length) of each test entry are sown between two infector rows of susceptible check SML 1082. YMD score is recorded on 1-9 scale (Singh and Bhan 1998) after first appearance of disease on susceptible check). Data is also recorded on per cent disease incidence (number of infected plants) and disease severity (number of infected leaves per plant). The formulas used for calculation of per cent disease incidence and disease severity were as follows:

$$\text{Per cent disease incidence (PDI)} = \left\{ \frac{\text{Number of diseased plants}}{\text{Number of total plants examined}} \right\} \times 100$$

$$\text{Disease severity} = \left\{ \frac{\text{Total number of leaves affected}}{\text{Total number of leaves per plant}} \right\} \times 100$$

Based on the disease severity, symptom severity grades, designated with numerical values of 0-4 and a scale of response value (0-1) corresponding to such grades was given to each entry. Coefficient of infection is calculated by multiplying the percent disease index to the response value. Disease reaction categories are assigned to each test entry on the basis of coefficient of infection as per scale given by Singh and Singh (2000).

Whitefly mediated inoculations under controlled conditions: Pure culture of whitefly (*Bemisia tabaci*) is maintained on cotton plants in pots and kept in cages made up of muslin cloth and black chart to provide dark condition for whitefly to establish on cotton plants. YMD infected plants from field are transplanted in pots and kept in insect proof cages. Non-viruliferous whiteflies from cotton are allowed to feed on YMD infected plant for 24 hours. After 24 hours acquisition access period, these viruliferous whiteflies are released on healthy mungbean plants of susceptible variety SML 1082 for 48 hours kept in insect proof cages separately. The inoculated plants expressing YMD symptoms are used as inoculum for screening. The viruliferous whiteflies are released on test entries for 48 hours of inoculation access period (IAP) at true leaf stage of seedlings. The YMD inoculum maintained on SML 1082 is also kept among test entries to enhance disease development. After IAP, whiteflies are removed and test entries are transferred to insect proof glasshouse. YMD score is recorded on 1-9 scale (Singh and Bhan 1998) after 2 days of inoculations and at every 2 days interval till 24 days.

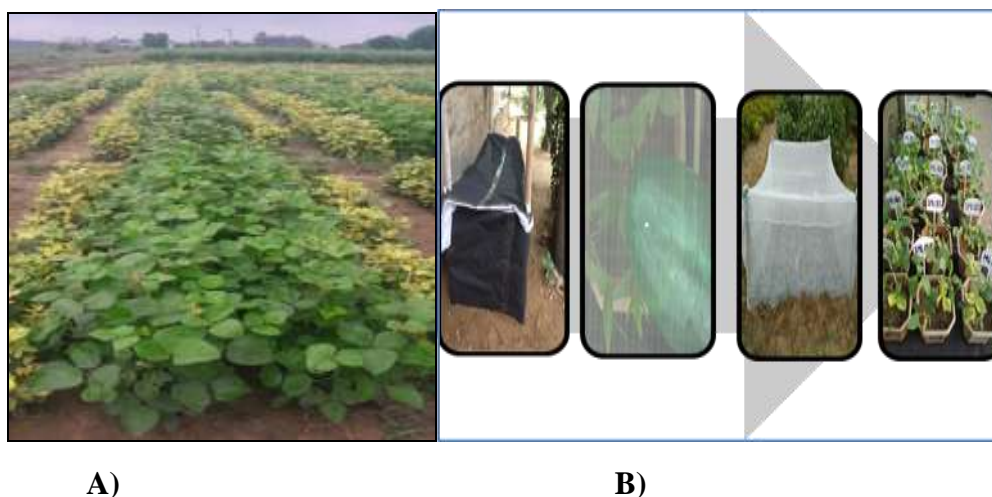


Fig 4: Modified double infector row and whitefly mediated inoculation technique
Important Symptoms of YMD

The YMD symptom appears on young leaves in the form of small yellow spots along the veinlet and later on covers the entire lamina. Later on, these spots expand and the whole leaf turns completely yellow. Generally the size of infected leaves is not much affected but sometimes puckering may be observed on leaves with reduction in size.

The diseased plant matures late and produces few flowers and pods. Infected pods show mosaic type of symptoms and their size is reduced.

Rating scale for YMD

Singh and Bhan 1998

Grade	Description	Reaction
1	No visible symptoms or minute yellow specks covering 1.1-5 per cent leaf area	Resistant(R)
3	Mottling of leaves covering 5.1-15 per cent leaf area	Moderately Resistant (MR)
5	Yellow mottling and discoloration of 15.1-30 per cent leaf area	Moderately Susceptible (MS)
7	Pronounced yellow mottling and discoloration of leaves and pods, reduction in leaf size and stunting of plants covering 30.1-75 per cent of foliage	Susceptible (S)
9	Severe yellow discoloration of leaves covering 75.1-100 per cent of foliage, stunting of plants and reduction in pod size	Highly susceptible (HS)

Scale for classifying reaction of YMD (Singh and Singh 2000)

Symptoms	Severity grade	Response value	Coefficient of infection (CI)	Disease reaction
Symptoms absent	0	0	0-4	Highly resistant
Very mild symptoms upto 25% leaves	1	0.25	5-9	Resistant
Appearance of symptoms in 26-50% leaves	2	0.5	10-19	Moderately resistant
Appearance of symptoms in 51-75% leaves	3	0.75	20-39	Moderately susceptible
Severe disease infection in symptoms (>75% leaves)	4	1	40-69	Susceptible
			70-100	Highly susceptible

Pigeon pea

Phytophthora stem blight (PB) is emerging as a potential threat to pigeonpea production, especially when excessive rainfall within a short span of time creates temporary flooding coupled with hot and humid weather. Field screening technique has been developed for screening against Phytophthora stem blight (PSB) of pigeonpea.

Field screening technique

Select a well-levelled, low lying isolated plot where flood irrigation is possible. Collect debris of PB infected pigeonpea plants and store it under dry conditions for use in the following season. Plant 2 rows of test lines (5 m long rows), spaced 40 cm apart following normal agronomic operations. Plant a highly susceptible cultivar such as AL 201 as indicator-cum- spreader rows after every four rows of test lines. Scatter the infected plant debris in the field at the time of sowing in furrows and maintain the soil moisture by irrigation. When crop is two month old, scatter fresh inoculum from early sown susceptible lines. Maintain the water logging conditions after 45 days of inoculation. Arrange an irrigation system as a standby. This must be used if dry weather prevails at the normal time of infection. Record disease incidence and repeat the entire process till the susceptible check shows >70% incidence.

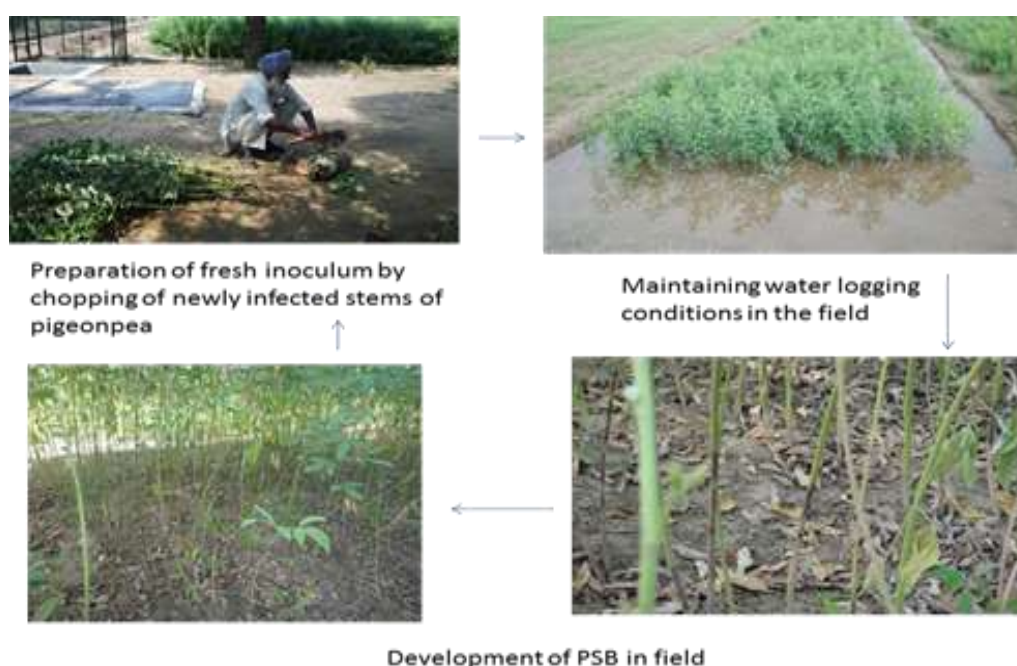


Fig 5: Field screening technique for PSB

Lentil: Among various diseases of lentil, rust is very dangerous. It causes about 25% to complete failure of the crop (Negussie and Pretorius, 2008). Rust symptoms appear on all aerial parts of the plant. Lentil rust is autoecious in nature and completes its life cycle on only one host. Regional Research Station, Gurdaspur (Punjab) serves as hot spot location for lentil rust. Infector row technique is used for screening against lentil rust.



Fig 6: Infector row technique for screening against lentil rust

Important symptoms of Lentil rust: The disease appears during flowering and early podding stage. First, Yellowish-white spermagonia appear on the abaxial surface of the leaflets and pods. Aecia develop singly or in small circular groups on abaxial surface and turn light brown eventually. Circular to oval shaped brown uredinia develop on both abaxial as well as adaxial surfaces of leaves, pods and stems.

Rating scale for lentil rust (Husain *et al* , 2008)

Grade	Typological description	Reaction
1	No pustule visible	Highly resistant
3	Few scattered pustule, usually seen after careful searching	Resistant
5	Pustules common on leaves and easily observed but causing no apparent damage	Moderately resistant
7	Pustules very extensive on all plant	Susceptible
9	Pustules very extensive on all plant parts, some death of leaves and other plant parts	

Contribution of Pulse pathology in varietal improvement programme - Disease resistant varieties

In chickpea, PBG 1, PBG 5 and PBG 7 are resistant to AB. Two varieties GPF 2 and PBG 1 are resistant to both AB and wilt complex. LL 931 and LL 699 varieties of lentil are tolerant to AB and rust. In mungbean, SML 1827, ML 2056, ML 267, ML 613, ML 818 and in urdbean, Mash 114, Mash 338 and Mash 1-1 are resistant to YMD.

Resistant donors identified at PAU centre

Over the years, several resistant donors have been identified in different pulse crops. Presently, our centre has maintained about 80 and 150 genetically diverse,

multiple disease resistant donors in chickpea and mungbean, respectively. In chickpea, two germplasm lines;

GL 84100 and GL 87045 have been registered with NBPGR with accession numbers INGR 13008 and INGR 13009 respectively for AB resistance. Two wild derivative lines; GLW 69 and GLW 91, derived from an interspecific cross (ICCV96030 x *C. pinnatifidum* 188) have been identified as resistant sources of AB and BGM. Recently, four mungbean lines viz., ML 1194, ML 1299, ML 1354 and ML 1464 have been deposited to NBPGR for MYMV resistance. The identified donors for resistance to different diseases in chickpea and mungbean are given in Table 1.

Table 1: Donors identified in chickpea and mungbean

Crop	Disease	Identified donors
Chickpea	AB	<i>Desi</i> : GL: 23094, 26083, 26105, 28295, 10006, 29098, 21107, 84100, 87045, 90168, 91058 <i>Kabuli</i> : GLK: 26167, 24092, 24096, 26153, 26167, 26171 Wild derivative lines : GLW : 69, 91 Wild accessions: <i>C. judaicum</i> : 182, 185, 185 B, ICC 17148, ILWC 61, <i>C. bijugum</i> ILWC7/S-3, <i>C. pinnatifidum</i> :199, 188, <i>C. echinospermum</i> 35/S-1
	Wilt and root rots	GL : 22044, 24080,26054 (<i>desi</i>), GLK: 22117, 23001, 26171, 28127 (<i>Kabuli</i>)
	BGM	<i>C. judaicum</i> 182,185, ILWC 61, ILWC 19-2, ILWC7/S-3, <i>C. pinnatifidum</i> 199, 188, ILWC 9, ILWC 212, <i>C. echinospermum</i> ILWC 39, 35/S-1 9, GLW 69, GLW91
Mungbean	YMD	ML: 11, 319, 729, 818, 1108, 1109, 1129, 1165, 1194, 1229, 1256, 1259, 1262, 1263, 1265, 1270, 1278, 1290,1294, 1299, 1349,1352, 1354, 1361,1392, 1409, 1430,1451, 1459, 1464, 1469, 1474, 1582, 1720, 1707, 708, 1722, 1732, 1741, 1751,1921, 1946, 2000, 2004, 1946, 2037, 2056, 2060, 2078, 2081, 2082, 2083

AB- Ascochyta blight, BGM- Botrytis grey mould, YMD- Yellow mosaic disease

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EXPLOITING WILD RESOURCES FOR DROUGHT TOLERANCE IN SUNFLOWER

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Abiotic stresses not only determine the geographic and regional distribution of crops but also dictate if a potentially arable piece of land can actually be used for cultivation. According to an estimate, 24.2% of the world's geographic area is potentially arable. However, only 10.6% of the geographic area is under actual cultivation, while the rest is not available for cultivation due to one or more abiotic stresses (Singh, 2000). According to the same author, drought is the main abiotic factor, as it affects 26% of the arable area. Mineral toxicities/deficiencies are second in importance, while frost stands third. Drought is the most limiting of all abiotic stresses and it affects well over one third of the soils worldwide. Plants that manage to survive the effects of drought stress show a decrease in fertility, yield and product quality (Monti, 1986).

Sunflower is grown in a number of countries on so-called marginal soils, often in semi-arid conditions where almost every year an abiotic stress of one kind or another is present acting as a limiting factor on crop production. However, of all field crops, sunflower is best able to withstand stress conditions, primarily on account of the structure of its organs (Škorić *et al.*, 1988). Fick and Miller (1997) reported that sunflower is considered moderately resistant to drought and is often grown in hot, semi-arid climatic regions. Breeding for resistance to drought and high temperatures is an important objective in many sunflower programs. Drought is the main cause not only of differences between mean yield and potential yield but also of yield variations from year to year and therefore of yield instability (Monti, 1986).

DROUGHT: AN INTERNATIONAL DILEMMA IN SUNFLOWERS

It has been found that a world wide reduction in sunflower yield has been associated with drought (Dragovic and Maksimovic, 1995). According to a report, oilseed production in 2005 was down 2 percent from 2004 due to drier than normal weather. In Spain sunflower crops suffered substantially from the effects of drought with a 41 percent decrease in production (USDA, Agriculture Weather Facility). In Pakistan, a crippling drought for a period of three years from 2000-2002 resulted in a major reduction in sunflower area and production (Rauf and Sadaqat, 2007a). Similarly, Bosnjak (2004) analyzed water availability over a period of 39 years and found drought to be a predominant feature in summer, the results of which up to 50% yield losses in sunflower that could have been obtained in irrigated conditions. He also predicted that severe droughts may be expected in the future with global climatic changes.

EFFECTS OF DROUGHT STRESS

Sunflower is categorized as a low to medium drought sensitive crop. It has been found that both quantity and distribution of water has a significant impact on achene and oil yield in sunflower (Fereres *et al.*, 1986; Andrich *et al.*, 1996; Krizmanic *et al.*, 2003;

Reddy et al., 2003; Iqbal et al., 2005;). However, oil quality of sunflower has not been significantly affected by drought treatment (Petcu et al., 2001). Intensity of yield reduction by drought stress depends on the growth stage of crop, the severity of the drought and tolerance of genotype (Lorens et al., 1987). Although drought stress affects every developmental stage of sunflower, maximum reduction in yield was experienced when drought occurred during the reproductive stage (Karaata, 1991; Reddy et al., 1998; Reddy et al., 2003; Vijay, 2004). Karaata (1991) conducted experiments to identify highly drought sensitive growth stages of sunflower, and it was found that maximum reduction in yield occurred when drought was imposed during flowering. Similarly, Vijay (2004) studied the response of achene yield to irrigation at four stages: initial at 15-20 days after seeding (DAS), capitulum initiation (30-35DAS), flowering (50-60 DAS), and grain development (70-80 DAS). It was shown that maximum achene yield was obtained with irrigation at the flowering stage. Drought during the vegetative phase of the plants affects both final biological and economic yields. During vegetative development, it reduces the main stem height, stem diameter, number of nodes or leaves and leaf area (Agele, 2003; Turhan and Baser, 2004) while an increase in root length occurs at the expense of above-ground dry matter. This has been observed by a higher root-to-shoot ratio obtained under drought stress (Sobrado and Turner, 1986). The reduction in vegetative biomass results in lower plant surface area which reduces the radiation use efficiency and photosynthetic activities (Stockle and Kiniry, 1990; Badr et al., 2004; Germ et al., 2005). This finally lowers assimilation of photosynthates during the reproductive phase which reduces head diameter. Reduction in head diameter further decreases the number of rows per head and number of achenes per head and results in correlation of yield components to severity of drought (Feres et al., 1986; Gimenez and Feres, 1986; Alza and Fernandez-Martinez, 1997; Rauf and Sadaqat, 2007a). On the other hand, stress during the flowering stage causes abortion of ovaries, embryo, sterility of pollen and decreases in leaf area index. This reduces the number of achenes per head, 100-achene weight and fertile achenes per head (Baldini and Vannozzi, 1999; Reddy et al., 2003). It has been estimated that stress during the vegetative phase causes 15-25% yield reduction, whereas more than 50% reduction can occur if stress coincides with the flowering stage (Reddy et al., 2003). However, minimal damage has been found to occur if drought was imposed during the achene filling stage (Karam et al., 2007). At this stage, the plant responds to the stress by early, abrupt leaf senescence (Rauf and Sadaqat, 2007b) and mobilization of stem reserves to the developing achenes (Baldini and Vannozzi, 1999; Rauf and Sadaqat, 2007b). However, a reduction of 100-achene weight may occur due to lower photosynthate production as a result of excessive loss of leaves at this stage. At the cellular level drought stress causes shrinkage of cells, cell membrane injury, and production of free radicals that cause damage to the cellular apparatus (Terbea et al., 1995; Sgherri et al., 1996; Kang and Zhang, 1997).

BREEDING FOR DROUGHT TOLERANCE

Drought can be managed to minimize its deleterious effects. Agronomically, the best approach for the management of drought stress is to irrigate the field. Other drought management techniques include the use of mulches to reduce evaporation losses, better weed control, crop rotations, improved cultivation methods to enhance infiltration rate,

foliar sprays, efficient irrigation practices (e.g. drip, sprinkler) (Rachidi et al., 1993; Gajri et al., 1997; Unger and Howell, 1999). A basic philosophy for all these management practices is to relieve the deleterious effects of drought and to use the water efficiently. It has been found that all these agronomic practices can improve yield up to 15-25% under drought stress by improving water supplies (Edmeades & Bänziger, 1997). However, above mentioned agronomic practices increase costs and are dependent on input availability, infrastructure, access to market and skills in crop and soil management (Campos et al., 2004). As a result, such management practices cannot be exploited by the farmer with small holdings or who cannot afford these inputs. Drought can also be managed by modifying the plant morphology or incorporating some traits that help the plants to cope with drought stress successfully (Yordanov et al., 2000). Thus, genetic modification is usually the most successful and cheapest strategy to cope with drought. Since modifications within plant morphology and physiology introduced through breeding are heritable, once introduced into a breeding material it will be a permanent source of drought tolerance. Heritable changes within a crop with the aim of improving drought tolerance can be broadly considered as breeding for improved drought tolerance.

There are numerous strategies utilized in breeding for drought stress in a crop species such as induction of earliness for drought escape, modification of certain plant traits that leads toward drought resistance and introduction of drought tolerant traits associated with high yield. Strategy for breeding drought stress depends upon the intensity, frequency and timing of drought occurrence. Under typical Pakistani conditions rainfall is too low and agriculture is dependent on supplemental irrigations. To compound the problem, irrigation water availability has now become highly unpredictable due to a recent shortage of water. Since water availability is highly unpredictable and the sunflower flowering period is highly sensitive to drought, tolerance to drought at flowering must be developed to minimize the damaging effect of drought. A strategy to produce a high yielding genotype combined with traits leading toward drought tolerance during anthesis is desirable provided that traits conferring drought tolerance also show strong positive correlation with achene yield. A trait leading toward drought tolerance should be considered ideal if it shows strong positive correlation with achene yield (in case of sunflower), moderate to high heritability, cheaply and easily measured, and hopefully determined before flowering so that undesirable plants may be rogued before crossing.

GENETIC VARIABILITY: AN ESSENCE FOR DROUGHT TOLERANCE BREEDING

CULTIVATED GERMPLASM

Most cultivated hybrids or open pollinated varieties evolved under near optimum agronomic conditions and often have some common parentage and history of origin. Therefore, breeding for drought tolerance must expand genetic variability. This depends on the incorporation of diverse germplasm so that potential sources of drought

tolerance may be identified and subsequently incorporated to ensure yield when drought occurs. The first approach for the development of a drought tolerant line is to screen high yielding germplasm accompanied by superior yield contributing traits. It is likely that this germplasm may also contain extensive variation for stress tolerant traits (Vasal et al., 1997).

Over the years, experience of handling plant material for drought tolerance at different research institutes (CIMMYT and ICARDA) indicates that improvement in drought tolerance can be made by screening both local and introduced germplasm belonging to various origins (Beck et al., 1990; Edmeades and Bänziger, 1997). However, the usefulness of introduced material depends on the performance under local conditions. Therefore, it is important to make selection of introduced material under local conditions, and a decision should be made accordingly: either it should be used as the source populations per se or to cross them with locally adapted materials or both (Beck et al., 1990). Results showed significant variation existed between the sunflower genotypes for yield under drought stress. Fereres et al. (1986) conducted field experiments at Cordoba, Spain to evaluate the yield responses to drought of 53 sunflower genotypes and found substantial variability among genotypes both in dryland yield and under frequent irrigation. Similarly, water stress revealed a high genetic variability among traits related to water status, osmotic adjustment, root characteristics, gas exchange parameters, seedling traits and drought susceptibility index when a large number of sunflower genotypes belonging to diverse origins were evaluated (Seiler, 1994; Chimenti et al., 2002; Lambrides et al., 2004; Turhan and Baser, 2004; Kiani et al., 2007a; Rauf and Sadaqat, 2007a; Rauf and Sadaqat, 2008a).

WILD RELATIVES

Wild species are a potentially important source of abiotic tolerance; therefore, it may be desirable to introgress drought tolerant genes from wild relatives. The genus *Helianthus* is comprised of 51 species and 19 subspecies with 14 annual and 37 perennial species (Seiler, 2007). According to Korrell et al. (1997), wild sunflower contains considerable variability for disease, insect pest resistance, and tolerance of abiotic stresses like drought and salinity. Similarly, fatty acid composition and protein quality can also be modified by introgression from wild species.

For drought tolerance breeding, specie *Helianthus argophyllus* has been extensively used by the sunflower breeders (Baldini and Vannozzi, 1998; Baldini et al., 1999; Griveau et al., 1998). In comparison with cultivated sunflower inbred lines and inbred lines obtained by a divergent selection for physiological traits from *Helianthus argophyllus*, it was found that lines obtained from *Helianthus argophyllus* had the highest water use efficiency, the best drought susceptibility index (S), and high harvest index (HI) value under drought conditions (Serieys, 1997; Baldini and Vannozzi, 1998; Baldini et al., 1999). However, a large genetic distance between the cultivated and wild sunflowers has decreased the introgression of the drought tolerant gene from wild relatives (Mohan and Seetharam, 2005). In addition, introgression from most of the

wild species has also been found to be associated with the deterioration of oil yield in cultivated sunflower (Seiler, 2007)

STRATEGIES FOR THE GENETIC IMPROVEMENT OF DROUGHT TOLERANCE

IMPOSING DROUGHT STRESS

Since a field experiment assures optimum vigor, growth and reproduction of genetic material, for evaluations drought stress has been imposed in the field during the reproductive phase which has been considered as the best strategy for improvement of genetic material against this stress (Baldini et al., 1999; Rauf and Sadaqat, 2007a; Rauf and Sadaqat, 2008a). However, performance in the field under drought stress should be considered a cumulative effect of some other biotic and abiotic stresses. In sunflower, drought stress results in higher intensity of fungal diseases, inter genotype competition, bird attack, injuries due to heat stress, and reduced soil thermal conductivity when compared with irrigated conditions (Alexandrov and Koteva, 2001; Rauf et al. 2008b). A controlled experiment on the other hand provides better control over these variables, but does not indicate optimum growth, vigor and reproduction. In pot experiments soil masses are too low, while in hydroponic conditions growth is restricted due to toxicity caused by differential genotypic absorption of polyethylene glycol (Blum, 1979). Therefore, ideal experiments should be designed to optimize the results both in field and controlled conditions.

YIELD AS A SELECTION CRITERION

There are two thoughts regarding the selection for high yielding genotypes. According to one group, selection for high yield should be practiced under non stress conditions, where genotypes may show their full genetic potential and environmental effects are minimal. A genotype with alleles responsible for high yield under non stress conditions may also show promise under stress conditions due to its high residual yield (Blum, 1979). However, another group is of the opinion that selection for a high yielding genotype should be carried out under the target environment. The presence of significant genotype x environment (G x E) interaction in most studies shows the desirability of the second approach. A significant G x E means changes occur in relative performance of genotypes across environments. As a result, a genotype showing promise in one environment may not be exceptional in another environment. However, improvement by direct selection of yield under the target environment has been slow due to low narrow sense heritability of trait under stress. Since yield is a resultant trait that is maximized by cumulative effects of a large number of yield contributing traits. The contribution of these traits to the yield is expected to be different under variable environments. Based on this fact, better improvement of the yield may occur if selection is based on a yield contributing trait that shows highest association with yield in a specific environment. For drought tolerance breeding, emphasis should be on traits that confer drought tolerance in the plant and also show positive association with yield. Over the past decades, plant breeders have focused on

some traits that were incorporated to plant survival under stress conditions such as lower leaf canopy and reduced transpiration (Fischer and Wood, 1979; Karamanos and Papatheohari, 1999). Often, traits were not necessarily positively associated with high yield. As a result breeders continue to develop cultivars which yield poorly under stress conditions. Therefore, analysis of plant traits with significant effects on drought tolerance and high yield potential under stress conditions seems to be necessary (Richards, 2004).

APPROPRIATE TRAITS FOR DROUGHT TOLERANCE BREEDING

SEEDLING TRAITS

Selection of suitable genotypes on the basis of seedling traits has been considered a reliable technique for evaluating a large number of genotypes for drought tolerance (Tomar and Kumar 2004; Basal et al., 2005; Longenberger et al., 2006). The effects of stress, environment and genotype on seedling traits have been identified and well documented. Pace et al. (1999) reported that drought-stressed seedlings showed some increase in root length but a reduced diameter. Prior et al. (1995) showed that inadequate soil moisture reduced root elongation, while Plaut et al. (1996) found that soil moisture deficit reduced root length and density. A number of different seedling traits have been suggested as important relative to drought tolerance. These include lateral and tap root weight, lateral root number and root-to-shoot ratios (Cook 1985; Pace et al., 1999). Singh et al. (1999) adopted a simple method of screening germplasm lines by seedling survivability, which accurately discriminated between drought tolerance and susceptibility under artificial moisture stress conditions. Rauf et al. (2008a) conducted experiments under two moisture regimes to study their modifying effects on phenotypic expression and combining ability of seedling traits and their genotypic correlation with achene yield. The moisture stress regime was favorable for the expression of additive genetic variability. All seedling traits except shoot length showed a significant correlation with achene yield, which also signified their importance for improving achene yield under drought regimes. From a breeding point of view, it was shown that seedling recovery % and root weight were useful traits for evaluating sunflower genotypes for drought tolerance.

HARVEST INDEX

A higher harvest index was shown as a better translocation of photosynthates to the reproductive part under drought stress (Rauf and Sadaqat, 2008b). The suppressing effect of drought stress on the harvest index has been reported previously in sunflower, and only drought tolerant genotypes showed stability or increase in the value of this trait, indicating harvest index as a useful trait in discriminating between drought sensitive or tolerant lines (Baldini and Vannozzi, 1998; Fereres, 1986). The harvest index has also shown a significant positive relationship with achene yield under drought stress, signifying the importance of this trait for improving achene yield under stress conditions (Reddy et al., 1995; Baldini et al., 1999). Moreover, significant genetic variability and high narrow sense heritability for harvest index has been reported in

sunflower (Alza and Fernandez-Martinez, 1997; Baldini and Vannozzi, 1998; Reddy et al., 2003; Rauf and Sadaqat, 2008b). However, narrow sense heritability was found to be higher than achene yield within a drought stress environment but found to be lower than achene yield when calculated combined across environments showing specificity of this trait under drought stress (Alza and Fernandez-Martinez, 1997). Rauf and Sadaqat (2008b) compared high and low osmotic adjustment genotypes and found superiority of high osmotic adjustment genotypes due to higher harvest index.

DROUGHT SUSCEPTIBILITY INDEX

Drought susceptibility index (DSI) is calculated on the basis of a ratio obtained from yield under drought stress condition to the yield under non stress condition (Fischer and Maurer, 1978). It has been calculated to determine the difference of yield potential under irrigated conditions and yield under drought for each genotype (Vannozzi et al., 1999). A higher value of susceptibility index indicates higher susceptibility of a genotype to the stress. Conversely, Andrich et al. (1996) calculated drought resistance index on the basis of a ratio between the weights of the kernels of the mean achenes grown in un-irrigated and irrigated conditions, with a value close to 1 indicating resistant cultivars and a reduced ratio indicating drought sensitivity. A significant negative relationship or genetic correlation has been observed between the drought susceptibility index and yield under drought stress conditions, indicating that the drought susceptibility index may prove a very useful selection criterion for drought tolerance breeding in sunflower (Feres et al., 1986; Baldini et al., 1992; Alza and Fernandez-Martinez, 1997; Rauf and Sadaqat, 2007a). However, a significant correlation between the DSI and yield under non stress conditions was absent (Feres et al., 1986; Baldini et al., 1992; Rauf and Sadaqat, 2007a). This indicated that yield under irrigated conditions could not be considered a prediction of its yield under drought conditions, and that yield under irrigated conditions should not be used as a selection criterion to improve yield under drought (Baldini et al., 1992). Therefore, yield in low and high yielding environments can be considered as two separate traits that are not necessarily maximized by identical sets of alleles (Falconer, 1990). Consequently, plant breeding strategies should be different when targeting stress and non-stress environments (Ceccarelli et al., 1991; Ceccarelli et al., 1998).

ROOT SYSTEM

The root system of a plant is important when considering drought tolerance breeding, since certain root characteristics such as root length, root biomass, and lateral root density would determine the efficiency of water extraction from soil. A deeper root system would allow water extraction from lower soil profiles and, thus, it is expected that the plant will perform better under moisture stress when its growth is dependent on water stored deeper in soil. Sunflower has a deep and extensive root system that can extract water up to 270 cm (Gimenez and Feres, 1986; Connor and Sadras, 1992; Rachidi et al., 1993). Its root growth depends upon several factors such as plant genotype, water availability, plant population and soils. Limited research literature is available on the intraspecific variations for the root system in sunflower (O'Toole and Bland, 1987), since determination of root characteristics in the field is very laborious

and time consuming. Few reports have characterized heritable variation in sunflower for root length and any greater root length associated with longer growth duration (Feres et al., 1986; Schneider, 1992; Rauf and Sadqat, 2008b). Angadi and Entz (2002) compared root system characteristics and water extraction patterns of dwarf hybrids with hybrids of standard height. Most of the dwarf sunflower hybrids showed lower root length, root length density and root distribution, thus showing a positive correlation between plant height and root characteristics studied. Other studies showed effect of water availability on root characteristics. An increase in root length and root-to-shoot ratio in response to moisture stress along with the decrease in lateral root length as an adoptive measure for drought stress can be generalized from experiments conducted on sunflower (Petcu and Stanciu, 2001; Gomes et al., 2005; Rauf and Sadaqat, 2008b). Soil characteristics such as compaction and depth also have significant impacts on root growth. A decrease in root area and biomass had been observed with the increase in soil depth and compaction under moisture stress conditions (Petcu and Petcu, 2006). Soil compaction limits root growth by directly posing physical injuries and indirectly by reducing water infiltration. Petcu and Petcu (2006) showed maximum root area and biomass of sunflower in soil depth of 0-10 cm along with a progressive decrease in the values of both these traits as the depth and compaction increased under water stress.

LEAF HYDRAULICS

Leaves can be considered as a sink for water absorbed by the plant; therefore, leaf water status may accurately define the demand and supply of water. Characteristics of leaves that define moisture content of leaves may also accurately discriminate between drought tolerance and susceptibility. These traits include relative water contents, leaf water potential, turgor pressure, osmotic potential and stomatal conductance. These physiological traits are collectively called 'water relations'. In sunflower, genotypes selected for these elevated physiological traits under drought had greater vegetative growth, elevated water consumption and increased root-to-shoot ratio. They avoid drought stress by continued water uptake from deeper soil layers (Perniola, 1994; Baldini et al., 1997). Among the traits used for the evaluation of plant water-status, relative leaf water contents (RWC) gives best measure of the level of the water deficit in the plant at a specific time-point. As RWC is related to cell volume, it may closely reflect the balance between water supply to the leaf and transpiration rate (Sinclair and Ludlow, 1985). Significant variability and heritability has been reported in sunflower for this trait (Baldini et al., 1997). Other traits such as leaf turgor, water and osmotic potential are related to the osmotic adjustment (Perniola, 1994). Osmotic adjustment (OA) is a phenomenon in which plants accumulate a certain type of compatible solute under stress conditions to lower its potential. As a result of this lower potential, plant may regain its turgor and attract more water from the soil. Compatible solutes help in maintaining the cell turgor, protect against denaturing of important structural molecules and cell organelle, and their high concentration does not cause toxicity in the cell. There are many molecules which may be considered as candidates of compatible

solutes such as K⁺, Ca⁺⁺, proline, HSPs, and dehydrins. However, in sunflower large number of studies depicted that proline has been an important osmolyte under drought stress. It was shown that its content tended to increase in young leaves in response to drought stress (Terbea et al., 1995; Badr et al., 2004; Cechin et al., 2006). Significant genetic variability between genotypes has been observed in sunflower for OA (Chimenti and Hall, 1993; Chimenti and Hall, 1994; Angadi and Entz, 2002; Rauf and Sadaqat, 2008a). In sunflower, different studies have shown a significant relationship of OA with leaf turgor, RWC and root growth, which signified the importance of OA in maintaining leaf turgor and relative water contents through better root growth (Conroy et al., 1988; Rauf and Sadaqat, 2008a; Rauf and Sadaqat, 2008b). As a result, plants were able to maintain a photosynthetic rate and stomatal conductance at lower leaf water potentials (Maurey et al., 2000). The value of OA as a yield contributing trait has been established in sunflower. It is inferred from the studies that sunflower genotypes with higher OA extracted more water from the soil during the stress period due to greater root growth. They had greater shoot biomass and harvest index (HI) at physiological maturity and greater grain yield. There was no effect of OA on these variables in the irrigated controls (Chimenti et al., 2002; Rauf and Sadaqat, 2008a; Rauf and Sadaqat, 2008b).

MOLECULAR BREEDING FOR DROUGHT TOLERANCE IN SUNFLOWER

The advent of emerging and novel technologies of plant cell and molecular biology provided us a powerful means to supplement and complement the traditional methods of plant improvement. Therefore, in the recent past much of the research has been devoted to isolation and testing the expression of drought tolerant genes. Novel stress responsive genes were identified following subtractive hybridization of cDNA synthesized from RNA isolated from drought stress and unstressed plants. Identified genes related to drought tolerance such as SunTIP, HaDhn1, HaDhn2, Sdi (sunflower drought induced), Gdi15, Hahb-4 and HAS1 or HAS1.1 showed an abundance of their transcript levels under drought stress, and it was proposed that they play a role in the sunflower response to drought (Ouvrard et al., 1996; Cellier et al., 1998; Sarda et al., 1999; Gopalakrishna et al., 2001; Liu and Baired, 2003; Dezar et al., 2005; Herrera Rodriguez et al., 2007). Furthermore, they only showed expression in drought tolerant types when compared to drought sensitive genotypes (Roche et al., 2007). Expression of these genes was not confined to a single organ. However, gene sdis and Ha- RPS28 showed highest expression in fully expanded leaves and they were linked to the production of certain ACC oxidase antioxidant or dehydrins while HAS1 and HAS1.1 showed more expression in root when compared with leaves. A few genes were shown to effect the expression of other genes to induce drought tolerance. Manavella et al. (2006) found that plants over-expressing Hahb-4 were less sensitive to external ethylene and entered the senescence pathway later. Expression of this transcriptional factor has a major repressive effect on genes related to ethylene synthesis, such as ACO and SAM, and on genes related to ethylene signalling, such as ERF2 and ERF5. Drought stress triggers the production of abscisic acid. A part from its differential

effect on biomass partitioning (Rauf and Sadaqat, 2007b) it also mediates the expression of certain drought tolerant genes such as HaDhn2, Sdis (sdi1, sdi5, sdi9, sdi6, sdi8), Ha-RPS28, and Hahb-4 (Ouvrard et al., 1996; Cellier, 1998). However, Giordani et al. (1999) indicated the existence of two regulation pathways of HaDhn1a transcripts accumulation, an ABAdependent and an ABA-independent one, which may have cumulative effects. Its expression was not uniform during day/night cycle and maximum HaDhn1 transcript accumulated during the mid day time (Cellier et al., 2000). Genes related to drought stress such as dehydrins (Dhn1) have also been used to study the phylogenetic and genetic variability between cultivated and wild sunflower for this gene. The expressed protein showed diversification of biochemical properties between annual and perennial *Helianthus* species. However, cultivated sunflower contained lower genetic variability for dehydrin genes than wild sunflower (Giordani et al., 2003; Natali et al., 2003). It was also concluded that the stress responsive gene (Dhn1) can also be used to study the phylogeny of *Helianthus*. The concept of DNA-based markers has revolutionized our ability to directly access any part of the plant genome, and has led to new opportunities such as screening a large number of segregating population for QTL's related to the drought tolerance without actually exposing them to drought and independence of plant growth stage. In nature, the vast majority of physiological traits are quantitative trait loci (QTL). Quantitative traits show continuous phenotypic distributions affected by a large number of loci and show significant interaction with environment (Tanksley, 1993). However, in sunflower very few studies have been carried out to determine the development of a molecular marker for QTL's related to drought tolerance (Jamaux et al., 1997; Hervé et al., 2001; kiani et al., 2007b). Hervé et al. (2001) used the AFLP linkage map for the identification of QTL related to net photosynthesis, stomatal movements and water status (transpiration and leaf water potential). Using an AFLP linkage map, 19 QTL were detected explaining 8.8–62.9% of the phenotypic variance for each trait. Among these, two major QTL for net photosynthesis were identified on linkage group IX. One QTL co-location was found on linkage group VIII for stomatal movements and water status. Coincidental locations for QTL regulating photosynthesis, transpiration and leaf water potential were described on linkage group XIV. On the other hand, Kiani et al. (2007b) detected 24 QTLs under well watered conditions, of which 5 (about 21%) were also detected in the water-stressed treatment. The percentage of phenotypic variance explained by the QTLs ranged from 6% to 29%. Among the eight QTLs detected for OA, four of them (50%) were co-located with the QTLs for turgor potential (Psi(t)) on linkage group IV (OA.4.1), with the QTL for osmotic potential at full turgor (Psi(sFT)) in wellwatered RILs on linkage group VII (OA. 12.2) and with QTLs of several traits on linkage group V (OA.5.1 and OA.5.2). The four other QTLs for OA (50%) were very specific. However, they concluded that these QTLs were identified in greenhouse conditions and the usefulness of these QTLs for marker-assisted selection should therefore be evaluated under field conditions and validated in other genetic backgrounds. Similarly, Jamaux et al. (1997) identified RFLP and STS molecular markers of relative water loss and osmotic adjustment by RAPD bulked analysis.

CONCLUSIONS

- Owing to the structure of its major organs (root, stem, and leaves), sunflower is more resistant to abiotic stresses than other field crops
- In overall sunflower breeding for resistance to abiotic stresses, the greatest progress has been made in selection for drought resistance. This has been done using a large number of parameters, most of them physiological.
- The best method for sunflower screening for drought resistance is the use of the stay-green character.
- Wild *Helianthus* species are used as sources of genes for resistance to drought. The most widely utilized of these species is *H. argophyllus*, which has been used to develop new sunflower germplasm by interspecific hybridization. Several other wild sunflower species should be included in these efforts as well.
- The screening of cultivated sunflower genotypes, interspecific hybrids and wild sunflower species at the molecular level has given significant results lately.
- Sunflower resistance to salinity can be significantly increased using the wild species *H. paradoxus*.
- Sunflower selection for resistance to mineral deficiency and mineral toxicity has so far produced modest results.

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EVALUATION OF MULTIPLE INSECT-PEST RESISTENCE IN RICE- GREENHOUSE & FIELD STUDIES

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Introduction

Rice, *Oryza sativa* L. is the staple food for over half of the world's population. It accounts for 35-75% of the calories for more than 3 billion Asians. Globally, it provides 27% of dietary energy, 20% of dietary protein and 3% of dietary fat. Rice demand globally was 676 million tonnes in 2010, which will further rise to 852 million tonnes in 2035 due to change in food intake by the people. To meet this challenge rice production on existing land must be increased (Khush, 2013). India ranks first in the world in rice area with 43 million hectare with a production of 110 million tonnes, but our productivity is only 3.01 t/ha, which is below the world average. In Punjab rice is cultivated over an area of 30.65 lakh hectares with total production of 199.72 lakh tonnes (Anonymous, 2019). Among more than 100 species of insects known to attack this crop, about 20 are of economic importance (Pathak and Dhaliwal, 1981). The cumulative losses due to insect-pest damage in rice were estimated at 25 per cent (Dhaliwal *et al.*, 2010). It being a crop of warm humid environment and this is also conducive for development and proliferation of insect-pests. Among different insect-pests, stem borers like yellow stemborer, *Scirpophaga incertulas* (Walker); white stemborer, *Scirpophaga spp.* (Walker); pink stemborer, *Sesamia inferens* (Walker); sap suckers like brown planthopper, *Nilaparvata lugens* (Stal); whitebacked planthopper, *Sogatella furcifera* (Horvath); and leaf feeder like leaf folder, *Cnaphalocrocis medinalis* (Guenee) cause extensive damage to the crop (Sarao, 2015). Efficient screening against insect-pests is mandatory for introgression of resistant plant breeding material into pre-breeding or hybridization programmes to develop varieties resistant to insect-pests (Sarao *et al.*, 2017).

Types of host plant resistance

Painter (1951) described plant resistance as the relative amount of heritable qualities that influence the ultimate degree of damage done by the insect. In practical agriculture, resistance represents the ability of a certain variety to produce a larger crop of good quality than do ordinary varieties at the same level of insect population. He grouped mechanisms of resistance into three main categories, viz., nonpreference, antibiosis and tolerance. Kogan and Ortman (1978) proposed the term antixenosis to describe the host plant properties responsible for nonpreference. Antixenosis refers to undesirability i.e. avoidance by the insect whereas antibiosis refers to unsuitability i.e., adverse effects on the insect after feeding on the host plant, while, tolerance refers to the ability of the host plant to withstand an insect population sufficient to damage severely the susceptible plants. It is generally attributed to plant vigour.

Cultivars differ in their degree of resistance as there may be a gradation from extreme resistance to extreme susceptibility (Russell 1978). Resistance is classified as low, moderate or intermediate, and high. Resistance at different rice growth stages has different names. Seedling resistance is detected and measured in the greenhouse seedbox screening of seedlings. It may persist as the plants mature. Adult or mature plant resistance is manifested in older plants whose seedling stage may be susceptible. There are types of apparent resistance, not heritable, which should not be confused with true resistance. Painter (1951) used "pseudoresistance" to describe resistance due to transitory characters in potentially susceptible plants. The types he listed are:

- **Host evasion:** The host plant passes through the susceptible stage quickly or when insect populations are low. A crop which is in the field when insect populations are low or matures before the insect population reaches damaging levels shows host evasion.
- **Escape:** A particular host plant is neither infested nor injured despite the local presence of the insect pest. The study of progeny of such plants is establish their true expression of resistance or susceptibility.
- **Induced resistance:** Some environmental conditions, such as soil fertility or water temporarily increase the level of resistance.

Mechanisms of resistance

Mechanisms of resistance have been grouped into three categories by Painter (1951). Nonpreference/antixenosis and antibiosis refer to the response of the insect to the plant; tolerance refers to the response of the plant to the insect.

- **Nonpreference/antixenosis:** Nonpreferred plants lack the characteristics of hosts for insect feeding, oviposition, or shelter. Because nonpreference pertains to the insect rather than the plant host and is not parallel to antibiosis. Kogan and Ortman (1978) proposed the term *antixenosis* for nonpreference. Antixenosis (against the guest) means the plant is considered a bad host.
- **Antibiosis:** Antibiosis is an adverse effect on the biology (survival, development, or reproduction) of the insect.
- **Tolerance:** A tolerant plant can produce good yield even while it supports an insect population that would severely damage and decrease the yield of a nontolerant plant. Much genetic variability occurs within rice insect populations. Tolerance is useful in pest management programmes due to certain distinct advantages (Panda and Khush, 1995).
- Economic threshold level is high in tolerant varieties than the susceptible varieties, this require less insecticide applications and hence it promotes biocontrol in the ecosystem.
- Tolerant varieties do not provide any selection pressure and thus useful in preventing the development of insect biotypes.

- In varieties with all the three mechanisms, tolerance increase the yield stability by providing at least moderate level of resistance when vertical genes providing a high level of resistance through antixenosis and antibiosis breakdown to new biotype.

Importance of insect resistant varieties

The incorporation of insect resistance into modern varieties is a major objective of most rice breeding programs. Insect-resistant rice varieties are important for several reasons:

- Growing insect resistant variety is generally free to farmers as any amount spent for insect-pest controls with insecticides greatly lowers the farmer's profit.
- Prophylactic applications of insecticides without the knowledge of economic threshold level of insect-pest populations/damage add to the cost of production and environmental pollution. Even misuse of insecticides may cause poisoning accidents.
- Varietal resistance is compatible with all other control tactics like chemical control, biological control, and cultural control.
- Some predators are more effective against insect-pests feeding on the resistant varieties.
- Resistant varieties increase the susceptibility of insect pests to insecticides.

Germplasm screening protocol: The basic requirements for the efficient screening methods are:

i). Diverse germplasm: It consists of

- improved modern cultivars presently under cultivation
- unimproved or purified cultivars no longer in cultivation
- mutants developed by mutagenic treatments as well as those of spontaneous origin
- wild species
- land races

ii). Target insect-pest of synchronize stage: To start the insect culture, collect nymphs and adults from infested rice fields. Use an aspirator if the population is very low. If a large population is present, tap infested plants over a container or an insect net. Adults can be collected from light traps. Progeny of these collected insects can be used for the screening purpose reared on susceptible plant (TN1).

iii). Screening technique: Germplasm screening for insect-pest resistance can be done in the greenhouse or in the field. Greenhouse screening is the rapid method for evaluating a large number of breeding lines, but the resistant entries should also be screened in the field before releasing them as varieties. Special methods have been developed to increase field populations to provide sufficient insect pressure for valid screening. The different efficient screening techniques against different insect-pests are given below:

I). Greenhouse screening/ artificial screening

a). Plant and leaf hoppers: The conventional seedbox screening or standard seedbox screening test (SSST) test is a common method used for greenhouse screening. It is a rapid method of screening large volumes of material for qualitative resistance. This test is used for brown planthopper, whitebacked planthopper, green leafhopper and gall midge. In the SSST, seeds of test material are sown in a single row of 3.5 cm apart in a seedbox of about 60 × 40 × 10 cm size. Suitable susceptible and resistant checks are sown in similar rows in the same box (Table 1). Susceptible check (TN1) is sown as outer row which also act as spreader row, in the centre of the box half susceptible and half resistant material row is also sown. These boxes are placed in the water through or galvanized iron trays containing water.

Table 1. Varieties/ lines used for screening insect-pest resistance

Insect	Susceptible check (s)	Resistant check (s)
Brown planthopper	TN1	Ptb 33, RP 2068, Rathu Heenati
Whitebacked planthopper	TN1	MO 1, Ptb 33, IR 2035-117-3, ARC 6248
Green leafhopper	TN1	Vikramarya, IR29
Stem borer	TN1, Rexoro	TKM 6, IR 40, W 1263
Leaf folder	TN1	TKM 6, W 1263

Seven days after sowing, seedlings is thinned to about 20 plants per row and infested with about 8-10 (WBPH) and 3 (GLH) 2nd – 3rd instar nymphs per seedling. In case of BPH twelve day old seedlings is infested. When susceptible checks is killed (usually after about 1 week), damage score on each line is recorded on 0-9 scale, when 90 to 100 per cent plants of TN 1 is dead following Standard Evaluation System (SES) of rice (Anonymous, 1996) (Table 2). Mean score of each line is classified as resistant (0-3.49), moderately resistant (3.50-5.49) and susceptible (5.50-9.00) following Sarao *et al.*, (2016).

A second method, the modified seedbox screening test (MSST), is used to overcome some limitations of SSST and for better understanding of “field resistance,” that is, when resistance is maintained or increases as plants age. In this method other practices are similar to that of SSST, but plants are infested at 20 days after sowing with 3 to 5, 2nd – 3rd instar nymphs per plant. Cover the whole seedbox of infested seedlings with a screen cage (65 × 45 × 90 cm) to prevent the insects from escaping from the tray. Plants are evaluated at the time that susceptible checks are killed, using the same scale as in the SSST. In MSST, usually mortality of the susceptible check is caused by F₁ population of planthoppers, that is, the original nymphs mature and reproduce in the seedbox, and their offspring kill the plants (Velusamy *et al.*, 1986). These two methods have been extremely useful for rapid, high-throughput, and inexpensive screening of the necessarily large amounts of material required to find and

isolate resistance genes. Furthermore, they incorporate “free choice,” that is, the target insects can choose between the different varieties under test before initiating feeding (SSST and MSST) or oviposition (MSST) behaviour.

Table 2. Standard Evaluation System (SES) of rice against planthoppers and leaf hopper

Scale	Seeding damage		
	BPH	WBPH	GLH
0	No visible damage	No visible damage	No visible damage
1	Partial yellowing of first leaf	Very slight damage	Slight yellowing of first leaf
3	First and second leaves of most plants partially yellowing	First and second leaves with orange tips; slight stunting	First and second leaves yellowing
5	Pronounced yellowing & stunting or about 10-25% of the plants wilting or dead	More than half the leaves with yellow orange tips: pronounced stunting	All leaves yellow, pronounced stunting or both
7	More than half of the plants wilting or dead and remaining plants severely stunted or dying	More than half of the plants dead; remaining plants severely stunted and wilted	Half of the plants dead: remaining plants severely stunted and wilted
9	All plants dead	All plants dead	All plants dead

b). Yellow stemborer

In case of Yellow stemborer, to obtain larvae for artificial infestation, egg masses from the field or adult females in light traps at night must be collected. The germplasm lines 14 days after transplanting (DAT) must be infested with ten first instar larvae/hill. A fine camel hair brush must be used to place the larvae on the youngest leaf or auricles of one tiller.

c). Leaf folder

1. Apply additional 40 kg Urea/ha on 30, 40 & 50 DAT than the recommended dose to get higher leaf folder infestation.
2. At 25 DAT, cover testing entries with a nylon net and release leaf folder adults. Collect adults from neighbouring fields or laboratory/ glass house culture. Release adults two times, once at 30 DAT and second at 40 DAT @ 100 adults per release. Let the adults remain inside the net to lay eggs for a week and then remove the net.
3. Take observations twice, at 60 DAT and 80 DAT. In each entry, select 10 plants at random. Count the total number of leaves and damaged leaves (consider as

damaged leaf only if one-third of the leaf area is damaged). Calculate per cent damaged leaves in each entry.

d). Gall midge

1. Sowing time should be so that the seedlings is of 10-15days old when adults intended for infestation emerge.
2. At 12-15 DAS, cover the seedlings with nylon mesh to prevent escape of adults. Infest each box with 30 females and 15 males.
4. Transfer the seedboxes to a mist chamber 2 days after infestation (DI). If a mist chamber is not available, transfer the seedboxes to an open shallow tray and spray water every 2h during the day for 2 days.
5. Five days later (or 7 DI), transfer the seedboxes to a water pan tray and raise the water level to 3 cm above the base of the plant. This will prevent parasitization and predation of the gall midge larvae.
6. At 20-30 DI, examine plants and determine total number of plants and number of infested and healthy plants.

II). Field Screening protocol

For greenhouse and screenhouse screening, insects are usually reared and plants artificially infested. Some difficult-to-rear insects can be collected in the field or at lights at night. These insects themselves, or their progeny, can serve for screening studies. It is done by either taking the test varieties to the hotspots or by manipulating the cultural or other practices to increase the insect-pest population in the field.

1. Techniques for field evaluation of rice for insect-pest resistance have been done by taking the testing material to the fields (hot-spots) where population of these insect-pests will be high. Select a screening site and time of planting when insect-pest population will be abundant. In Punjab field screening for WBPH resistance is done at Patiala.
2. A major portion of the screening plot can be planted to a highly susceptible variety as a border row, this will build up the population. To increase the chance of insect buildup, susceptible plants are infested with artificially reared or field-collected insects.
3. Knowledge of ecological conditions affecting the target insect is necessary. Follow cultural practices which favours pest buildup. Wide spacing causes higher whorl maggot damage while, narrow spacing results in BPH buildup. A high N rate favours hoppers, leafroller and stemborer buildup. Most rice insects are sensitive to water management. Water in the field is necessary for adequate planthopper population, whorl maggot and caseworm population.
4. Lights are commonly used to attract gall midge adults and some time to BPH to fields for screening.

5. Ratooning provides a second chance if the crop escapes infestation because insect populations are low. It is a best technique against insects which attack only at the tillering stage. Gall midge develops only in growing plants. If the gall midge does not appear in sufficient numbers during the tillering stage, plants can be cut and the ratooned plants will still be susceptible.
 6. Apply resurgence-inducing insecticides. Application of resurgence-inducing insecticides to susceptible border rows increases BPH and WBPH populations. This method is used at IRRI for the field screening of BPH. Application of the right insecticide at the right time can also increase leafhopper populations.
- ❖ Planthopper populations are often too low or too unevenly distributed for reliable field screening. This can be overcome by causing BPH population increases through the application of resurgence-inducing insecticides (spray of 0.002% deltamethrin) (Chelliah and Heinrichs 1980; Heinrichs *et al.*, 1982; Reissig *et al.*, 1982).
 - ❖ **Field screening using the polyethylene barrier technique:** Some time hopper population have been so low that the resurgence technique described in the preceding paragraph did not increase populations to a level where hopperburn occurred. Kalode *et al* (1982) modified the resurgence technique in which he used a polyethylene sheet around small field plots to prevent movement of BPH nymphs outside the plot and to prevent predators such as spiders from entering the plot.
 - ❖ Other cultural practices for screening of stemborers and planthoppers is given below:

Table 3. Field screening of rice germplasm against insect pests

Cultural practice	BPH, WBPH and GLH	Stem borer
Planting date	Adjust to obtain high infestation of the pest	Two plantings, one normal and another one month after. Idea is to catch the infestation of both vegetative and heading stages of the crop
Plant spacing	10 x 10 cm for planthoppers and 20 x 15 cm for GLH	20 x 20 cm
Plot size	Two rows of test variety alternating with two rows of susceptible check TN1. All round test entries, plant 10 rows of tall susceptible long duration variety	Two rows of 20 hills per variety/culture
Special instructions	Collect the insect from the adjacent areas and release them uniformly in the field if level of infestation is low	Collect stem borer egg masses and implant one egg mass/5 hills to initiate infestation
Observations	Insect count on 10 plants/variety 2-3 times at 10 days interval during peak infestation period. Or damage score (0-9)	Count the total number of tillers and no. of dead- hearts on 10 hills/ variety at 30 & 50 DAT (days after transplanting). At heading stage record white ear heads and total panicle bearing tillers

Priorities for insect resistance breeding

Several criteria's must be considered while deciding which insect pests are to be included for screening and what resources are to be spent on each insect pest for screening. Manpower, facilities, and financing must be sincerely allocated.

- In general, screening should be undertaken against target insect for which there is a good possibility of success in selecting resistance sources and transferring the resistance into good agronomic types.
- The extent of economic losses caused by the target insects should be known. Their geographic distribution within the area of concern and the periodicity of outbreaks should be considered.
- Using marker assisted selection, two or more resistance genes preferably with different mode of action need to be pyramided particularly for BPH.
- Researchers must study the facilities, land and manpower required. For some insects (such as hoppers) greenhouse screening is the best method. Screening for yellow stemborer requires a big greenhouse and adequate manpower for manual plant infestation).
- The availability of right stage of insects for screening is important. If greenhouse screening is conducted, an efficient rearing program is required. If field screening is done, a high natural natural population is necessary coincided with the germplasm growth stage.

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DIRECT SEEDED RICE: OPPORTUNITIES AND CHALLENGES

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Rice (*Oryza sativa* L.), providing staple food for about 50% of the world population, is cultivated on an area of about 150 mha in the world (9% of total cultivated area). Due to diverse ecologies in India, rice is cultivated throughout the year in one or other part of the country on an area of about 44.6 mha with a productivity of 2.96 t/ha. In Punjab, rice was cultivated over an area of 2.9 mha (6.5% of India) producing 19 million tonnes of rice (12.7% of India) with a productivity of about 6.5 t/ha during *kharif* 2017. Punjab, a tiny state with geographical area of 1.53% of India, is contributing 30-48% to the national buffer stock and playing a key role in food security of India (Chauhan *et al* 2012). Therefore, sustainability of rice production system in Punjab is important for ensuring food security of India.

Among the rice production systems, Puddled transplanted rice (PTR) is the most widely adopted system while globally; about 23% rice is direct seeded rice (Rao *et al* 2007). Similarly in India, including Punjab state, rice is mainly grown by transplanting seedling into flooded puddled fields. Punjab state of Indian subcontinent has been playing an important role in agricultural transformation of country and has been considered as most fertile plains for sustaining the livelihood of people (Dhillon *et al* 2010). With the increase of burgeoning population, the demand of food is also increasing, while at the same time natural resources of the Punjab state are deteriorating at an alarming rate. The conventional system of rice production (PTR) in Punjab is water, labor and energy intensive, thereby, raising the question of sustainability of rice production system and environment. It has been reported that PTR system requires up to 5000 litres water to produce 1 kg of rice (Bouman 2009). Rice is a major freshwater user and consumes about 50% of total irrigation water used in Asia (Barker *et al* 1998) and accounts for about 24-30% of the withdrawal of world total freshwater and 34-43% of the world's irrigation water (Bouman *et al* 2007). The excessive pumping of groundwater for rice cultivation under Punjab conditions has resulted in decline of water table by 0.4-1.0 m per year, leading to increased pumping cost and water scarcity (Hira, 2009, Brar *et al* 2012). Repeated puddling destroys soil structure and creates shallow hard pan, which, besides affecting the performance of rice as well as succeeding wheat crop, also make the conditions favorable for emission of methane (CH₄), thereby, contributing to global warming. PTR system of rice growing is not only water-guzzling but also cumbersome and laborious. Rice transplanting requires 150-200 man-hr ha⁻¹, which is about 25% of the total labour requirement for the rice crop production. Moreover, the unavailability of sufficient labor at appropriate time sometimes delays the transplanting of rice beyond optimum time. Under Punjab conditions, the delay in transplanting from June 15 to July 5 caused a yield decline of

7-16% (Mahajan *et al.*, 2009). Further, reduced labor availability is increasing the cost of transplanting and squeezing the farmer's profit. Moreover, manual transplanting also results in low (18-20 plants m⁻² against recommendation of 33 plants m⁻²) and non-uniform plant population, which is responsible for low crop yields (Mahajan *et al.*, 2009). Hence, the productivity and sustainability of rice production systems in Punjab are threatened because of increasing scarcity of resources, especially water and labour; changing climate; the emerging energy crisis and rising fuel prices; the rising cost of cultivation and emerging socio-economic changes such as urbanization, migration of labour, preferences of non-agricultural work, concerns about farm-related pollution. Therefore, to sustain the long-term production of rice; more efficient alternative methods of rice productions are needed. Under these circumstances, the direct seeding of rice, paves the way for efficient use of land, labour and water resources.

Direct seeding of rice refers to the process of establishing a rice crop from seeds sown in field rather than by transplanting seedlings from nursery. It has been recognized as the principal method of rice establishment since 1950's in developing countries. Direct-seeding is cost-effective, can save water through earlier rice crop establishment and alternate wetting and drying cycles, and allows early sowing of succeeding crop (Kumar *et al.*, 2015). The comparative performance of DSR v/s PTR can be evaluated on the basis of grain yield, irrigation water applied and water use efficiency, labor use, emission of GHG, economics and effect on succeeding crops. DSR is both cost and labour-saving technology and it gives similar or even higher yields than PTR (Mahajan *et al* 2013). Comparative yields in DSR can be obtained by adopting various cultural practices viz., selection of suitable cultivars, proper sowing time, optimum seed rate, proper weed and water management. Studies of Gill *et al.*, 2006 at PAU, Ludhiana indicated higher grain yield in DSR (4.83t/ha) as compared to PTR (4.28t /ha), which was mainly attributed by the more numbers of effective tillers and higher 1000-grain weight under DSR. It has been reported that best yield of DSR can be realized by sowing the crop on June 10 (Gill et al 2006). However, the recent studies have shown that short duration, early maturing cultivars viz PR 115 and PR 126 can be sown up to June 25. It has also been reported that DSR is subjected to greater weed competition than transplanted rice because weeds emerge before or at the same time as the rice. Therefore, heavy weed infestation is a major problem in direct seeded rice and its success lies in effective weed control measures (Rao *et al.*, 2007), as failure to eliminate weeds may result in low or no yield. However, Punjab Agricultural University, Ludhiana is instrumental in development of various weed management technologies for DSR. Proper package of practices for weed management in DSR have been developed and given to the farmers of state. Similarly, many studies have reported the saving of irrigation water up to 30 % and cost saving of USD 100 ha⁻¹ (Mahajan *et al* 2013) and labor saving of 7-29 % (Kumar *et al* 2015). In general, DSR has the potential to provide several benefits to farmers and the environment over PTR. These includes labour saving, sowing in stipulated time frame because of easier and faster planting, early crop maturity by 7-10 days which allows timely planting of subsequent

crops and efficient water use. More profitability especially under assured irrigation facilities, better soil physical conditions and less methane emission are also some of the potential benefits of DSR.

Status of DSR in Punjab

The technology of DSR has been successfully adopted by farmers of Punjab state. The area under DSR reached a peak during 2015 (1,65,000 ha), which was almost nil few years back, hence, growers are showing keen interest in this technology. Efforts are being made by different extension agencies to convince the mass of farmers for adoption of this technology at large. This process of adoption can be supported by maintaining LONG-TERM SITES in different regions of the state, so that local farmers can develop confidence in the long-term viability of this technology. Moreover, Punjab Agricultural University, Ludhiana has identified some 'niche' areas for DSR, as it cannot be adopted everywhere. More 'niches' in the state can be identified on the basis of soil type, geological parameters and socio-economic status of the farmers. The ever worsening labor, water and energy crisis will also lead to fast adoption of DSR in Punjab.

Reasons for slow adoption of technology/ Constraints associated with DSR

Despite many ecological, economical and physical benefits of DSR, the adoption level of technology is not catching pace with potential benefits. In this regard, the interaction with the farmers revealed the following points for non-adoptability of technology.

1. Weeds are the most important constraint to the success of DSR. The weeds pose to be more problematic in DSR than in puddled transplanting because (1) The emerging weeds are more competitive as compared to the simultaneously emerging DSR seedlings and (2) lack of water layer in DSR make the crops more prone to initial weed infestation. The research has shown that uncontrolled weed can reduce the yield of DSR by about 20-85% (Rao *et al* 2007). Besides this, shift in weed flora with a shift from PTR to alternative rice establishment methods is another factor limiting the adoption and profitability of DSR (Singh *et al* 2009). Gill *et al* (2013) reported rapid shifts in weed flora with the change in crop establishment systems. Clear associations were found with grass spp. *Leptochloa* showing strong preference for DSR and sedge *Cyperus difformis* for the PTR. *Echinochloa* has the ability to infest both DSR and PTR crops with slightly higher densities observed under DSR. Along with rice weeds such as *Echinochloa sp* and *Leptochloa*, non-rice grasses like *Dactyloctenium aegyptium* and *Eragrostis sp* are rapidly becoming major weeds of DSR. It is also reported that more reliance on herbicides for weed management in DSR would lead to the problem of herbicide resistance and also emergence of weedy rice (*O. Sativa*, f. sp), is a serious concern to rice production in areas where DSR widely replaces PTR. Some reports of increase in soil-borne pathogens such root-knot nematodes (RKNs) pose a severe constraint.

2. Some reports of increase in soil-borne pathogens such root-knot nematodes (RKNs) pose a severe constraint. Prasad, 2011 conducted studies at Philippines and reported that RKNs were found to be most damaging pathogen for aerobic rice causing crop failure in untreated plots.
3. It is also of serious concern that although DSR can help in reducing CH₄ emissions, but aerobic soil conditions can increase N₂O emissions, which is having a more GWP than CH₄.
4. It has also been reported that shift from PTR to DSR system altogether changes the nutrient dynamics mainly because of the difference in land preparation and water management techniques (Wade *et al.* 1998). The effect is more pronounced w.r.t. phosphorus, zinc and iron. Availability of Fe is often particularly high in anaerobic soils because of low redox potential but in aerobic soils, Fe may become limiting. Moreover, with the adoption of DSR at large, new researchable issues will come up, which will further help in development and refinement of technology.
5. The availability of short duration rice varieties, which are able to give high yield even under very late transplanting conditions favours PTR.
6. The unavailability of canal or tube well water during 1st fortnight of June for pre sowing or follow up irrigation of DSR hamper the adoption of technology.

In spite of some controversies associated with DSR, comparable grain yields and additional resource conserving as well as environment related benefits can be accrued from DSR, provided **recommended model production technology** is adopted by growers.

Model Package of DSR developed by PAU, Ludhiana

On the basis of series of on-station and on farm field experiments conducted from 2006-2010 under a research project “Zero tillage rice establishment and crop weed dynamics in rice and wheat cropping system in India and Australia” at Punjab Agricultural University (Bhullar *et al* 2012) as well as on the basis on ongoing research projects on DSR, a model package of practices to be adopted by growers has been developed. The various cultural practices to be adopted for realizing potential yield from DSR are as follows:

S. No.	Production technology	Details
1.	Laser leveling	-Precise leveling is pre-requisite for direct seeding. -Enhances water and resource use efficiency
2.	Selection of varieties	-Short and medium duration rice varieties having early vigour should be preferred. - PR 115 and PR 126 varieties of non-basmati rice, and Pusa Basmati 1121 and Pusa basmati 1509 varieties of basmati rice are most suitable for direct seeding.

S. No.	Production technology	Details
3.	Sowing time	-The sowing of short duration rice cultivars can be done up to third week of June, while sowing of basmati cultivars (Pusa basmati 1121 and Pusa basmati 1509) can be done up to the end of June.
4.	Seed drill	-Drills with inclined plate metering mechanism are most suitable for DSR. -These types of drills help in maintaining row to row and seed to seed spacing with little breakage. The row to row spacing should be 20 cm.
5.	Seed rate and treatment	-Seed rate @ 20-30 kg /ha is adequate. -Treat the seed with fungicides like streptocycline 1 g+bavistin 10 g/10 kg seed to reduce seed and soil borne diseases.
6.	Nutrient management	-Soil test based nutrient application is best approach for realizing higher productivity, nutrient use efficiency and environment benefits. -In absence of soil test report, on medium fertility soils, apply 150 kg N ha ⁻¹ to coarse rice varieties at 2, 5 and 9 weeks after swing (WAS), and 60 kg N ha ⁻¹ to basmati varieties at 3,6 and 9 WAS. -Generally Punjab soils have sufficient amount of potassium to meet the crop requirement. Also, if recommended dose of P ₂ O ₅ has been applied to wheat crop, then there is no need to apply phosphatic fertilizers to DSR. -However, if on soil test basis some soils indicate low status, then it can be applied as per soil test report. -Zinc sulphate (21%) @ 62.5 kg ha ⁻¹ or Zinc sulphate (33%) @ 40 kg ha ⁻¹ should also be applied on the basis of soil test report. -To ameliorate Iron deficiency, spray 0.5-1.0% FeSO ₄ at weekly interval. Soil application of Iron is ineffective.
7.	Irrigation management	-If pre-sowing irrigation is given, then first post-sowing irrigation should be applied 7-15 days after sowing, with subsequent irrigations at an interval of 5-10 days. However, if dry sowing is done, then the first irrigation should be given immediately after sowing the crop. -Water stress must be avoided during critical stages of seedling emergence, active tillering, panicle initiation and flowering.

S. No.	Production technology	Details
8.	Weed management	<p>-Effective weed control is pivotal for DSR.</p> <p>-Cultural methods of weed control like selection of cultivars with early vigour and weed competitiveness, stale seed bed, surface mulch, cover crops (viz., <i>Sesbania rostrata</i>, <i>Phaseolus radiatus</i> and <i>Vigna unguiculata</i>) and brown manuring can help to reduce weed pressure.</p> <p>-Pre-plant application of non-selective herbicides</p> <p>-Sequential application of Pre- and post-emergence herbicides according to the prevalent weed flora.</p> <p>-Integrated weed management is key to higher profitability and sustainable weed management.</p>

*Direct seeding should be done on medium to heavy textured soils as direct seeding in light soils suffers from iron deficiency, leading to yield penalty and sometimes crop failure.

By adopting the technologies developed by PAU, the growers can increase the overall profitability of rice production system along with potential resource saving.

Researchable issues on DSR

- Identification and development of weed competitive and iron efficient cultivars
 - Management of diverse weed flora (Need to minimize yield losses and weed control costs)
 - Planting in zero tillage brown manured fields
 - Irrigation management for different soil types
 - Strategies to reduce N₂O emissions
 - Use of biotechnology tools to resolve issues like lodging, nematode infestation, diseases, etc.
- and many more.....

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ARABIDOPSIS: A MODEL TO UNDERSTAND MOLECULAR INTRICACIES OF HOST-PATHOGEN INTERACTIONS

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Journey of an inconspicuous weed to a glorious model

Since Mendel's time Pea (*Pisum sativum*) and Maize (*Zea mays*) were considered the ideal crops to study crop genetics and *Arabidopsis thaliana*, a dicot flowering weed belonging to the Cruciferae family, was nowhere in picture. In 1907, Friedrich Laibach while searching for a plant having fewer numbers of large chromosomes suitable for cytogenetic analysis included *Arabidopsis* in his study for the first time. But, due to the small-sized chromosomes, *Arabidopsis* was left out until Laibach re-focused his attention on it in 1943. Owing to its short generation time, small size, large progeny, self-pollinating lifestyle with possibility of out-crossing Laibach proposed it as a good genetic model. Albert Kranz and Laibach, further contributed to *Arabidopsis* research by developing a repository of natural accessions (750) of *Arabidopsis* from all around the world. Another plant geneticist, George Redei, standardized mutagenesis protocols for *Arabidopsis* and generated a collection of X-ray induced mutants and in 1955, Langridge identified and characterized the first auxotrophic mutant in higher plants. Thereafter, the use of *Arabidopsis* mutants to dissect physiological and biochemical pathways underlying various biological processes gained momentum (Meyerowitz 2001). Prof. Koornneef's group at Wageningen Agricultural University used *Arabidopsis* mutants in a major way to construct its detailed genetic map, thus further facilitating research in *Arabidopsis* genetics (Koornneef and Meinke, 2010). Around the same time, many researchers started using *Arabidopsis* mutants to characterize important biochemical processes like photorespiration, further emphasizing usefulness of this plant in genetic analysis. A small genome size relative to other crop models made mapping and gene cloning comparatively convenient in *Arabidopsis*. The next big step in *Arabidopsis* research was the discovery of a simple, convenient *Agrobacterium tumefaciens* mediated transformation of germinating seeds which opened the floodgates for developing various tools for genetic analysis (Feldmann and Marks, 1987). Clough and Bent (1998) further simplified plant transformation by devising the "floral dip" method. All these advances, together, brought this weed into limelight as a model plant in the field of plant genetics. *Arabidopsis* was chosen as a model based on the principles of evolution that – the fundamental genetic, cellular, molecular and physiological framework is conserved across the plant kingdom, despite the diversity observed, and can be unraveled by studying a model species in details. This belief has been further strengthened by the comparative genetics and genomic studies. It was also believed that using a model system where making discoveries or testing a hypothesis is faster will enable crop improvement in a shorter span of time.

Relatively smaller genome size (approx. 125 Mb) of *Arabidopsis*, brought it the fame to be the first plant genome to be completely sequenced in the year 2000 under an international *Arabidopsis* Genome Initiative (AGI). Analysis of the genome using various gene-finding algorithms, along with supporting data from vast experimental evidences like EST sequences, MPSS tags, cDNA clones, etc. predicted about 33,000 gene models (TAIR 10). The genome analysis also revealed that it is enriched for genes with an average size of 5 Kb and had very little repetitive DNA as compared to any other higher plant, which facilitated molecular studies and map-based cloning. Release of its genome sequence acted as a catalyst for commencement of various projects on functional genomics, which led to generation of a vast stockpile of resources.

Furthermore, a 1001 genome project (www.1001genomes.org) for *A. thaliana* proposed to sequence genomes of other ~1000 accessions which would contain sequences not present in the reference genome (Weigel and Mott, 2009). This multi-national effort sheds light on local polymorphism patterns and chromosomal-scale differences, and was directly useful in QTL and association mapping as well.

Resources for functional genetics in *Arabidopsis thaliana*

With the ever-expanding wealth of genomic data produced by genome sequencing projects, the next essential step is to decipher the gene function. Multiple tools and techniques have been developed for *Arabidopsis* with a focus on dissecting and defining its gene function and interactions in a given biological process.

Forward genetic approach is the classical phenotype-based approach for screening for mutants in the biological pathway or process of interest. Large-scale forward genetic screens have provided a basis for the discovery of a multitude of new genes and pathways fundamental to various aspects of plant biology. Various libraries of mutants for forward genetics generated in *A. thaliana* are available as a public resource maintained at Arabidopsis Biological Resource Center (ABRC) Ohio State University.

Reverse Genetics: As a part of the extensive effort to experimentally validate the function of all the predicted genes an indexed T-DNA mutant library with atleast two independent insertion mutation in each gene are available for the scientific community as a valuable resource for several reverse genetic studies. Additionally, the Arabidopsis TILLING (**T**argeting **I**nduced **L**ocal **L**esion **I**n **G**enomes) project exploits the available annotated genome sequence to provide allelic series of point mutations for the general Arabidopsis community. The robustness of TILLING strategy observed in Arabidopsis has led to the extension of this strategy to other crop plant for functional genomics and even crop improvement (Cooper et al., 2013).

Another effort towards the functional genetics i.e. the **AGRIKOLA project** (*Arabidopsis* Genomic RNAi Knockout Line Analysis) under the European leadership created a genome-scale library of constructs was for the silencing of most genes in the model plant *Arabidopsis thaliana*

The fast rate of discovery of the gene – trait association in *Arabidopsis* has been the most pivotal point for its development as a reference species.

Following the availability of complete genome annotation, multiple omics approaches – transcriptomics, and proteomics has added comprehensive and quantitative data to establish functional modules based on co-expression, co-regulation, physical association/ interaction, tissue specificity etc providing great insights into gene expression networks.

Arabidopsis as a model for studying host -pathogen interaction:

Two main strategies have been deployed to develop pathosystems with *Arabidopsis thaliana*: 1) search for naturally infected *A. thaliana* in the field and under glass house conditions and 2) pathogens infecting other crop species are used to inoculate *A. thaliana* to see if they can cause a disease. *Arabidopsis* has been a host for several groups of pathogens like viruses, bacteria, oomycetes and fungi.

The next step is to look for variation in the response of different *Arabidopsis* accessions to a given pathogen isolate, such that the genetic basis underlying the variation may be characterized. The existence of compatible and incompatible or susceptible and resistance responses facilitates the isolation and characterization of the so-called resistance (R) genes. This strategy have been successfully used to isolate R genes against bacterial pathogens *Pseudomonas syringae* pv tomato, *Pseudomonas maculicola*, *Xanthomonas campestris* pv *campestris*, oomycete-*Hyaloperenosporoparasitica*, fungi – *Cladosporium*, *fusarium* etc and viruses. Once the R gene is identified in *Arabidopsis* its functional homologues are sought for in the crop plant for example in brassica, bean, pea etc. The *Arabidopsis*- *Albugo* pathosystem has led to identification and molecular characterization of White Rust Resistance 4 (WRR4), a TIR-NB-LRR gene. WRR4 confers broad-spectrum resistance against *Albugo* spp. and has the potential for use in *Brassica* crops. Borhan et al., 2010 developed transgenics in *Brassica juncea* and *B. napus* expressing WRR4 from *Arabidopsis*. These transgenics show resistance to white rust. Similarly, Bouwmeester et al. (2011), identified *Arabidopsis* Lectin Receptor Kinase 1.9 (AtLecRK 1.9) could recognize the infection caused by *Phytophthora infestans*, a notorious pathogen of potato causing late blight. Interfamily transfer of the gene AtLecRK 1.9 to potato and tobacco could confer resistance against *Phytophthora* and, hence, can be used in breeding strategies to develop durable late-blight resistant Solanaceous crops (Bouwmeester et al., 2014).

Besides identification of R genes, studies on *Arabidopsis* has also led to an in-depth knowledge of cellular and molecular mechanism underlying resistance/susceptibility to a broad class of pathogens. For example, the involvement of signaling pathways controlled by SA, JA, and/or ET and their involvement in controlling the outcome of *Arabidopsis*- interaction with different biotroph, necrotroph and hemi-biotroph pathogens. Identification of NPR1 (non-expressor of PR 1), a master regulator of the SA pathway has been exploited in multiple crop system to confer resistance to pathogens (Lin et al., 2004; Parkhi et al., 2010). Virulence of Potyvirus class of viruses

depends on eIF4E, a translation initiation factor essential for translation initiation in plants. eIF4E was first identified in *Arabidopsis* as a major susceptibility factor against single stranded positive sense RNA (ssRNA⁺) viruses and there after this was found to be true for other crop species as well (tomato and capsicum). This information thus opens up a possibility to discover or engineer resistance alleles to these important group of viruses (Bastet et al 2017).

We in our lab use *Arabidopsis* as a model host to study *Alternaria* blight resistance. *Alternaria* blight of brassicas caused by *Alternaria* sp. is a serious disease of Indian rapeseed mustard that affects not only crop yield but also the oil quality. So far, there are no usable sources of resistance identified in the cultivated brassicas that can be used in breeding programs. A comparative study of disease development and fungal growth in brassica and *Arabidopsis* revealed parallels between the two hosts suggesting *Arabidopsis* can be a good surrogate host to dissect the genetics of resistance against *Alternaria* blight (Mandal et al 2018). We challenged *Arabidopsis* natural accessions (124 accessions) with *A. brassicae* and observed a huge amount of variation in responses varying from highly susceptible to highly resistant. Using the available 250K SNP data for these accessions, we proceeded with GWAS analysis using a linear model (LM) and a mixed linear model (MLM). Multiple genomic regions containing potential candidate genes were identified suggesting that the resistance is quantitative in nature. The candidate list contains various genes including receptor-like proteins, lipases, ion channels and protein kinases. The integration of association mapping with reverse genetics has helped to identify four candidate genes that might be involved in the host response to invading necrotrophic pathogens (Rajarammohan et al 2017a). Interestingly gene controlling stomatal aperture involved in disease resistance correlated the role of stomatal opening and severity of the disease.

Additionally, a parallel traditional biparental mapping population was also developed from parents with contrasting responses to *A. brassicae* infection. Analyzing these populations also pointed at the involvement of multiple QTLs in conferring to resistance against *A. brassicae* (Rajarammohan et al 2017b). Fine mapping and identification of candidate genes will be useful in improving resistance against *Alternaria* blight in Brassicas.

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SCREENING TECHNIQUES FOR DISEASE RESISTANCE IN MAIZE

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The rate of progress toward development of stable and durable disease resistance in maize depend on availability of methodologies and protocols for reliable differentiation of resistant and susceptible genotypes. Establishing adequate and uniform disease pressure is crucial for progress. Low disease pressure will result in too many escapes and non-reliable results, and thus slow down the rate of genetic gain toward development of stable resistance. Conversely, too high a disease pressure will eliminate low levels of resistance inherent in adapted germplasm, drastically narrowing the germplasm base. Therefore, establishing adequate levels of disease pressure is critical for reliable differentiation of test entries.

The locations with high levels of natural infection referred as “hotspots”, are used effectively to identify disease resistance and promising germplasm in a breeding program. However, natural infection is variable from year to year under the influence of climatic conditions and crop management practices. Therefore, artificial inoculation methods provide more uniformity in the evaluation process and allow the elimination of highly susceptible genotypes from the breeding programme. These methods can be utilized at all stages of the selection process, and can effectively assist in the selection of more resistant genotypes frequently before flowering. The techniques for preparing of inoculums for various maize diseases and field inoculation methods/techniques and disease rating scales are described here, which can be carried out by anyone who are interested in selection for resistance.

1. FOLIAR DISEASES

a) Leaf blights -

i) Maydis Leaf Blight (MLB) *Drechslera maydis* (*Cochilobolus heterostrophus*)

Symptoms: This disease appears during the first week of July throughout the State in moderate to severe form. Disease appears as spindle shaped water soaked chlorotic lesions on the leaves which later turn necrotic. In extreme cases, several such lesions coalesce to give blightened appearance of the leaf. Sometimes, the symptoms appear on leaf sheaths, cob husks and ears.

ii) Turicum leaf blight (TLB) *Exserohilum turcicum* (*Setosphaeria turcica*)

Symptoms: The disease is recognised by long elliptical greyish or tan lesions. When fully expanded, the spots may be 1½” by 6” in size. These lesions appear first on the lower leaves and as the season progresses, the lesion number increases and all the leaves are covered. Heavily infected fields present a scorched appearance.

Inoculation procedure: Standard resistant and susceptible checks need to be included in all evaluations so that the level of infection can be compared. About an inch layer of sorghum grains (nearly 40 to 45 g) are dispensed in conical flask (500 ml), soaked in water for about 3-4 hours and excess water is drained off. The flask containing sorghum grains is autoclaved twice, seeded with fungus under aseptic condition and kept for incubation at 25-27°C. After incubation of about a fortnight, the impregnated sorghum grains are dried by spreading them on a clean paper sheet in shade at room temp. After drying, prepare a fine powder of these grains with the help of mixer-grinder and put a pinch of this powder in the leaf whorl. The inoculum should be directed into the whorl of the plant followed by spraying 10 -12 ml of water in the whorls by means of sprayer so as to maintain adequate moisture for longer period to permit spore germination. Inoculation should be done in late afternoon to avoid the maximum day temperature during incubation period. Second inoculation can be followed, if the symptoms do not appear even after a week of first inoculation.

Recording disease reaction: Disease reaction to MLB and TLB can be determined using 1-9 scale based upon disease ratings and disease leaf area (DLA) at 30-35 days after inoculation (Hooda *et al* 2018).

b) Banded leaf and sheath blight (BLSB) (*Rhizoctonia solani* f.sp. *sasakii*)

Symptoms: Disease appears on basal leaf sheaths as water soaked, straw colored, irregular to round spots alternating with dark brown bands. Under favorable conditions, wave pattern of disease development is visible on leaves, sheaths and husk leaves. Later as the infection becomes older, sclerotia develop on sheaths, husk leaves and cobs. Developing ears are completely damaged and dry up prematurely with cracking of husk leaves.

Inoculation procedure: Fresh culture of the fungus is prepared in petriplates from last year's infected leaves. Purify the culture by single hyphal tip method. The inoculum is mass-multiplied on maize-sand media. Inoculation should be done after 30-40 days of sowing by artificial sheath inoculation method (Pascual and Raymundo, 1989). Inoculum should be placed at junction of sheath and leaf.

Recording disease reaction: Disease reaction can be determined using 1-9 scale based upon disease ratings and disease leaf area (DLA) at 45 days after inoculation (Hooda *et al* 2018).

c) Rusts

Common rust (*Puccinia sorghi*)

Symptoms: Oval to elongate cinnamon brown pustules appear scattered over upper and lower surfaces of the leaves. The pustules rupture and expose dusty red urediniospores, which disperse by wind and have the ability to infect healthy leaves directly. As the pustules mature, they turn brownish black and release the dark-brown overwintering teliospores. In severe epidemics, pustules may also appear on the ears and tassels as well.

Inoculation procedure: Inoculum should be collected during the previous year, from naturally infected leaves showing large number of pustules. And these spores can be multiplied by inoculating in any susceptible maize cultivars and incubated in the greenhouse or inside a plastic house with adequate ventilation. The urediniospore from these plants are then collected and used for inoculum. The plants are inoculated at around 6-8 leaf stage and inoculation can be repeated within 2 weeks. The spore (urediniospore) suspension @ 60, 000 spores/ml is applied in the whorl using a syringe, atomizer or a knapsack sprayer (same as leaf blights). During application, the spore suspension is agitated or stirred continuously because the spores (urediniospore) tend to stick or clump together on the upper surface of the water.

Recording disease reaction: Evaluations and disease recording should be done 2-3 times during the cropping season. Reactions are evaluated using a scale of 1-9 (Hooda *et al* 2018). In addition, observation on pustule type (e.g. large, sporulating or small chlorotic, non-sporulating types) may also be recorded to measure specific resistance.

d) Downy mildews

i) Brown Stripe Downy Mildew (*Sclerophthora rayssiae* var. *zeae*)

Distribution - Himachal Pradesh, Sikkim, W.B., Meghalaya, Punjab, Haryana, Rajasthan, Delhi, Uttarakhand, Bihar, MP and Gujarat. The disease is favoured by cool and moist condition.

Symptoms: The symptoms start as small brownish lesions on leaves which on maturity extend in both the directions of leaf lamina and results into long stripes limited by veins. A characteristic downy growth is visible on the lower surface of leaves prior to flowering. The infected plants produce smaller cobs with lighter grains.

Inoculation procedure: Since the pathogen is an obligate parasite and could not be cultured artificially so the artificial disease pressure can be created by placing the powdered infected maize leaves containing oospores collected during the last season in furrows just before planting. Artificial epiphytotic condition can also be created by putting 2-3 cm pieces of freshly infected leaves containing sporangia of the fungus in the whorls of the seedlings. This should be done during cloudy weather in the evening between 5 pm and 7 pm at 24 and 30 days after planting.

Recording disease reaction: Reactions are evaluated using 1-9 scale (Hooda *et al* 2018) which is modified scale of Payak and Sharma (1983).

ii) Sorghum downy mildew (*Peronosclerospora sorghi*)

Distribution - Disease is found in Guajrat, Maharashtra, A.P., Karnataka, Tamil Nadu. Plant less than one month old is highly susceptible to this disease. The favorable condition for the disease is mild temperature (20-25° C) in presence of free water.

Symptoms: In systemic infection, the disease appears in the seedlings soon after their emergence. The affected seedlings have pale yellow, narrow leaves covered with a fine

downy growth consisting of the conidial stage of the fungus. Infected plants become chlorotic, stunted and sterile and have striped leaves.

iii) Rajasthan downy mildew (*P. hetropogoni*)

Distribution – The disease occurs only in Rajasthan. The favorable condition for disease development is free water with slightly low temperature during night (19-26° C). The oospore survives in alternate host i.e. *Hetropogon contortus* and *H. melanocarpus* in non cropping season.

Symptoms: The typical symptoms are "half diseased leaf" which are characterised by the pale appearance of bases of second and third diseased leaves of the seedling. On infected leaves, yellow stripes at the base also extends upto upper green portion. Severely infected plants give yellowish appearance even from a distance. Most of the infected plants die at about knee-high stage. Under humid conditions whitish fluffy growth due to abundant fructification of the fungus can be observed on the lower and upper leaf surfaces.

Inoculation procedure: Spreader rows of highly susceptible variety (CM-500) are sown 15-20 days prior to the sowing of test entries. These susceptible plants are sprayed with water in the evening around 6 pm so that leaves have thin film of water for good sporulation. A total of 25 litre inoculum suspension can be prepared from 375 diseased leaves. The spore suspension (inoculum) should be sprayed with the help of hand compression sprayer only and the process should be completed by 4.00 am. By 6 am, water spray should be given to the inoculated plot to maintain/create the required humidity artificially.

Recording disease reaction: Disease incidence can be recorded at 35 days after sowing. When infection is <10% of total population – resistant; 10-25% disease - moderately resistant; 25.1-50.0- moderately susceptible and more than 50% - susceptible.

2. STALK ROTS

a) Pre-flowering stalk rot -Bacterial Stalk rot (*Dickeya zeae*)

Distribution - Disease is found in Sikkim, H.P., W.B., Punjab, Haryana, Rajasthan, Delhi, Uttarakhand, Bihar. The disease is most prevalent and destructive in the areas with high rainfall and and prone to flood condition. The disease is favoured by high temperature (30-35°C) and poor air circulation.

Symptoms: The basal internodes develop soft rots and emit characteristic fermenting odour. The rind loses its natural green colour and becomes pale straw colored as if boiled in water. Ear shoots and cobs occasionally get infected directly, but these droop down and hang limply on the infected plant. Ultimately the stalk breaks and the plant collapses.

Inoculation procedure: A virulent isolate of *Dickeya zeae* pathotype should be selected for inoculation. The desired inoculum can be multiplied on nutrient broth for

48 hours at 30° C. The inoculum can be diluted so that the concentration of bacterium could be maintained approximately $1 \times 10^{7-9}$ /ml. The appropriate time for the inoculation is when crop is at pre-silking stage. To inoculate the plants, a diagonal hole, deep upto the pith, should be made in the middle of second internode from the ground with the help of jabber. One ml of bacterial suspension is injected in the plant through the hole by hypodermic syringe. If necessary, second inoculation can be done after one week in third internode from the ground.

Recording disease reaction: The inoculated plants are rated individually for their disease reaction using 1-9 scale (Hooda *et al* 2018). Disease assessment can also be made on the basis of percentage of plants toppled/rotten in each entry and disease reaction is interpreted as $\leq 10\%$ of total population – resistant; 10.1-25% disease - moderately resistant; 25.1-50.0- moderately susceptible and more than 50% - susceptible.

b) Post Flowering stalk rots-

i) Charcoal rot (*Macrophomina phaseolina*) - The disease is common in Jammu & Kashmir, W.B., Punjab, Haryana, Rajasthan, Delhi, U.P., M.P., AP, Karnataka and Tamil Nadu. Disease is prone to hot and dry weather condition. Soil temperature above 37°C is favorable for the disease.

ii) Fusarium stalk rot (*Fusarium verticillioides*) -The disease is common in Rajasthan, Uttar Pradesh, Bihar, Andhra Pradesh. Dry and warm (28-30°) temperature early in the season followed by wet weather two to three weeks after silking favours the disease condition.

iii) Late wilt (*Cephalosporium maydis*) - The disease is common in Hyderabad, UP, Rajasthan. The disease develops more rapidly in light and sandy soils.

Symptoms: The symptoms are more conspicuous after flowering and towards maturity when plants show premature drying. The pathogen commonly attacks the roots, collar region and lower internodes. The affected internodes lose their normal green colour, causes the pith to become soft and spongy, resulting in deterioration of the vascular system. The disease induces rapid wilting and premature drying at or after flowering. The infected stalks show reddish browning of parenchymatous tissues.

Inoculation procedure: Isolations are done by plating surface sterilized small pieces of infected tissues on potato dextrose agar medium. Mass inoculum is prepared on toothpicks, which are boiled thoroughly in water to remove resin, gum or any toxic substances that might inhibit the growth of the fungus. Keeping the tapering end upwards, the dried toothpicks are staked loosely in screw capped jars. The level of potato dextrose broth is adjusted to one-third length of toothpicks. Subsequently, the sterilized jar are incubated 28° C for one week. Abundant mycelial growth will spread on the toothpicks. Inoculations should be done on 45-50 days old plants just after flowering stage, in the lower internodes (second) above the soil level. The toothpick is

inserted diagonally after pricking and making 2 cm hole with the help of jabber in the desired internodes.

Recording disease reaction: Disease symptoms appear in the inoculated plants about 20-25 days after inoculation. The disease intensity and severity is recorded following 1-9 rating scale as given by Payak and Sharma (1983).

3. EAR ROTS

The ear and cob rots are caused by species of *Fusarium*, *Aspergillus*, *Penicillium*, *Diplodia*, *Cephalosporium* and *Botryodiplodia theobromae*. The pathogens are isolated and identified from infected kernels.

Symptoms: Gray-green, olive, yellow-green or yellow-brown powdery mold growth appear on and between kernels. *Fusarium* ear rot is characterized by cottony mycelium growth that typically occurs on a few kernels or is limited to certain parts of the ear. Mycelium is generally white, pale pink or pale lavender. Infected kernels typically display white streaking (also known as 'starburst' symptoms) on the pericarp and often germinate on the cob. Typically, infection occurs close to ear tips and is commonly associated with damage and injury caused by ear borers. *Penicillium* ear rot is characterized by a distinct light blue-green powdery mold that grows between kernels and on the ear surface. Under severe infestation, the entire ear appears withered and is characterized by mycelium growth between kernels.

Inoculation procedure: Spore concentration of 5×10^5 spores/ml is prepared from pure cultures of respective fungi growing on PDA medium. Ears are inoculated at 7-10 days after pollination using a spore suspension with 2×10^5 spores/ml. The most susceptible stage of the plant is period between 0-14 days post female flowering time. One ml of the spore suspension is injected at 7-10 days after silking, in the silk channel using a hypodermic syringe.

Recording disease reaction: The disease intensity and severity is recorded following 1-9 rating scale as given by Henry *et al* (2009).

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EXPLOITING SECONDARY GENE POOL OF COTTON

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Cotton belongs to genus *Gossypium* that includes more than 50 species which are distributed in arid to semi-arid regions of the tropics and subtropics (Wendel and Cronn, 2003). Recently, two more tetraploid cotton species namely *G. ekmanianum* (Krapovickas and Seijo, 2008; Grover *et al*, 2015) and *G. stephensii* (Gallagher *et al*, 2017) have been recognized. Forty-five cotton species are diploid ($2n=2x=26$) and seven are allotetraploid ($2n=4x=52$). *G. arboreum* and *G. herbaceum* are the two diploid cultivated cotton species popularly known as Old World, Asiatic or *desi* cottons. Among the tetraploid cottons, *G. hirsutum* and *G. barbadense* are cultivated. *G. hirsutum* is the dominating cotton species accounting for about 95 per cent of the total cotton production (Trapero *et al*, 2016). India is the only country to grow all the four species of cultivated cotton and the interspecific hybrids.

Single-letter genome symbols have been designated to related clusters of *Gossypium* species based on observations of pairing behavior, chromosome sizes, and relative fertility in the interspecific hybrids. Presently, eight diploid genome groups (A through G and K) are recognized (Endrizzi *et al* 1985, Stewart 1995). One can refer to a recent review for the designations of individual *Gossypium* genomes and chromosomes (Wang *et al* 2018). *Gossypium* species can be further grouped into three gene pools based on ability to generate fertile hybrids between donor and recipient species and frequency of homeologous recombination (Harlan and de Wet 1971). By following these criteria, Stewart (1995) assigned the *Gossypium* species into primary, secondary and tertiary gene pools. The wild, commensal and feral forms of *G. hirsutum* and *G. barbadense*, including other tetraploid species constitute the primary gene pool. The secondary gene pool comprises of A and D genome progenitors of allotetraploid subgenomes and B and F genomes whose chromosomes are structurally similar to A and D genomes (Table 1). Secondary gene pool species are diploid, so initial interspecific hybrid with tetraploid cotton is a sterile triploid with only few rare exceptions. Tertiary gene pool comprises of C, E, G and K genomes which are difficult to hybridize with tetraploid cotton and level of genetic recombination is low.

Diploid cottons were the predominantly cultivated cotton species in India. However, due to several reasons including the commercialization of bollworm resistant transgenic Bt *G. hirsutum* hybrids, area under *desi* cottons has largely been displaced. Nevertheless, breeding programmes of *G. arboreum* are active at many centres in the country. Several reports suggesting narrow genetic base of dominating cotton species *G. hirsutum* are available. Cultivated diploid cottons are important reservoir of genes of economic importance. For example, Asiatic cottons have contributed high fibre strength and resistance to *Pectinophera gossypiella*, *Puccinia cacabata*, *Xanthomonas campestris* pv. *malvacearum* (Fryxell, 1984; Meredith, 1991). *G. arboreum* is also known to be resistant to cotton leaf curl disease (Singh *et al.*, 1997, Rashida *et al.*,

2005; Gupta *et al.*, 2006), a serious threat to American cotton cultivation in North zone cotton growing states of India and Pakistan. Asiatic cottons are sources of resistance to the sucking pest complex (hoppers, thrips, mites and whiteflies) (Mehetre *et al* 2004). Thus, it has been suggested that assessment of genetic diversity of *G. arboreum* is important for all the cotton breeding programmes including those of American cotton (Stanton *et al.*, 1994).

Table 1: *Gossypium* species belonging to secondary gene pool (Wang *et al* 2018)

Genome group	Number of species	Species presently recognized	Geographic distribution
A	2	<i>G. arboreum</i> , <i>G. herbaceum</i>	Wild forms known only from Southern Africa, but with an indigenous domesticated range encompassing parts of Africa, the middle east, and Asia
B	3-4	<i>G. anomalum</i> , <i>G. triphyllum</i> , <i>G. capitis-viridis</i> , (<i>G. trifurcatum</i>)	Africa, Cape Verde Islands
D	13-14	<i>G. thurberi</i> , <i>G. armourianum</i> , <i>G. harknessii</i> , <i>G. davidsonii</i> , <i>G. klotzschianum</i> , <i>G. aridum</i> , <i>G. raimondii</i> , <i>G. gossypoides</i> , <i>G. lobatum</i> , <i>G. trilobum</i> , <i>G. laxum</i> , <i>G. turneri</i> , <i>G. schwendimanii</i> , (<i>G. sp.nov.</i>)	Primarily Mexico, with range extensions into Peru, the Galapagos Islands, and southern Arizona
F	1	<i>G. longicalyx</i>	Endemic to East Africa

Some of the successful examples of introgression in cotton include: Beasley (1942) transferred gene for superior lint strength in *G. hirsutum* through amphidiploid of *G. thurberi* x *G. arboreum*. Knight successfully transferred bacterial blight resistance genes from *G. arboreum* into tetraploid cotton by creating a synthetic

tetraploid followed by successive back crossing to the tetraploid parent (Knight 1948, 1953). Resistance to rust caused by *Puccinia cacabeta* in *G. anomalum* and *G. arboreum* was reported by Blanks and Leathers (1963). Wheeler *et al* (1999) reported that *G. arboreum* was resistant to fungus *Thielaviopsis basicola* causing black root rot. *G. arboreum* lines immune to grey mildew caused by *Ramularia areoala* are available (Mohan *et al* 2000, Mukewar and Mayee 2001). Sacks and Robinson (2009) introgressed resistance against *Rotylenchulus reniformis* nematode into tetraploid cultivated cotton through crossing a resistant *G. arboreum* accession with a hexaploid bridging line.

Successful introgressions between *G. arboreum* and *G. hirsutum* have been reported (Deshpande *et al*, 1992; Kulkarni and Khadi, 1998; Deshpande and Baig, 2001). DLSA 17, an *arboreum* variety with better fibre quality has been released. Deshpande and Baig (2001) have reported the identification of segregants with increased boll weight, staple length and ginning outturn derived from interspecific crosses of *G. arboreum* and *G. hirsutum*.

Pima cottons possess superior fibre properties than the Upland cottons. Saha *et al* (2004) reported the development of substitution lines in the *hirsutum* background where a chromosome/chromosome arm of *hirsutum* genome was substituted by a corresponding chromosome/chromosome arm of *barbadense* genome. The lines with substitutions for chromosomes 15, 18, 14sh and 22sh had reduced seed cotton yield and lint yield. Lines with alien chromosomes 2, 6, 16, 18, 5sh, 22Lo, and 22sh had improved lint percentage. Lines with substitution of chromosome 25 had reduced micronaire and increased fibre length. All the substituted chromosomes except 2, 4 and 6 had reduced boll weight. Lines with substituted 14sh, 15sh and 25 had increased fibre length. The results provided information on the association of specific chromosomes with genes agronomic and fibre traits. These new genomic resources will, provide additional approaches for improvement of Upland cotton and will enable the development of chromosome specific recombinant inbred lines for higher resolution mapping.

Nazeer *et al* (2014a) explored the possibility of transferring virus resistance genes from the wild species *G. stocksii* into *G. hirsutum*. Interspecific F₁ hybrid between the two species was produced after attempting 438 pollinations. 3.4% boll setting and 42.9% germination was obtained during this hybridization programme. The F₁ did not show any symptom of Cotton leaf curl disease (CLCuD) in the field, tested by grafting with CLCuD susceptible rootstock. It was concluded that it is possible to transfer CLCuD resistance and high fiber strength from *G. stocksii* to *G. hirsutum*.

A new synthetic allotetraploid (A₁A₁G₂G₂) between *G. herbaceum* and *G. australe* was produced by Liu *et al* (2015) with the objective to transfer “Glandless-seed and Glanded plant” trait to the upland cotton. The putative interspecific F₁ hybrid plant appeared to be highly male and female sterile. The sole putative hybrid plant was propagated by grafting and treated with 0.10% colchicine for 24 h during squaring stage. In the sixth year, one branch of the hybrid plant had produced three bolls and a total of 19 S₁ seeds were obtained from these bolls by self-pollination in 2012. This

interspecific tetraploid hybrid had partial fertility. In 2013, one S₁ plant was rescued which set five bolls to give 22 seeds. S₂ seeds were planted. The interspecific incompatibility, to some extent, had been alleviated in the S₂ generation. Both S₁ and S₂ were new synthetic allotetraploid plants.

With the commercialization of transgenic Bt cotton, menace of bollworms in cotton has largely been managed. However, cotton leaf curl disease (CLCuD) has become a constant threat to successful American cotton cultivation in the North Indian cotton growing states. Substantial yield losses and adverse effects on fibre quality due to CLCuD have been reported in the susceptible American cotton varieties. The losses to Pakistan economy are calculated to have been US \$ 5 billion in the five years between 1992 and 1997 (Briddon and Markham 2000). CLCuD is caused by a whitefly *Bemisia tabaci* (Genn.) transmitted Gemini virus complex. Most of the established resistant/tolerant cultivars/germplasm of American cotton have become susceptible to this disease. This shows the ability of begomoviruses and their associated satellites to rapidly evolve (adapt) in response to selection pressure such as host plant resistance (Amrao *et al* 2010).

Therefore, identification and use of new sources of CLCuD resistance has become a very important research activity. In this context, *G. armourianum* (DD), a related non-progenitor wild cotton species and a synthetic amphiploid derived from *G. arboreum* and *G. thurberi* cross were evaluated for their reaction to CLCuD at Punjab Agricultural University (Pathak *et al* 2016). Both of these stocks were observed to be free from disease under natural conditions. For artificial screening, whiteflies were initially allowed to suck the sap of CLCuD infected American cotton plants for 24 hours. Then, the viruliferous whiteflies were collected from the diseased plants and were allowed to feed on the plants of synthetic tetraploid and *G. armourianum* Acc. PAU 1. No symptoms of CLCuD on these stocks were observed throughout the crop season, whereas susceptible variety F 846 manifested severe symptoms of the disease such as vein thickening, curling of the leaves, leaf enation etc. Total genomic DNA from the diseased American cotton plants (positive control), synthetic tetraploid and *G. armourianum* was isolated. Virus specific primer was used to amplify the viral DNA from the samples. Virus specific bands were observed in the positive control and the synthetic but not in *G. armourianum*. Results indicate that the synthetic tetraploid is a symptomless carrier/tolerant, whereas, *G. armourianum* is resistant to CLCuD. An interspecific hybrid between *G. hirsutum* (female) and *G. armourianum* (as pollen parent) was developed without any difficulty and characterized (Kaur *et al* 2016). However, BC₁F₁ was obtained with great difficulty. BC₂F₁ population showed segregation for CLCuD resistance and susceptibility. Similarly, crosses between synthetic amphidiploid (as the female parent) and natural allotetraploid *G. hirsutum* as the pollen parent were developed. The introgressed lines from both of these donors have been developed and are under evaluation.

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MOLECULAR BREEDING EFFORTS IN DEVELOPING DROUGHT TOLERANT AND DRY DIRECT SEEDED ADAPTED RICE VARIETIES

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The climate change process leading to increased drought and high temperature is predicted to adversely affect the rice production. Water scarcity affects both rainfed and irrigated ecosystems at different scales and leads to yield decline. Unpredictable rainfall in the beginning of the rainy season, increased water shortage and high temperature at reproductive stage, reduced water and nutrient availability and increased cost of irrigation and non-availability/increased cost of labor demands development of climate adapted short duration rice varieties with traits providing flexibility to be successfully grown under variable situations ranging from dry direct seeded to wet direct seeded to transplanted situations based on rainfall pattern and labor availability. In the present scenario, emerging problems of decline in the natural resource base, low efficiency of inputs use and increase in food demand coupled with the climate change forced to develop early maturing, drought/heat tolerant/resistant high yielding varieties that can be effectively cultivated with 40% less water compared to presently available medium to long duration varieties.

Recently, 14 major QTLs with large effects on grain yield under drought (*qDTY*) have been identified at IRRI. 7 of these *DTY* QTLs- *qDTY_{1.1}*, *qDTY_{2.2}*, *qDTY_{3.1}*, *qDTY_{3.2}*, *qDTY_{4.1}*, *qDTY_{6.1}*, and *qDTY_{12.1}* have shown consistent effect across genetic backgrounds and ecosystems (Bernier et al. 2007, Venuprasad et al. 2009, Vikram et al. 2011, Mishra et al. 2013, Yadaw et al. 2013, Swamy et al. 2013, Sandhu et al. 2014, Dixit et al. 2017). Rice drought breeding at IRRI has witnessed some of the successful example of marker assisted introgression of drought QTLs in elite backgrounds. Two to three QTLs have been introgressed in the background of popular high yielding varieties IR64 and Swarna and drought tolerant versions of IR64, Swarna with yield advantage of 0.8-1.0 t ha⁻¹ under drought have been developed. Pyramiding of more complementary *DTY* QTLs shall provide opportunity to further enhance the yield under drought and also understand the interactions between them. The development and release of marker-assisted breeding product for the rainfed lowland areas in IR64 and Swarna-Sub1 backgrounds (Sandhu et al. 2019) are the successful examples that should encourage the rice breeders to deploy the identified QTLs in the breeding programs targeting yield improvement under abiotic stresses.

To large extent the interaction among different QTLs combination is unknown except for some that have been recently developed and evaluated. The developed drought tolerant lines have shown better adaptability under heterogeneous environment due to plastic nature of their roots to adapt to varying aerobic to anaerobic cultivation situations. Deciphering of root plasticity traits and proper management decisions in the

favour of plants through agronomic interventions can contribute to improve yield stability under different moisture regimes (Sandhu et al. 2016). Apart from this, the identified *DTY* QTLs linked with early days to flowering and can be successfully used to develop short duration varieties of 105-110 days without any yield decline to replace the presently grown long duration varieties of 130-140 days which are highly susceptible to drought. 115-120 days duration version of 140 days varieties Swarna and Samba Mahsuri have recently been developed without any yield decline. Four of the *qDTY* QTLs- *qDTY_{1.1}*, *qDTY_{2.2}*, *qDTY_{6.1}* and *qDTY_{12.1}* are known to be associated with increased yield under dry direct seeded/aerobic situation. Similarly, *qDTY* QTLs- *qDTY_{1.1}* and *qDTY_{4.1}* are reported to be associated with tolerance to high temperature at reproductive stage. The role of epistatic interactions among the introgressed drought QTLs affecting the performance of introgression lines with same QTL combinations has been well documented in various marker-assisted introgression studies conducted at IRRI (Dixit et al. 2012, Shamsudin et al. 2016, Vikram et al. 2016, Sandhu et al. 2018, Yadav et al. 2019). The positive interaction of *qDTY_{12.1}* with *qDTY_{2.3}* and *qDTY_{3.2}* (Dixit et al. 2012), *qDTY_{2.2}* and *qDTY_{3.1}* with *qDTY_{12.1}* (Shamsudin et al. 2016) and *qDTY_{4.1}* and *qDTY_{9.1}* locus with *qDTY_{7.1}* (Sandhu et al. 2018) reported to increase grain yield under drought significantly

Puddled transplanted (manually) rice (PTR) is widely practiced in South Asia. Despite multiple benefits associated, PTR is highly labor, water, and energy intensive system. Labor and water are becoming scarce and consequently expensive. PTR is also a major methane emitter (10-20% of total global annual methane emission). Moreover, drudgery associated with manual transplanting- a job largely done by women farmers is a concern. Furthermore, puddling creates soil physical conditions which adversely affect the performance of succeeding non-rice crop in World's major crop rotations (rice-wheat and rice-maize) grown on millions of hectares. All these factors are making PTR less- sustainable, profitable and attractive to farmers especially women and youth. IRRI has been engaged in refining direct seeded management technologies, traits, QTLs, genes identification research since the last six years. QTLs for higher grain yield (*qGY_{1.1}*, *qGY_{6.1}*, and *qGY_{10.1}*) as well as other traits related to direct seeding-early uniform germination (*qEMM_{1.1}*, *qEMM_{11.1}*), early vigor (*qEVR_{9.1}*), better nutrient uptake (*qRHD_{5.1}*, *qN_{5.1}*, *qP_{5.2}*, *qPU_{5.2}*, *qFe_{5.2}*, *qFeU_{5.2}*, *qAl_{5.2}*, and *qAlU_{5.2}*) and lodging resistance (*qLDG_{3.1}*, *qLDG_{4.1}*) have been identified which can be used for developing high yielding varieties suitable for direct seeded aerobic situations (Sandhu et al. 2015). The available QTLs and proper genetic x environment x management interactions provide unique opportunity to better address the evolving climate related uncertainties such as unpredictable rainfall pattern, irrigation water shortage, low nutrient availability, high temperature as well as labor unavailability scenario. The developed short duration, water efficient, drought and high temperature tolerant nutrient efficient rice varieties for both irrigated and rainfed environments can be cultivated with flexibility under dry direct seeded/wet direct seeded/ transplanted depending upon rainfall.

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HOW TO ANALYZE GENE FUNCTION: TOWARDS GENOME EDITING WITH NEW DIRECTIONS AND CHALLENGES

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Abstract:

The evolution of tools to study gene function ranged from classical mutagenesis approach to RNA interference and high-throughput functional screen using virus induced gene silencing. RNAi has been used in forthright manner to knock down expression of targeted genes. However, RNAi is limited to particular organisms which has suitable host machinery and sometimes exhibited off-target effects and toxicity. With the advancement of functional tool such as virus induced gene silencing, have largely encouraged researchers to perform genetic screen for the given trait and phenotype in a high throughput manner.

Recently a new technology based on Prokaryotic Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) and CRISPR-associated protein (Cas) system has been identified as an effective tool for genome engineering. It is highly specific, climbable, inexpensive, very easy to engineer and provides potential platform for targeted gene editing or regulation (Cong et al., 2013). About 90% archaea and 40% bacteria possess CRISPR/ Cas systems to confer resistance against foreign DNA elements like viruses (Makarova et al., 2011). This system utilizes small base-pairing RNAs to target and cleave foreign DNA elements in a sequence-specific manner (Wiedenheft et al., 2012). Various CRISPR systems are reported in different organisms, in which type II from *Streptococcus pyogenes* is simplest one. It requires only a single gene encoding the Cas9 protein and two RNAs, a mature CRISPR RNA (crRNA) and a partially complementary trans-acting RNA (tracrRNA) for RNA-guided editing of foreign DNAs (Gasiunas et al., 2012; Jinek et al., 2012). Processing of mature crRNA needs RNase III and tracrRNA (Deltcheva et al., 2011). However, this requirement is now avoided by using an engineered small guide RNA (sgRNA), which mimics the tracrRNA-crRNA complex (Jinek et al., 2012). sgRNA binds to target DNA by base pairing. The special endonuclease Cas9 recognizes the sgRNA bound target DNA and causes double-strand breaks (DSBs) (Jore *et al.* 2011). The binding specificity is mainly determined by base pairing and a short DNA motif known as protospacer adjacent motif (PAM) sequence (NGG) juxtaposed to the target region (Marraffini and Sontheimer, 2010). Thus, the CRISPR system only requires a minimal set of two molecules: Cas9 protein and sgRNA, and therefore holds the potential to be used as a host-independent gene-targeting platform. Very recently, it has been demonstrated that the Cas9/CRISPR can be harnessed for site-selective RNA-guided genome editing in bacteria, yeast, animals and plants as well (Kumar & Jain, 2015; Mali et al., 2013; Sander & Joung, 2014; Upadhyay et al., 2013).

The CRISPR-Cas system is simple in designing as compared to other editing tools it is very effective and good for complex genome like wheat. Interestingly, using CRISPR/Cas system, we can target many genes (multiplexing). This result suggests that the method can be useful in deletion of target regions from the genome and the

development of knockout mutants. Certain off target mutations have also been reported but that off target mutations can be avoided by careful selection of the target sequences. Instead of targeting the conserved and semi-conserved regions, unique sequences should be preferred during CRISPR designing, especially toward 3' side. Selection of CRISPR-Cas target site is also an important point to be considered. Although, it is simple, which needs direct analysis of DNA sequences for the presence of specific 23 nucleotides (including 'NGG' PAM at 3'end). A few tools like CRISPR Design, CRISPR Target, ZiFiT and SSFinder are recently reported for the determination of target sites (Biswas et al., 2013; Upadhyay and Sharma, 2014). Due to the simplicity in designing sgRNA and applicability to a wide variety of plants and animals, this RNA guided genome editing CRISPR-Cas system will be very useful tool for reverse genetics as well as functional genomics.

Other functional tools such as zinc finger nucleases and transcription activator-like effector nuclease are also been exploited for editing of plant genomes with respective advantages and limitations. The expansion of genome editing tool within a short span of five years from model plants to crop plants have open new avenues and opportunities in plant biology. CRISPR based technologies are powerful tools for genome editing in animals, plants, and microorganisms. CRISPR/Cas9 systems have emerged as important tool resource in the area of plant biotechnology. Advancement in crop biotechnological tools including plant regeneration, genetic transformation and advanced functional tools provided the required impetus for trait development. Therefore, new functional tools including genome editing is an excellent resource to demonstrate gene function/s linked with traits in major crop plants. In addition to that innovation to generate non-transgenic plants has provided the much-needed boost to possibly avoid biosafety related issues. These editing tools have provided the hallmark to perform gene knockouts, precise modifications etc. in the important nutritional crops like wheat and rice. During my talk we recount the different functional tools has been used for reverse genetics approaches especially in crop plants to understand and address few interesting phenomena including disease resistance, nutritional traits, yield etc. Multiple examples were cited and other ongoing gene editing activities in NABI was discussed.

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CLIMATE CHANGE VIS-À-VIS PLANT-INSECT INTERACTIONS

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1. Introduction

Climate is the synthesis of weather conditions in a given area, characterized by long-term statistics (mean values, variances, probabilities of extreme values etc.) for the meteorological elements in that area. The quantities measured are surface variables, such as temperature, precipitation, and wind. Climate regulates food production, water resources and influences energy use, disease transmission, and other aspects of human health and well-being. Over the years, various human activities including large scale deforestation, industrial pollution led to increased concentration of greenhouse gases like carbon dioxide (CO₂), methane (CH₄), Chlorofluorocarbons and nitrous oxide (N₂O) in the atmosphere. This increase in concentration of gases largely contributes to global warming. As a result of global warming, global average temperature is increasing, rainfall distribution and intensity are getting altered, frequency of floods and droughts are increasing. Alteration in all these climatic parameters over a period of time is collectively known as climate change. Current estimates of changes in climate indicate an increase in global mean annual temperatures of 1°C by 2025 and 3°C by the end of the 21st century. The amounts of CO₂ will double between 2025 and 2070, depending on the level of emission of greenhouse gasses (UNFCCC 2007). Global warming and climate change will have major implications for ecosystem services, water availability, crop production, food security and on the composition of fauna and flora. Climatic variables such as temperature, rainfall, humidity, and atmospheric gases interact with plants in numerous ways with diverse mechanisms. These changes are affecting plants directly in terms of tissue and organ-specific photosynthetic allocation, and indirectly through change in geographic distribution and population dynamics of the pest species. There may be effect on productivity of crop plants due to higher levels of CO₂ that increases the photosynthetic rates and biomass production. However, increase in crop production may be offset through high temperatures and reduced water availability. An increase of 6°C in temperature, and precipitation deficit of 300 mm reduced the maize yield by 36 percent in the European Union (Ciais *et al* 2005). Increased temperatures will also increase the pest population, and water stressed plants at times may result in increased insect populations and pest outbreaks. It is proved that global warming is having adverse effects on the productivity of cereals and other crops and at the same time the insect pests becoming more abundant. The change in climate triggers the change in cropping pattern and major insect pests such as cereal stem borer, pod borer, aphids and whiteflies move to temperate regions causing greater damage to the crops there. Moreover, losses due to insect damage are likely to increase as a result of changes in crop diversity and increased incidence of insect pests due to global warming. This will alter the crop yield, and also influence the effectiveness of insect-pest management practices. Impact of Climate Change on Arthropods (insects, spiders,

and mites) are the most abundant and diverse group of organisms. Insect herbivores are likely to suffer direct or indirect effects of global climate change, directly through changes in physiology, behavior and life history parameters while indirectly through changes experienced by host plants in their morphology, biochemistry, physiology and patterns of richness, diversity and abundance.

2. Effect of elevated Temperature and CO₂ on crop pests

Increased temperature will cause insect pests to become more abundant and almost all insects will be affected by changes in temperature. The various effects of temperature on insects include limitation of geographical range, overwintering, population growth rates, number of generations per annum, crop–pest synchronization, dispersal and migration, and availability of host plants and refugia. In-season effects of warming include the potential for increased levels of feeding and growth, including the possibility of additional generations in a given year. Increased global temperature will also influence the phenology of insects including early arrival of insect pests in their agricultural habitats, emergence time of a range of insect pests and pest outbreaks. This will affect the crop yield and availability of food grains and threaten food security. The rising temperature can cause range expansion of insects, increased adaptation for introduced insects and modification of ecosystem allowing some insects to population out breaks and some insects to extinction. Survey of around 1100 insect species revealed that the climate change and global warming will lead to the extinction of about 15-30 per cent of these species by 2050 (Hance *et al* 2007). Mountain species and those restricted to high latitudes are most likely to become extinct as a result of climate change. Four butterfly species have disappeared from lower latitudes during the past 25 years in the United Kingdom, with climate change being responsible for at least half of the population extinctions. The current extinction rates are 100–1000 times greater than what they were earlier, and nearly 45–275 species are becoming extinct every day. Due to early occurrence of spring, the range limits of different species are expanding north wards by 6.1 km/decade. Species of butterflies are shifting pole ward and up in the elevation in Europe and North America as temperature there has increased. The pole ward range expansion of several species of damsel flies, cockroaches, grasshoppers and locusts and increase in the population of several species of forest butterflies and moths including gypsy moth *Lymantria dispar* (Linnaeus) in central Europe in the early 1990s have been linked to increased temperature. Potato psyllid, a destructive pest migrated to California during 1999 or 2000 is causing higher losses in pepper, tomato and potato industries (Liu & Trumble, 2007). For instance, green stinkbug *Acrosternum hilare* (Say) in England and Japan has demonstrated dramatic shifts of range of 185 miles in past 25 years with an increase of 2 °C. Similarly, in Europe, corn borer, *Ostrinia nubilalis* (Hübner) has shifted northward of more than 1000 km (Porter *et al* 1991). In the same way, Edith's checker spot butterfly *Euphydryas editha* (Boisduval) has exhibited its population expansion 21 northward in the USA (Parmesan, 2006). Furthermore, mountain pine beetle (*Dendroctonus ponderosae* Hopkins) has extended its range northward of more than 180 miles in last 15 years in the Rocky Mountains

(Logan & Powell, 2001). For a 3°C temperature increase in Japan, Mochida (1991) predicted expanded ranges for tobacco cutworm, *Spodoptera litura* (Fabricius), southern green stink bug, *Nezara viridula* (Linnaeus), rice stink bug, *Lagynotomus elongatus* (Dallas), Lima-bean pod borer, *Etiella zinckenella* (Treitschke), common green stink bug, *Nezara antennata* (Scott), soybean stem gall midge, *Asphondylia* sp., rice weevil, *Sitophilus oryzae* (Linnaeus), and soybean pod borer, *Leguminivora glycinivorella* (Matsumura), but a decreased range for rice leaf beetle, *Oulema oryzae* (Kuwayama), and rice leaf miner, *Agromyza oryzae* (Manukata). For all insect species, higher temperature, below the upper threshold limit would cause more rapid development and hence increase in pest populations as the time to reproductive maturity will be reduced considerably. Yamamura and Kiritani (1998) estimated that with every 2°C rise in temperature, multivoltine insects may have 1-5 additional generations. Similarly, Kiritani (2006) reported additional generations in the range of 1.17 ± 0.45 for 41 species of rice pests due to rise of 2°C in mean temperature. Climate change has also impact on period of activity of arthropods. Period of activity of many insects might increase in warmer winters and has been an important factor in increased population of *Pieris brassicae* (Linnaeus) on Cole crops, and cereal aphids, *Macrosiphum miscanthi* Takahashi, *Rhopalosiphum maidis* Fitch, *Shizaphis graminum* Rondani, *Sitobion avenae* Fabricius on wheat, barley and oats in north India (Arora & Dhawan, 2012). Global warming will lead to earlier infestation by *Heliothis zea* (Boddie) in North America (EPA, 1989), and *Helicoverpa armigera* (Hübner) in North India (Sharma, 2010), resulting in increased crop loss. An increase of 3°C in mean daily temperature would cause the carrot fly, *Delia radicum* (Linnaeus), to become active a month earlier than at present (Collier *et al* 1991), and temperature increases of 5 to 10°C would result in completion of four generations each year, necessitating adoption of new pest control strategies. An increase of 2°C will reduce the generation turnover of the bird cherry aphid, *Rhopalosiphum padi* (Linnaeus), by varying levels, depending on the changes in mean temperature (Morgan, 1996). An increase of 1 and 3°C in temperature will cause northward shifts in the potential distribution of the European corn borer, *O. nubilalis* of up to 1220 km, with an additional generation in nearly all of the regions (Porter *et al* 1991).

Although the Global warming with increased levels of Green House Gases causes a reduction in the pest performance of some insects, in many insect pest cases increased pest status are reported by many researchers. This is evident particularly in notorious pests such as Japanese beetle, *Popillia japonica* (Newman), Diamond backed moth, *Plutella xylostella* (Linnaeus), pyralid rice pest, *Chilo suppressalis* (Walker), gypsy moth, *L. dispar*, corn flea beetle, *Chaetocnema pulicaria* (Melsheimer), mountain pine beetle, *D. ponderosae*, European corn borer, *O. nubilalis*, and Colorado potato beetle, *Leptinotarsa decemlineata* (Say). These pests are reported to increase their herbivory, longevity, fecundity and voltinism as an indirect effect of climate change on host plants like more carbohydrate accumulation in relation to nitrogen,

reduction in the level and effectiveness of deterrent chemicals against insect herbivory and signaling phytochemicals towards natural enemies for the rescue.

3. Impact of Climate Change on Specific Functional Groups of Insects

3.1 Pollinators

Climate change will strongly dismantle the relationship between the flowers and pollinators and may create the asynchrony between flower opening and pollinator emergence. A strong increase in temperature has negative impacts on foraging by pollinators. Decline in the population of pollinators lead to decreased pollination will have tremendous impacts on world food production. The population abundance, geographic range and pollination activities of important pollinator species like bees, moths and butterflies are declining considerably with changing climate (FAO, 2008). The geographical distribution of Africanized honey bee had extended as far as Argentina and the USA (Mesfin, 2010). Climate change might also disrupt the synchrony between the flowering period of plants and the activity season of pollinators. Hegland *et al* (2009) reported that both *Apis mellifera* (Linnaeus) and *Pieris rapae* (Linnaeus) advanced their activity period more than their preferred forage species, resulting in a temporal mismatch with some of their main plant resources. However, Kudo *et al* (2004) found that early-flowering plants in Japan advanced their flowering during a warm spring whereas bumble bee queen emergence appeared unaffected by spring temperatures. A change in foraging community composition has been observed for different weather conditions and it has been reported that honey bees dominated good weather while bumblebees dominated bad weather conditions. Berry clusters when exposed during good weather had more than five times mature seeds that weighed twice as earlier and had double the fruit set (Tuell & Isaacs, 2010). Similar impact of climate change in the form of late cold in the month of April altered pattern of blossoming, bearing and reduced pollination activity of bees affected apple production. Similarly, Thomson (2010) reported that the lack of synchrony between lily and bumblebees has worsened in recent years. If winter precipitation patterns and extreme storm events increase as predicted, early blooming lilies will be severely limited by frosts and the lack of bumblebees. Memmot *et al* (2007) simulated the effects of increasing temperatures on a highly resolved plant-pollinator network. They found that shifts in phenology reduced the floral resources available for 17 to 50 percent of the pollinator species. A temporal mismatch can be detrimental to both plants and pollinators. Intensively managed monocultures usually provide floral resources for a limited time period. The survival rate and population size of the main pollinators may decrease if the foraging activity period is initiated earlier than the flowering period of the crop species. A loss of important pollinators early in the season will reduce crop pollination services later in the season. In such cases, introducing alternative food sources might be an option for farmers.

Insect Pests Insect life stage predictions are most often calculated using accumulated degree days from a base temperature and biofix point. There is a general

belief that the effect of temperature on insects largely overwhelms the effects of other environmental factors. While researchers have found that moisture and CO₂ effects on insects can be potentially important considerations in a global climate change setting. Kiritani (2006) reported a sequential change in the rice pest status due to climatic warming in Japan since 1945. The dominance of pyralid moths from 1945 to 1965 was successively replaced by delphacid and cicadellid homopterans in 1965-1995 and then recently by various kinds of rice bugs. Population outbreaks due to enhanced fecundity under warm condition are attributed for the widening of distribution range of the pyralid rice pest, *Chilo suppressalis* Walker in Hokkaido Island of Japan (Kiritani and Morimoto, 2004). Similar outbreak of a noctuid moth, *Tricho plusiani*, on vegetables in Japan is suspected to be the after effect of global warming (Yase 2005). Yamamura *et al* (2006) analyzed the 50-year dynamics of three insect pests attacking paddy namely rice stem borer, green rice leafhopper and brown planthopper by annual light-trap catches. Results indicated that the number of light-trap catches of stem borer and leafhopper in summer increased with increased temperatures in the previous winter. On the other hand, increased temperature could decrease pest insect populations. Some insects are closely tied to a specific set of host crops. Temperature increases that cause farmers not to grow the host crop any longer would decrease the populations of insect pests specific to those crops.

3.2 Parasites and Predators

The same environmental factors that impact pest insects can impact their insect predators and parasites as well as the disease organisms that infect the pests, resulting in increased attack on insect populations. At higher temperatures, aphids have been shown to be less responsive to the aphid alarm pheromone they release when under attack by insect predators and parasitoids – resulting in the potential for greater predation (Awmack *et al* 1997). Some insects are sensitive to precipitation and one would expect the predicted more frequent and intense precipitation events forecasted with climate change to negatively impact these insects. Other insects such as pea aphids are not tolerant of drought (Macvean and Dixon, 2001). Some researchers have found that rising CO₂ can potentially have important effects on insect pest problems. During the early season, soybeans grown in elevated CO₂ atmosphere had 57 percent more damage from insects (primarily Japanese beetle, potato leafhopper, western corn rootworm and Mexican bean beetle) than those grown in today's atmosphere (Hamilton *et al* 2005).

3.3 Insect vectors of plant pathogens

Changing climate conditions can contribute to a successful spread of newly introduced viruses or their vectors and establishment of these organisms in areas that were previously unfavorable. A number of plant viruses are transmitted by vectors, many of them are not able to establish at current climate conditions. The most important vectors such as aphid, whitefly, thrip, and leaf hoppers which are associated with potyviruses, begomoviruses, tospoviruses and phytoplasma, have emerged during

the last 24 two decades. Plant virus diseases and vectors are strongly influenced by weather and climate. The temperature and moisture conditions interacting with seasonal phenology, and stress on the host determine infection severity and distribution. Climate change increases or decreases environmental conduciveness, the shift in selection pressure on the host populations could result in shifts in the diversity of resistance genes present. Recent observations have shown that resistance to bhendi yellow vein mosaic virus tends to break when okra is grown at higher temperatures. The number of disease epidemics has dramatically increased in recent years, as have the threat of emerging new diseases and the reemergence of other diseases. Some of the recent examples are incidence of thrips transmitted tospoviruses and whitefly transmitted begomoviruses in chilli, cucurbits, okra and tomato. Different biotypes of an aphid or whitefly species have been associated to outbreaks and expansions of viral diseases. Amongst the insects that are commonly associated with virus transmission, aphids are of particular interest for a number of reasons. Aphids generally have a low developmental temperature threshold and a short generation time, so that when they continuously reproduce in a parthenogenetic manner, they achieve 18 generations a year in British conditions. Yamamura and Kiritani (1998) suggested that aphids are amongst the insects best adapted to take advantage of a warming climate, and could go through an extra five generations a year following a warming of 2°C. Increased temperatures, particularly in early season, have been reported to increase the incidence of viral diseases in potato due to early colonization of virus bearing aphids, the major vectors for potato viruses in Northern Europe. Warmer autumns and winters will increase the risk for insect transmission of viruses into winter crops, such as winter wheat, winter barley and winter oilseed rape. They are now sown when the number of active insect vectors has decreased significantly. Wheat dwarf virus (WDV) is transmitted in a persistent manner by the leafhopper, *Psammotettix alienus* (Dahlbom). A study in Sweden showed that the catches in autumn of adult *P. alienus* in fields of winter wheat increased with higher temperatures. Numerous factors have been cited as potential drivers of the emergence of viral outbreaks, including pathogen introduction through global traffic, changes in vector populations, genetic recombination, new farming techniques, changes in weather conditions. As a first and necessary step in obtaining baseline information about climate change impact on different virus diseases and vectors, a survey should be conducted among growers and plant protection officers. The survey will define a list of most important climate-related plant viruses and vectors for specific regions. Awareness of forthcoming significant climate change it is necessary to work out the impact to be able to make predictions.

3.4 Insect vectors of humans and animal diseases

In the view of climate change, vector-borne diseases of man and animals with vectors and reservoirs developing in natural habitats are of special interest. Climate change creates new risks, for human exposure to vector-borne diseases (VBDs) which are transmitted to humans through the bites of insects that carry the disease-causing pathogens. Common vectors include mosquitoes, ticks, and flies. Climate change

creates 25 new uncertainties about the spread of VBDs such as the Zika virus, dengue fever, malaria, and Lyme disease by altering conditions that affect the development and dynamics of the disease vectors and the pathogens they carry. While the direction of change is difficult to predict, there is evidence that certain vectors, such as *Aedes mosquitos*, are mostly changing their range. For example, dengue fever, chikungunya and West Nile virus are emerging in areas where they were previously unknown and it may be due to increasing temperatures, increasing global travel and trade. Rising global temperatures can lengthen the season and increase the geographic range of disease-carrying insects. As temperatures warm, mosquitoes and other warm-weather vectors can move into higher altitudes and new regions farther from the equator. Increased rainfall, flooding and humidity creates more viable areas for vector breeding and allows breeding to occur more quickly, as eggs hatch faster in hotter climates. For example, officials braced for an increase in risk for Zika and West Nile virus infections after the massive flooding event in Louisiana in August 2016, which increased the breeding habitats for *Aedes* mosquitoes. Disease flare-ups at the human-animal-ecosystems interfaces are particularly prominent in pig and poultry production, with Influenza A as a primary example. A number of new emerging diseases due to climate change are finding their way from wildlife reservoirs via pigs to humans. Fruit bats may transmit Henipah viruses (HeV) or Reston Ebola virus (REBOV). Other impacts of climate change regarding the vector of animal borne diseases include northward expansion of the traditional vector, changes in vector abundance, change in vector/pathogen interaction, vector activity, survival of the pathogen in a vector population. The management of this growing number of veterinary and medical threats requires adjustment of natural resource management practices, land use and food and agriculture.

3.5 Activity and abundance of Natural Enemies

Quantifying the effect of climate change on the effectiveness of natural enemies will be a major concern in future pest management programs. Relationships between insect pests and their natural enemies will change as a result of global warming, resulting in both increases and decreases in the status of individual pest species. Oriental armyworm, *Mythimna separate* (Walker), populations increase during extended periods of drought, which is detrimental to its natural enemies, followed by heavy rainfall (which is favorable to the armyworm) (Sharma *et al* 2002). Increasing climatic variability has resulted in a reduced level of parasitism of caterpillars, which in turn may increase the frequency and intensity of herbivore outbreaks. The coccinellid predator, *Leis axyridis* (Pallas), of cotton aphid, *Aphis gossypii* (Glover), consumed more prey under higher CO₂. The pentatomid bug, *Oechalia schellenbergii* (Guerin-Meneville), exhibited increased predation of cotton bollworm, *H. armigera*, feeding on peas under elevated CO₂ because the pea plants had reduced nitrogen content when grown under high CO₂ (Coll & Hughes, 2008). With the modification in temperature, behaviour of predators can be stimulated or dispirited. For example, below 11°C, reproduction rate of pea aphid at which lady beetle (*Coccinella septempunctata*

Linnaeus) can prey it exceeds while the situation is reversed above 11°C. 26 On the other hand, at higher temperatures the natural enemies of spruce budworm (*Choirestoneura fumiferana* Clemens) become less operative (Harrington *et al* 2001).

Quality of insect hosts may also affect parasitoid fitness, particularly in parasitoids whose hosts continue to feed after parasitization as fecundity of the parasitoid is positively correlated with size and host quality. However, increased abundance of the braconid parasitoid, *Aphidius picipes* (Nees), was recorded on *S. avenae* parasitism under elevated CO₂ compared to the insects raised under ambient CO₂. Whereas, under 750 ppm CO₂ concentration involving *H. armigera* larvae reared on milky grains of wheat, no significant change in parasitisation rate of its larval parasitoid, *Microplitis mediator* (Haliday) was found. The development of the parasitoid wasp, *Glyptapanteles liparidis* Bouché, of gypsy moth, *L. dispar*, feeding on three different tree species fumigated with 540±20 ppm CO₂ was not adversely affected by changes in food quality when compared to ambient CO₂. Exposure to temperature extremes even for short periods is likely to influence parasitoid survival and host searching ability. Temperature influenced the fecundity and sex ratio of *Campoletis chloridae* Uchida, an ichneumonid larval parasitoid of *H. armigera*. Host location of the egg parasitoid, *Trichogramma carverae* (Oatman and Pinto), decreases sharply at temperatures above 35°C, while fecundity reductions of up to 50% are commonly observed at temperatures >30°C. In cassava, parasitism of mealy bugs is reduced under conditions of water stress associated with drought due to improved immune response of mealy bugs on water stressed plants, leading to an increased rate of encapsulation. The interactions between insect pests and their natural enemies need to be studied carefully to devise appropriate methods for using natural enemies in pest management programmes under changed climate.

3.6 Other arthropod groups

Climate changes can influence soil microarthropod community abundance and composition directly by altering soil microclimate and indirectly by altering resource availability and the composition of the soil food web. Mites and Collembola are the most important and abundant soil microarthropod groups. Since the concentration of CO₂ in the soil is already high the direct effects of climate change on soil inhabiting insects are unlikely. But the indirect effects will be pronounced in the due course of time. Under elevated CO₂ conditions, the Collembola population was found to increase in the laboratory studies. Considerable reduction in the collembolan population associated with the lichen communities has been observed because of decline of isotomid, *Cryptopygus antarcticus* (Willem) at higher temperatures. Similarly, forensic entomology using insects and other arthropods in criminal investigations will be affected by changing climate resulting in declined accuracy and reliability. The blowflies are of prime importance for forensic entomologists as one of the secret weapons in the fight against crime. With temperatures warming, the range of different species of blowflies is expanding. The calliphorid, *Chrysomya albiceps* (Diptera: Calliphoridae) is generally described as a tropical and subtropical species that can be

found in Africa, southern Europe, Arabia, India, and Central and South America in recent years has shifted to central Europe from the southern 27 regions. This could fundamentally change the life cycle and throw off forensic timing. Conclusions based on temperature and species distribution data is a key in forensic entomology. On the other hand, several reports have shown that the climate change is affecting the beneficial insects as well. The finest and most expensive silk in the world produced in Assam, the golden silk is under the threat of climate change. Unlike the mulberry silkworm it is grown outdoors being highly exposed to weather extremes. Unpredicted rain fall pattern, rise in temperature and persistent floods are threatening the cultivation of golden silk. The case of lac is also not different. India is the largest producer of lac in the world giving livelihood to the farmers of West Bengal, Chhattisgarh and Jharkhand. The change in temperature and rainfall pattern affects the sexual behavior and mortality rate of lac bugs which intern hits the lac production in the country.

4. Effect of climate change on plant leaf chemistry

Insects consuming foliage produced under elevated CO₂ often find this food source less suitable than foliage developed under current ambient levels. When consuming high CO₂ foliage, pupal and adult weights reduce (Robinson *et al* 2012). An unambiguous indicator that elevated CO₂ reduces insect growth rates by altering the chemical and physical properties of foliage is that insects consume more high-CO₂ foliage, an average of 14% more, to maintain lower growth rates. Increased consumption of low-quality food to meet critical nutrient limitations is referred to as “compensatory feeding” and may portend greater herbivore damage to both managed and natural ecosystems as CO₂ continues to increase (Cornelissen, 2011). In plants using the C3 photosynthetic pathway, the rate of photosynthesis and biomass accumulation increases strongly with rise in CO₂ (Long *et al* 2004). This affect the suitability of leaves to herbivorous insects. Carbohydrates, including starch and soluble sugars, typically increase in foliage grown under elevated CO₂, which leads to decrease in leaf N content (Stiling and Cornelissen, 2007; Robinson *et al* 2012). Increased carbohydrates (23%–50%) and reduced protein levels (10%) drive an average 19% increase in plant C:N ratio (Robinson *et al* 2012). Because chewing insects in particular are strongly limited by the N content of their diets, they must consume more high-CO₂ foliage to meet their nutritional needs.

Physical characteristics of leaves, including specific leaf area, surface waxes, secretory canals, and leaf toughness, also respond to elevated CO₂ and affect herbivory. Specific leaf area (SLA), the ratio of leaf area to leaf mass, decreases (toughness increases) under elevated CO₂ (Stiling and Cornelissen, 2007; Robinson *et al* 2012), directly reducing the suitability of foliage to insects by diluting the leaf N content. Elevated CO₂ causes smaller changes in leaf chemistry in gymnosperms than angiosperms and little or no change in leaf carbohydrates in plants using C4 photosynthesis. In addition, the reductions in N are considerably smaller in N-fixing than non-N-fixing species (Robinson *et al* 2012).

Elevated temperature can also influence the food suitability to insect herbivores directly by modulating leaf carbohydrate concentration through its effect on photosynthesis and respiration and indirectly through its influence on plant development. Elevated temperature negated the increase in leaf carbohydrates caused by elevated CO₂, but the simultaneous effect of both elements of global change was to amplify decreases in leaf N, causing substantial increases in leaf C:N ratio (Zvereva and Kozlov, 2006). The coevolutionary arms race with herbivorous insects has produced a dazzling array of secondary defensive compounds (allelochemicals) in plants. Many of these compounds are continuously present in low levels in plant tissues (constitutive) but rise to high concentrations (induced) in response to herbivory. No consistent trend has been identified in the response of allelochemicals, including glucosinolates, tannins, and terpenoids, to global change, and taxon specificity reigns supreme (Bidart-Bouzat and Imeh-Nathaniel, 2008), some generalizations are emerging. On average, growth under elevated CO₂ stimulates the production of phenolics, tannins, and flavonoids and suppresses the production of terpenoids (Robinson *et al* 2012). This differential response suggests that elevated CO₂ favors the shikimic acid pathway leading to the production of phenolics and suppresses the mevalonic acid and methylerythritol phosphate pathways leading to the production of terpenoids (Lindroth, 2010). In members of the mustard family (Brassicaceae), including *Arabidopsis* (*Arabidopsis thaliana*), elevated temperature increases the production of isothiocyanates, hydrolysis products of some glucosinolates, but decreases nitriles (Bidart- Bouzat and Imeh-Nathaniel, 2008). Elevated temperature also increases the production of biogenic volatile compounds (BVOCs; Peñuelas and Staudt, 2010). These volatile compounds emitted by plants, including isoprene, monoterpenes, sesquiterpenes, green leaf volatiles, methyl salicylate, and many others, aid in plant defense, pollination, and communication. The emission of BVOCs announces that a plant is under attack and that a meal awaits any predator answering the call (Arimura *et al* 2009).

It is important to note here that most experimental approaches to studying plant and insect responses to global change have not been done in an evolutionary context and thus do not address how organisms will adapt to rapid increases in CO₂ and temperature (Shaw and Etterson, 2012). For example, *Plutella xylostella* caterpillars reared for multiple generations on a high-carbohydrate diet that mimics foliage grown under elevated CO₂ developed the ability eat excess carbohydrates without the fitness costs of excess fat storage (Warbrick-Smith *et al* 2006). So, a complete analysis of the response of plants and insects to global change will need to consider potentially important mechanisms of adaptation.

5. Effect of climate change on plant defence to herbivory

Mechanical damage and insect oral secretions, singly or in combination, initiate the defense response in plants. Insect oral secretions are detected by putative receptors and amplify mitogen-activated protein kinases, which control the main molecules involved in signaling JA (jasmonic acid), as well as ET (ethylene) and SA (salicylic

acid), hormones that regulate plant development and chemical defenses (Wu and Baldwin, 2010). Phloem-feeding insects, such as aphids (Aphidoidea) and whiteflies (Aleyrodidae), induce SA-dependent responses, whereas wounding caused by chewing insects is associated with rapid accumulation of JA and ET at the wound site (Wu and Baldwin, 2010). However, chemical responses of damaged plants are an integral component of adaptive antiherbivore defense that abiotic factors, such as high temperature or elevated CO₂ environment, disrupt by altering plant signalling, that in turn, affects defensive pathways and the plant-insect interaction balance (Zavala *et al* 2008; Niziolek *et al* 2012). In tomato (*Solanum lycopersicum*), elevated CO₂ enhances induced defenses derived from the SA-signaling pathway, such as the pathogenesis related proteins, and reduces JA-signaling and defences (Sun *et al* 2011; Guo *et al* 2012). Moreover, whereas enriched CO₂ atmosphere increased the susceptibility of soybean to Japanese beetle and western corn rootworm (*Diabrotica virgifera virgifera*) by downregulating JA and ET, elevated CO₂ increases SA levels in field-grown soybeans and in leaves and rhizomes in two varieties of ginger (*Zingiber officinale*; Zavala *et al* 2008; Ghasemzadeh *et al* 2010). The concomitant effects of elevated CO₂ on SA and JA/ET in soybean increased the concentration of SA regulated phenolics, such as flavonoids with antioxidant properties (quercetin, kaempferol, and fisetin), but decreased the concentration of JA-regulated antiherbivore defenses, such as the isoflavonoid genistein and Cys protease inhibitors (Zavala *et al* 2008; O'Neill *et al* 2010). Elevated CO₂ decreased the emission of JA-regulated terpene volatile compounds in cabbage (*Brassica oleracea*) and reduced the host searching efficiency of the specialist parasitoid *Cotesia plutellae* (Vuorinen *et al* 2004).

High temperature affects the BVOC emissions in response to insect attack. The combination of high temperature and simulated herbivory in corn resulted in greater JA-regulated BVOC emission than when each stress was applied separately (Gouinguéné and Turlings, 2002). In contrast with elevated CO₂, high temperature increases JA/ET and SA (Clarke *et al* 2009), which also results in high levels of chemical defences (e.g. trypsin and CystPIs; Mosolov and Valueva, 2011). High temperatures increased allelochemicals regulated by JA/ET, such as trypsin and CystPIs, in many plant species (Mosolov and Valueva, 2011). In addition, high temperature induces the production of antioxidant flavonoids and the pathogen-related response proteins, both regulated by SA (Djanaguiraman *et al* 2011). These chemical responses regulated by phytohormones to high temperature explain the higher resistance of plants to both insects and pathogens when grown in a high-temperature environment (Arimura *et al* 2009; Maimbo *et al* 2007). However, the less explored interactive effects of elevated CO₂ with high temperature on plant allelochemical production suggest that the up-regulation of JA/ET by high temperature would eliminate the susceptibility of plants to herbivore attack produced by elevated CO₂ alone (Niziolek *et al* 2012).

6. Conclusion

Climate change now a days is globally acknowledged fact. Global warming and climate change will trigger major changes in arthropod diversity, geographical distribution of insect pests, insect population dynamics, herbivore–plant interactions, activity and abundance of natural enemies, and efficacy of crop protection technologies for pest management. This intimate relationship between plants and insects will in some cases results in devastating crop losses and, in other cases, facilitates agricultural production. How elevated CO₂ and temperature independently affect the primary chemistry of leaves, including leaf carbohydrate contents and leaf N, is relatively well understood, as is how these changes affect the performance of many herbivorous insects. Atmospheric CO₂ and temperature, however, are increasing in parallel, and even for primary plant chemistry, our knowledge of the interactive effects of these two elements of global change remains shallow.

Understanding how global change will affect the ecological and evolutionary relationships between plants and insects remains challenging as these interactions integrate all behavioral, physiological, and molecular interactions at lower organizational scales. Whereas many or most generalist herbivores may be preadapted via host switching to changes in plant phenology and hence resource availability, many or most specialists may face local extinction due to ecological mismatching. Impacts of climate change on crop production mediated through changes in populations of serious insect-pests need to be given careful attention for planning and devising adaptation and mitigation strategies for future pest management programmes. These strategies can be a promising programme for crop-health management and sustainable ecosystem from insects under changing climate.

Suggested Readings

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STATISTICAL PROCEDURES IN AGRICULTURAL EXPERIMENTAL TRIALS

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Crop improvement is a continuous process and development of new cultivars better than the existing ones is the ultimate objective of plant breeding. Most of the economically important traits in crop improvement programmes are quantitative in nature and needs to be estimated with higher precision. In order to find significant differences in the quantitative trait values, several statistical procedures are required including analysis of variance (ANOVA), analysis of covariances, multivariate analysis etc. ANOVA is an important procedure to find differences in experimental material which are being evaluated in experimental designs along with elite cultivars termed as checks. In this regard, statistical procedures in general and experimental design in particular are of great importance.

The choice of the experimental design depends critically on the number of entries being evaluated and availability of seeds for replications. In early stages of the experiment, plant breeder has a large number of treatments (usually > 20) with sufficient seeds to be evaluated with respect to check cultivars. At these stages, the randomized complete block design (RCBD) has always been preferred due to its ease in the layout and data analysis. On the other hand, a plant breeder develops different mapping populations including Recombinant Inbred Lines (RILs), Backcross Inbred Lines (BILs) etc. depending upon the purpose of study. The size of such populations is also large enough for precise evaluation in RCBD. Whereas, the evaluation of more than 20-25 entries in RCBD increases soil heterogeneity within large size blocks, thereby increasing the experimental error (Cochran and Cox, 1957). As a result, the error sum of squares is large compared to the sum of squares attributable to the model and hence, small treatment differences may not be detected as significant. The analysis of data also reveals that the coefficient of variation (CV) in many of such experiments is high and as a consequence, the precision of treatment comparisons is low. So there is a need to shift the plant breeding field experiments from RCBD to such experimental designs that would increase the efficiency of evaluation of large number of treatments by reducing the soil heterogeneity and increasing the precision of treatment comparisons. The Incomplete Block Designs (IBD) are the type of designs that could better replace the RCBD. As compared to RCBD, IBD gets its name from the incomplete set of treatments in each block. In this design, plots are grouped into blocks that are not large enough to contain all the treatments, thereby a large number of entries can be evaluated in number of incomplete and more uniform blocks. These designs were introduced by Yates (1936) in order to eliminate heterogeneity to a greater extent as compared to RCBD and Latin squares, when the number of treatments is large. In field trials, more uniform blocks can be formed by conducting uniformity trials in the preceding years to determine the fertility pattern of the field through a contour map.

The precisions of the estimate of a treatment effect depends on the number of replications of the treatment, large is the number of replications, more is the precision. Similar is the case for the precision of estimate of the difference between two treatment effects. If a pair of treatment occurs together more number of times in the design, the difference between these two treatment effects can be estimated with more precision. To ensure equal precision of comparisons of different pair of treatment effects, the treatments are so allocated to the experimental units in different blocks of equal sizes such that each treatment occurs at least once in a block and each pair of treatments has the same number of replications. When the number of replications of all pairs of treatments in a design is same, then the class of IBD is called Balanced Incomplete Block (BIB) designs and when there are unequal number of replications for different pair of treatments, then the designs are called as Partially Balanced Incomplete Block (PBIB) designs. In plant breeding, the minimum number of replications required for balance is often too large to be practical due to large number of treatments. Another class of IBD is the resolvable incomplete block designs also called as lattice designs. Lattice designs are the type of IBD, where the blocks must be capable of arrangement in complete replications of all the treatments and plant breeder has more flexibility choosing the number of replications depending upon the availability of resources (Patterson and Williams, 1976). In the article, we will further discuss the different types of Lattice designs, their method of construction and analysis using SAS (SAS Institute Inc.) software.

Lattice Designs

Lattice Designs are the most useful incomplete block designs for the plant breeders. In lattice design, blocks are grouped such that each group of block constitute one complete replication of treatments. We can call a group of incomplete blocks that contain one complete replication of treatments as superblock. These designs provide more flexibility in managing an experimental trial on replication basis. Based on the number of replications, Lattice could be simple lattice if the design has two replications of the treatments; triple-lattice if it has 3 replications and so on. In general, if the number of replications is m , it is called m -ple lattice (Hinkelmann and Kempthorne, 2005). Lattice designs can also be analyzed as RCBD, if the blocking turns out to be ineffective. Based on the number of treatments, lattice designs can be square lattice, rectangular lattice and α -designs.

Square Lattice Designs

The characteristic features of these designs are that the number of treatments " t " should be equal to square of the number of blocks " k " so that $t=k^2$. A balanced square lattice with k^2 treatments has $r = k+1$ replications and are arranged in k ($k+1$) blocks. The numbers of replication of partially balanced square lattices are less than required for balanced square lattices. So, each replication has k blocks and contains every treatment. In this design, every pair of treatments occurs together once in the same incomplete block. Let " λ " be an integer number indicating how many times each treatment occurs together in same block, and the relationship among the number of treatments t , block size k , and number of replications r . Numerically, it is defined as $\lambda = r(k-1)/(t-1)$ (Montgomery, 2005).

Design Construction

Assume there are t treatments labelled as $1, 2, \dots, k^2$ with treatment numbers arranged in a $k \times k$ square referred as standard array. For example, in a 3×3 square, there are nine treatment numbers arranged in a specific order such that each row of the square array is considered as a block containing three treatments. Standard array forms the Replication I of square lattice (Table 2). To construct Replication II, each column of the array for Replication I is taken to form the three blocks in Replication II (Table 2). Replications III and IV are formed using two mutually orthogonal Latin squares (MOLS). Two Latin squares are said to be mutually orthogonal, when superimposed, all ordered pairs are distinct (Table 1). From the standard array, upon superimposing, the treatment numbers that fall on the same letter in a Latin square are taken to form a block. For example, from latin square 1 (Table 1), treatment numbers 1, 6 and 8 fall on the letter A, so treatments 1, 6 and 8 are in the same block in Replication III (Table 1). Similarly, B falls on 2, 4, 9 and C falls on 3, 5, and 7 that form other two blocks. Analogous to Replication III, Replication IV is constructed from the second Latin square. Another method of constructing lattice square designs can be found in Federer and Wright (1988) who proposed a simple method for constructing lattice square designs with greater than 3 numbers of blocks.

Table 1. Standard array and two mutually orthogonal Latin squares (MOLS)

Standard Array			Latin Square I			Latin Square II			Superimposed		
1	2	3	A	B	C	A	B	C	(A,A)	(B,B)	(C,C)
4	5	6	B	C	A	C	A	B	(B,C)	(C,A)	(A,B)
7	8	9	C	A	B	B	C	A	(C,B)	(A,C)	(B,A)

Table 2. 3×3 balanced lattice ($t = 9, k = 3, r = 4, \lambda = 1$)

Block	Replication I	Block	Replication II	Block	Replication III	Block	Replication IV
B ₁	1, 2, 3	B ₄	1, 4, 7	B ₇	1, 6, 8	B ₁₀	1, 5, 9
B ₂	4, 5, 6	B ₅	2, 5, 8	B ₈	2, 4, 9	B ₁₁	2, 6, 7
B ₃	7, 8, 9	B ₆	3, 6, 9	B ₉	3, 5, 7	B ₁₂	3, 4, 8

Randomization

Randomization is important for giving equal chance to assignment of treatment to the experimental block. Randomization of square lattice design should be carried out with following steps: (i) Allot the treatments to treatment numbers at random; (ii) Randomize the replications; (iii) Randomize the blocks separately and independently within replication; (iv) Randomize the treatments separately and independently within each block.

Steps (ii) and (iii) give each treatment an equal chance of being allotted to any experimental unit. If differences among blocks are large, the error variance per plot for the mean of a group of treatments that lie in the same block may be considerably higher than the average error variance. This additional randomization ensures that the average error variance may be used, in nearly all cases, for comparisons among groups of treatments.

Statistical Analysis using SAS

The LATTICE procedure in SAS is very handy programme to analyse data from balanced square lattices, partially balanced square lattices, and some rectangular lattices. The LATTICE procedure determines the type of lattice design from the data set and gives the message if data is not valid. The data must consist of variables named "Group", "Block", "Treatment", and "Rep". The values of "Group" are 1, 2...r, where r is the number of replicates in a balanced design. The variable "Rep" is needed when there are more than 1 repetition of the entire basic plan. The values of "Rep" are 1, 2....p, where p is the number of replications of the entire basic plan. Hence, the experiment has a total of $r \times p$ replications. The variable "Treatment" should be written as shown. Details can be found in SAS/STAT 9.2 User's Guide (http://support.sas.com/documentation/cdl/en/statug/63033/HTML/default/viewer.htm#lattice_toc.htm).

The SAS script of square lattice design is:

```
Proc lattice data;
```

```
Var [variables];
```

```
Run;
```

The output of the Lattice procedure using Proc lattice displays the Analysis of variance (ANOVA) table and related statistics. ANOVA includes Replications, Blocks within Replications (adjusted for treatments), Treatments (unadjusted), Intra-block Error, Randomized Complete Block Error as the sources of variation. The Blocks within Replications sum of squares is divided into "Component "A" and Component "B". Both the components may show difference, if there is repetition of the basic plan. The variation due to Randomized Complete Block Error is the sum of variation due to Blocks within Replications and Intra-block Error. However, this SAS procedure does not give the adjusted treatment sum of squares. In addition, it also gives the efficiency of lattice design as compared to RCBD and adjusted means due to block effects. The related statistics include the LSD values and variance of mean difference in the same and different blocks.

Rectangular Lattice Design

To avoid the restriction of using perfect square number of entries in square lattice design, Harshbarger (1946) introduced rectangular lattice design, with details and extensions provided in further series of papers by Harshbarger (1947, 1949, 1951) and Harshbarger and Davis (1952). The design was a useful addition to the square lattice design. A rectangular lattice design is a resolvable incomplete block design for t treatments in r replicates of k blocks of size $k - 1$, where $t = k(k - 1)$ and $2 \leq r \leq k$, for

some integer k . The design has the property that any pair of treatments occur together in at the most one block. The design is constructed from a set of $r - 2$ mutually orthogonal $n \times n$ Latin squares.

Method of Construction

There are several methods of constructing rectangular lattice designs. Here we describe the simple method suggested by G.S. Watson (Robinson and Watson, 1949). This method uses a Latin square with k number of rows and columns, in which every letter in the diagonal is different. For constructing rectangular lattice with $t=12$, $k=4$, $r=3$, first write down a Latin square with different letters assigned to diagonal elements and give a number to all letters except those in the leading diagonal, as illustrated below for a 4×4 Latin square (Table 3).

Table 3. 4×4 Latin square with assigned numbers

A	B ¹	C ²	D ³
D ⁴	C	B ⁵	A ⁶
B ⁷	A ⁸	D	C ⁹
C ¹⁰	D ¹¹	A ¹²	B

The numbers in the same row of the latin square will form blocks of Replication I, whereas the numbers in the same column will form the Replication II. The Replication III will be formed by all numbers that have the same latin letter (Table 4).

Table 4. 4×3 rectangular lattice ($t= 12$, $k = 4$, $r = 3$)

Block	Replication I		Block	Replication II		Block	Replication III
B ₁	1, 2, 3		B ₅	4, 7, 10		B ₉	6, 8, 12
B ₂	4, 5, 6		B ₆	1, 8, 11		B ₁₀	1, 5, 7
B ₃	7, 8, 9		B ₇	2, 5, 12		B ₁₁	2, 9, 10
B ₄	10, 11, 12		B ₈	3, 6, 9		B ₁₂	3, 4, 11

By using the first 2 replications from any plan we obtain a rectangular lattice in 2 replications, which by means of repetitions can be used for an experiment in 4, 6, 8, etc., replications, by using all 3 replications of the plan we have designs for 3, 6, 9 etc., replications.

Statistical Analysis using SAS

The statistical analysis using SAS can be done with Lattice procedure described above.

Alpha (α) designs:

Square and Rectangular lattices designs are more restrictive to incorporate a statutory number of treatments. The requirements to evaluate non-statutory number of treatments led to the development of generalized lattice designs (Patterson and Williams, 1976).

Alpha design is a class of resolvable block design that is more flexible as compared to square and rectangular lattice designs for number of treatments to be evaluated in the experiment. The original catalogue of designs provides designs suitable for any number of treatments up to 100, grown in 2, 3 or 4 replications. The experimenter is able to specify the size of the block and hence to fix the number of blocks per replicate. In addition, if the total number of treatments does not factorise exactly, the design will permit some blocks to contain more or fewer treatments than all the other blocks. For example, an alpha design of 46 treatments can be constructed with 8 blocks in each replication, of which 6 blocks will contain 6 treatments and rest 2 blocks will have 5 treatments. Alpha designs are not balanced which means that not all the possible pairs of treatments occur together within an incomplete block thus reducing the efficiency of pairwise comparison. However in general, plant breeder wants to compare new selections/varieties with already existing varieties or checks on the basis of performance. For such experimental situations, one may take an alpha design in new selections/varieties and reinforce each block of the design by the check varieties. Thus new selections can be compared with the check variety which is the foremost concern of the breeder rather than all possible comparisons among new selections/varieties. Moreover, in experiments on RILs and NILs with large number of entries, reduction in experimental size is the main issue rather than pair wise comparisons. In that case alpha designs are the best suited, where one can incorporate good number of entries as single plot within incomplete blocks with minimum number of replications reducing the experimental size.

Method of Construction

Alpha designs are described as 0, 1 designs if pairs of varieties concur once or never. They are described as 0, 1, 2 designs if, in addition, some pairs of varieties concur twice and so on. The steps below can generate alpha design with number of treatments t , for which $t = sk$ where s is the block size (number of treatments per block) and k is the number of blocks per replicate. For example, a design for 200 varieties can be produced in $k=20$ blocks per replicate, each with $s=10$ treatments per block.

i) α -array: It is a $k \times r$ array of elements consisting of residue modulo class of s , i.e., the elements can be 0, 1, 2, -----, $s-1$, i.e. if $s = 3$, the $k \times r$ array will have (upto $s-1$) 0, 1, 2 as its elements (Table 5). Under a suitable relabeling or permutations convert the α -array to reduced array where the first row and first column consist of zero as elements. We shall construct the α -designs with the help of the reduced array. We can determine the concurrences of the different pairs by the α -array.

ii) Intermediate array (α^*): Construction of the intermediate array is the intermediate or second step to develop α -designs that helps to generate the replications. One can have $(s-1)$ new columns from a column of reduced array by developing the column cyclically modulo s . By this way we can construct $r(s-1)$ new columns by cyclically developing each column of the α -array modulo s (Table 5). This arrangement $k \times sr$ of array is called intermediate array.

iii) α -designs: Now, take the intermediate array and add $\{1+(j-1)s\}$ to the elements of j^{th} row of that array ($j=1,2,---k$), this gives us an array of size $(k \times sr)$ (Table 6). For

example, take the first row of the intermediate array (Table 5) written as (0,1,2), add $\{1+(j-1)s\} = 1$ ($j = 1$ for first row and $s = 3$), this will give 1,2,3 as the first row of replication 1 (Table 6). Similarly, taking one to one correspondence between the treatments and the elements of the final array, we get an α -designs with parameters $t = ks$, $b = rs$, r , k .

Given the generating array α , the intermediate array α^* and hence designs are easily constructed as per the steps given above.

Table 5: Construction of reduced α array and Intermediate array α^* for generating α -design with $t=24$, $k=4$, $r=3$ $s=6$, $b=18$

Reduced array α		
0	0	0
0	3	4
0	1	5
0	4	3

Intermediate array α^*																			
0	1	2	3	4	5		0	1	2	3	4	5		0	1	2	3	4	5
0	1	2	3	4	5		3	4	5	0	1	2		4	5	0	1	2	3
0	1	2	3	4	5		1	2	3	4	5	0		5	0	1	2	3	4
0	1	2	3	4	5		4	5	0	1	2	3		3	4	5	0	1	2

Table 6: Generating α -design from table 5.

Replicate	R1						R2						R3					
Block	B ₁	B ₂	B ₃	B ₄	B ₅	B ₆	B ₇	B ₈	B ₉	B ₁₀	B ₁₁	B ₁₂	B ₁₃	B ₁₄	B ₁₅	B ₁₆	B ₁₇	B ₁₈
	1	2	3	4	5	6	1	2	3	4	5	6	1	2	3	4	5	6
	7	8	9	10	11	12	10	11	12	7	8	9	11	12	7	8	9	10
	13	14	15	16	17	18	14	15	16	17	18	13	18	13	14	15	16	17
	19	20	21	22	23	24	23	24	19	20	21	22	22	23	24	19	20	21

Concurrences: The number of concurrence of two treatments is the number of blocks containing pair of treatments. In the above example pair of treatments 1 and 7 concur once, while treatments 1 and 3 do not concur at all. A design with concurrence g_1, g_2, \dots, g_n will referred as $\alpha(g_1, g_2, \dots, g_n)$ design; the above example is therefore an $\alpha(0,1)$ design.

Softwares for generating alpha lattice designs

The software packages can be a great help to construct alpha lattice designs. A catalogue of designs for number of varieties up to 100 has been published, and can be accessed from within GenStat (<http://www.edgarweb.org.uk/>). For larger number of

varieties, the packages Gendex (<http://designcomputing.net/gendex/alpha/>), CropStat7.2 (<https://drive.google.com/file/d/0Bw-PTBz1SHmsWWIRMXdybWJWT0k/view>) or CycDesignN (<http://www.vsni.co.uk/downloads/cycdesign>) and will create the designs. The R package “Agricolae” (<https://cran.rproject.org/web/packages/agricolae/index.html>) and SAS procedure “Proc Plan” (<http://www.stat.ncsu.edu/people/dickey/courses/st711/Demos/>) can also be used for constructing optimal or near optimal alpha lattice designs.

Statistical analysis using SAS

The statistical analysis of alpha lattice design is discussed here using an example. The data used for analysis consists of evaluation of 15 rice varieties in alpha lattice design for tiller number (hypothetical example). The linear model for SAS lattice design can be written as

$$Y_{ijk} = \mu + \tau_i + \gamma_j + \rho_{k(j)} + \varepsilon_{ijk},$$

where Y_{ijk} is the phenotype of i^{th} treatment in j^{th} replication and k block; τ_i = treatment effect $i=1, 2, \dots, k$; γ_j = replicate effect $j=1, 2, \dots, r$; $\rho_{k(j)}$ = block within replicate effect $k=1, 2, \dots, s$; ε_{ijk} = random error. The SAS procedures *Proc GLM* can be used if all the effects in the above model are fixed effects, whereas, *Proc Mixed* can be used if some effects are random in nature.

SAS code for fixed effects:

```
Proc glm;           /* procedure generalized linear model*/
Class rep blk trt;
Model y = rep blk(rep) trt ;      /* 'y' is any trait, 'blk(rep)' shows blocks within
replications*/
Lsmeans trt/PDIFF=all lines adjust=tukey;      /* 'PDIFF=all' will generate
pairwise
Run;                                     differences of all lsmeans, Tukey
test is used for multiple
comparisons of means, lines is
used to show the alphabets
showing significant differences*/
```

SAS codes for mixed effects including both random and fixed effects

```
Proc mixed; /* computes REML and ML estimates of variance parameters, generally
preferred over ANOVA*/
Class rep blk trt;
Model y = trt;
Random rep blk(rep)/solution; /* replications and blocks within replications are
taken as
ods output solution=y;          random effects, 'solution' will produce parameter
estimates
```

```

Store y;                                ods output makes dataset 'y' of estimates, and
stores the results in 'y'*/
Run;
Proc plm source= y;    /* uses the dataset 'y' and do the post fitting statistical
analysis*/
Lsmeans trt /pdiff=all lines adjust=tukey;
Run;

```

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DOUBLE HAPLOIDS FOR ACCELERATING BREEDING IN WEHAT

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Accelerated breeding is an urgent target in major crops to cope with typical emergent situations such as climate change and new pathotypes. We are also witnessing an unprecedented proliferation of plant breeding objectives. Further, the breeding time frames have shrunk and acquired a project mode. The concept of doubled haploids provides a potential solution but the available technologies, based on tedious *in vitro* steps, fall way short of required throughput. Doubled Haploid breeding is a technique to boost the crop production. Unlike to conventional breeding, this technique can reduce the time gap for breeding new varieties. Homozygous DH lines can be usefully deployed to elucidate the behavior of traits controlled by recessive alleles, genome characterization, genetic linkage mapping for QTL analysis, genetic transformation and proteomic studies. Among the different methods for DH generation, anther culture techniques play an immense role in rice improvement programme. However, this technique has not been put use in India to the maximum advantage due to lack of expertise and recalcitrant to androgenic response in *indica* rice. *Indica* varieties showed recalcitrant androgenic response due to early anther necrosis, poor callus proliferation and albino plant regeneration.

Application of wheat x maize system to wheat breeding The wheat x maize approach of doubled haploid production is finding increasing use in wheat breeding programmes world wide. The technique is an integral part of the wheat breeding methodology at Australian Grain Technology Pty. Ltd., the largest wheat breeding company in Australia (Kuchel *et al.*, 2005). Public sector institutes in Australia e.g., Plant Breeding Institute, South Australian Research and Development Institute and Agriculture Western Australia are also employing this technique. Wheat variety Bond CL was developed by Colorado State University using wheat x maize crosses and released in 2004 (Haley *et al.*, 2006). In Japan, wheat cultivar Sanukinoyume 2000 is one of the cultivars developed by using the wheat x maize system (Yuichi *et al.*, 2002). At CIMMYT, Mexico, this system of DH production is being used to generate populations for gene mapping and genetic analysis. Since 1997, 27 DH cultivars were released in Canada (De Pauw *et al.*, 2010). In 2009 DH cultivar Lillian occupied the largest area under any single variety in Canada and accounted for more than a third of the western Canadian wheat acreage. Lillian was developed using marker assisted selection to improve protein content with the *Gpc-B1/Yr36* introgression from *Triticum dicoccoides*. In 2010, DT801 first DH variety of durum wheat was released in Canada. The system is also being applied to selected crosses from wheat breeding programme at PAU, Ludhiana (Singh *et al.*, 2012). A DH wheat variety has been developed in India by CSKHPKV, Palampur for cultivation in the state of Himachal Pradesh. Doubled haploid technology has existed for a number of years and is widely used in Europe, Canada and Australia. Oklahoma State University released Gallagher, a DH in 2012 (Brett Carver, 2016). Carver also illustrates that he is on fourth cycle of doubled

haploid plant breeding, and has always gotten something of value out of the process. From the 1,100 crosses produced at OSU every year, Carver selects about 10 based on what has the best chance of producing a usable variety and applies DH technology. Twenty-five percent of his most elite material will be processed with DH. Kentucky Small Grain Growers Association approved following DH lines during 2015 BW965, BW966. Longreach Plant Breeder, a wheat breeding company in Australia primarily work with doubled haploids with Longreach Reliant a recent DH variety released in 2016. The cost of producing a DH globally is around 30-35 dollars. At Washington State University cost of DH plant is 15 \$ for partners and 25\$ for commercial breeders. The Plant Breeding institute, Cobbity in Australia produces DH at ~ 25\$ each and produce ~8000 DH lines per year while the biggest service provider in wheat DH is Heartland Plant Innovations Inc. (HPI) in Kansas. They produce up to 50,000 DH plants per year. The cost per DH is 35-50\$ for partner and commercial breeders. The cost of producing a DH in a developing country can be less provided the protocol does not require continued controlled conditions. The working conditions for DH production needs to be hybrid of field and growth chambers for limited duration and DH lines are produced in batches. On a small scale cost at our centre are estimated to be five dollars per plant where working episodes however will be restricted to 3-4 per year. Claiming a successful variety from DH programme in wheat requires large number of DH lines per cross. The number can vary from 200-300 but less than this, obtaining a commercially viable product may be difficult

Application of maize x maize haploid inducer (TAIL) system: Another *in vivo* system of haploid induction has been available in maize since long when a highly efficient haploid inducer line Stock 6 was identified by Coe, 1959 and development of a technique for rapid screening of haploids in the seed stage only (Coe and Sarkar, 1964) by linking endosperm/embryo colour with haploidy. In maize, Stock-6 based inducer lines (Coe 1959) possessing 8-10% haploid induction rate (HIR) is used as a male parent and crossed with F1 hybrids/OPVs. The haploids are produced due to high hetero-fertilization. Most of the haploid inducers are based on the anthocyanin colour marker, R1-nj-which is a dominant marker that facilitates easy identification of haploids at phenotypic level. R1-nj expresses in embryo (scutellum) as well as outermost layer of endosperm i.e. aleurone. Seed with coloured endosperm (at crown) and colourless embryo is indicative of haploidy, while the colour at both endosperm and embryo indicates crossed diploid seed. Further, seed and seedling traits have been identified for differentiating haploid and diploid seed for facilitating use of DH technology in germplasm with dominant colour inhibitor (2013, Chaikam et al. 2017). Identification of additional seed and seedling marker for segregating of haploid and diploid seed will enable application of DH technology to Indian germplasm with dominant colour inhibitor. The spontaneous chromosome doubling rate of maize haploids is very low (0.4%). Thus, artificial chromosome doubling is necessary for an efficient large-scale application of *in vivo* haploid induction. The highest doubling rates are achievable by immersing 2-3 day old seedlings in a colchicine solution. Several institutions current use colchicine as a source of chromosome doubling agent in DH production. In general, 8-10% doubling of chromosomes through colchicine treatment

is achieved. Further, various chemical compounds viz., pronamid, APM, trifluralin, pendamethalin and oryzalin have been reported to be efficient mitotic inhibitors (Hantzschel and Weber, 2010). These chemicals are less-expensive and -toxic than colchicine, and are easier to handle and dispose. Several commercial breeding companies apply such proprietary artificial chromosome doubling treatments (Geiger and Gordillo, 2009). Two major loci viz., *qhir1* and *qhir8* on chromosome 1 and 9, respectively, have been found to play a major role in haploid induction, with a contribution of 66% and 20% R², respectively. Of the two, *qhir1* has been fine mapped and recently a gene at this locus has been cloned. The genetic basis of haploid induction behavior in the commercially successful Stock-6 based maize system has been recently reported by three independent research teams within the space of one month (Kelliher et al 2017, Liu et al 2017 and Gilles et al 2017). It was shown that a four bp frameshift mutation in a pollen specific phospholipase gene is responsible for haploid induction. RNAi, TALENS and CRISPR-Cas suggest of a loss of function in this gene (named MATRILINEAL1 or NOT LIKE DAD) as the trigger for HI behaviour. The gene is a pollen-specific phospholipase, termed as Matrilineal (MTL), Phospholypase A (ZmPLA1) and Not Like Dad (NLD) by Kelliher et al. (2017), Liu et al. and Gilles et al., respectively. The mutant allele carries a 4-bp insertion leading to a frame-shift mutation, thus a truncated protein is produced. The transcript is detected only in anther at the second mitosis stage (two-nuclei stage), which reached the highest level at the three-nuclei stage.

Prospects of developing same species haploid inducers:

Haploids can be obtained from the progeny of crosses between parents from different species by a process of selected chromosome elimination (Forster et al, 2007; Kasha and Kao, 1970; Laurie et al., 1990; Wezdzony et al, 2009). Uniparental chromosome elimination can occur due to difference in the timing of mitosis, asynchrony in nucleoprotein synthesis or formation of multipolar spindles, different genome ratios, a spatial separation between genomes at interphase and/or at metaphase. Chromosomes destined for elimination appeared to not segregate properly at metaphase and lagged at anaphase. This process was first discovered in barley (*H. vulgare* x *H. bulbosum*) (Kasha and Kao, 1970). Haploid production by intra-specific hybridization is the predominant way in maize (Chang and Coe, 2009; Geiger, 2009). This method was first reported in 1959 with the discovery of maize haploid inducer Stock 6, producing 2%–3% maternal haploids when outcrossed as a male. Over the years, haploid induction rates (HIR) increased to 8%–10% due to development of high inducing lines, such as WS14, RWS, UH400, BHI306 and CAU5 (Wu et al., 2014; Xu et al., 2013).

The path-breaking report for development of haploid inducers (through CENH3 manipulations in *Arabidopsis*) was published by Ravi and Chen in 2010. Soon after, CENH3 was also implicated in chromosome elimination in *bulbosum* system (Sanei et al 2011). Distant CENH3s (e.g., *Zea mays*) were seen to complement the *Arabidopsis* null mutants but cause haploid induction (HI) in crosses (Maheshwari et al 2015) on account of a divergent CENH3 N-terminal tail. Kuppu et al (2015) showed that point mutations including preexisting ones in ‘Histone Folding Domain’ could confer HI in *Arabidopsis*. In crop species, CENH3 modifications were seen to generate HI trait in

maize (Kelliher et al 2016). Britt and Koppu (2016) called CENH3 an emerging player in haploid induction technology and strongly recommended non transgenic approaches involving alien gene introgression and mutagenesis for creating haploid inducers

Doubled haploids coupled with Marker assisted selection

MAS allows breeder to discard a large number of plants with undesired gene combination, pyramid beneficial genes in subsequent generations, minimize field testing and reduce the number of generations (Collard and Mackill, 2008; Dwivedi *et al.*, 2015). Scenario for marker assisted selection has improved by great leaps with mapping and tagging of new genes and availability of linked selectable molecular markers for each. The feasibility of combining MAS and DH to accelerate breeding programmes has been explored (Howes et al. 1998; Yonezawa and Ishii 2005; Gupta et al. 2010) and the two approaches can be combined successfully. Conventionally, DH based mapping populations have helped in generating molecular tags of many genes and large number of QTLs. The combination of MAS and DHs offers new opportunities for increasing genetic gain and shortens the time required to cultivar breeding. Wessels and Botes (2014) developed a series of DH wheat lines containing rust resistance genes. MAS was used for the selection of resistance genes, and DH technology was used for the generation of homozygous lines. With the increase in number of genes and traits, three way and four way crosses become necessary, subjecting such crosses to F_2 decreases the chances of recovery of desired progeny by $2n$ times where n is number of genes being incorporated. The strategy that we follow for DH and MAS involves, multiple elite lines having single or two genes proven for yield. These would serve as the parents for initial cross. Such elite x elite parents F_1 s are subjected to DH production and first cycle DH are then subjected to three way or four way crosses for further pyramiding and incorporation of 4 and more genes together. The F_1 s generated by intercrossing two DH lines are then again subjected to DH production. The products can be obtained at multiple stages (cycle 1 and 2) giving it required flexibility for number of traits and number of cycle of DH. Multiple trait incorporation can be possible only through a minimum of two cycle of DH. Figure 4 is an example of incorporation of 8 genes and gives the frequency or proportion by which DH would scale over the segregating generation.

The scheme balances the DH production and MAS work in such way the resource are conserved without compromising the number and worth of final product. This strategy is currently being implemented at PAU following the progress of continuous work that we have put in developing a DH production protocol suited to our conditions, the wheat breeding programme is just at the verge to take final leap into a DH based acceleration.

A quick backcross breeding for maximum recovery (97%) of recipient parent genome (RPG) within two backcross was given by Randhawa et al (2009). They adopted a four stage strategy, where large number of BC_1F_1 (2000-3000) seed is generated. Plants carrying introgressed gene are identified using flanking markers. Post foreground selection, the chromosome carrying the gene(s) is further recovered with respect to recurrent parent using 25-30 equally spaced markers. Selected plants are then analyzed for recovery of non carrier chromosomes. For this, each chromosome is surveyed with

4-5 markers which are monomorphic to recipient parent. Two to four plants were identified having maximum RPG were backcrossed again and BC₂F₁ following the four step marker assisted selection. The team bred stripe rust resistance into wheat variety 'Zak' through marker analysis of 2,187 backcross-derived progeny that resulted in the recovery of a BC₂F_{2:3} plant with 97% of the recurrent parent genome. In contrast, only 82% of the recurrent parent genome was recovered in phenotypically selected BC₄F₇ plants developed without MABS. In India, HD2932 was improved, by Indian Institute of Agriculture Research, New Delhi, for multiple rust resistance by marker-assisted transfer of genes Lr19, Sr26 and Yr10 following background recovery by Mallick et al (2015).

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MOLECULAR BREEDING METHODS IN GUAVA FOR YIELD AND QUALITY TRAITS IN CHANGING CLIMATIC CONDITIONS

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Fruit crops in general have long juvenile period and big plant size limiting the size of bi-parental populations to be used in genetic linkage mapping. Genetic diversity during the selection and domestication of fruits has further reduced the genetic base. Interestingly, fruit crops are cross-pollinated bringing an opportunity in disguise that they have fast Linkage Disequilibrium decay. Mapping in fruit crops is capitalized on the account of treating F1s as a backcross population apt for mapping. Instead of one, here two linkage maps are developed each individually for a single parent. Second parent here is treated as dummy and allelic segregation of single parent involved in cross is scored in the F1 population. This strategy has been named as pseudo-test cross strategy. However, QTLs identified using such approach are not very stable. So, we need to generate MetaQTLs to detect the actual effect of such QTLs (Tan *et al* 2019). For MetaQTL analysis we need to generate multiple independent crosses among parents differing in that particular trait. Several such contrasting combinations among individual parents are developed to identify the robust associations. So we basically need large number of molecular markers to map the QTLs and involve it in marker assisted breeding.

Generating large number of markers is now possible with the re-sequencing of genomes and mapping data from individual strains/ parents to a reference genome assembly (Slovak *et al* 2019). However even if we are lacking a reference genome we can still use denovo assembly generation and map the high throughput sequencing data generated through reduced representation library sequencing schemes like GBS, RAD and ddRAD (Generally referred to as GBS). However GBS schemes have their major limitations as large amount of sequencing data are missing at same genomic spots for several individuals. Missing data needs an imputation step and can be performed only in cases where we have a reference genome available. However if we have a reference genome available we can go for high throughput re-sequencing of select genotypes and generate SNP dataset and develop SNP arrays/SNP chips (Roorkiwal *et al* 2018). The DNA extracted from association panel or a bi-parental population can be flooded over a SNP chip containing several thousand SNPs and traits can be mapped to the genome at a very high resolution. A good example of mapping fruit shape and non-acid trait is in Peach with the help of 9K SNP chip developed by re-sequencing genomic DNA from several accessions. Reducing the juvenility in fruits is also been achieved by overexpressing the Flowering Locus T (FT) or its downstream genes like *Apetala* and *Leafy* in citrus and apple (Kotoda *et al* 2010, Yamagishi *et al* 2011)

Guava is a very important fruit crop in changing climatic conditions as it is widely adapted to low fertility lands and harsh weather conditions in tropics and sub-tropics of south-east Asia. Genomic resources in guava “apple of tropics” are scanty.

Being the second most important fruit crop of Punjab, at PAU we have taken an initiative to develop Genomics platform in this crop. We have generated the first transcriptome in guava by sequencing the RNA from leaf, shoot tips, flower buds and fruits at various development stages in the widely grown cultivar “Allahabad Safeda”. Allahabad Safeda is a non-coloured fruit cultivar with green skin and white flesh. Genotypes with coloured flesh and/or skin are available. To understand the colour development in guava we have compared the RNA-Seq of coloured vs non- coloured fruits among genotypes and have identified the molecular mechanism behind this important phenomenon.

However to develop large number of markers we have to develop the reference genome of guava. The recent funding from DBT will help PAU to generate the reference genome of guava using high throughput Next generation sequencing technology. We also have good diversity among genotypes of guava at PAU. Selected panel of 21 genotypes are being re-sequenced to develop high through-put markers. However this diversity panel size is small. So, we have developed several F1 populations by crossing the parents of contrasting traits so that mapping of fruit quality traits be performed. Mapping the traits of breeder’s interest will identify the associations making marker assisted breeding in guava possible.

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MAPPING QTLs FOR COMPLEX QUANTITATIVE TRAITS IN PLANTS

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BACKGROUND

Between Mendel's presentation of his experiments with peas to Brunn Natural History Society in 1865 and recognition in 1900 of the basic importance of his findings by Erich von Tschermak, Hugo de Vries, Carl Correns independently, Francis Galton tried to elucidate the principles of hereditary transmission. But the choice of material as well as the characters of study was opposed to that of Mendel and for that reason, Galton could not bring out as impressive laws of heredity as that of Mendel. He described the inheritance to be largely but not wholly particulate. Galton's theory of Natural inheritance helped his follower to prove natural selection of continuous traits and his and Karl Pearson's viewpoint resulted in severe controversy around 1900 with the non-believers of Biometry. Karl Pearson was greatly inspired by Galton and developed number of techniques for analysis of continuous traits like correlation and regression that serve today as well. This group of Biometricians (Weldon, Pearson) lead by Sir Francis Galton considered Mendelian principles to be inappropriate to explain genetic basis of continuous variation and rather speculated the involvement of blending inheritance for quantitative characters. William Bateson and his group, on the other hand, considered only qualitative characters to be heritable with little chance of blending inheritance. The conflict between these two groups had a strong bearing on Darwin's theory of Evolution which was based on permanence of small changes brought about by natural selection which largely was supposed to operate on continuous type of variation. The subject of Quantitative Genetics originated out of a historic conflict between two schools of thought regarding the relevance of Mendelian principles of inheritance to explain variation for quantitative characters.

The quantitative characters were proposed to be controlled by relatively large number of genes (multiple factors) with small, similar and cumulative effect termed 'polygenes' (Mather 1949), which is likely to be influenced by environmental factors. In 1906, Yule hypothesised the existence of genes with cumulative action, and the experimental evidence for their existence was provided by Nilsson-Ehle in 1908. Subsequently, between 1910 and 1916, East and Emerson collected extensive data in support of polygenic inheritance in maize and tobacco. The findings from these and subsequent studies made it clear that the continuous variation characteristic of quantitative traits resulted from the large number of polygenes involved in their control and the environmental influences on their phenotypic expression. For most of the period up to 1980, the study of quantitative traits has involved statistical techniques based on means, variances and covariances of relatives. These studies provided a conceptual base for partitioning the total phenotypic variance into genetic and environmental variance, and further analysing the genetic variance in terms of additive, dominance and epistatic effects. From this information, it became feasible to estimate the heritability of the trait and predict the response of the trait to

selection. It was also possible to estimate the minimum number of genes that controlled the trait of interest. However, little was known about what these genes were, where they are located, and how they controlled the trait(s), apart from the fact that for any given trait, there were several such genes segregating in a Mendelian fashion in any given population, and in most cases their effects were approximately additive (Kearsey and Pooni, 1996). Sax's (1923) demonstrated with experiment on beans that the effect of an individual locus affecting seed weight, a quantitative trait could be isolated through linkage with genetic marker and all of the markers used by Sax were morphological seed markers with complete dominance. In the 1940s and 1950s, a series of elegant experiments with *Drosophila* by Mather and co-workers showed that genes controlling quantitative traits were distributed over many chromosomal sites. But during 1930-80s, there were extremely few successful detections of marker-QTL linkage in crop plants and of these, even fewer were repeated. The major limitation was the lack of availability of adequate polymorphic markers.

QTL MAPPING - REVIVAL OF QUANTITATIVE GENETICS

Two major developments during the 1980's changed the scenario: (i) the discovery of variability at the DNA level that could be used as markers; and (ii) development of statistical packages that can help in analysing variation. We now refer the polygenes by a more catchy acronym, 'QTL' (Quantitative Trait Loci), a term first coined by Gelderman (1975). A QTL is defined as "*a region of the genome that is associated with an effect on a quantitative trait*". Conceptually, a QTL can be a single gene, or it may be a cluster of linked genes that affect the trait. In the last two decades, phenomenal improvements in molecular marker technology has greatly facilitated the QTL mapping. The report by Paterson et al, 1988 on mapping of QTLs governing fruit size, pH, and soluble solids in tomato is one of the first studies that used DNA markers for QTL mapping. Over the years, not only a very large number of QTLs governing various quantitative traits but also several different types of QTLs have been discovered and mapped. QTLs have been grouped into different categories on the basis of their effect size, effect of the environment on their expression, the type of effect produced by them, and the manner of their action. Main effect QTLs produce direct effect on the expression of the concerned traits, while epistatic QTLs interact with the main effect QTLs to influence the trait phenotype. A main effect QTL is described as a major QTL if it explains 10 % or more of the phenotypic variance for the trait, while a QTL with a smaller effect size is termed as minor QTL. Most quantitative traits are governed by few major QTLs and many minor QTLs. Many QTLs affect the expression level, i.e., the level of RNA transcript produced in a tissue, of various genes; such QTLs have been designated as expression QTLs (eQTLs) or regulatory QTLs. Metabolic QTLs (mQTLs) control metabolic traits, i.e., rates of various metabolic reactions and metabolite levels. In most crop species, plant breeders would have already exploited the major QTLs. In addition, marker-assisted selection for major QTLs is relatively easy.

PRINCIPLES OF QTL MAPPING

QTL is a region of genome that is associated with effect on quantitative trait. The basic principle of determining whether a QTL is linked to a marker is to

partition the mapping population into different genotypic classes based on genotypes at the marker locus, and then apply correlative statistics to determine whether the individuals of one genotype differ significantly with the individuals of other genotype with respect to the trait being measured. QTL mapping is thus to estimate linkage relationship between QTL and marker.

SALIENT REQUIREMENTS FOR QTL MAPPING

1. A suitable mapping population generated from phenotypically contrasting parents
2. A saturated linkage map based on molecular markers
3. Reliable phenotypic screening of mapping population
4. Appropriate statistical packages to analyze the genotypic information in combination with phenotypic information for QTL detection

APPROACHES OF QTL MAPPING

- 1) Single Marker Analysis (SMA)
- 2) Interval Mapping (IM)
 - a) Simple Interval mapping (SIM)
 - b) Composite Interval Mapping (CIM)
 - c) Inclusive Composite Interval mapping (ICIM)
 - d) Multiple Interval Mapping (MIM)
 - e) Bayesian Mapping

SINGLE-MARKER ANALYSIS

Single-marker analysis (SMA) is the simplest and the earliest used method of QTL detection (Soller and Brody 1976). In this method, each marker is separately tested for its association with the target trait. The phenotypic means for the plants placed in the different marker genotype groups are compared to detect a QTL at or near the site of the marker. The significance of differences between the means of the marker classes can be tested by Student's t-test, analysis of variance, linear regression analysis, likelihood ratio test, or maximum likelihood estimation. SMA is computationally the simplest; can be performed using common statistical software and does not require marker linkage maps. But SMA suffers from some serious limitations as (1) the likelihood of detection of a QTL with a given effect size depends upon magnitude of r (recombination rate); (2) The position of QTL in the genome remains unknown; (3) This method cannot determine whether one or more QTLs are associated with a marker; (4) The method has low QTL detection power; (5) it gives many "false-positive" signals.

SIMPLE INTERVAL MAPPING

Lander and Botstein (1989) developed the interval mapping (IM) procedure, which is generally known as simple interval mapping (SIM). SIM requires a marker linkage map for QTL search as it uses neighbouring marker pairs to define marker intervals and search QTLs within these intervals. The SIM model considers at a time a

single QTL affecting the concerned trait, and each marker interval is analysed independent of the other marker intervals. The SIM genetic model is as follows: $y_i = \mu + ax_i + e_i$; where y_i is the trait phenotype of i th individual, μ is the grand phenotypic mean, a is the QTL effect, x_i is the indicator of QTL genotype, and e_i is a random error term mean 0 and variance σ^2 . The conditional probabilities of different genotypes (x_i) of the presumed QTL within each marker interval can be estimated on the basis of genotypes of the marker pair defining the concerned interval. A linear regression program that maximizes the likelihood function is used to obtain the maximum likelihood estimates (MLEs) for μ , a , and σ^2 with the above estimates of x_i . The MLEs are the values of these parameters (μ , a , and σ^2) that maximize the likelihood of obtaining the observed phenotype data with the given marker genotype data. Now MLEs for these parameters are estimated under the assumption that there is no QTL in the marker interval so that the value for ' a ' becomes zero. The above two MLEs are used to estimate the LOD score, which indicates the likelihood of a QTL being present in the marker interval. SIM has more precision and detection power than SMA, but suffers essentially the same shortcomings of SMA.

COMPOSITE INTERVAL MAPPING

Composite interval mapping (CIM) combines interval mapping with multiple regression analysis (Jansen 1994; Zeng 1994). CIM controls the effects of QTLs present in other marker intervals of the same chromosome and other chromosomes that increases the precision of QTL detection. CIM first carries out single-marker analysis; it then typically builds up the model as multiple QTL model using stepwise or forward regression method. In this approach, the marker with the highest LOD score is selected first; then the marker with the second highest LOD score is added, and the two markers are re-evaluated for significance. If both the markers remain significant, the marker with the next highest LOD score is added to the model, and the significance of the three markers is re-evaluated. In this manner, all the markers that remain significant when brought together are fitted into the model as cofactors, and the entire genome is scanned for QTL detection and mapping. When CIM is implemented properly, it is the best interval mapping method based on linear regression model and maximum likelihood principles. The chief limitation of CIM algorithm is (1) the arbitrariness in selection of the cofactors for QTL analysis; (2) CIM is unable to detect QTL X QTL interaction; as a result, it is inefficient when epistasis is present.

INCLUSIVE COMPOSITE INTERVAL MAPPING

The inclusive composite interval mapping (ICIM) uses a modification of the CIM algorithm, which uses all the marker information to build the linear regression model of CIM (Li et al. 2007a). Since the number of QTLs would be lower than the number of markers used for QTL analysis, standard stepwise regression analysis is used to discover the markers that are the most important for the QTL analysis; this in turn identifies the significant QTLs affecting the trait. The markers having significant regression coefficient estimates are selected as background markers or cofactors; this is done only once during the entire analysis, and the regression coefficients for the remaining markers are set at zero. ICIM is not much affected by the choice of probability levels for the inclusion/exclusion of cofactors, but a lower probability level

would reduce the chances of detecting false-positive QTLs. Stepwise regression analysis is used to estimate the effects of significant markers before the interval mapping of Lander and Botstein (1989), is applied to the adjusted phenotypic data. In the case of a QTL that is located in the center of a marker interval, the effect of QTL is equally distributed between the two markers defining the interval, and the QTL maps in the middle of the interval. But when a marker is located close to one of the two markers, this marker will absorb most of the effect of this QTL, and it will map onto this marker. ICIM is capable of detecting dominance and two-gene epistasis. The form of ICIM is simpler and the speed of convergence is faster than those of CIM, while it retains the optimal properties of CIM. ICIM shows visibly high LOD scores that improves the QTL mapping power and the precision of ICIM over CIM. The results obtained from ICIM were more or less comparable to those from more complex and time consuming Bayesian models of QTL mapping.

MULTIPLE INTERVAL MAPPING

The multiple interval mapping (MIM) approach is devised for simultaneous QTL mapping in multiple marker intervals (Kao et al. 1999). MIM avoids the complicated procedure used in CIM for the selection of background markers, but it uses several selection methods like forward search method and forward and backward selection methods to search for the best genetic model. However, the different model selection methods usually lead to different results. MIM is able to take into account QTL X QTL interactions, if present, among the multiple QTLs included in the model. The chief limitations of MIM are (1) As the number of QTLs included in the model is increased, there is an exponential increase in the number of parameters; (2) the selection of appropriate model from among the innumerable models that are possible is a challenge since an appropriate and reliable criterion of model selection is difficult to develop.

BAYESIAN MULTIPLE QTL MAPPING

Bayesian QTL mapping has been designed for the detection of multiple QTLs. It treats the number of QTLs as a random variable and uses reversible-jump Markov Chain Monte Carlo (MCMC) procedure for specific modelling (Banerjee et al. 2008). In a Bayesian model, a prior distribution is selected, from which the posterior distribution is derived, and inferences are drawn from the posterior distribution. Both CIM and Bayesian methods use maximum likelihood functions. The advantages of prior distribution decline with the increase in sample size. Therefore, for most biparental mapping populations (population size in hundreds), the Bayesian method may offer little advantage over the conventional mapping, particularly when high-density maps are available, and the genotype data are nearly complete. The Bayesian mapping methods are flexible in handling the ambiguity related to the QTL number, locations of the QTLs, and missing genotypes of QTLs. Bayesian models estimate the probability that a QTL exists in a given marker interval; this feature is regarded as the major advantage of these methods. The Bayesian mapping has not been used widely partly because of (1) difficulties in choosing a prior distribution; (2) complexities of computation, including that of the posterior distribution; (3) lack of user-friendly softwares.

APPLICATIONS OF QTL INFORMATION

QTL analysis with molecular markers has enormously accelerated the development of quantitative genetics during the past 15 years. It is now possible to obtain approximate locations for agronomic QTL, estimate their effects, and bracket them with markers to facilitate marker-assisted introgression and breeding. In rice, two yield QTLs viz. *yld 1.1* and *yld 1.2* have been mapped and introgressed into elite lines, further increasing their yield potential (Luo et al, 2009). Finer QTL mapping with NILs and other introgressed segments can now lead to accurate candidate gene location, cloning and transformation in key crop and model species. These techniques offer considerable promise for future plant breeders. The introgression of QTLs into elite lines / germplasm, and marker-aided selection (MAS) for QTLs in crop improvement has been undertaken in the crop like Maize, Rice, Tomato and Wheat. The plant breeders may need not to know the precise location of QTL as the large effect QTL can be introgressed using marker assisted back crossing (MABS). The methods available will enable them to pick such useful QTL, which could well have been missed by conventional phenotypic selection. Identification of putative QTL locations and DNA markers linked to QTLs have opened up opportunities for isolation and molecular characterization of QTLs via map based cloning. Further developments in this area will help us to understand the nature and functions of complex quantitative traits.

Genetic Architecture of Economically Important Traits in Plants

Genetic architecture is defined by knowledge of all the major and minor genes controlling a trait. Genetic architecture can be simple and complex depending upon number of major and minor genes controlling the trait. Traits like disease resistance and insect resistance can be categorized as simple, where few major genes control most part. Most of the economically important traits in plants (eg. yield, drought tolerance, heat tolerance, nitrogen use efficiency, water use efficiency etc) that are of interest to plant breeder now a days are complex in nature and controlled by number of sub component traits with limited contribution to the trait. Besides most plant QTL studies in the past have been based on individual biparental populations. Inferences from these studies suggested that these plant population's segregate for limited number of small effect QTL plus very few large effect QTL. Further QTL were not often consistently detected across mapping populations. So researcher now shifted to study complex trait genetics in large populations. Such type of QTL mapping studies carried out in Maize revealed a genetic architecture of complex traits like seed oil content and grain yield which are characterized by many genes with relatively small effect. , and QTL of moderate and even small effects are now being resolved to the gene level.

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PLANT RESISTANCE TO PHYTOPHAGOUS INSECTS IN GRAIN LEGUMES

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Grain legumes such as chickpea, pigeonpea, green gram and black gram are the major sources of dietary protein in many parts of the globe. However, these crops suffer from various ecological and biological constraints. Being an important source of dietary protein, grain legumes attract many phytophagous insect pests that cause considerable damage to these crops, thus, affecting their productivity. Grain legumes are host to a wide range of insect pests such as gram pod borer (*Helicoverpa armigera*), spotted pod borer (*Maruca vitrata*), whitefly (*Bemisia tabaci*), tobacco caterpillar (*Spodoptera litura*), tur pod fly (*Melanagromyza obtusa*), pod bug (*Clavigralla gibbosa*), bruchids (*Callosobruchus* sp.), thrips (*Megalurothrips distalis*), etc (Taggar 2017). Development of resistant varieties in legume crops is an important component of integrated pest management (IPM) programme. Host plant resistance (HPR) to herbivores is considered to be an effective, economical, and environment friendly method of pest management. Development of grain legume cultivars resistant or tolerant to insects has a major potential for use in integrated pest management, particularly under subsistence farming conditions. Resistant varieties derived through conventional plant breeding, marker-assisted selection, introgression of genes from wild relatives into cultivated lines or developed through genetic transformation can provide an effective weapon for pest management in grain legumes, particularly against the pod borers.

In spite of the importance of HPR as a component of IPM, breeding for resistance to insects has not been as successful as breeding for disease resistance (Sharma 2002). This is largely because of the relative ease with which insect control is achieved through insecticide use, and the slow progress in developing insect-resistant cultivars as a result of the difficulties involved in ensuring adequate insect infestation for resistance screening (Sharma 2009). Screening for resistance to insects under natural conditions is a long term process because of the variation in the insect density across seasons & locations and staggered flowering of test material. Knowledge concerning the period of maximum insect abundance and hot-spots is the first step to initiate work on screening and breeding for insect resistance in grain legumes (Sharma *et al.* 2014). Considerable progress has been made in identification of crop cultivars with tolerance to major insect pests infesting grain legumes. Research at PAU has focused on standardizing and developing techniques for resistance to *major insect-pests of grain legumes*, both under natural and artificial infestation conditions (multiple- and no-choice tests) (Taggar 2017). The basis and mechanisms of host-plant resistance have been elucidated against major insect-pests of pigeonpea, chickpea and black gram (Taggar and Gill 2014; Wubneh and Taggar 2016b). Efforts have been made to

understand the biophysical and biochemical mechanisms underlying HPR to major insect pests of grain legumes.

Delayed/early plantings of the crop and use of infester rows of a susceptible cultivar of the same or of a different species can be used to increase *H. armigera* infestations under natural conditions in chickpea (Sharma *et al.* 2014). Artificial infestation with laboratory-reared insects can be used to overcome some of the difficulties encountered in screening the test material under natural infestation. Caging the test plants with the larvae in the field or greenhouse is another dependable method of screening for resistance to *H. armigera*. Chickpea plants infested with 10 neonate or three third-instars per plant at the flowering stage can be used to screen for resistance to this pest. For valid comparison, resistant and susceptible checks of appropriate maturity should be included, and infested at the same time as the test genotypes (Sharma *et al.* 2014). Detached leaf assay can be used to evaluate a large number of chickpea and pigeonpea lines for resistance to *H. armigera*. Infesting plants with two third instar larvae of *M. vitrata* at flowering stage has been found to be effective in screening pigeonpea genotypes (Wubneh and Taggar 2016a). Black gram genotypes can be effectively screened against whitefly, *B. tabaci* at 3rd trifoliate leaf stage using 100 whitefly adults per plant under screen-house conditions (Taggar *et al.* 2013).

Grain legume germplasm with resistance to insect pests has been identified, but the sources of resistance have not been used extensively in breeding programs (Clement *et al.* 1994; Sharma and Ortiz 2002). Insect resistance-breeding programs are underway for a few crop pests only. Entomologists and plant breeders have experienced difficulties in screening and selecting for resistance to target pests, in part, because of the lack of uniform insect infestations across locations and seasons. In addition, it is difficult to rear and multiply some of the insect species on synthetic diets for artificial infestation (Sharma 2002). Cultivars with resistance to insect pests have been identified in pigeonpea, chickpea, cowpea, black gram, green gram, and field pea. However, the levels of resistance are low to moderate (Sharma and Pampapathy 2004). More information is needed on mechanisms of resistance, the genetic regulation of resistance traits, the underlying biochemical pathways and their physiological effects (Simms 1992). One might expect a negative correlation between the potential yield of a cultivar and its level of resistance to the target pest. This is illustrated by the failure to evolve insect resistant varieties in soybean, pigeonpea, chickpea, etc. Yield of promising soybean lines at the advanced stages of testing usually falls short of the performance of commercial varieties. Although HPR promises to contribute a great deal to pest management in several crops, progress has been slow mainly because of low yield potential of the resistant varieties (Newsom *et al.* 1980; Sharma 1993).

Cultivars with resistance to insect pests can play a pivotal role in pest management in grain legumes, but only if breeding programs utilize identified sources of resistance. Resistance genes from closely related wild relatives of grain legumes should also be utilized wherever possible. Genetically engineered plants with different insecticidal genes can also play a crucial role in IPM. Molecular marker-assisted

selection has the potential to pyramid resistance genes and other desirable traits to magnify the value of host plant resistance in grain legume IPM. The need of the hour is to transfer insect-tolerance genes into high-yielding cultivars with adaptation to different agro-ecosystems (Sharma 2009). Present research focuses on deploying and evaluating genes from the wild relatives of these crops, and novel genes, such as those from *Bacillus thuringiensis* to make HPR an effective weapon to minimize the losses due to insect pests. A thorough understanding of HPR can be utilized for interpreting the ecological interactions between plants and phytophagous insects and for exploiting it in pest management in legume crops. Molecular markers can be used to accelerate the pace of introgression of insect resistance genes into high yielding cultivars. Conventional plant resistance to insects can also be deployed in combination with novel genes to make plant resistance an effective weapon for pest management and sustainable crop production.

Insect-resistant varieties exercise a constant and cumulative effect on insect populations over time and space, have no adverse effects on the environment, reduce the need to use insecticides, have no extra cost to the farmers, and do not require inputs and application skills by the farmers (Sharma 2009). Therefore, plant resistance to insects should form the backbone of pest management programs for integrated pest management.

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EXPLOITATION OF GRAIN DEVELOPMENT RELATED GENES FOR TERMINAL HEAT STRESS IN WHEAT

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Wheat (*Triticum aestivum*) provides one fifth of the dietary energy and serves as a staple food for about 40% of the world population (<http://faostat.fao.org/>). It accounts for 17% of the total cultivated area with annual production of 713 million tons per year. India is second largest producer of wheat after China with a global share of 11.78 percent in the year 2015-2016 (Department of Agriculture and Cooperation). Uttar Pradesh, Punjab, Haryana, Madhya Pradesh, Rajasthan, Bihar and Maharashtra are the major (80%) wheat growing states in the country from the point of both area and production. Wheat plant faces temperature stress during its life cycle at one or the other stage. However, about 90 percent wheat is exposed to terminal heat in India. During the hot year the crop suffers yield losses to the extent of 10-15 percent, which is one of the major threat to the national food security due to increasing pressure of the population and inclination towards wheat consumption. To increase grain yield, it is imperative to understand the genetic, molecular, and physiological mechanisms of the genes controlling important agronomic traits.

Starch is the most significant reserve of carbon and energy in plants. Major component of seed endosperm is starch, which accounts for 56–74 % of the available carbohydrates in the grain of most food crops including cereals (Koehler and Weiser 2013). It is widely known that the following four enzymes play a key role in starch biosynthesis: (1) ADP glucose pyrophosphorylase (AGPase), (2) starch synthase (SS), (3) starch branching enzyme (SBE) and (4) starch debranching enzyme (SDBE). AGPase converts glucose-1P into ADP-glucose using ATP, which is then converted into amylose starch using starch synthase (SS), and into amylopectin starch using starch branching enzyme (SBE). Therefore, it is of importance to know these four enzymes, that would help plant breeders to understand and use to enhance plant yield. Of these four enzymes, AGPase and SS have attracted the major attention. AGPase catalyses the first key enzymatic step (synthesis of ADP-glucose) and has been described as a rate-limiting enzyme in the biosynthesis of starch. It has long been in 1990's that the enzyme is rate-limiting enzyme for starch synthesis from the results of transgenic cereals (e.g., maize, wheat and rice), overexpression of modified forms of AGPase resulted in enhanced rate of starch synthesis, but also in increased final grain yield (Stark et al. 1992; Giroux et al. 1996; Greene and Hannah 1998a; Smidansky et al. 2002, 2003; Sakulsingharoj et al. 2004; Obana et al. 2006; Wang et al. 2007; Li et al. 2011; Hannah et al. 2012). This is further strengthened by the fact that null mutants showed decreased starch content (Johnson et al. 2003) and deregulation of AGPase activity leading to increase in seed yield (Giroux et al. 1996; Cross et al. 2004). Physiologically, crop yield depends on conversion of photosynthate into the dry matter in the seed, which is dependent upon the source sink relationship. Plastidial AGPase in

the leaf and the cytosolic AGPase in the endosperm function independently to produce starch, which is then accumulated in the seed endosperm (Huang et al. 2014). AGPase activity, which is regulated by two key allosteric regulators, 3-PGA acting as an activator and Pi acting as an inhibitor, when deregulated leads to increase in seed yield. It has also been shown that the activity of AGPase goes down under high temperature, that necessitates the development of thermo tolerant crops through the use of thermo tolerant variants of AGPase. Attempts to study the thermo-stable forms of the enzymes has been made but not fully understood. The grain weight is known to be significantly reduced under heat stress due to reduction not only in the rate and duration of starch synthesis. Despite the considerable information availability in literature, there is no detailed study about the number of gene copies available in wheat, a polyploidy species. ADG-glucose is both synthesized in cytosol and plastids. Both the AGPase act in the tetramers composed of two similar smaller units and two similar large subunits. Large subunits has mainly been shown to synthesise starch for endosperm, and small units are responsible for synthesis in plant biomass.

AGPase is responsible for catalyzing the first committed step in starch biosynthesis by synthesizing the activated glucosyl precursor, ADP-glucose for elongation of glucan chains and subsequent branching. SSs elongate α -1,4 glucan chains by catalyzing the transfer of the glucosyl moiety from ADP-Glc to the non-reducing end of a pre-existing glucan chains through α -1,4-glucosidic linkages. Starch branching enzymes simultaneously cleave short α -1,4 glucan chains and reattach them with an α -1,6-glucosidic linkage to other chain within the starch molecule leading to a branched structure with additional non-reducing ends for subsequent extension by SSs. Starch debranching enzymes are believed to be responsible for maintaining ordered structure in amylopectin molecules by trimming the irregularly arranged glucan chains. Short glucan chains are degraded by disproportionating enzymes leading to formation of glucose molecules which are ultimately used either for the synthesis of ADP-glucose or as a source of energy for metabolism.

Based on amino acid sequence similarities, five distinct classes of SSs have been identified in higher plants: the starch synthases I-IV (SSI, SSII, SSIII and SSIV) and granule-bound SS (GBSS). Genome sequence information further revealed that duplication of several SS-encoding genes has generated 10 isoforms (Li *et al.*, 2003; Hirose and Terao, 2004; Fujita *et al.*, 2006). They are highly similar in the C-terminal region, spanning over an approximately 450 amino acid residues comprising the catalytic and starch-binding domains, but differ significantly in the sequences of their N termini, with SSIII having the longest N-terminal arm. Genetic analyses in plants that accumulate storage starch indicate that at least three of the SS classes provide unique functions in starch biosynthesis: GBSS (Shure et al., 1983; Klösgen et al., 1986); SSIII (Gao et al., 1998; Cao et al., 1999); and SSII (Craig et al., 1998; Morell et al., 2003; Zhang et al., 2004). Whole-genome sequence analysis in unicellular green algae indicated that the algae possess most of the SS classes found in higher plants (Ral *et al.*, 2004), suggesting that gene duplication events for the genes have predated the evolution of higher plants. Mutational analyses showed that the elimination of particular class of SS leads to alteration of one or more starch characteristics, such as

structure of amylopectin, its ratio with amylose; composition, morphology, shape, size, number, or even the initiation of starch granule (Maddelein et al., 1994; Ball et al., 1996; Denyer et al., 1996; Craig et al., 1998; Yamamori et al., 2000; Umemoto et al., 2002; Morell et al., 2003; Delvalle et al., 2005; Zhang et al., 2005, 2008; Konik-Rose et al., 2007; Roldan et al., 2007; Szydlowski et al., 2009; Li et al., 2011; Lin et al., 2011; McMaugh et al., 2014). The five classes of SS are broadly conserved in plant species and their functional analysis from aforesaid study suggests that their particular function has been evolved during the course of evolution. A model to account for the roles of different SSs in determining the lengths of Ap chains proposes that SSI is primarily responsible for synthesis of the shortest chains, SSII lengthens these to provide most of the chains that crystallize, and SSIII produces longer length chains extending between clusters (Commuri and Keeling, 2001; James et al., 2003).

In addition to its role in shaping glucan structure primarily through enzymatic extension of linear glucan chains, the SSIII isoform also has influence over final molecular architecture of the starch indirectly through its interaction with other starch-metabolizing enzymes. This idea is supported in part by previous biochemical analysis of enzyme activities in maize (*Zea mays*) kernels in which the loss of SSIII activity in double mutants involving *du1* is accompanied by the additional loss of a specific BEII activity (now known to be BEIIa; Preiss and Boyer, 1979). Furthermore, combinatorial genetic effects are produced in several maize double mutants involving *du1*, such that a more extreme kernel phenotype results than that exhibited by either single mutant. Such effects, which are observed with *du1* in combination with *sugary1* (*su1*), which codes for the DBE ISA1 (Cameron, 1947; Mangelsdorf, 1947), *amylose extender* (*ae*), which codes for BEIIb (Creech, 1965), or *waxy* (*wx*), which codes for GBSS (Creech, 1965; Shannon and Garwood, 1984), suggest either that physical interactions occur between the two gene products or that the mutations affect parallel or sequential steps in the starch synthesis pathway.

High temperature during grain filling stage is an important yield limiting factor in wheat (Chinoy, 1947). The main effect of high temperature after anthesis is reduction in grain size. It has been reported⁶ that single grain weight falls by as much as 4 per cent for every 1°C rise in temperature above 18°C. Physiologically the rate of grain filling reflects the rate of biochemical reactions involved in the synthesis of reserves. As starch constitutes around 70 per cent of dry matter in cereal grains, the synthesis and deposition of starch may be an important determinant of the size of the grain. Indeed, it has been suggested that control of starch synthesis in wheat endosperm is vested largely in the activity of soluble starch synthase (Jenner, 1993). Reports are there that soluble starch synthase (SSS) is extremely sensitive to high temperature (Rijven, 1986). The effect of high temperature on these enzymes was extensively studied in wheat and maize, and activity and/or gene expression for soluble SS was impaired under elevated temperature (Hawker and Jenner, 1993; Singletary et al., 1994; Hurkman et al., 2003). Even when adequate assimilates are available, starch accumulation in wheat grains can be reduced by over 30% at temperatures between 30°C and 40°C, with the most critical stage being in early grain filling (Stone and Nicolas, 1995). Enzymes involved in starch accumulation are a key target for improving adaptation to warmer climates.

Considering above facts about the number of functions, functional subunits, temporal and spatial expression, there expected to be multiple copies of each of the above genes. Systematic study on the AGPase, starch synthesis and alpha amylase enzyme has been made. The following references may be referred to study the work done at Punjab Agricultural University, Ludhiana: Chaudhary Charan Singh University, Meerut: Dr Rajindera Prasad, Central Agricultural University, Smastipur.

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BREEDING FORAGE CROPS FOR BIOTIC AND ABIOTIC STRESSES

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Oats rank around sixth in world cereal production statistics, following wheat, maize, rice, barley and sorghum (FAO 2015). Currently, oat remains an important grain and forage crop in many parts of the world grown on 13.2 million hectares with a grain production of 26.2 million metric tons in 2003 (USDA, Foreign Agricultural Service. Commodity production, supply, and disposition database <http://fas.usda.gov/psd>). The Russian Federation is the largest producer followed by Canada and the USA. Land area devoted to oat has fallen substantially the past several decades with oat being displaced by higher value crops, such as soybean in the USA. Oat grain has always been an important form of livestock feed, and provides a good source of protein, fibre and minerals, but world oat grain production declined as farm mechanization increased between 1930 and 1950. In many parts of the world, oats are grown for use as grain as well as for forage and fodder, straw for bedding, hay, haylage, silage and chaff. Livestock grain feed is still the primary use of oat crops, accounting for an average of around 74 per cent of the world's total usage.

In India, breeding of oats begun in nineteen-eighties and is grown as the most important cereal fodder crop in North Western, Central India, extending to the eastern regions. India has a wide range of research centres and agro-ecological zones to develop suitable varieties for different zones as well as different systems of fodder production. India has a well-managed system for evaluation of breeding lines for release and notification for cultivation by farmers.

It has excellent growth habit, good quality herbage and recovers quickly after cutting. Oat requires a long and cool season for its growth; therefore, it is successfully grown in the plains and hilly areas of the country. In India it is grown in Punjab, Haryana, Jammu & Kashmir, Himachal Pradesh, Uttar Pradesh, Madhya Pradesh, Rajasthan, Maharashtra and Bengal. Variety of soils is suitable for oats cultivation. Reasonably fertile and well-drained soil is suited if temperature and moisture conditions are favourable; although maximum oat yields are usually not achieved until sufficient lime is added to bring the soil pH up to range of 5.3-5.7. Oat has been shown to tolerate acid soils with a pH of 4.5. The total area covered under oat cultivation in the country is about 5,00,000 ha. Oats have assumed considerable importance in India as fodder as well as grain for animal feed particularly calves and young stock, horses, poultry and sheep.

As a fodder crop, it has excellent protein quality, fat and mineral content. It is a palatable, succulent and nutritious crop. The average yield varies from 45 to 55 tons of green fodder per hectare. It forms an excellent combination when fed along with other winter legumes like berseem, lucerne, senji, shaftal and wild pea or vetch. When mixed with berseem, oat provides balanced feed to milch animals. Its straw is soft and superior to wheat and barley.

Oat Diseases: An Overview

a) *Losses due to oat diseases*

Oat grain has always been an important form of livestock feed and serves as a good source of excellent protein, fibre and minerals. However, the world oat production has declined from 26.30 million metric tonnes in 2003 to projected production of 23.16 metric tonnes in 2018/2019 (USDA, Foreign Agricultural Service, Commodity production, supply, and disposition database, owing to several factors including various biotic and abiotic stresses.

Roughly, direct yield losses caused by pathogens, animals and weeds are altogether responsible for losses ranging between 20 and 40 per cent of global agricultural productivity (Teng and Krupa 1980; Teng 1987; Oerke et al. 1994; Oerke 2006). The phrase ‘losses between 20 and 40 per cent’ therefore inadequately reflects the true costs of crop losses to consumers, public health, societies, environments, economic fabrics and farmers. On an average it is estimated that 20–30 per cent losses occur due to diseases, while yield loss will be complete in case of severe disease epidemics. Plant protection in general and the protection of crops against plant diseases in particular have an obvious role to play in meeting the growing demand for food quality and quantity (Strange and Scott 2005).

In 350 B.C. Theophrastus, the father of botany, first recorded occurrence of plant diseases and differences among oat plants with respect to the disease reactions. Among the diseases in oats that significantly reduce production the world over, the most important ones are crown rust, stem rust, powdery mildew, *Fusarium* head blight, leaf blotch, smut and barley yellow dwarf virus (BYDV). Diseases reduce total biomass production by either causing death of plants, killing of branches, general stunting, damage to leaf tissues or damage to reproductive organs including fruits and seeds.

The crown rust disease is considered the most serious and destructive disease. The annual yield losses averaged 5.1 per cent on account of this disease during the period 2001–2005 in Canada (Chong et al. 2011), with highest losses of 11.2 per cent and 8.8 per cent in 2001 and 2005, respectively (McCallum et al. 2007). The *Fusarium* head blight (scab) is yet another destructive disease of oat. There are several examples of major famines or food losses of crop plants associated with pest and disease epidemics in the past. The prevention of epidemics and ultimately the reduction of losses in yield have been of great concern.

b) *Disease Susceptibility in Oats and Severity*

The optimum conditions for a disease to occur and develop are a combination of three factors – susceptible host, virulent pathogen and favorable environmental conditions. In traditional agriculture owing to the presence of genetic heterogeneity and natural biological control, the natural population and wild species of crop plants rarely shows epidemics. On the contrary, modern agriculture technology has introduced important changes: (1) it has narrowed down the genetic base of cultivars, which alters the dynamic imbalance between host and parasites, which in turn results in epidemics; (2) it

has generated more or less continuously distributed populations and has changed the whole ecosystem, creating habitats profoundly altered for host and parasites.

Many diseases cause serious direct damage, mainly by reduction of the fodder yield. Among them diseases such as crown rust, stem rusts and leaf blotch caused by *Pyrenophora* spp., *Septoria* spp. and BYDV, respectively, cause severe direct damage through reduction of the fodder yield, while other diseases like scab and ergot cause indirect damage by compromising the quality of the product. Based on the causal organism, the diseases occurring in oats can be categorized into three classes based on the type of pathogen (Table 1).

c) Status of oat genetic resources to combat disease

Crop genetic resource refers to the biological diversity existed among the crop plants found in a distinct ecosystem of habitats. Genetic resources are the rich source of genetic diversity and serve as an essential raw material for improving crops and developing new value-added products. A wide spectrum of genetic diversity exists in oats with respect to morphological differentiation at both genus and species level. Based upon agro-morphological parameters, several researchers have described oats' genetic resources for the benefit of human kind. The collection of genotypes as well as the conservation of gene pools of cultivated and wild species is essential for genetics and plant the world's oat collections have been estimated to be about 131,000 accessions stored by 125 institutions in 63 countries which are considered as eighth most numerous germplasm collections after wheat, rice, barley, maize, bean, sorghum and soybean. The largest world collection of cultivated oats is maintained by Canada (~40,000), followed by the USA (~22,000) and Vavilov Institute of Plant Industry (VIR, Russia) (~12,000) which has a collection of about 10,000 accessions of 4 cultivated and 2000 accessions of 21 wild species. About 2 per cent of total oat

Table 1: Diseases of common occurrence in oats

Type of disease	Diseases	Causal organism
Fungal	Crown rust	<i>Puccinia coronata</i> f. sp. <i>avenae</i>
	Stem rust	<i>Puccinia graminis</i> f. sp. <i>avenae</i>
	<i>Helminthosporium</i> leaf blotch	<i>Drechslera avenae</i>
	<i>Septoria</i> leaf blotch	<i>Septoria avenae</i>
	Powdery mildew	<i>Erysiphe graminis avenae</i>
	Loose smut	<i>Ustilago avenae</i>
	<i>Fusarium</i> head blight (Scab)	<i>Fusarium graminearum</i>
	Anthracnose	<i>Colletotrichum graminicola</i>
Bacterial	Halo blight	<i>Pseudomonas coronafaciens</i>
	Seed and seedling diseases	<i>Bipolaris sorokiniana</i> , <i>Fusarium</i> and <i>Pythium</i> spp.
	Bacterial stripe blight	<i>Pseudomonas syringae</i> pv. <i>striae</i>
Viral	Soil-borne oat mosaic	<i>Oat mosaic virus</i>
	Barley yellow dwarf	<i>Barley yellow dwarf virus</i>

accessions (2110) of world's collection (WC) are held in India. In India, National Bureau of Plant Genetic Resources (NBPGR), New Delhi, and Indian Grassland and Fodder Research Institute (IGFRI), Jhansi, are maintaining 940 (13 species) and 450 oat accessions, respectively.

Conservation of wild gene pool of any crop plant is of utmost importance as they carry valuable genes for desirable traits such as yield, quality and biotic and abiotic stresses for crop improvement programmes. Wild species are helpful in providing basic information on species relationship and evolution pattern of crop plants. More than 24 per cent of accessions in world's oat collections are classified as wild species. Mostly the wild species of oats comprised of numerous hexaploid species which are included into primary gene pool. Nearly 31,000 accessions of oat wild species are maintained in 29 oat collections, of which 13 hold more than 20 accessions (Brazil, Canada, China, Germany, Israel, Morocco, Norway, Poland, Russia, Spain, Sweden, UK and USA) (FAO/WIEWS). The cultivated species of oats are *A. sativa*, *A. byzantina*, *A. strigosa* and *A. abyssinica*. Around 75,000 accessions of cultivated species are conserved in the world collections (Boczkowska et al. 2016).

d) *Harnessing genetic variation for disease resistance*

Genetic variation is the prerequisite in any breeding programme. The breeding of disease-resistant varieties of crop has perhaps received more attention than any other phase of plant breeding. Use of fungicide and other methods of disease control has given effective control of diseases. However, host plant resistance is the most preferable means of crop protection of all kinds as it combines the advantages of cost-effectiveness and ecological soundness. In case of breeding resistant varieties to diseases and pests, it is imperative to search for source of resistance, i.e. the donors from which the resistant gene(s) may be transferred.

The supply of genes for resistance, for disease(s), insect pest(s) and nematode(s), is the first concern in an ongoing resistance breeding programme. The primary and secondary centre of origin (gene centres) of cultivated plants is the best places to find genuine resistance to common diseases and pests. Resistance to diseases may be obtained from germplasm collection, wild/weedy relative species, mutations, somaclonal variations and unrelated organisms. Often the genes from wild species have resistance against a wide range of races. Such genes have been called 'super genes'. Therefore, resistance available from wild relative is attractive even when other sources of resistance are available. I. A. Watson (1970) noted that the new races/biotypes of parasites overcome the resistant gene(s) being used in the cultivars. Thus, it is emphasized that the wild relative or species become increasingly important sources of germplasm in the breeding of many crops.

Several resistance genes against the major diseases, i.e. crown rust, stem rust, powdery mildew, BYDV, etc., from oat gene pool (Table 2) have been discovered in over 31 wild oat species. *A. sterilis* exhibits multiple resistance to several oat diseases, i.e. crown rust, stem rust, powdery mildew and cereal cyst nematode. Powdery mildew resistance has been transferred into variety HiFi through synthetic amagalon of *A. magna* × *A. longiglumis* cross. During 1925–1946, several disease-resistant

cultivars were developed utilizing the Victoria and Bond which are derived from wild red oat and cultivated hexaploid species *A. sativa*. This served as a source for development of many disease-resistant oat varieties for different regions.

Table 2: Source of disease resistance among wild oat species

Species	Genome	Sources of resistance to					
		Powder y mildew	Crown Rust	Stem rust	BYDV	Smu t	Septoria leaf blight
<i>A. bruhnsiana</i>	Cv	+	+				
<i>A. ventricosa</i>	Cv	+	+	+			
<i>A. clauda</i>	Cp	+	+	+	+		
<i>A. pilosa</i>	Cp	+	+	+			
<i>A. prostrata</i>	Ap	+	+				
<i>A. damascena</i>	Ad	+	+	+	+		+
<i>A. longiglumis</i>	Al	+	+	+	+		+
<i>A. canariensis</i>	Ac		+	+	+		+
<i>A. wiestii</i>	As	+	+			+	+
<i>A. hirtula</i>	As	+	+	+	+		+
<i>A. atlantica</i>	As	+					+
<i>A. strigosa</i>	As	+	+	+	+	+	+
<i>A. barbata</i>	AB	+	+	+	+	+	+
<i>A. vaviloviana</i>	AB	+	+		+	+	+
<i>A. abyssinica</i>	AB	+	+	+		+	
<i>A. agadiriana</i>	AB?	+					
<i>A. magna</i>	AC		+	+	+		
<i>A. murphyi</i>	AC	+	+		+		+
<i>A. insularis</i>	AC?		+	+			
<i>A. macrostachya</i>	CC?	+	+	+	+		+
<i>A. fatua</i>	ACD	+	+	+	+	+	+
<i>A. occidentalis</i>	ACD	+	+	+	+		
<i>A. ludoviciana</i>	ACD	+	+	+	+		+
<i>A. sterilis</i>	ACD	+	+	+	+	+	+

Source: Loskutov and Rines (2011)

e) Marker-Assisted Breeding

With the discovery of DNA markers, plant breeding has experienced a new technological revolution by the development of a large array of DNA markers which makes breeders task easy for the selection of complex traits especially those which are difficult to assess phenotypically. Marker-assisted breeding (MAB) serves as boon to breeders to carry out effective and speedy selection based upon the DNA markers. With the development of a wide variety of DNA markers and genetic maps, MAB can be used for traits conditioned by qualitative as well as quantitative genes. By

Table 3: Molecular markers linked with crown rust and stem rust resistance

Gene	Marker	Linked marker/QTL
<i>Crown rust</i>		
<i>Pc38</i>	RFLP	Cdo673, wg420
<i>Pc39</i>	RFLP	Cdo666
<i>Pc48</i>	RFLP	cdo337
<i>Pc54</i>	RFLP	cdo1435B
<i>Pc58a,b,c</i>	RFLP	PSR637, RZ516D
<i>Pc59</i>	RFLP	Cdo549B
<i>Pc68</i>	RAPD	ubc269
	SNP	Pc68-SNP1, PC68-SNP2
	AFLP	U8PM22, U8PM25
	SDS-PAGE	AveX, AveY, AveZ
	RGA/RFLP	Orga1
	SCAR	ubc269a SCAR
<i>Pc71</i>	RFLP	cdo783, cdo1502
<i>Pc81,82</i>	AFLP	isu2192, OP C18
<i>Pc83,84,85</i>	STS	Agx4, Agx9, Agx7
<i>Pc91</i>	RFLP	UMN145
	DArT	oPT-0350
	SCAR	oPT-0350-cdc
	KASP	oPT-0350-KOM4c2
<i>Pc92</i>	RFLP	OG176
<i>Pc94</i>	AFLP	AF94a
	SCAR	SCAR94-1, SCAR94-2
	SNP	Pc94-SNP1a
<i>Pca</i>	RGA/RFLP	Isu2192
		L7M2.2
		B9-1
<i>Pcx</i>	RFLP, RAPD	Xcdo1385F, XpOP6(A), Xacor458A
<i>Stem rust</i>		
<i>Pg3</i>	RAPD	ACOpR-1, ACOpR-2
	SCAR/CAPS	Pg3 SCAR/CAPS
<i>Pg4</i>	SCAR/CAPS	Ubc254s SCAR
<i>Pg9</i>	Acid-PAGE	avenin band
	RFLP, RAPD	Xcdo1385F, Xacor458A
	SCAR/CAPS	Pg9 SCAR/CAPS
<i>Pg13</i>	SDS-PAGE	56.6-kDa polypeptide locus
	RFLP, RAPD	Xmog12B, Xacor254C
	SCAR	Pg13SCAR

Source: Gnanesh et al. (2014)

practising MAB in breeding programmes, the rate of genetic gain is twice the genetic gain obtained from traditional phenotypic selection. It includes several breeding strategies such as marker-assisted selection (MAS), marker-assisted back- crossing (MABC), marker-assisted gene pyramiding and marker-assisted recurrent selection (MARS). The success of marker-assisted breeding depends on the availability of a tightly linked trait-molecular marker (disease resistance gene).

Since oat genomic resources are not as developed as in other cereal crops (rice, wheat, maize, etc.), the DNA marker system is less developed too. The first molecular marker RFLP developed by Botstein et al. (1980) has been utilized by many oat researchers and strengthens the linkage and comparative mapping for the discovery of crown rust and stem rust resistance genes. Marker-assisted breeding for disease resistance and other agronomic traits has been well discussed by Rines et al. (2006) and Kapoor and Batra (2016).

f) Genomics Perspectives and Future Scope for Disease Resistance Breeding

Staple crops such as wheat, rice, barley, maize, pearl millet and sorghum are enriched with a large number of genomic resources in comparison to oats. The progress made in molecular genetic research in oats in comparison to other staples is less owing to the genomic complexity and non-availability of complete genome sequence. DNA marker-based genetic linkage maps developed in various oat genetic populations reveal marker-trait association useful for the identification of genes/ QTLs for their further utilization in marker-assisted breeding. But most of the identified genes/QTLs/markers in oats are linked to agronomic and nutritional value traits as the crop is considered as an important food grain crop owing to its high protein content.

Although several linkage maps have been developed in oats by many scientists using numerous mapping populations till date, only one consensus map (Chaffin et al. 2016) is available in oats which depicts the genetic locations of several resistance genes. A major reason for this lacuna may be the lack of oat genome sequence which could provide insights into the plant architecture and genomic relationship between different oat genomes. Deducing the complete oat sequence is a challenge for scientists mainly because of polyploidy nature of oats. Highly precise and reliable next-generation sequencing DNA markers like SNP (single nucleotide polymorphism) which are widely used in the present era of genomics can however prove useful in delineating the genome sequence of oats. With the advancement of new sequencing technologies and a rapid development in bioinformatics, complete oat genome sequencing is no longer out of reach. High-throughput genotyping is a pre-requisite for marker-assisted breeding (MAB), genomic selection (GS), genome-wide association studies (GWAS), TILLING which is the next-generation mutagenesis technique and the CRISPR/Cas9, most recently developed genome editing platform. In this regard sequencing of oat genome would be highly beneficial. It will enable fine mapping and cloning the disease resistance genes which is a challenge to DNA marker technology aimed for disease resistance.

Marker-assisted breeding (MAB) is the most suitable methodology in plant breeding for disease resistance breeding. It is highly useful in the selection of desirable

individuals with major disease resistance genes/QTLs. However, minor genes/ QTLs also played a major role in disease resistance and tend to produce more durable varieties. As the genetic architecture of resistance shifts from single major R genes to a diffused architecture of many minor genes, the best approach for molecular breeding will shift from marker-assisted selection to genomic selection.

Genomic selection (GS) or genome-wide selection (GWS) is also a form of marker-assisted selection which is based on the statistical prediction models and selection methodology. These statistical models will be able to predict accurately for disease resistance and will outperform the multiple linear regressions applied in marker-assisted breeding. GS has become feasible in plants due to the discovery and development of a large number of SNP markers by genome sequencing (Dhillon and Chhuneja 2014). Thus, use of GS in oats for disease resistance becomes a powerful approach in oat breeding programmes. Many QTLs have been identified using the bi-parental population utilizing DNA markers in crop plants, but such QTLs have limited application in MAS as parental genotypes because these genotypes are often not representatives of germplasm pool which is actively used in breeding programmes, and markers linked to QTL are not always transferable to other genetic backgrounds (Snowdon and Friedt 2004). Genome-wide association studies (GWAS) emerged as an alternative approach which has overcome the limitations of bi-parental linkage mapping. GWAS is most commonly used for detecting the variants for complex human diseases. Recently, it has been utilized in maize, rice, wheat and sorghum for the identification of marker-trait association for agronomic traits (Huang et al. 2010; Jia et al. 2013; Li et al. 2013; Morris et al. 2013), but there are some reports available where GWAS have been utilized for identifying disease resistance genes in maize, rice and wheat. In oats, few GWAS are available where marker-trait associations have been determined for grain quality traits (Newell et al. 2012; Asoro et al. 2013). But there are some studies where GWAS have been utilized for the identification of QTLs/loci linked with disease resistance. Klos et al. (2017) identified 29 SNPs on 12 linkage groups related to crown rust reaction and *Pc48*, *Pc58a*, *Pc68*, *Pc71*, *Pc91* and *PcKM* QTLs shown to be linked with seedling resistance genes using genome-wide association mapping (GWAM). Presently, GWAS has become a potential approach which will open new frontiers in disease resistance research in oats.

TILLING (targeting induced local lesions in genomes) is a reverse genetic approach which utilizes traditional mutagenesis to discover spontaneous mutation. It is helpful in generating an allelic series of genes for a particular trait of interest. TILLING has been exploited for many agronomical traits in many crop plants. There is a plenty of scope of utilization of TILLING for the identification of genes involved in regulatory pathways of defense related genes in oats. Till date, there are no reports available on utilization of TILLING in oats for disease resistance. Recently, CRISPR/Cas9, a new genome editing technology, is used worldwide among the plants as well as animals for different traits for their improvisation. Once oat genomes become a reality, there would be ample opportunity for precise site-directed mutagenesis using CRISPR/Cas9. CRISPR/Cas9 system can also be utilized for defect elimination at specific position in oat genome which regulates pathogenesis genes and

would be useful for correcting disease resistance in susceptible oat cultivars. Considering the above, delineating the complete genome sequence in oats would open up new vistas in disease resistance breeding and help accelerate ‘precision oat breeding’.

g) **Conclusion**

Oat genome sequencing would pave new pathways for breeders to develop a large number of sequence-based markers such as SNPs which will help in identifying the disease resistance genes through exploiting linkage disequilibrium mapping and genomic selection. Exploring new genome editing techniques would not only allow precise breeding but also provide a remarkable new opportunity for oat breeders. Integrating traditional breeding methodologies with modern genomics-assisted breeding to develop consensus linkage maps would open new vistas for the identification and precise mapping of major as well as minor genes/QTLs governing resistance against the economically important diseases. Meticulous planning and effective utilization of oat genetic resources would therefore provide ample scope for breeders to develop disease resistance cultivars in oats.

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MEMBRANE STABILITY IN SCREENING FOR ABIOTIC STRESS TOLERANCE

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General Information

Being a crucial component of cell viability, membranes serve as first living barrier to incoming signal molecules. Regulation of molecular trafficking by this component of cell helps in maintenance of cellular redox homeostasis and hence electrochemical potential. Eukaryotic cells are not only unique in having cell membranes but also large endomembrane system which result/limit cellular compartmentalization as subcellular organelles. Some organellar membranes form extensive intra-membrane network to maximize their functional efficiency as in plastids and mitochondria. But the buffering environment maintained by cellular membranes is severely affected under abiotic stress. Despite acting as check barrier, cell membranes face stress signals at the first step. Their response to stress signals (heat, cold, drought, salinity, light etc.) is reflected in membrane composition, fluidity/permeability and ultimately functional efficiency in regulating inflow outflow of charged and neutral metabolites.

Methods to assess membrane stability

In order to assess membrane stability or functional permeability, which upon impairment causes cellular death, various biochemical measures are used. These methods allow us to screen membrane efficiency of large set of germplasm in a short time duration and cost effective manner. Some of these measures are:

Electrolyte leakage: It is one of the common functional assays for determination of membrane permeability. The main assumption in leakage method is: 'Higher the stress intensity, more is membrane injury' and hence more is the leakage. Resistant genotypes with stable membranes have higher threshold optima for stress level and can adapt themselves by maintaining membrane functionality and exhibit reduced leakage. Several advantages of this method include cost effectiveness, simple instrumentation i.e. conductivity meter, screening of large set of germplasm in short duration etc. Although this method does not give us an idea about contribution of each cellular compartments and nature of ionic species contributing to total leakage; yet this physiological measure is routinely used in breeding material to assess membrane integrity.

Procedure: The method of electrolyte leakage is divided in three steps:

- 1) Washing
- 2) Incubation
- 3) Boiling

Among all plant tissues, leaf tissue is generally used to assess membrane permeability, as they are seat of light perception and act as source sites of photosynthesis. Impairment in membrane integrity of this plant tissue results in less

current photo-synthates and ultimately productivity loss to crop plants. Leaf discs or segments (10-20) of known length/diameter (1 or 2 cm) are cut from sample and then washed with distilled water. Washing treatment results in ion leaching from worn out cells at surface of disc/segment. So the error contribution to total leakage is minimized through this step.

After washing, leaf segments are incubated in known volume of deionised water for 24 hrs to estimate the ion leakage. Depending upon damage to membrane, leakage can be less or more. After the incubation step, conductivity of this solution containing leached ions is measured; which is known as initial conductivity (C_i). The leaf segments along with ion leachate are then autoclaved or boiled at 100°C for 2-3hrs. Due to boiling, membranes in tissue segment become leaky and release all of the stored ions in leachate solution. Conductivity of this solution is termed as Final conductivity (C_f).

Electrolyte leakage (%) is calculated as: $C_i/C_f \times 100$

Lipid peroxidation

Exposure of membranes to stress condition often results in production of reactive oxygen species (ROS) which perturb membrane functionality. These ROS induce free radicals production which causes peroxidation of membrane lipids. Malondialdehyde is one such end product formed during peroxidation of polyunsaturated fatty acids. It forms pinkish-red chromogen upon reaction with thiobarbituric acid (TBA) whose absorbance can be measured at 532 nm. To avoid overestimation in absorbance by phenolics, anthocyanins, sugars etc. at 532nm, absorbance value at 600 nm is subtracted from OD at 532 nm.

Reagents Required

0.1% TCA

0.5% TBA in 20% TCA

Procedure

100 mg of fresh leaf tissue is homogenized in 2 ml of 0.1% TCA using a pestle and mortar. The homogenate is centrifuged at 10000 g for 5 min. To 1ml aliquot of the supernatant, 4 ml 20% TCA containing 0.5% TBA is added. The mixture is then heated at 95°C for 30 minutes and quickly cools it in an ice bath. The reaction mixture is then centrifuged at 10000 rpm for 10 min. The absorbance of the supernatant at 532 nm is read and the value for the non-specific absorption at 600 nm is subtracted. The concentration of MDA is calculated using its extinction co-efficient of $155 \text{ mm}^{-1} \text{ cm}^{-1}$.

2, 3, 5- Triphenyl tetrazolium chloride (TTC) assay

Cell membranes always act as hallmark to determine cell adaptability under adverse conditions. If remain stable, these help in maintenance of cellular homeostasis. But when perturbed, membrane dysfunction can lead to cellular death. Thus membrane stability can help in assessment of cell viability under normal and stressed condition.

Vital stains such as TTC, Evan's blue are generally used to assess cell viability. Living cells of tissue convert tetrazolium chloride in to red coloured formazan by activity of mitochondrial NADH reductase. The formazan produced can be extracted from tissue sample by dipping the TTC treated tissue in alcohol. The absorbance of alcoholic phase having formazan gives us an idea about percent viability of given tissue sample.

Procedure: The sample leaf tissue is cut in segments (3.5cm each) and is placed in two sets of test tube containing 100µl of water. One set is kept at 25⁰C and other set is placed in a water bath at 49⁰C for 90 min. Immediately after treatment, 10ml of TTC solution (0.8% TTC in 0.05 M NaPO₄ buffer, pH 7.4 and 0.5 ml/l Tween 20) is added in each tube. The tubes are vacuum infiltrated to allow complete absorption of TTC solution by leaf tissue and incubated in dark for 24h at 25⁰C. After incubation, the leaf segments are removed and rinsed with distilled water. Leaf segments are then placed individually in separate tubes containing 3 ml of 95% ethanol, for 24 h at 25⁰C in the dark. The level of acquired high temperature tolerance is determined by measuring the percentage reduction of TTC to formazan at 530 nm using the following formula:

$$TTC = (OD_h / OD_c) \times 100$$

Where OD_h and OD_c corresponds to absorbance of ethanolic extract from tissue segment incubated at 49⁰C and 25⁰C at 530 nm.

ABIOTIC STRESSES AND THEIR MANAGEMENT FOR OPTIMUM PRODUCTION IN BRASSICAS

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Agricultural productivity is affected by a number of abiotic stresses. These may include deficit or excess water availability, flashfloods, high salt levels in soil as well as in irrigation water and extreme temperatures. In addition, mineral deficiency or toxicity is frequently encountered by plants in agricultural systems. In many cases, different abiotic stresses challenge plants in combination. For example, high temperatures and scarcity of water are commonly encountered in periods of drought and can be exacerbated by mineral toxicities that constrain root growth. Further, plants are also exposed to salinity, drought and frost-like conditions in combination in some of the cases. Higher plants have evolved multiple, interconnected strategies that enable them to survive abiotic stresses. Across a range of cropping systems around the world, abiotic stresses are estimated to reduce yields to less than half of that possible under ideal growing conditions. Traditional approaches to breeding crop plants with improved stress tolerance have so far met with limited success, in part because of the difficulty of breeding for tolerance traits in traditional breeding programmes. Desired traits can be crossed into crop species from different donors. Since different crops and their genotypes show differential levels of tolerance to many abiotic stresses, efforts are underway to unravel their molecular basis for abiotic stress tolerance to speed up the development of improved genotypes with increased stress tolerance.

In *B. juncea*, drought, high temperature and salinity are the major abiotic stresses affecting crop productivity. Most of the drought-prone areas are found in arid, semi-arid and sub-humid regions, which experience less than average annual rainfall. Further, salt-affected areas in India have been assessed to cover 6.73 Mha equivalent to 2.1% of the geographical area of the country. Out of 6.727 mha salt affected soils 2.956 mha are saline and 3.771 mha are sodic (Shahid et al 2018). There are also large areas across the world underlain by brackish groundwater resources. The crop Brassica also experience low temperatures and also frost, though only occasionally, during the growing season. Crops also experience high soil temperature affecting seed germination and crop establishment. The climatic variations further accentuate the stresses experienced by the plants. Of late, the climatic variations being observed include frequent variations, both high as well as low and declining number of rainy days but with higher intensity. Germination of seeds and emergence of seedlings is considered to be a very sensitive and critical stage in Brassicas, which ultimately influences crop productivity through decreased plant stands. Accordingly, germination, emergence of seedlings and their good growth are pre-requisite attributes for increasing mustard production under rainfed situations as well as in semiarid saline soils and under prevailing high soil temperature during normal sowing time. There is a distinct possibility for the improvement of these traits in Brassica oilseeds, as large genetic variation with regards to these traits exists

Low light stress: Light plays key role in net primary productivity. Light availability varies with plant population, spatial arrangement and canopy structures (Liu *et al* 2012). Foliar traits like leaf area index and leaf mass per unit area are the important factors influencing leaf light harvesting capacity and photosynthetic potentials. Light interception also varies with the crop development. Dry matter production is always positively related to light interception. During different growth phases of Indian mustard, photosynthetically active radiation is affected at the canopy level as a consequence of by mutual shading between plants and at the plant level by self-shading caused by the upper leaves, flowers and pods. Indian mustard faces low light stress due to cloudy weather, severe winter, foggy and frost conditions leading to yield losses. Shading markedly influenced morpho-physiological traits leading to suppressed sink strength/flowering, reproductive phase and increased vegetative phase (Sharma *et al* 2014).

Drought stress: Rainfed crops may suffer from stringent droughts of fluctuating and unpredictable intensities and drought-induced adverse effects by secondary or even tertiary pathways. These pathways could be through transpirational or heat complexes, drought tolerance is probably the most difficult plant trait to be improved through conventional plant breeding. The challenge is even greater for developing drought-tolerant plants for water-limited environments where occurrence, timing and severity of drought may fluctuate from one zone to the next and also over the years. Furthermore, it induces large impacts on emergence, growth, production and quality of produce through phenological, physiological and biochemical pathways (Kaur and Sharma, 2015). More precisely, depressed seedling emergence with early seedling mortality may lead to sub-optimal plant stand, delayed growth and removal of sinks in extreme situations and may lead to heavy irreversible production losses. The convenient and traditional solution to drought lies in providing irrigation water to the crop plants experiencing drought experienced by the plants. Of late, the climatic variations being observed include frequent variations, both high as well as low and declining number of rainy days but with higher intensity. Physiological changes in water potential and relative water content of the water-stressed leaves through osmoregulation and osmotic adjustment have been observed in Brassicaceae. In the process of physiological adaptation, maintenance of turgor pressure appears to be the central process.

The role of antitranspirants in checking transpiration rate is well documented. Different techniques are used in crop production to conserve water and increase water use efficiency in order to tackle water scarcity. One of them is planting method which affects the plant population and nutrient availability. Most commonly used method for water conservation are drill sowing, raised bed planting and furrow planting. Seedling establishment is a phenological stage at which drought stress could be damaging. Broadcasting in Brassicas results in uneven distribution and leads to imbalances availability of water, space and nutrients and poor seedling establishment and ultimately lower yield. Drill sown seeds shows better emergence and crop stand as compared to broadcast method. Furrow sowing results in better growth and higher yield as compared to ridge sowing method. Water conservation is also high in furrow sowing, which gave 13.7% more seed yield and 13.2% more WUE as compared to

ridge sowing. More water conservation is due to less water evaporation. PGRs regulate the germination, formation and distortion of roots, leaves and stem elongation and fruit ripening etc. Crop plants mitigate adverse effects of water deficit to some extent by exogenous application of salicylic acid, gibberellic acid, putrescine and cytokinins. Potassium (K) is one of the major plant nutrients and is involved in drought mitigation by regulating turgor pressure, photosynthesis, translocation of assimilates to various organs and enzyme activation.

Temperature stress: Crop plants depict high productivity when grown in ambient temperatures, optimum for various growth and physio-metabolic processes. Temperature higher than optimum (5-7⁰C) above the optimum decreases rate and duration of metabolic process and therefore yield. Brassica species are known to be heat susceptible at seedling emergence if planted at greater than 30⁰C mean day/night temperature. Light textured arid soils show very high seedling mortality, leading to lower plant stands. Hence, tolerance to heat at seedling emergence stage in Brassica will facilitate an extended planting period increasing the cropped area and utilization of conserved soil moisture received due to late rains. Therefore under resource constraint farming systems, it is desirable to breed for heat tolerance towards critical sensitive growth stages. High temperature induced yield losses could be due to accelerated senescence, reduced photosynthetic rates but increased respiration and inhibition of metabolic processes of seed development like starch synthesis. Crop species including Brassica adapt to heat stress membrane stability and accumulation of proline. Due to intensive cropping systems planting of Rapeseed mustard gets delayed and the crop experiences high temperature (>30⁰C) during seed filling stage, hampering translocation and resulting in higher shriveled seeds leading to yield penalties. Improved genotypes have been developed in *B. juncea* that can tolerate high temperature during germination and later growth stages and should be preferred over other high yielding varieties. Two foliar applications of salicylic acid improved seed filling in *B. juncea* (Sharma et al 2013).

Compatible solutes, small organic molecules that help in osmotic adjustment under stressful environment. They scavenge the ROS, helps in osmoregulation and stabilization of cell membrane proteins and lipids. These osmoprotectants include glycine betaine, trehalose, proline etc. Foliar application of these solutes is a potential strategy to minimize the adverse effects of stressful environments.

Frost: Frost is a sudden crop killer with devastating potential, especially in the north and northeastern parts of India where temperature unexpectedly drops below 0⁰C. During December and January. When the frost occurs, the temperature invariably drops below 0⁰ C, however it is not essential to have high relative humidity. It has direct effect on the flower and silique development and prevents seed formation, thereby affecting crop productivity, causing considerable yield loss. The conditions favoring frost development generally occurs in the night, during such conditions crops can be saved by burning waste material, dung or straw on the windward side of the field in the evening so that temperature of surroundings is increased. Irrigate the field at 10-15 days interval when temperature is low, if possible raise a protective vegetation taller than the mustard to prevent direct impact of cold wave. In the areas, where frost is

regular phenomena ,frost tolerant varieties like Urvashi,RGN-48,RK9001 and RH8816 should be grown. Crop can be protected from frost by chemical spray of dimethyl sulphoxide, dithane or 0.15% of H_2SO_4

Fog: Rapeseed mustard is also affected by fog. The conditions for fog build up is ideal when the sky is clear, wind does not blow and RH is more than 75% .Temperature should not necessarily below $0^{\circ}C$.Fog develops even at temperature of $10-12^{\circ}C$. Fog affects photosynthetic activity due to non availability of sunlight which affects crop growth and ultimately oilseed yield.

Salinity: Saline soils and poor quality water for irrigation are the main causes of salt stress. In hot and semi-arid regions, where drainage is poor and the surface evaporation is high, the soluble salts formed by weathering process excessively accumulate on the surface of soil. As the salts accumulate beyond the tolerable limit of the crops, the plant growth is affected adversely and the soil becomes unfertile. Such soils are called saline soils. In saline soils soluble salts accumulate on the surface as thin white crust after evaporation, hence called white alkali soil. These white salts are chlorides, sulphates and nitrates of calcium, magnesium, potassium and sodium. Saline soils are slightly alkaline having pH <8.5 and exchangeable sodium $<15\%$ but EC of saturation extract $>4mmhos/cm$ at $25^{\circ}C$.Due to high concentration of salts, the osmotic pressure of soil solution is higher than the root cells, thereby root cells instead of absorbing water from soil lose water leading to water and nutrition imbalance in plants, thus adversely affects plant growth. The impact of saline water on the crop appears to be similar to that of drought. Leaves become short, thick and dark green in appearance in comparison to crop irrigated with good quality water Indian mustard (*B. juncea*) yield higher than other species under salinity (Sharma et al 2019). Salt tolerant varieties like CS52, CS54, CS58 and Narendra Rai have better tolerance potential can be grown in such condition. Salts are removed from rhizosphere by leaching where water table is low. Another method of reclamation is scrapping which is expensive. In the area of sufficient irrigation and efficient drainage facilities, flushing off soluble salts can be done.

Alkali soils are formed in arid and semiarid climate where exchangeable sodium form hydroxide on hydrolysis .The hydroxide of sodium produces alkalinity beyond pH 8.5.The strong alkali dissolves organic matter which on evaporation leaves black residue on the soil surface; therefore these soils are called “black alkali”. The EC of saturation extract of such soils is $<4mmhos/cm$ at $25^{\circ}C$ and exchangeable sodium $>15\%$.Alkali soil is highly deflocculated or dispersed due to large amount of sodium hydroxide and soil becomes compact. Aeration and movement of water are poor due to compactness, making tillage difficult and the crop root penetration is restricted. Alkali soils are rendered less injurious by treating the soils with chemical amendments. Gypsum is a cheap and effective amendment for reclaiming alkali soils. Farmyard manure, compost, green manures etc. can be used as organic amendments integrated with chemical amendment.

Oilseed Brassicas are cultivated under wide ecological diversities that include irrigated/rainfed and salt affected soils experiencing, a vast diversity of agro climatic conditions. The crop is exposed to extreme variations in temperature, high soil

temperature at sowing time, low temperature and even frost during the reproductive phase along with the changing climatic conditions. A large chunk of area comes under arid and semiarid regions which are prone to drought and salt stress. In India, Brassicas are mostly cultivated under residual /conserved moisture conditions with low productivity. In the absence of good quality water crop is irrigated with brackish or poor quality water at critical crop growth stages. All these factors decline the crop and oil yield. These abiotic stresses can be managed by exploring genetic resources and agronomic factors. Donor lines have been identified for different abiotic stresses and are being used in the breeding programmes for developing tolerant varieties. A number of improved varieties have been developed which perform better under different kinds of stresses resulting in lower reduction upon exposure to stress compared to high yielding varieties. New molecular approaches with marker assisted selection, identification of QTLs for drought, salt, frost and high temperature besides transgenic approaches would help improve the tolerance potential of rapeseed mustard.

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DEPLOYMENT OF DISEASE RESISTANCE IN INDIAN MUSTARD: CHALLENGES AND OPPORTUNITIES

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India is the third largest producer of Rapeseed & mustard followed by Canada and China with a total area of 6.7 mha contributing to total production of 6.4 million metric tons (MMT) in 2017-18 (Anonymous, 2018a). Indian mustard (*Brassica juncea*; AABB; 2n=36), commonly called as mustard, is predominant species among Rapeseed-mustard group of crops and accounts for more than 90% to its total acreage in India (Anonymous, 2018b). It is an important crop of Indian oilseed sector due to its role in meeting domestic demand and achieving self-sufficiency for edible oils in the country.

Despite the availability of high yielding modern day varieties, with a potential yield of about 4t/ha, the average yield is hovering around 1.0 to 1.1t/ha from more than decade. Many biotic and abiotic factors are responsible for this low productivity. Furthermore, majority of mustard cultivating farmers in the country are resource poor and about 24 per cent of the area under this crop, in the country, does not have facilities for even a single irrigation to apply. Therefore, majority of mustard cultivating farmers don't want to invest much on inputs. Mustard crop, on the other hand, is attacked by many disease-causing organisms including bacteria, fungi and viruses etc. These pathogens cause large crop losses and, thus, always challenged the productivity and sustainability of Indian oilseed sector. Use of chemicals against pathogens leads to environmental pollution and health hazards, therefore, development of cultivars with in-built resistance is the most effective, most economical and environment friendly method of disease control. Genetic resistance in plants can be considered a major form of biological control of diseases and development of resistant cultivars have been used effectively for the control of diseases across the crops.

Since the rediscovery of Mendalism, our knowledge about pests, their nature and mechanism has improved a lot. Van der Plank (1963, 1968) classified resistance on the basis of interactions between host plants and their pathogens into two major types, namely, vertical resistance and horizontal resistance. VR in many plant host-pathogen relationships is hypersensitive, race specific, and governed by the gene-for-gene system (Hulbert *et al.*, 2001). In contrast, HR is quantitative, presumably race-non-specific, and controlled by polygenes. Assessment of genetic variation for avirulence in plant pathogens is a pre-requisite for identifying resistance genes and their systemic deployment. Furthermore, it is necessary to know the genetic control of resistance (monogenic/oligogenic/polygenic) its reaction to different races of pathogen (similar/differential), relative durability of resistance (low/high), host expression of resistance (seedling stage/adult plant) and rate of evolution of new pathotypes

(slow/fast evolution of races) etc. before planning an effective resistance breeding programme.

Mustard crop is being cultivated under diverse climatic conditions and, thus, affected by number of diseases. More than 22 diseases have been reported to affect rapeseed-mustard group of crops in India. However, only few of them are considered as major diseases on the basis of economic yield losses and their distribution in the country. The details of these diseases and their causal organism which are affecting mustard crop in India are listed below:

Table 1: Economically important diseases of mustard and losses caused by them

Disease	Pathogen	Extent of losses Caused (%)
White rust	<i>Albugo candida</i> (Lev.) Kunze	17-37
Sclerotinia Stem rot	<i>Sclerotinia sclerotiorum</i> (Lib.) DeBary	Upto 35
Downy mildew	<i>Hyaloperonospora brassicae</i>	17-37
Powdery Mildew	<i>Erysiphe cruciferarum</i> Opiz ex. Junell	17 (along with 6% reduction in oil content)
Alternaria blight	<i>Alternaria brassicae</i> (Berk.) Sacc.	10-70

Created from “Manual on Management of Rapeseed-Mustard Disease”, DRMR Bharatpur

The Challenge:

Genetic variability in Brassicas and their related species has been studied by a number of workers and sources of resistance or partial resistance against all the above mentioned pathogens has been identified. Despite that, efforts directed toward development of resistant varieties could not be much successful. Till now, hardly any cultivar possessing resistance against major disease(s) has been accepted by the Indian farmers. Resistant sources and varieties succumb to regional pathotypes/races and observed susceptible reaction variably. Due to this fact the resistance in the developed variety is challenged by farmers and scientific community.

Pathogen biology and epidemiology has been well understood, even then, resistance has been identified using regional isolates and limited efforts were made to characterize the pathogens prevalent in India. The pathogen variability has been reported by many workers despite that host differential sets has not been developed for most of these pathogens and, thus, genes could not be identified and designated. Due to these reasons limited efforts were made to develop genomic resources enabling precise transfer of resistance in any genetic background. Furthermore, the reliability of identified molecular markers is always challenged during genotyping of populations using different isolates every time. Therefore, Brassica breeders are relying mainly on selecting resistant segregants under disease epiphytotic conditions without having much knowledge about race spectrum and genes. Under such situation pyramiding of genes is

not possible due to limited knowledge about the underlying genes, their precise location on chromosomes, their reaction to different pathotypes/races and availability of reliable molecular markers linked to them.

Problems and Opportunities:

White rust:

White rust caused by *Albugo candida* (Pers.ex Lev.) Kuntze is highly destructive disease of Indian mustard. Both the vegetative and reproductive phases of the plants are affected by this fungal pathogen. Infection at the vegetative phase results in the appearance of white pustules, predominantly on cotyledonary leaves and symptoms start from the abaxial surface of leaves. The pathogen can spread systemically and cause severe malformation of the inflorescence through hypertrophy and hyperplasia resulting in stag head formation. Severe infection is reported to cause yield losses up to 89.8 per cent (Saharan and Verma, 1992; Punjabi *et al.*, 2010). At least 13 races of *A. candida* have been identified on the basis of their specificity to different crucifer species of which race-2 predominantly infects *B. juncea* (Verma *et al.*, 1999). However, this species specific race pathogenicity is not absolute. Most races can also infect related Brassica species, especially those sharing their genome with the hosts from which they were originally collected.

Genetic analysis of available white rust resistance has elucidated a digenic mode of inheritance with duplicate gene action in *B. napus* and monogenic dominant resistance in *B. juncea*, *B. rapa*, *B. carinata* and *B. nigra*. At IARI, New Delhi we have observed differential host pathogen interactions by using different genotype with wide origin and isolates of pathogen collected from different parts of India. Therefore, it becomes imperative to identify different resistance genes from different resistant hosts and pyramiding them in single nuclear background for achieving durable resistance. Furthermore, knowledge about the genomic regions controlling trait of interest, such as white rust resistance, and their close association with molecular markers would help in precise transfer of this trait to the improved genetic background using marker assisted selection (MAS).

Sclerotinia Stem Rot:

Sclerotinia stem rot (SR) is pathogenic to > 400 plant species (Purdy, 1979; Boland and Hall, 1994) and > 500 plant species (Sharma *et al.*, 2015) worldwide. The pathogen poses a hazard to many dicotyledonous and monocotyledonous species (Boland and Hall, 1994; Bolton *et al.*, 2006). There are several studies where resistance against SR has been reported in mustard. *B. juncea* cv. rugosa genotypes have been reported to possess resistance against SR in the field as well as in green house conditions (Singh *et al.*, 1994). Nine genotypes viz., Cutton, ZYR 6, PSM 169, PDM 169, Wester, PYM 7, Parkland, Tobin and Candle also showed resistance to Sclerotinia rot (Shivpuri *et al.*, 1997). Pathak *et al.* (2002) reported PCR-10, RW-8410, RW-9401 and RGN-8006 consistently proved promising against *S. sclerotiorum* of mustard. Varuna albino, RAUD 25, BioYSR, PHR 2, Purple mutant, Montara, Ringot 1, Brassica I, Brassica II, EC 126743, EC 126745, EC 322090, EC 322091 and Kiran showed moderately resistant reaction (10-20 % D.I) against stem rot disease (Rakesh,

2014). Singh et al (2010) identified RH 13, Ringot 1, B. juncea I, B. juncea II from China and JM 018 from Australia possessed resistance against SR. Although in most of the above studies resistance reaction in form of low severity has been reported, however, absolute resistance against this pathogen has not been identified.

Studies on SR have mainly focused on the understanding the inheritance, however, no *R* genes have been cloned for this pathogen (Wu et al., 2016). Although, monogenic control of single dominant genes against *S. sclerotiorum* has been found outside the *Brassica* genus, such as in common bean (*Phaseolus vulgaris*; Schwartz et al., 2006). It is more likely that monogenic control is present in *Brassicaceae* where only a HR and/or lesions ≤ 2 mm diameter have been observed on stems (Uloth et al., 2013, 2015a; You et al., 2016) or cotyledons (Garg et al., 2008, 2010b; Uloth et al., 2014; Ge et al., 2015). On the other hand, candidate *R* genes against *S. sclerotiorum* have been identified and physically mapped on the *B. napus* genome (Li et al., 2015). Candidate *R* genes, belonging to the CC-NBS-LRR class, were also identified on chromosome A9 in *B. napus* (Mei et al., 2013). Yet, the majority of the QTL for *S. sclerotiorum* have been identified in the C genome (C9 and C6) (Li et al., 2015). The genetic control of resistance genes is further complicated by the influence of inoculation methods (Zhao et al., 2004; Yin et al., 2010) and physiological traits. Keeping this in mind, future efforts for deployment of resistance in *B. juncea* should focus on:

1. Search of resistant sources against Sclerotinia rot in oilseed brassicas and its wild relatives
2. Elucidate the genetic variability existing in the pathogen enabling better understanding of Host-Pathogen interactions.
3. Identification and map *R* gene(s)/QTLs in *B. juncea* nuclear background
4. Conducting gene expression studies in pathogens and host infecting susceptible, tolerant and resistant genotypes enabling better understanding of biochemical basis of resistance and identification of genes/QTLs responsible for their changed expression.
5. Possibility of induced resistance and systemically acquired resistance (SAR) need to be explored.

Powdery Mildew:

Erysiphe sp. are obligate, ascomycete parasites that cause powdery mildew disease on a variety of crops. The powdery mildew of Indian mustard, caused by *Erysiphe cruciferarum* Opiz. Ex. Junell., was once considered to be of minor importance but, in the recent years it became widespread throughout the mustard growing regions of India particularly in the states like Uttar Pradesh, Rajasthan, Jammu & Kashmir, Haryana and Gujarat (Mohitkar et al., 2012) and in other non-traditional areas such as Maharashtra, Karnataka and Andhra Pradesh. All the Indian mustard cultivars currently grown in different states of India are susceptible to highly susceptible to this disease. This fungus can infect any above ground plant part and can cause heavy yield losses by reducing plant growth and consequently, the quantity and quality of seeds (Dange et al., 2002; Kumar and Saharan, 2002). The weather conditions prevailing during February-March were reported to be congenial for

powdery mildew development thereby leading to poor seed weight, seed germination and seedling growth rate. In these situations, the farmers either go for chemical spray or let the disease uncontrolled.

In India, earlier attempts to find resistant sources in Indian mustard against powdery mildew disease could not succeed as all the *B. juncea* genotypes evaluated were found to be highly susceptible. However, the resistant sources were reported in other related species i.e. *B. carinata*, *B. napus*, *B. alboglabra*, *B. japonica*, *Sinapis alba* and *Eruca sativa* (Narain and Siddiqui 1965, Kumar *et al.*, 2002 and Mohitkar *et al.* 2012). At global level also, resistance has been reported in closely related species viz., *Arabidopsis thaliana* (Xiao *et al.* 2001), *B. rapa* (AA), *B. oleracea* (CC) and *B. napus* (AACC) (Li *et al.* 2016) and no reports are available about resistance in *B. juncea* (AABB). In absence of information on sources of resistance in *B. juncea* no systematic works were undertaken to understand the inheritance and, thus, transfer it from other related species despite the fact that many of these species are easily crossable. Furthermore, the work on understanding the pathogen variability has not been systematically undertaken.

Keeping in view the economical importance of powdery mildew disease in mustard, research initiatives have begun at ICAR-Indian Agricultural Research Institute (IARI), Regional Station, Wellington, which is a hot spot for this disease *inter alia* many other crop diseases (Nanjundan *et al.*, 2017). A set of more than 1020 Indian mustard germplasms with diverse origin were evaluated for their reaction to *E. cruciferarum* isolate designated as PMN (Powdery Mildew Nilgiri) during the *khari*f 2016. The large scale screening resulted in identification of 12 Indian mustard germplasm lines possessing variable degree of resistance. Out of these one germplasm line viz., PMW 18 possessed very high degree of resistance against powdery mildew disease. Utilizing this resistance source populations were generated and the work on revealing the inheritance is in progress.

Since resistance is available in Indian mustard and related species, future efforts should be directed towards identification of gene(s) and their stability across the mustard cultivating areas *vis a vis* understanding the variability among the isolates. Information, thus, generated shall help in deployment of powdery mildew resistance genes over time and space and also open avenues for their pyramiding.

Downy Mildew:

Peronospora parasitica now known as *Hyaloperonospora brassicae*, causes downy mildew disease in species of *Brassica*, *Raphanus*, *Sinapis* and other genera within the *Brassicaceae*. The disease is more common among young plants with infection appearing as yellow speckling of the upper surface of seedling leaves, and white mildew on the lower surface. Severely affected seedlings remains stunted or die. The disease appears also on the adult plant stage and infect both leaves and siliquae. This disease reduces the photosynthetic activity in the plants leading to reduced yield and oil content.

Inheritance of specific resistance to *H. parasitica* has been reported in *B. juncea* (Nashaat and Awasthi, 1995; Nashaat *et al.*, 2004; Chattopadhyay and Séguin- Swartz,

2005). Efforts were also made delineate pathotypes of *H. parasitica* (Sherriff and Lucas (1990), however, success has not been achieved yet. Mohammed et al. (2017) on the other hand reported existence of very high levels of pathotype- independent resistance in Australian *B. napus* rapeseed varieties.

Although, five *R* genes from *Arabidopsis* viz., *RPP2* (Sinapidou et al., 2004), *RPP5* (Parker et al., 1997), *RPP8* (McDowell et al., 1998), *RPP1* (Botella et al., 1998) and *RPP13* (Bittner- Eddy et al., 2000) (Botella et al., 1998) have been cloned but no *R* gene has been cloned from *Brassica* species. However, *Ppa3*, a single dominant *R* gene against this pathogen was mapped in *B. oleracea* using molecular markers (Singh et al., 2012). The major locus *Pp523* on has been identified to confer resistance at the adult stage in *B. oleracea* (Farinhó et al., 2004; Carlier et al., 2012) and *RPP31* gene expressing adult plant resistance in *A. thaliana* is also mapped (McDowell et al., 2005). Since many *R* genes have been cloned or mapped in *Arabidopsis* and other related species, the orthologous genes in *B. juncea* can be surveyed and incorporated into mustard genotypes.

Sporadic efforts were made to classify the isolates into pathotypes/races and only in few studies the existence of separate races was reported (Natti et al., 1967; Mohammed et al., 2017; Jensen et al., 1999a). These studies has not allowed comparison of pathotypes across genotypes of same species, crops and locations. Till now, it has not been possible to universally characterize *H. parasitica* pathotypes due to unavailability of a set of host differentials. Furthermore, differential host plant interaction (at seedling and adult plant stage) are expected (Dickson and Petzoldt, 1993; Coelho et al., 1998), even though only limited efforts were made to establish it. Once, the pathotypes/races were identified on host differentials the gene for gene hypothesis can be established, which further facilitate the incorporation of resistance genes/QTLs in any genetic background and also provide opportunity to pyramid them.

Alternaria blight:

Alternaria blight disease is the most catastrophic and pervasive disease of oilseed brassicas reported over a wide geographical area all over the world and is one of the most important disease of Indian mustard. It affects both quantitative and qualitative changes in seed yield and quality by reducing size, seed discoloration and reduced oil content (Kaushik et al., 1984). The immense variation available among representative isolates of *A. brassicae* indicates their ability to adapt to varied climatic situations (Meena et al., 2012). Since this disease affects all the parts leaf, stem and siliquae on different time periods, therefore chemical management is not cost effective.

Till now no genotype with absolute resistance against *A. brassicae* is available among the cultivated species. However, some related wild species have been found to express resistance reaction against this pathogen. At ICAR-National Institute for Plant Biotechnology, (earlier NRCPB), Delhi a number of potential donors viz., *Brassica desnottesii*, *Camelina sativa*, *Coincya pseudoerucastrum*, *Diplotaxis berthautii*, *D. catholica*, *D. cretacea*, *D. eruroides* and *Erucastrum gallicum* imparting resistance against Alternaria blight has been identified (Sharma et al. 2002). Since resistance is

not available in the cultivated species, hence, wild species could be used for introgression of resistance to Indian mustard.

D. eruroides x *B. rapa* hybridization supplemented by embryo rescue led to development of amphihaploids and after colchicine doubling stable amphidiploids were obtained (Bhat et al. 2006). This amphidiploid was crossed reciprocally with improved *B. juncea* genotype RLM 198. The F₁ was highly male and female sterile. A few BC₁F₁ progenies obtained after pollination with *B. juncea* were used for another round of backcrossing. The BC₂F₁ plants were selfed and subsequent generations were advanced through selfing. Male/female sterility persisted to various degrees up to the BC₂F₄ stage (Vasupalli et al. 2017). The material is in the BC₂F₇ stage, even then segregation for resistance and variable degree of fertility is observed among the recombinants. Once the resistance is stably integrated in *B. juncea* background its inheritance and molecular mapping will become possible. More efforts of this kind need to be made by involving other wild/ weedy relatives for search of resistance and identification of novel genes.

Summary:

Major challenges and opportunities to improve disease resistance are outlined and discussed. To initiate any resistance breeding programme good knowledge about the source of resistance, inheritance of resistance and information about the underlying genes is necessary. On the other hand, understanding about the pathogen, its life cycle and pathogenic variability is of utmost important. Delineating pathotypes/races, on the basis of host-differentials, is pre-requisite for identification of R genes and establishing gene for gene hypothesis. This shall further help in separating the effect of genes and revealing their host-stage specific differential expression. Combining race-specific and race-nonspecific genes together in the one cultivar will significantly improve durability of R genes deployed within *Brassica* species. Recently, genome of *Brassica juncea*, *B. napus*, *B. rapa* and *B. oleracea* is sequenced which will further allow discovery of novel R genes and enhancement of our understandings about genetic relationships among different R genes within and between members of the *Brassica* species. Availability of the DNA sequence of Brassicas and progress in functional genomics will further lead to the identification of many novel genes which can putatively be involved in pathways of different stress tolerance mechanisms and can be proposed as candidates. Hybridization between divergent groups in the Brassicaceae, which harbor numerous R genes, is already demonstrated in India and outside, which will further complement the incorporation of identified genes/QTLs from one species to another. Once the identified genes/QTLs are tagged and mapped with molecular markers their pyramiding in single genetic background would be possible. This shall also open new opportunities for development of multiple disease resistant Indian mustard genotypes.

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MARKER-ASSISTED BREEDING FOR BIOTIC AND ABIOTIC STRESSES IN CHICKPEA

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Chickpea (*Cicer arietinum* L.) is a self-pollinated diploid ($2n = 2x = 16$) crop with genome size of 738 Mb (Varshney *et al* 2013), originated from South-eastern Turkey (Ladizinsky 1975). It has 650 genera which includes 18,000 species grown in the semi-arid regions of the world (Polhill *et al* 1981). Globally, India produces about 70% of the world's total production of chickpea (FAOSTAT 2015). Major chickpea producing countries are India, Pakistan, Australia, Turkey, Myanmar, Iran, Ethiopia, Mexico, Canada and the United States. Chickpea productivity consistently increased in India and Mexico, while it declined in Turkey, Pakistan, and Iran (Upadhyaya *et al* 2012). India ranks first in terms of production and productivity, followed by Pakistan, Turkey, Iran, Myanmar, Ethiopia, Mexico, Australia, Canada and the United States.

Chickpea has two categories: the small-seeded with a brown-coloured seed coat 'desi' and the large-seeded with a cream or beige-coloured 'kabuli'. *Desi* chickpeas are mostly grown in India, Pakistan, Myanmar, Australia and Bangladesh, whereas *kabuli* chickpeas are predominantly cultivated in Turkey, Ethiopia, Syria, Spain, Canada, the United States, Mexico and Portugal (Thudi *et al* 2014). Chickpea seed contains 17–31% protein and biological activity of protein ranged from 52 to 78%, thus it has prime significance for human food and animal feed. Chickpea also contain significant amount of essential amino acids like leucine, isoleucine, lysine, valine and phenylalanine in the readily digestible seed protein (Williams and Singh 1987, Karim and Fattah 2006). Chickpea contains minerals such as Ca, Mg, Zn, K, Fe, and phosphorus (Jukanti *et al* 2012) and vitamins and is also free from anti-nutritional factors which make it nutritionally more valuable and thereby highly preferable by consumers. Chickpea has superior fibre content over many cereals and improves insulin secretion and controls blood sugar levels after consumption. Apart from the essential components of human dietary and health requirements, chickpea also has the capability of symbiotic nitrogen fixation and thereby enrich the soil fertility.

The estimated potential of chickpea yield is 6 t/ha under optimum growing conditions whereas currently average chickpea yield is 0.9 t/ha globally which is much lower than the estimated potential. Most of the chickpea area is cultivated under rainfed condition, surviving on receding soil moisture. Chickpea yield is adversely affected by various abiotic and biotic stresses like drought, heat, salinity, cold, *ascochyta* blight (AB caused by *Ascochyta rabiei*), *fusarium* wilt (FW *Fusarium oxysporum* f. sp. *ciceris*), pod borer (*Helicoverpa armigera*) and *botrytis* gray mold (BGM caused by *Botrytis cinerea*).

Chickpea productivity is highly hampered due to more rigorous temperature regimes and dry soils (heat and drought stresses). Water deficit stress affects several physiological processes relates with crop growth and developments (Turner and Begg 1978). Extensive artificial irrigation is required to achieve acceptable harvest yield in many of the chickpea cultivating regions (Bakht et al. 2006). However, prolonged practice of artificial irrigation results in increased soil salinization and thereby contributing toward declining productivity. Due to the effect of various stresses on yield, it is highly required to initiate serious efforts in the direction of developing improved varieties or alternate strategies which permit sustainable chickpea production under adverse environmental conditions. New breeding approaches has been developed to improve crop productivity under adverse environmental conditions which requires a better understanding of the genetic mechanisms involved in crop's response. A network of specialized genes induces in response of plant stress which is controlled through intricate regulation by specific transcription factors (Chen and Zhu 2004). Thus, the application of genomics-assisted breeding (GAB), a holistic approach combining genomics with breeding and physiology (Varshney et al. 2005), provides strategies for improving component traits related to biotic and abiotic stresses tolerance (Mir et al. 2012).

The conventional breeding efforts need to be supplemented by genomics-assisted breeding (GAB) for solving the growing demands for food grains due to ever-increasing population (Varshney *et al.* 2005). Chickpea was considered as an orphan legume only a few years ago. But now, the availability of chickpea genome sequence information (Varshney *et al.* 2013) and large-scale genomic resources have turned the crop into a resource rich crop. Thus, trait mapping and molecular breeding such as marker-assisted backcrossing (MABC), marker-assisted recurrent selection (MARS) and advanced backcross quantitative trait loci (AB-QTL) analysis are being practiced in chickpea. Large-scale molecular markers have been developed recently by using the advent of next generation sequencing (NGS) technologies. These genomic resources have been used for construction of saturated genetic maps and identification of linked markers associated with traits of interest (Varshney 2016).

Biotic stresses in chickpea

Despite of its economic importance, neither the area under cultivation nor productivity has increased to meet the current demands. This sluggish pace of production trend is due to several biotic and abiotic constraints that have been challenging the crop (Thudi et al., 2014). Among the biotic constraints, ascochyta blight caused by the necrotrophic fungal pathogen *Ascochyta rabiei* (Pass.) Lab., fusarium wilt (FW) caused by *Fusarium oxysporum* f. sp. *ciceris* and botrytis grey mould caused by necrotrophic fungal pathogen *Botrytis cinerea* Pers. ex. Fr. are the most devastating fungal diseases of chickpea worldwide. Pod borer is one of the most devastating insect-pests in chickpea. Ascochyta blight can infect chickpea plants at any growth stage from plant emergence to seed maturity, however, more susceptible at flowering and podding stages results in substantial yield loss and poor seed quality

(Sharma et al., 2010). While, all the aerial parts of chickpea are susceptible to the botrytis grey mould with growing tips and flowers being the most vulnerable (Bakr and Ahmed 1992; Grewal et al., 1992; Haware and McDonald 1992; Haware 1998; Bakr et al., 2002). In addition, the scenario of changing climatic conditions in the form of increased temperature and erratic rainfalls especially in semi-arid regions has enhanced the occurrence of fusarium wilt (Sharma and Pande 2013). For effective control of these disease infection, fungicide applications were used which results insensitivity of pathogen isolates against several fungicides (Chang et al. 2007; Wise et al. 2009). Therefore, development of varieties resistant to ascochyta blight, fusarium wilt and botrytis grey mould is considered as the most effective and sustainable approach. Globally, several sources moderately resistant to ascochyta blight, fusarium wilt and botrytis grey mould have been identified as complete resistance are lacking and successfully used in chickpea breeding (Sharma and Ghosh 2016). However, rapid adaptation of these pathogens to the resistance is becoming an alarming problem.

Chickpea is known to have a narrow genetic base as compared to most other legumes (Stephens et al., 2014). Due to relatively low levels of polymorphism, inter-specific crosses between *C. arietinum* and *C. reticulatum* have been the primary focus for genetic studies (Singh et al., 2008). The amount of polymorphism in an inter-specific mapping population varied from 16% to 36% whereas 9.5% only in an intra-specific mapping population (Nayak et al., 2010). High-resolution genetic linkage maps can also be constructed by exploiting the inter-specific polymorphisms between *C. arietinum* and *C. reticulatum* (Thudi et al., 2011). Variation detection based on SNPs has also shown the similar trends. Thus, an inter-specific mapping population from the cross between *C. arietinum* and *C. reticulatum* has been used in our study to identify the key genomic regions providing resistance against ascochyta blight, fusarium wilt and botrytis grey mould.

In case of ascochyta blight, the exact genetic and molecular mechanism of partial resistance against *A. rabiei* infection is still unknown. Depending on the isolates of *A. rabiei* and the method of disease scoring, both qualitative and quantitative modes of inheritance for resistance against ascochyta blight have been reported in chickpea. Resistance to ascochyta blight was initially characterized as monogenic with additional modifier genes. Single dominant or recessive gene controlling ascochyta blight resistance in both *desi* and *kabuli* types was reported (Dey and Singh 1993; Tewari and Pandey 1986). However, recent studies using recombinant inbred lines demonstrated continuous distribution of disease response and suggested a polygenic inheritance. In case of fusarium wilt, genomic resources have been deployed in chickpea to locate and tag the molecular markers linked to disease resistance (Gaur et al. 2012b, 2014). For fusarium wilt, eight races (races 0, 1A, 1B/C, 2–6) have been identified and the genetics of resistance to six races has been reported as monogenic, digenic or quantitative (Singh et al. 1987; Kumar 1998; Tullu et al. 1999; Rubio et al. 2003; Tekeoglu et al. 2000; Sharma et al. 2004, 2005). Genetic control of resistance to races 1B/C and 6 is not reported so far. The genes/QTLs for resistance to six races (0, 1A, 2–5) of fusarium

wilt pathogen have been mapped on to the chickpea genetic map (Li et al. 2015). In case of botrytis grey mould, the limited reports available on genetics of BGM resistance suggests that the resistance is controlled by few genes. A single dominant gene 'Bor1' for resistance was identified by Tiwari et al. (1985), while two genes with dominant and recessive epistasis (13:3 ratio) were reported by Rewal and Grewal (1989) and duplicate dominant epistasis (15:1 ratio) by Chaturvedi et al. (1995). Deployment of resistant genotypes could be an effective way to minimize yield losses due to AB, FW and BGM.

Chickpeas can be damaged by nearly 60 insect species, of which *H. armigera* are the most devastating insect pests in the field. *H. armigera* infestation begins at 25–30 days after sowing, and maximum damage is inflicted during flowering. The neonate larvae are voracious eaters and prefer young leaves and flowers, while the late instar larvae bore into the immature pods. *H. armigera* have a facultative diapause (delay in development due to external stimuli), which enables them to survive adverse weather conditions in both winter and summer. A number of cultural practices were adopted to reduce the damage caused by *Helicoverpa*, including time of sowing, spacing, fertilizer application, deep plowing, interculture, and flooding. These cultural practices are labor-intensive, and their adoption depends on the crop husbandry practiced in a particular agro-ecosystem. The application of insecticides is common, and more than 50% of all of the insecticides are used to control pod borers in India and China. However, the ecological effects of chemical pesticides on the environment and the evolution of resistance within the insect population are unavoidable. Microbial pathogens, such as *H. armigera* nuclear polyhedrosis virus (HaNPV), entomopathogenic fungi, and *Bacillus thuringiensis* (Bt) formulations have been used to control this pest. The activity of HaNPV and Bt spray is reduced, due to highly acidic exudates from the trichomes of chickpeas. Several chickpea genotypes have low-to-moderate levels of resistance against pod borers, but these levels are unstable because of the variable insect population and climatic conditions. Transgenic chickpeas that are resistant to *H. armigera* were generated using the cry1Ac gene. However, the deployment of transgenic crops expressing only one cry gene may lead to the evolution of resistant *H. armigera* populations. A second gene, cry2Aa, was introduced in chickpeas to generate a stacked product using the Cry1Ac lines.

Abiotic stresses in chickpea

Among abiotic stresses, drought, heat and salinity are considered as major constraints that affect the chickpea production. In arid and semi-arid regions of the world, one of the major constraints to chickpea production and yield stability is drought stress particularly at the end of the growing season (Krishnamurthy *et al* 2010). Drought causes considerable annual yield losses up to 50% in chickpea and whereby the productivity remained persistent for the last many decades (Ahmad *et al* 2005, Varshney *et al* 2010). Drought tolerance is a generic term for a highly complex phenomenon of plant responses. Improvement in drought tolerance is laggard owing uncertain water availability across the marginal lands, non-uniform evaluation methods,

lower genotypic variance for seed yield under stressful conditions (Ludlow and Muchow 1990) and complex genetic background of traits (Turner *et al* 2001). Thereby, it is a challenge to improve drought tolerance by conventional breeding due to dependence on improved yield under stressful condition, quantitative nature of traits and prevalence of linkage between desired and undesired genes (Richards 1996, Flowers *et al* 2000).

Different drought tolerance indices are used widely for selection of relative drought tolerant genotypes, comparing the relative performance under normal and stress conditions (Maqbool *et al* 2015). Genotype \times environment interaction hampers the direct selection of genotypes for higher yield in association with ineffective control of stress level under field conditions (Maqbool *et al* 2015). Due to instability and poor heritability, breeding for yield under drought conditions by means of conventional approaches has not been fairly successful over the years. Under such circumstances, molecular breeding seems to be a better strategy that can be deployed by targeting drought tolerance component traits with the help of molecular markers. QTL mapping based on SSR primers helped in establishing association of genotype and phenotype (Huttel *et al* 1999, Winter *et al* 2000, Tar'an *et al* 2007, Rehman 2009, Varshney *et al* 2009, Rehman *et al* 2011). Moreover, for utilizing molecular breeding for developing superior genotypes of chickpea, understanding genetic basis and identification of molecular markers for drought tolerance module traits are prerequisites.

The damage due to drought is compounded by heat stress in the warmer Mediterranean regions and regions like South Asia where temperature rises towards flowering therefore making it difficult to distinguish the damage caused by the individual stresses, which further slows down the progress made on heat tolerance. Studies on the impact of climate change on chickpea production underlined the effect of warmer temperatures on crop development and following yield. For example, the yields of chickpea deteriorated by up to 301 kg/ha per 1 °C rise in mean seasonal temperature in India. It is anticipated that climate change would result in temperature rise by 3-4 °C over current levels by 2050 (Basu *et al* 2009). Chickpea is usually grown in winter season in Northern India, but it experiences a high temperature (>35 °C) during the reproductive phase. Since the last few years, an obvious change has been witnessed in chickpea production in the Indian subcontinent. A big shift in chickpea area from cooler northern India to relatively warmer central and southern India has been observed (Gaur *et al* 2007). The identification of heat tolerant genotypes thus helps to adapt chickpea to warmer temperatures.

Flowers are the most sensitive organs of plant to heat stress (Wery *et al* 1993, Toker and Canci 2006). Pod formation and seed set are adversely affected if temperature rises above the threshold level during the flowering or reproductive period, leading to reduction in grain yield (Summerfield *et al* 1984, Wang *et al* 2006, Basu *et al* 2009). Adverse effects of high temperature occur in seed germination, respiration, membrane stability, photosynthesis, hormone level, nutrient absorption, protoplasmic movement, quality of seeds, fruit maturation, fertilization, materials transport,

withering, burning of lower leaves, desiccation of poorly developed plants, stunting flower and pod abortion, reduced root nodulation, nitrogen fixation and seed yield (Kurdali 1996). Pollen viability, fertilization and seed development are also adversely affected by heat stress which leads to the reduction in harvest index. Heat tolerance is a complex trait and thus, an effective and simple screening method having well-defined traits for selecting heat-tolerant genotypes under field conditions is indispensable for breeding heat tolerant cultivars (Devasirvatham *et al* 2012).

Chickpea is grown mainly in arid and semi-arid regions; the soils in these areas are susceptible to salinization, yet chickpea is relatively salt-sensitive (Vadez *et al.* 2007; Flowers *et al.* 2010). Salinity adversely affects chickpea germination (Khalid *et al.* 2001), plant establishment (Al-Mutata 2003) and vegetative growth (Lauter & Munns 1986; Dua & Sharma 1997); however, the reproductive processes are considered the most salt sensitive (Vadez *et al.* 2007; Samineni *et al.* 2011; Vadez *et al.* 2012; Turner *et al.* 2013). Reproductive success (i.e. seed yield per plant) is a function of flower number and successful fertilisation of the ovule, pod development, pod retention, seed number per pod, and seed growth (Flowers *et al.* 2010). A few studies have reported that salt-tolerant chickpea lines tend to produce more flowers (Datta *et al.* 1987; Vadez *et al.* 2012) and maintain more pods, indicating that the conversion of flowers into pods is a salt sensitive process (Vadez *et al.* 2007; Samineni *et al.* 2011; Turner *et al.* 2013).

The processes of reproductive development most affected by salinity are not known for chickpea and have rarely been studied for plants generally. However, it is well established in chickpea that there is a specific sensitivity to salinity around reproduction, as yields were similar when the salt was applied at sowing or at the beginning of flowering (Vadez *et al.* 2012). Samineni *et al.* (2011) showed that the reproductive stage was not affected by changes in pollen viability, but *in vitro* pollen germination was severely inhibited by addition of NaCl to the germination media indicating that if Na⁺ and/or Cl⁻ accumulate to high levels in floral tissues this may compromise pollen tube growth and fertilisation. Turner *et al.* (2013) documented increased pod abortion in salt sensitive genotypes, but pollen viability, *in vitro* pollen germination and *in vivo* pollen tube growth were not affected by salinity, suggesting that the reduction of number of filled pods arose either from the lack of ovule fertilisation or from the failure of the fertilised ovule to develop and grow into a seed (and/or associated structures). The mechanism(s) by which salinity affects reproductive processes in chickpea remains unclear.

Genomics-assisted breeding (GAB) approaches for biotic and abiotic stress tolerance

GAB refers to integration and use of genomic tools in breeding practices for developing superior lines with enhanced biotic or abiotic stress tolerance and improved yield. GAB helps in establishing relationship between genotype and phenotype for crop improvement by exploiting several approaches like genomics, transcriptomics and

proteomics. Next-generation sequencing (NGS) platforms (Varshney et al. 2009) and high-throughput genotyping technologies (Varshney 2011) provides the genome-wide marker profile for prediction of phenotype of individuals. Marker-assisted backcrossing (MABC) approach has been considered a good approach for retaining the desirable gene combinations or gene pyramiding. However, marker-assisted recurrent selection (MARS) has been proposed as better approach than MABC. In addition of this, genomic selection (GS) approach is emerging as a powerful approach for identifying desirable progenies for making the crosses. Advanced back-cross QTL (AB-QTL) approach is being used for simultaneously identification and transfer of superior alleles for a given trait from the wild species to a leading cultivar. In such cases, advanced back-cross QTL (AB-QTL) approach (Tanksley and Nelson 1996) has been proposed by for simultaneous discovery and transfer of superior alleles from wild species to develop improved lines.

MABC is suitable to introgress 1–3 gene combinations in chickpea. However, introgression of large number of genes in one genetic background through backcross programme utilizing low-cost genotyping system remains a challenging task. MABC involves introgression of specific trait(s) from a donor parent into the genetic background of a recurrent parent (generally leading variety) using molecular markers. This approach can also be used to generate near-isogenic lines (NILs) or chromosome segment substitution lines (CSSLs) for genomics research (Varshney et al. 2010). QTLs for resistance to AB have been located and validated on LG4 (QTLAR1 and QTLAR2), LG2 (QTLAR3) and LG3 (QTLAR4) of the chickpea map using different mapping populations. Castro et al. (2015) studied phenotypic classical selection and MAS to select blight-resistant genotypes and compared the effectiveness of both methods. The phenotypic evaluation showed that the resistance to AB could be recessive in the material employed. Phenotypic selection in $F_{2:4}$ and $F_{2:5}$ generations leads to an increase in the frequency of the allele associated with the resistance of the markers CaETR and GAA47 (linked to QTLAR1), indicating the usefulness of these markers for MAS. The markers TA72 and SCY17 (linked to QTLAR2) could be also useful for MAS, but the high distorted segregation towards the susceptible parent in the region where these markers were located could explain their low effectiveness. Tar'an et al. (2013) used MABC to introgress resistance to AB and double pod into adapted chickpea cultivars. They carried out backcrosses between moderately resistant donors (CDC Frontier and CDC 425-14) and the adapted varieties CDC Xena, CDC Leader and FLIP98-135C. They included two backcrosses and selection for two QTLs for AB resistance (Abr QTL3 and Abr QTL4 on LG4 and LG8, respectively) and a locus associated with double podding on LG6. An average of three markers per chromosome and the average distance of 25 cM between two markers were used. This procedure allowed selecting plants with 92% of the recipient genome by BC_2F_1 . The selected plants possessed the majority of elite parental type SSR alleles on all fragments analysed except the segments of LG4, LG6 and LG8 that possessed the targeted QTL. Therefore, using MAS and two generations of backcrosses followed by one generation

of selfing, Tar'an et al. (2013) obtained an improved version of CDC Xena, CDC Leader and FLIP98-135C. This practical example of MAS clearly illustrates the superiority of using MABC compared to conventional backcrossing due to solving the problem of linkage drag.

In the case of *fusarium* wilt, MABC approach is being used for introgressing resistance to two races (*foc* 2 and *foc* 4) independently and pyramiding of resistance to two races (*foc* 1 and *foc* 3) for FW and two QTLs conferring resistance to AB. More recently, five resistant lines representing *foc2* gene introgressed into the background of Pusa 256 were reported with the help of foreground selection aided by two SSR markers (TA 37 and TA110) (Pratap et al. 2017). *Fusarium* wilt (FW) and *Ascochyta* blight (AB) are two major constraints to chickpea production therefore, stepwise MABC approach was adopted to develop dual resistance C 214, an elite chickpea cultivar. To develop resistant lines, *foc* 1 locus for FW and two quantitative trait loci (QTL) regions, ABQTL-I and ABQTL-II, for AB were targeted for introgression. Foreground selection with six markers linked to *foc* 1 and eight markers linked to both QTL regions was used for selection of plant with desirable alleles in different segregating generations. In addition to foreground, background selection was performed for selection of plant with high recurrent parent genome recovery, with evenly distributed 40 SSR markers. After three backcrosses and three rounds of selfing, 22 BC₃F₄ lines were generated for FW and 14 MABC lines for AB (Varshney et al. 2014). Phenotyping of these lines has identified three resistant lines for FW and seven resistance lines for AB. MABC efforts also focused on introgression of QTLs responsible for drought stress tolerance. As part of trait mapping, a genomic region on LG04 was identified as “*QTL-hotspot*” explaining up to 58% phenotypic variation for several root traits that control the yield under rainfed condition. Efforts to introgress this genomic region into elite chickpea genotype JG 11 were initiated using MABC approach as described by Varshney et al. (2013). Introgression lines generated after three backcross and two rounds of selfing (BC₃F₃) showed improved performance with 12% (under rainfed) to 24% (under irrigated) higher yield. After multilocation field evaluation, 10 introgression lines have been identified as superior and are being sent for AICRP trial for release in India. Inspired by success of JG11, efforts have already been initiated to introgress this genomic region in several other elite chickpea varieties. In parallel, “*QTL-hotspot*” genomic region has been transferred in two more leading chickpea varieties (Chefe and KAK 2) by using MABC approach (Thudi et al. 2014).

In MARS, QTLs are identified in the breeding population derived from good × good crosses. Subsequently, the lines carrying superior alleles for maximum QTLs are crossed to pyramid superior alleles in one genetic background. Recombined lines are then subjected to a final phenotypic screening to select the best lines for multi-location field testing to release them as varieties. MARS is particularly useful for capturing the several genomic regions especially to target more number of minor as well as major QTLs. Some efforts have been initiated to use MARS in the case of chickpea, for assembling favorable alleles for drought tolerance using ICCV 04112 × ICCV 93954

and ICCV 05107 × ICCV 94954 crosses. For exploiting MARS, two crosses JG 11 × ICCV 04112 and JG 130 × ICCV 05107 were made to combine the favorable alleles of yield QTLs from the respective parents with an assumption that each population had a different set of QTLs involved. 188 F₃ plants each in two crosses were genotyped using SSR markers and F_{3:5} progenies were evaluated at multi-locations. QTL analysis of phenotyping and genotyping data resulted in identification of few major and several minor QTLs contributing to yield, and yield contributing traits. Based on the QTL(s) information for seed yield, harvest index, biomass, and seed size in different F₅ progenies, 4 lines were selected in JG 11 × ICCV 04112 and 3 lines in JG 130 × ICCV 05107 having different combinations of favourable alleles for recombination cycle. Multiple cycles of MARS increased the frequency of favourable alleles associated with agronomic traits. The selected lines were subjected to 1st and 2nd recombination cycles. F₁ plants having all favourable alleles of QTLs from both parents for seed yield and other yield-related traits were identified in JG 11 × ICCV 04112 and JG 130 × ICCV 05107, respectively. Finally, F₁ plants with all favourable alleles in homozygous condition were grown. Each selected F₁ plant was advanced to F₄ generation separately for field evaluation. On the other hand, for comparing the advantage of MARS over the traditional method of recurrent selection, top 8 high-yielding F₅ progenies were also selected and intercrossing is being completed in each cross. Practical utility of this scheme in regular breeding programs will depend on the genetic gain achieved in terms of selection efficiency, selection accuracy, marker-trait associations, and distribution of favourable alleles between the selected parents used for crossing.

The incorporation of multiple parents ensures the population segregates for multiple QTLs for multiple traits. In this direction, MAGIC lines were developed in chickpea using eight diverse genotypes selected from South Asia and sub-Saharan Africa. In this development scheme, more number of recombinations and greater genetic diversity in MAGIC lines greatly help in detection of QTLs with high precision. Further, MAGIC lines can act as a base for gene discovery, characterization, and deployment of genes for understanding complex traits (Glaszman et al. 2010). The parents (ICC 4958, ICCV 10, JAKI 9218, JG 11, JG 130, JG 16, ICCV 97105, and ICCV 00108) of the MAGIC lines from Ethiopia, Kenya, and India were crossed in direct fashion excluding reciprocals. Twenty-eight two-way, 14 four-way, and 7 eight-way crosses were made to develop a MAGIC population. Over 1200 F₂ plants from 7 eight-way crosses were advanced to F₈ using single seed descent (SSD) method and seed was harvested from 1136 F_{7:8} progenies. These progenies were evaluated for two years (2013 and 2014) under field conditions with rainfed and irrigated treatments. Large variability in phenology (days to flowering: 34– 69 d) and yield-related traits (seed yield: 170– 4554 kg/ha); 100 seed weight: 10–45 g) was observed under two treatments. Several promising lines with significantly higher seed yield than the best parents were also identified. On the other hand, eight parents used in the development of MAGIC lines were genotyped using 70 SSR and 747 SNP markers. In addition, the parental lines were resequenced (with 5.79–16.08X coverage) and variable number of SNPs and indels were identified among the parental lines by aligning to CDC Frontier reference genome.

Advanced-backcross QTL based breeding (AB-breeding) approach is the most suitable for introducing novel alleles from wild relatives to the cultivated species in a controlled manner. In AB breeding approach, a selected wild species is backcrossed to a cultivar or a variety and then, selection is imposed in segregating BC₂F₂ or in BC₂F₃ population to identify and preserve individuals with desirable traits in the population. Both genotyping and phenotypic data are generated with this segregating BC₂F₂ or BC₂F₃ and, these data sets will be subjected to QTL analysis to identify QTL, QTL associated markers and, also to check whether any of these QTL are involved in trait improvement in the progenies that are preserved. Therefore, AB-QTL strategy involves the parallel discovery and transfer of desired QTL from an unadapted germplasm into selected breeding lines (Tanksley and Nelson 1996). In addition, it also provides an opportunity to select for QTL-near isogenic lines (NILs) in a short time span. Using QTL-NILs, the QTL effects can be established and NILs may serve either as improved varieties or as parents for use in hybridization programs and for studies related to heterosis. In the case of chickpea, attempts were made for making wide crosses using cultivated and wild chickpea (*C. arietinum* × *C. reticulatum*) which resulted in selection of progenies with increased seed yield (Jaiswal et al. 1987). Similarly, Singh and Ocampo (1997) identified transgressive segregation for agronomic traits in an F₂ population of a cross, *C. arietinum* × *C. reticulatum*. These reports offer some opportunities in exploiting genetic variation present in wild species of chickpea and hence, fresh initiatives have been initiated at ICRISAT to introgress stress resistance through AB-breeding approach.

Genomic selection (GS) approaches targets identification of superior lines with higher breeding value in a breeding program based on genome-wide marker profile data. As breeding values are estimated using the genome-wide marker data, these are generally referred as genomic-estimated breeding values (GEBVs). GS can reduce the selection cycle length of a breeding program that could take several seasons to develop reliable phenotypes. However, use of appropriate statistical model is very critical for estimating the GEBVs with higher precision. Among different models of GS, GEBVs predicted using either best linear unbiased prediction (BLUP) or Bayesian methods are more effective according to simulation studies. Though GS has not been used in chickpea at present, GS seems to be a deployable approach in coming future at least in chickpea. A set of 320 elite chickpea lines was selected and genotyped using DArT markers at ICRISAT for exploiting GS approach. This set was phenotyped at Patancheru and New Delhi for two seasons for yield and yield-related traits. Phenotyping data along with genome-wide marker profile data was used with six statistical GS models to estimate the prediction accuracies (Roorkiwal et al. 2016). Promising results of GS were evident in chickpea from higher prediction accuracies (up to 0.91) obtained for yield-related traits using six different GS models (Roorkiwal et al. 2016). However, low accuracies were observed for seed yield under rainfed environments. In a more recent GS study in chickpea, Li et al. (2018) suggested incorporating information about the significant markers (GWAS results) to different GS models to increase prediction accuracies. As demonstrated in chickpea, the ability of GS to consider multiple variables simultaneously allowed breeding programs to gain

higher prediction accuracies through inclusion of $G \times E$ effects (Roorkiwal et al. 2018). This study also reported higher prediction accuracies with DArT Seq system as compared to the GBS.

Conclusion

Several sources of resistance to biotic and abiotic stresses have been identified among wild *Cicer* accessions and efforts are on to develop pre-breeding populations to facilitate greater access and use of such lines in chickpea breeding. Chickpea has witnessed tremendous growth in establishment of genomic resources and utilization of these genomic resources for developing biotic and abiotic stress tolerance. Availability of whole genome sequence and different re-sequencing efforts has allowed the development of high-throughput genotyping platform. A combination of linkage mapping and a candidate gene approach has been the most successful method of identifying the causal genes for the trait of interest in chickpea. QTLs analysis in chickpea has been fruitful, and important genomic areas in the chickpea genome have been related to important biotic, abiotic and yield-related traits. Markers linked to some of those QTLs have been successfully applied in breeding programmes originating new varieties with the desired traits. The availability of the whole-genome sequences in *kabuli* and *desi* chickpea types opens new possibilities to survey or re-localize QTLs. The analysis of either segregating populations or genomic resources using next-generation sequencing (NGS) technologies produces genome-wide high-density single-nucleotide polymorphisms (SNPs) with a relatively low cost, allowing to identify haplotype blocks significantly correlated with quantitative trait variation (Barabaschi et al. 2016). Compiled information applied in multiparent populations such as MAGIC will provide a unique opportunity to explore environmental interactions and better predict allelic effects in diverse backgrounds (Huang et al. 2015). The chickpea genome sequencing has provided the much needed thrust to genomics based breeding approaches. Further, the re-sequencing of the germplasm will help in better understanding of the diversity present in *Cicer* species. MABC is being routinely used for developing superior varieties by targeting simple traits. Recently, GS has also gained momentum with its capability to target complex traits. Developing the MAGIC population for addressing the issue of narrow genetic diversity is an excellent approach. New genomic advances, many of which are already being developed, will make it easier for breeders to obtain new cultivars with improved characteristics, either by facilitating selection or by improving the variation available by using precision breeding approaches.

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DISEASE IDENTIFICATION AND SCREENING TECHNIQUES FOR BIOTIC STRESSES IN GROUNDNUT AND SESAME

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Yield losses in any crop are produced by diverse causes and one of the more important is due to diseases. In Groundnut, the major diseases are Collar rot caused by *Aspergillus niger*, Stem rot caused by *Sclerotium rolfsii*, dry root rot caused by *Macrophomina* spp. and leaf spots caused by *Cercospora*. The recognition of peanut genotypes being tolerant to them and simultaneously having higher production potentials should benefit growers and breeders to carry out the proper variety for sowing or for further The cultivation of resistant and tolerant peanut varieties does not only eliminate the crop losses caused by disease, it also contributes to reduce costs related to fungicide sprayings and other control methods. The hazard of pollution of the environment with toxic chemical compounds can also be discarded, that otherwise would have to be used in the crop for diseases control. These diseases can be easily recognized by some symptoms caused by them.

Collar rot: The most important disease of groundnut in Punjab, collar rot shows two type symptoms i.e. pre-emergence and post emergence. In pre-emergence seed rot infected seeds fail to germinate and are covered with masses of black conidia which impart them a sooty appearance. Collar rot is the most serious disease of groundnut in early stage of growth and the emerged seedlings are affected at collar region causing yellowing of lower leaves, slow death due to blighting effect on the shoot, finally leading to death of crown portion the most serious disease of groundnut in early stage of growth.

Screening: Pots are inoculated at three days before sowing with collar rot pathogen (*A. niger*) multiplied on sorghum grains. Forty grams of sorghum grains containing pathogen is applied to the soil at 10 cm below the surface layer and covered with soil. After inoculation, the pots are watered daily to maintain soil moisture. The soil is used in a ratio of 1 part sand and 2 parts soil. There are sown three seeds per pot for each accession equi-distantly at a depth of 4 cm.

Observations on per cent disease incidence are recorded at 60 days after sowing (DAS)

Stem rot: Signs of infection include the presence of coarse white strands of mycelium growing in a fan-shaped pattern on lower stems, leaf litter, or soil. Strands of coarse white mycelium may also be visible on the surface of pods. Occasionally, mycelium takes on a tufted appearance or forms sheaths around the main stem. In dry weather, white to pale buff mycelium may be appressed to lower stems, looking like small patches of paint and later, tan to brown round sclerotia 0.5 to 1.5 mm in diameter may be present on the mycelium, lesions, or soil, or on litter near infected plants.

Screening : stem rot pathogen, *S. rolfsii* is multiplied on sorghum grains and added to the pots at 45 DAS. The pots are immediately watered for two consecutive days after inoculation. The pots are maintained on a greenhouse bench at 26±2 °C and at RH

90% until harvest. Stem rot disease severity is measured for every 15 days starting from the day of inoculation.

Stem rot disease severity was measured on 1-5 scale

wherein, 1= Healthy plant

2= Lesions on stem only

3= Up to 25% of the plants symptomatic (wilted, dead or decaying)

4=26- 50% of the plants symptomatic and

5=> 50% of the plants symptomatic

Dry Root rot: In the early stages of infection, reddish brown lesion appears on the stem just above the soil level. The leaves and branches show drooping, leading to death of the whole plant. The decaying stems are covered with whitish mycelial growth. The death of the plant results in shredding of bark. The rotten tissues contain large number of black or dark brown, thick walled sclerotia. When infection spreads to underground roots, the sclerotia are formed externally as well as internally in the rotten tissue. Pod infection leads to blackening of the shells and sclerotia can be seen inside the shells.

Screening can be done in sick field and observations on percent incidence are taken.

Tikka Leaf Spots: (*Cercospora arachidicola*, *Phaseoisariopsis personata*): The disease occurs on all above ground parts of the plant, more severely on the leaves. The leaf symptoms produced by the two pathogens can be easily distinguished by appearance, spot colour and shapes. Both the fungi produce lesions also on petiole, stem and pegs. The lesions caused by both species coalesce as infection develops and severely spotted leaves shed prematurely. The quality and yield of nuts are drastically reduced in severe infections.

Screening: The mixture of sand: vermiculite mixture (1:1 v/v) is prepared and steam sterilized at 15 lbs pressure and 121° C for 1 h in two cycles of autoclave. The excised explants from all three genotypes are planted in sterile culture (1.5 cm thick sand) in plastic trays (40 x 30 cm) and transferred to dew chamber. Immediately after planting the detached leaves in the tray are sprayed with LLS inoculum (30,000 conidia ml⁻¹). The dew chamber conditions are maintained at a temperature of 24° C and 85% relative humidity. Further the trays are covered with transparent plastic sheets to maintain suitable conditions for disease development. Water is sprayed on the culture, leaves and the sheets once daily up to 8 days. Disease development is monitored every alternate day from 5 to 40 days after inoculation (DAI).

Each entry is raised in two rows of 3 m length with a row to row spacing 45 cm apart with 15-20 cm interplant distances.

Data collection on leaf spot disease was based on a subjective visual score as follows:

1 = highly resistant,

1 - 3 = resistant,

4– 5= moderate resistance,

6 – 7 = susceptible and

8 – 9 = highly susceptible (ICRISAT, 1995).

Disease score indicates

0%= highly resistant,

1 - 20% = resistant,

21 – 50%= moderate resistance,

51 – 70% = susceptible and

71 – 100% = highly susceptible. Data on leaf spots is collected at 75 and 85 days from planting.

Rust: (*Puccinia arachidis*): The disease attacks all aerial parts of the plant. The disease is usually found when the plants are about 6 weeks old. Small brown to chestnut dusty pustules (uredosori) appear on the lower surface of leaves. The epidermis ruptures and exposes a powdery mass of uredospores. Corresponding to the sori, small, necrotic, brown spots appear on the upper surface of leaves. The rust pustules may be seen on petioles and stem. Late in the season, brown teliosori, as dark pustules, appear among the necrotic patches. In severe infection lower leaves dry and drop prematurely. The severe infection leads to production of small and shriveled seeds.

In Groundnut, the major losses are caused by soil borne diseases which are best screened in sick fields where groundnut is grown year by year. There is huge build up of inoculum and the genotypes can be screened there. However if there is constraint of space and germplasm number is too large, then Pot screening for soil borne or detached leaf technique for foliar diseases can be done.

Sesame: Sesame is another important oilseed crop with its oil with dietary and medicinal properties. It is affected by few major diseases among which Phyllody is the destructive one and there is no germplasm reported to be having resistance.

Phyllody: All floral parts are transformed into green leafy structures followed by abundant vein clearing in different flower parts. In severe infection, the entire inflorescences is replaced by short twisted leaves closely arranged on a stem with short internodes, abundant abnormal branches bend down. Finally, plants look like witches broom. If capsules are formed on lower portion of plant they do not yield quality seeds.

Phytophthora blight: Disease can occur at all stages of the plant. Initial symptom is water soaked spots on leaves and stems. The spots are chestnut brown in the beginning later turn to black. Premature leaf fall occurs. In humid weather, severity of disease increases, main root is affected, diseased plants are easily pulled out and produce shriveled seeds and gives blighted appearance.

Alternaria blight: The pathogen attacks all parts of the plant at all stages. Small, dark brown water soaked, round to irregular lesions, with concentric rings, 1-8 mm in diameter appear on the leaves and under excessive atmospheric and soil humidity the spot increases in size and number. The lesions may also appear on the midrib and veins of the leaves. Milder attacks cause only defoliation, in severe cases the plant may die

Screening: In sesame major disease Phyllody can be best screened under field conditions by sowing at a time when vector population is in abundance. However, for soil borne diseases, a sick field screening is more appropriate whereas for foliar disease we can spray the spore suspension on seedlings or 30 days old crop for Alternaria or Cercospora.

RECENT ADVANCES IN BREEDING FOR BIOTIC AND ABIOTIC STRESS TOLERANCE IN SOYBEAN

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1. Introduction

Soybean (*Glycine max* L. Merrill) is the world's most important seed legume, which contributes to 25 % of the global edible oil, about two-thirds of the world's protein concentrate for livestock feeding. Soybean meal is a valuable ingredient in formulated feeds for poultry and fish. The cultivation and use of soybean could be traced back to the beginning of China's agricultural age. Chinese medical compilations, dating back 6,000 years, mention its utilization for human consumption (Krishnamurthy and Shivashankar (1975). To the populace of China, Japan, Korea, Manchuria, Philippines, and Indonesia, for centuries, soybean has meant to be meat, milk, cheese, bread and oil. This could well be the reason, why in these countries, it has earned epithets like "Cow of the field" or "Gold from soil" (Horvath 1926). Owing to its amino acids composition, the protein of soybean is called a complete protein. Its nutrition value in heart disease and diabetes is well known. It is significant that Chinese infants using soybean milk in place of cow's milk are practically free from rickets. Today, USA, Brazil, and Argentina are the "Big-3" producers of the world. Versatility of soybean was recognized in the West quite recently. Around 1921, China produced about 80 % of the world's soybean (Bisaliah 1988). In India, Soybean was introduced from China in tenth century AD through the Himalayan routes and also brought in via Burma (now Myanmar) by traders from Indonesia. As a result, soybean has been traditionally grown on a small scale in Himachal Pradesh, the Kumaon Hills of Uttar Pradesh (now Uttaranchal), eastern Bengal, the Khasi Hills, Manipur, the Naga Hills, and parts of central India covering Madhya Pradesh. It has also been reported that the Indian continent is the secondary center for domestication of the crop after China [Hymowitz (1990), Khoshoo (1995), Singh and Hymowitz (1999)]. At present, India ranks fifth in the area and production in the world after USA, Brazil, Argentina, and China. The contribution of India in the world soybean area is 10 %, but the contribution to total world soybean grain is only 4 % indicating the poor levels of productivity of the crop in India (1.1 t/ha) as compared to other countries (world average 2.2 t/ha). The main reason of low productivity is that soybean is grown as a rain fed crop in India. A number of biotic and abiotic stresses are also attributable to the poor productivity of soybean in India which are discussed below.

2. Biotic stresses

The biotic stresses are diseases and pests faced by the crop. Among diseases, soybean cyst nematode, phytophthora, soybean rust and soybean mosaic virus are of global importance whereas yellow mosaic virus, soybean mosaic virus, rust and other diseases of regional significance are given in Table 1. The pest of the crop in India are stem fly (*Melanagromyza sojae* Zehntner), Tobacco caterpillar (*Spodoptera litura* Fabricius), Green semiloopers (*Chrysodeixis acuta* Walker, *Gesonía gemma* and

Diachrysis orichalcea Fabriciussensu Hübner), Girdle beetle (*Obereopsis brevis* Gahan), Pod borer (*Helicoverpa armigera* Hubner) and White fly (*Bemisia tabaci* Gennadius). The present article deals with measures to combat major biotic and abiotic stresses faced by soybean using plant breeding and molecular techniques.

2.1 Soybean cyst nematode

Soybean cyst nematode (SCN, *Heterodera glycines* Ichinohe) is among the most economically destructive pests of soybean in the world. In the US, an estimate shows that SCN causes 2 to 6% annual yield loss worth \$1 billion each year (Wrather *et al* 2003). Early inheritance studies indicated that SCN resistance is genetically controlled by three recessive genes designated rhg1, rhg2, and rhg3 (Caldwell *et al* 1960) and one dominant gene designated Rhg4 (Matson and Williams 1965). An additional dominant gene was later identified in PI88788 and designated as Rhg5 (Rao-Arelli 1994). But genetic analysis of different sources of resistance showed that inheritance of SCN resistance was oligogenic and complex (Anand and Rao-Arelli 1989). Multiple alleles at a single locus could be involved in SCN resistance (Hancock *et al* 1987; Hartwig 1985). Among the resistant sources identified, soybean line 'Peking' and plant introduction 'PI88788' were used to incorporate resistant genes into new varieties in soybean breeding programs (Arelli *et al* 2000). However, it was reported that certain field populations of SCN could overcome resistant cultivars, revealing the complex nature of host-pathogen interaction in soybean and SCN, which required the establishment of race-specific interrelationship for each resistant genotype and SCN race. Advances in molecular genetics and biotechnology make it possible to identify and map genomic regions conditioning SCN resistance. As one of early molecular genetics analysis of SCN resistance, Concibido *et al* (1994) reported two RFLP markers, pA85 and pB32, were significantly associated with SCN disease response and tentatively mapped to LGs A and K. In addition, a previously identified RFLP maker, pK69 (Boutin *et al* 1992), was also found to be associated with SCN resistance and mapped to LG G (Concibido *et al* 1994). Since then, several additional putative SCN resistant QTL were identified from a number of resistance sources, such as plant introductions, exotic germplasm, wild annual species (*Glycine soja*). While reviewing QTL analysis of SCN resistance, Concibido *et al* (2004) summarized a total of 16 putative QTL responsible for SCN resistance mapped on 17 different LGs of the soybean integrated molecular linkage groups (Cregan *et al* 1999a). Of these, the QTL on LG G has been well characterized and proven to be the most important QTL because it contains the gene rhg1, which is capable of resistance to most of existing SCN races, 1, 2, 3, 5, 6, and 14 (Concibido *et al* 1997; Yue *et al* 2001a; 2001b). In addition to RFLP, AFLP, and RAPD markers reported to be associated with different genomic loci on LG G (Chang *et al* 1997; Concibido *et al* 1994; Kabelka *et al* 2005; Meksem *et al* 2001), Cregan *et al* (1999b) mapped two SSR markers, BARC-Satt309 and BARC-Sat_168, at a distance of 0.4 cM to the gene rhg1. When mapping QTL for SCN resistance from PI90763 and PI404198A, Guo *et al* (2006) also found the same genetic linkage of these SSR markers on LG G. It indicated that marker-assisted selection for SCN resistance can be highly efficient by deploying a small set of the SSR markers flanking within interval of the rhg1 locus. The second important QTL on LG A2 was

located at the dominant gene Rgh4, which was reported to play a distinct role in resistance to SCN race 3 (Concibido *et al* 1994; Heer *et al* 1998; Webb *et al* 1995). Similar to genetic linkage of the gene rgh1, various DNA molecular markers were shown to be associated with this LGA2-QTL containing the Rhg4 gene (Concibido *et al* 2004). Especially, Cregan *et al* (1999b), Guo *et al* (2006) mapped the SSR marker Satt632 close to this genomic region. Using AFLP technology and bulk segregant analysis (BSA) to analyze resistant cultivar ‘Forrest’, Meksem *et al* (2001) constructed a high-density genetic map of AFLP markers for the intervals carrying the genes rgh1 and Rhg4 and concluded that these two genes together explained over 98% of the resistance to SCN race 3. With the availability of molecular markers closely linked to SCN resistance genes, such as Satt309, Satt163, Sat_168 (LG G), or Satt632, Sat_157 (LG A2), soybean breeders are capable of selecting resistant lines. However, in some occasions MAS for the rgh1 gene has some difficulty due to lack of polymorphisms between parental lines for markers linked to this locus. Lately, the development of SNP genetic markers in soybean (Zhu *et al* 2003) has provided soybean breeders an innovative approach to overcome the difficulty encountered. Wu *et al* (2005) reported that several SNPs were identified for the rgh1 and Rhg4 loci in 30 parental genotypes commonly employed in the SCN resistance breeding program. With a sequenced region containing the rgh1 gene, Hyten and Cregan (2006) demonstrated association analysis and discovered 67 SNPs throughout the region being significantly associated with SCN resistance.

2.2 Phytophthora

Phytophthora root and stem rot caused by fungal pathogen, *Phytophthora sojae* was reported to occur in soybean production regions of the world, such as USA, Brazil, Argentina, Australia, China, and South Korean (Grau *et al* 2004). To date, eight dominant genes, designated as Rps1 to Rps8, have been identified to convey resistance to 55 physiologic races of *P. sojae* (Burnham *et al* 2003). Among these, Rps1 and Rps3 were reported to have multiple alleles (Grau *et al* 2004). This indicated race-specificity of host resistance and genetic complexity of *P. sojae* population in soybean. The availability of genomic genotyping technologies has facilitated the identification and mapping of DNA molecular markers associated with resistance to Phytophthora root and stem rot on the soybean genetic linkage map (Cregan *et al* 1999a). Screening mapped RFLP markers in ‘Williams’ near-isogenic lines, Diers *et al* (1992) mapped the loci Rps1, Rps2, and Rps3 on linkage groups (LG) K, L, and E, respectively. However, Demirbas *et al* (2001) utilized SSR markers for genetic linkage analysis and mapped the loci Rps1, Rps2, Rps3, and Rps4 on LGs N, J, F and G, respectively. Both research groups reported genomic locations of Rps5 and Rps6 were unclear. Lohnes and Schmitthenner (1997) used another set of RFLP markers to map locus Rps7 in a ‘Clark x Harosoy’ isoline mapping population and reported this locus was closely linked to the RFLP marker R022-1 on LG N. The genomic location of the loci Rps1 and Rps7 on LG N was subsequently confirmed by Demirbas *et al* (2001) and Weng *et al* (2001), who utilized SSR markers and compared their linkage group to a consensus linkage group of the soybean (Cregan *et al* 1999a). Burnham *et al* (2003) reported a novel Phytophthora resistant locus, Rps8, in PI399073 from South Korea and mapped the locus on LG A2.

Subsequently, the locus was re-located on LG F based on linkage analysis with SSR and RFLP markers (Gordon *et al* 2006). Among single-dominant Rps genes, Rps1 locus was reported to carry five alleles (Rps1-a, Rps1-b, Rps1-c, Rps1-d, and Rps1-k) and confer resistance against a large number of *P. sojae* races (Schmitthenner *et al* 1994). Thus, many efforts have been made to investigate molecular basis of this locus. By the means of primary trisomic analysis of cytogenetics, Gardner *et al* (2001) was able to locate the locus Rps1-k on chromosome 3, corresponding to LG N of the integrated genetic linkage map of soybean. Bhattacharyya *et al* (1997) and Kasuga *et al* (1997) reported the high-resolution genetic and physical maps of the Rps1-k region and mapped three AFLP markers tightly linked to the locus Rps1-k at a genetic distance less than 0.5 cM. The authors concluded that the Rps1-k was located at the end of an introgressed region. From a disease resistance gene-like sequence, Bhattacharyya *et al* (2005) successfully cloned a family of CC-NBS-LRR-type sequences from the genomic region containing the locus Rps1-k. Among 12 candidate signaling proteins interacting with the resistance gene Rps1-k, Bhattacharyya and Gao (2006) reported that Rps1-k-2 interactor regulates GmMcII, a gene responsible for hypersensitive cell death, which is associated with the expression of Phytophthora resistance. Unlike the extensive studies conducted for single-dominant Rps genes, genetic information of partial resistance to *P. sojae* is limited. Burnham *et al* (2003) developed three RIL mapping populations derived from the crosses of three susceptible parents and a partial resistant cultivar Conrad. The authors reported two QTL associated with *P. sojae* resistance were mapped to LG F and D1b+W of the soybean linkage groups. However, the authors also indicated that genomic locations of the QTL were different from the regions where the known Rps genes were mapped. This will pose a challenge to soybean breeders. Employing single-dominant Rps genes for development of resistant cultivars has proven to be an economical and efficient measure to control this pathogen. However, as in many other host-pathogen systems, the fungal pathogen *P. sojae* has been reported to quickly adapt to specific Rps genes and as the result the host plant becomes vulnerable to a new physiologic race of the pathogen population. This is a great challenge to soybean breeders who have to continue accessing soybean germplasm to mine novel Rps genes for breeding programs. Besides single dominant gene-based breeding to control the disease, partial resistance has been shown to be effective against all races of *P. sojae* (Tooley and Grau 1986; Schmitthenner 1985). Anderson and Buzzell (1992) and Walker and Schmitthenner (1984) investigated resistance to *P. sojae* in several soybean lines and concluded that partial resistance is highly heritable and a quantitative trait. The authors suggested that selection for partial resistance combined with single-dominant Rps genes would provide long-term disease management of *P. sojae* in soybean. Linkage analysis of DNA molecular markers and various Rps genes conditioning resistance to *P. sojae* in soybean can be useful to soybean breeders and pathologists to implement cost-efficient and successful MAS for Phytophthora resistance.

2.3 Yellow mosaic disease

Yellow Mosaic Virus is one of the most destructive and widely distributed plant pathogenic viruses belonging to the family *Geminiviridae*. Two sources of YMV

resistance i.e. cultivated soybean (*G. max*) and wild progenitor (*G. soja*) has been reported. There are several reports on genetic nature of MYMIV resistance genes in *G. max*. It has been reported to be controlled by single recessive gene by Rani *et al* (2017a), double recessive genes by Singh and Mallick (1978), a single dominant gene by Talukdar *et al* (2013) and inhibitory gene action (Khosla 2018). In *G. soja*, it has been reported to be controlled by single dominant gene by Bhattacharya *et al.* (1999) and duplicate dominant nature of YMV resistance (Rani *et al* 2017b). BSA performed by Rani *et al* (2017a) using 144 polymorphic SSR markers, mapped resistant gene from *G max* on chr 6 (LG C2) within a 3.5-cM genome region between two SSR markers GMAC7L and Satt322 whose size was estimated to be 77.115 kb (position of 12,259,594–12,336,709 bp). In interspecific cross, Rani *et al* (2017b) mapped one of the MYMIV resistance genes on chromosome 8 closely linked to SSR marker BARCSOYSSR_08_0867 (15,434,295-15,434,316 bp) and another on chromosome 14 closely linked to two SSR markers BARCSOYSSR_14_1416 (47,686,933-47,686,956 bp) and BARCSOYSSR_14_1417 (47,738,940- 47,738,961 bp). BC₃ F₂ population derived from the same cross segregated in 3 resistant: 1 susceptible ratio and genotyping with linked markers showed that the population inherited resistance gene from chromosome 14 only. Khosla (2018) identified two major QTL, one on D1b LG flanked by markers BARCSOYSSR_02_0423 and BARCSOYSSR_02_0425 and other on C2 LG in the region flanked by markers Satt322 and Sat_076 in intraspecific cross, SL 958 x AGS 456.

2.4 Soybean mosaic virus

Soybean mosaic virus (SMV) is the most prevalent and destructive viral pathogen in soybean worldwide (Hill and Whitham 2014). Seven distinct strains (G1 to G7) in the United States while in China, 21 strains (SC1–SC21) have been classified. The relationship between G strains and SC strains has not been fully established yet. Multiple independent resistance loci with different SMV strain specificities have been identified. So far, three independent loci, *Rsv1*, *Rsv3*, and *Rsv4* in the United States and many *Rsc* loci in China, have been reported for SMV resistance. *Rsv1* was originally identified in the soybean line PI 96983 (Kiihl and Hartwig 1979), and it confers extreme resistance (ER) to SMV-G1 through G6 but not to SMV-G7 (Hajimorad and Hill 2001). Multiple *Rsv1* alleles including *Rsv1-y*, *Rsv1-m*, *Rsv1-t*, *Rsv1-k*, and *Rsv1-r* have been identified from different soybean cultivars with differential reactions to SMV G1–G7 strains (Chen *et al* 2001). *Rsv1* was initially mapped to soybean linkage group F on chromosome 13 (Yu *et al* 1994). Six candidate genes (1eG30, 5gG3, 3gG2, 1eG15, 6gG9, and 1gG4) in PI96983 were mapped to a tightly clustered region near *Rsv1*, three of them (3gG2, 5gG3, and 6gG9) were completely cloned and sequenced (GenBank accession no. AY518517-AY518519). Among the three genes, 3gG2 was found to be a strong candidate for *Rsv1* (Hayes *et al* 2004).

Many *Rsc* loci have been identified. The resistance genes *Rsc-8* and *Rsc-9* have been mapped to the soybean chromosomes 2 (MLG D1b+W) (Wang *et al* 2004). The interval of *Rsc-8* contains 17 putative genes and five of them, Glyma02g13310, 13320, 13400, 13460, and 13470 could be the candidates of *Rsc-8* (Wang *et al* 2011). The *Rsc-15* resistant gene was mapped between Sat_213 and Sat_286 with distances of 8.0 and

6.6 cM to the respective flanking markers on chromosome 6 (Yang and Gai 2011). The resistance gene *Rsc-7* in the soybean cultivar Kefeng No.1 was mapped to a region on soybean chromosome 2 (Fu *et al* 2006) and was subsequently narrowed down to a 158 kilo-base (Kb) region (Yan *et al* 2015). Within 15 candidate genes in the region, one NBS-LRR type gene (Glyma02g13600), one HSP40 gene (Glyma02g13520) and one serine carboxypeptidase-type gene (Glyma02g13620) could be the candidates for *Rsc-7*.

Soybean line PI486355 displays broad spectrum resistance to various strains of SMV and two independently inherited SMV resistant genes have been identified in PI486355 (Ma *et al* (1995). Cultivar Columbia is resistant to all known SMV strains G1-G7, except G4. Results from allelism tests demonstrate that two genes independent of the *Rsv1* locus are present in Columbia, with one allelic to *Rsv3* and the other allelic to none of the known *Rsv* genes (Ma *et al* 2002). Plants carrying both genes were completely resistant to both G1 and G7, indicating that the two genes interact in a complementary fashion (Ma *et al.*, 2002). Tousan 140 and Hourei, two soybean accessions from Japan, and J05, a accession from China, carry both *Rsv1* and *Rsv3* alleles and are resistant to SMV-G1 through G7 (Shi *et al* 2011). These results indicate that integration of more than one *Rsv* genes into one cultivar can confers a broader spectrum of resistance against SMV.

Table 1. Major Diseases of National and Regional Importance of soybean in India.

S. No.	Disease	Pathogen	Scouting	Yield loss (%)	Distribution
1.	Rust	<i>(Phakopsora pachyrhizi</i> H. Sydow & Sydow)	Flowering to pod filling stage	30-100	Maharashtra, Karnataka, M.P, NE states, Kerala, Rajasthan, A.P. and Tamil Nadu (regularly occurring in Maharashtra, Karnataka, and NEH region)
2.	Yellow mosaic	<i>Mungbean yellow mosaic virus</i> Nariani	Early vegetative stage to physiological maturity stage	15-75	Uttarakhand, M.P., U. P., Delhi, Rajasthan, Haryana, Punjab, Himachal Pradesh, Karnataka and NE states
3.	Soybean mosaic	<i>Soybean mosaic virus</i>	Late vegetative stage	25-50	Uttarakhand, M.P., Rajasthan, U.P., Karnataka, Delhi, A.P., Maharashtra, Haryana, Punjab, Himachal Pradesh, Jharkhand and NE states
4.	Collar rot or Sclerotium blight	<i>Sclerotium rolfsii</i> Curzi	1-4 week after planting & mid to late flowering	30-40	Delhi, Uttarakhand, M.P., Maharashtra, Rajasthan, Karnataka and NE states

S. No.	Disease	Pathogen	Scouting	Yield loss (%)	Distribution
5.	Charcoal rot	<i>Macrophomina phaseolina</i> (<i>Rhizoctonia bataticola</i>) (Tassi) Goid	Mid to late flowering	77	Uttarakhand, M.P., Delhi, Rajasthan, Maharashtra, Karnataka and NE states
6.	Rhizoctonia root rot and aerial blight	<i>Rhizoctonia solani</i> (<i>Thanatephorus cucumeris</i>) Julius Kuhn	1-4 week after planting & mid flowering to pod formation following rain.	35	Uttarakhand, M.P, Delhi, Maharashtra, Jharkhand, Chattisgarh and NE states
7.	Bacterial pustule	<i>Xanthomonas campestris</i> pv.glycines Pammel	35 days after sowing to throughout the season	20	Uttarakhand, M.P., Rajasthan, Karnataka, A.P., Maharashtra, Himachal Pradesh, Jharkhand and NE states
8.	Anthracnose	<i>Colletotrichum dematium</i> f. sp. truncatum (Pers.) <i>Colletotrichum truncatum</i> (Schwein.) Andrus & W.D. Moore	All season	16-25	Uttarakhand, M.P., Rajasthan, Karnataka, A.P., Maharashtra, Himachal Pradesh, Tamil Nadu, Kerala, NEH states, Delhi and Chattisgarh
9.	Myrothecium leaf spot	<i>Myrothecium leaf spot</i> <i>Myrothecium roridum</i> Tode	Flowering to pod filling stage	20-40	M.P., Rajasthan, Karnataka, A.P., Maharashtra and Uttarakhand
10.	Alternaria leaf spot	<i>Alternaria alternata</i> (Fr.) Keiss & A. <i>tenuissima</i> (Kunze) Wiltshire	Post flowering stage	15	Uttarakhand, M.P, Karnataka, Delhi and NE states
11.	Indian bud blight	<i>A strain of Groundnut bud necrosis virus</i>	Late vegetative to pod filling stage	22	M.P., Rajasthan, Delhi, Karnataka, Maharashtra and Chattisgarh
12.	Purple seed stain	<i>Cercospora kikuchii</i> T. Matsumoto & Tomoy	Beginning of seed set to throughout	15-30	M.P, Maharashtra, Delhi, Karnataka and NE states
13.	Brown spot	<i>Septoria glycines</i>	Mid & late flowering to seed set	8-15	Uttarakhand, M.P., Rajasthan, Maharashtra, Himachal Pradesh and NE states

S. No.	Disease	Pathogen	Scouting	Yield loss (%)	Distribution
14.	Frog eye leaf spot	<i>Cercospora sojina</i> K.Hara	Flowering to seed set	22	Himachal Pradesh, M.P., Karnataka, Uttarakhand, U.P., Rajasthan and NE states
15	Target leaf spot	<i>Corynespora cassiicola</i> (Berk. & M.A. Curtis) C.T.	. Mid & late flowering to seed set	18-32	Himachal Pradesh, Uttarakhand, Chhatisgarh and NE states
16.	No-podding/ Phyllody/Bud proliferation	Etiology not known	Flowering to pod set	20-30	M.P., Karnataka, Himachal Pradesh and Maharashtra
17.	Fusarium seedling /collar rot	<i>Fusarium equiseti</i> Corda	All season	64	Delhi and M.P.
18.	Nematode	Rotylenchulus reniformis Linford and Oliveira, Meloidogyne spp. Goldiand Pratylenchus spp.	Pre sowing/ soil samples	6-17	M.P., Rajasthan and Maharashtra

3. Abiotic Stresses

Major abiotic stresses affecting productivity of crop plants are drought, heat, water logging and salinity. It was reported that the average yield losses due to abiotic stresses are more than 50% for major crops (Boyer 1982). Crop improvement with traits that confer tolerance to abiotic stresses is commonly practiced using traditional and modern breeding, as well as through biotechnology. Global soybean production and crop quality are severely affected by various environmental stresses. Drought and salinity are most devastating stresses in most of soybean growing regions of the world. To develop soybean plants with enhanced tolerance to stress, a basic understanding of the physiological, biochemical and gene regulatory networks is essential. Various genomic and functional genomics tools have helped to advance our understanding of stress signal perception and transduction and the associated molecular regulatory network. These tools revealed several stress-inducible genes and various transcription factors that regulate the stress inducible systems. This section highlights the physiological factors associated with stress tolerance and related genetic information.

3.1 Water stress

3.1.1 Drought

Water stress including drought and flooding is considered as a major threat, limiting growth and yield of plants [Boyer 1983a; Thomas *et al* 2004]. Due to drought and

flooding stress, 40–60% yield losses have been reported in soybean (Ahmed *et al* 2013; Valliyodan and Nguyen 2006). Crop loss depends on plant growth stages and duration of drought. Drought stress during flowering and early pod development stages significantly increases the rate of flower and pod abortion, ultimately decreasing grain yield (Boyer 1983a; Westgate and Paterson, 1993; Liu *et al.*, 2004). Drought stress affects germination rate and early seedling growth of the plant (Kaya *et al* 2006; Okçu *et al* 2005). Under water deficit conditions, a significant reduction in germination, hypocotyl length, root and shoot fresh and dry weight were observed whereas the root length is increased (Zeid and Shedeed 2006). It also affects the carbon assimilation and phenology of the plant (Hirt and Shinozaki 2004). Prolonged drought stress at different growth stages has profound effect on soybean growth and yield [Boyer 1983b]. To counteract the adverse effects of drought, the soybean plant adopts three mechanisms i.e. escape, tolerance, and avoidance (Turner *et al* 2001). In the escape mechanism, the plant completes its life cycle before the onset of drought. Normally, the plants complete their life cycle very quickly and produce few seeds. For instance, early planting of soybean helps to avoid drought, and is largely practiced in the USA—planting in March to April affords escape from water stress (Boerma and Specht 2004; Purcell and Specht 2004). Drought avoidance is performed by maintaining high water potential, grow deeper in soil, leaf rolling, reducing water loss through stomatal closure and thick leaf cuticles i.e. stomatal control of transpiration rate and by reduction of water loss from tissues. The tolerance mechanism includes low tissue water potential, maintenance of turgor through osmotic adjustments [Kavar *et al* 2008, Nguyen *et al* 1997]. Approaches to breed for drought tolerance in soybeans have involved several mechanisms and include slow canopy wilting, prolific rooting, sustained nitrogen fixation, and selecting for higher yield under drought conditions.

3.1.2 Characters associated with water stress

Root-related traits are crucial in maintaining crop yield soybean under drought. Root depth, root angle ($>40^\circ$) and root branching density) are important root characters causing drought tolerance (Fenta *et al* 2014). Root-to-shoot ratio is also a good indicator for this character. On the other hand waterlogging stimulated the formation of aerenchyma and adventitious roots in soybean plants. Rapid shoot elongation is another escape mechanism for adaptation in waterlogging stress (Phukan *et al* 2014).

Water stress-responsive mechanisms have been studied at the physiological and biochemical level in soybean under drought and flooding stress. Several studies have provided strong evidence that drought-tolerant soybean genotypes exhibited a higher reduction in stomatal conductance than the susceptible one (Hossain *et al* 2014). In soybean, ABA is involved in the reduction of stomatal conductance and photosynthesis. Maintenance of cell turgidity and water-use efficiency are important indicators to cope with drought stress (Hossain *et al* 2015). Soybean introduction line PI 416937 is an excellent example of drought tolerance by limiting transpiration rate and maintaining a lower osmotic potential.

3.1.3 Screening techniques for drought tolerance

The yield reduction under drought conditions as compared to irrigated conditions is a field technique used for screening against drought. Terminal drought stress induced by spray of 0.2% potassium iodide (KI) at seed filling stage have been used for drought screening by many workers. Since field techniques are laborious and require full season for screening so laboratory techniques are useful for initial screening. Incorporation of polyethylene glycol (PEG) in nutrient medium is such technique. Screening for drought tolerance under hydroponics conditions is very efficient and cost effective technique to screen for drought. Recently this technique has been standardized at IARI, New Delhi. The technique involves initial acclimatization period of 4 days followed by exposure of roots to air for 4 hr 30 mnts for 6 consecutive days and final acclimatization of 2 days was successful to differentiate between tolerant and susceptible genotypes.

Table 2. A list of reported QTLs in soybean associated with drought tolerance.

Trait	QTL	Chr o.	Marker	Population	Ref.
Grain yield	Gm18	18	Sat_064	Archer×Minsoy, Archer × Noir I	Van Toai <i>et al</i> (2001)
Injury score, tolerance index	Gm5, Gm13	5, 13	Satt385, Satt269	A5403 × Archer, P9641 × Archer	Cornelious <i>et al</i> (2005)
Flooding tolerance	ft1	6	Satt100	Misuzudaizu × Gong 503	Githiri <i>et al</i> (2006)
Seed germination	Sft1, Sft2	12, 8	Sat_175, Satt 187	Peking × Tamahomare	Sayama <i>et al</i> (2009)
	Sft3, Sft4	4, 2	Satt 338, Sat_279		
Flooding tolerance and/or resistance to P. sojae	FTS-13	13	Sct_033, BARC024569-4982	PI 408105A × S99-2281	Nguyen <i>et al</i> (2012)
	FTS-11	11	BARC-016279-02316		
Joint waterlogging tolerance index	Wt1, wt 2	19	Satt229- Satt527 Satt527- Sat_286	Su88-M21 × Xinyixiaoheidou	Sun <i>et al</i> (2010)
Root length development/ Root surface area	Qhti-12-1	12	Satt052- Satt302	Iyodaizu × Tachinagaha	Van Nguyen <i>et al</i> (2017)

3.1.4 Genetic stocks for drought tolerance

Brazilian cultivars BR-4 and Ocepar 4 were considered as drought-tolerant. Several cultivated germplasm lines (*Glycine max*) including Williams, Jackson, Prima 2000, Jindou 21(C12), PI416937, PI 427136, PI 408105A, PI 471938, PI 424088, PI 081041, N04-9646, DT51, R02-1325, PI 561271, PI 567343, PI 407184, PI603910C, PI

567394B, PI 567651, Archer and Misuzudaiz have promising performance under water deficit conditions and can be used in breeding program (Hossain *et al* 2014; Fenta *et al* 2014; Valliyodan *et al* 2016). The drought tolerant lines PI 416937 and PI471938 are being extensively utilized to develop slow wilting varieties in USA. Wild soybean (*Glycine soja*), is a valuable genetic resource for the tolerance to water stress by reintroducing alleles. Wild soybean PI 483463 (*G. soja*) had favourable donor alleles for root angle, while PI 468917 predicted to contribute to slow wilting. The wild parent, PI 407162 had favourable alleles for fibrous roots, thus enhancing the soybean ability to survive under drought stress (Table 2). Other wild soybean accessions, PI 479751, PI 407229, PI 597459C, PI 424082, PI 378699A, PI 424107A, PI 366124, PI 378699A were also identified, which showed tremendous waterlogging tolerance than *G. max* (Valliyodan *et al* 2016).

3.1.5 Flooding

Flooding due to excess soil water is the second most damaging constraint on crop growth, after drought and affects about 16% of the production areas worldwide (Boyer 1982). Flooding causes premature senescence that results in leaf chlorosis, necrosis, defoliation, cessation of growth and reduced yield (Van Toai *et al* 1994). Waterlogging can reduce soybean yield 17–43% at the vegetative growth stage and 50–56% at the reproductive stage (Oosterhuis *et al* 1990). Yield losses are the result of reduced root and shoot growth, nodulation, nitrogen fixation, photosynthesis, biomass accumulation, stomatal conductance, and plant death due to diseases and physiological stress (Scott *et al* 1989; Oosterhuis *et al* 1990).

Flooding can be divided into either waterlogging, where only the roots are flooded or complete submergence where the entire plants are under water. Waterlogging is more common than complete submergence stress and is also less damaging. While soybeans die within one or two days of complete submergence (Sullivan *et al* 2001), plants develop adaptive mechanisms that allow them to survive long term waterlogging (Bacanamwo and Purcell 1999). Soybean responses to waterlogging can be divided into two phases such as the “reactive” phase, which occurs immediately after the stress and is followed by the second “acclimatized” phase. The duration of the reactive phase was 2–4 days shorter in the flood-tolerant genotypes than flood-susceptible genotypes, indicating that flood-tolerant genotypes acclimatized more quickly to flooding stress than the flood-susceptible genotypes (VanToai *et al* 2003).

During the last two decades, more information has accumulated from research on the molecular, biochemical and physiological responses of plants to the lack of oxygen than to flooding (Ricard *et al* 1994; Ismond *et al* 2003). Various reports on waterlogging tolerance in crop plants concluded that this trait is quantitatively inherited (Sripongpankul *et al* 2000; Setter and Waters 2003; Boru *et al* 2003). QTL for waterlogging tolerance associated with improved soybean growth (18%) and grain yields (180%) in waterlogged environments were identified (VanToai *et al* 2001).

3.2 Salt tolerance

Salt stress is one of the abiotic stresses that significantly reduce the yield of soybean. Over 25% of the world’s potential arable land is currently contaminated by salt.

Soybean is considered a salt-sensitive crop, with all developmental stages adversely affected by salinity stress (Phang et al., 2008). High salt levels generate a two-component stress on plants: an osmotic stress caused by reduced water availability in soil and an ionic stress due to imbalance of solutes in the cytosol (Blumwald et al., 2000; Conde et al., 2011). During soybean development, salt stress significantly reduces plant height and leaf size (Wang et al., 2001; Essa, 2002), decreases the number of internodes and pods (Phang et al., 2008), decreases protein content and seed quality and causes a reduction in chlorophyll content (Lu et al., 2009). Salt stress also significantly affects seed germination, seedling growth, biomass, and seed yield (Abel and MacKenzie, 1964; Katerji et al., 1998; Wang and Shannon, 1999; Essa, 2002). The increase in salt content in soil could cause a decrease of up to 40% in soybean yield (Chang et al., 1994). Development of salt tolerant cultivars will help reduce the detrimental loss of yield in soybean production in the areas with elevated salt levels.

3.2.1 Mechanism of salt tolerance

Soybean plants have developed several mechanisms to cope with salt stress conferring a wide spectrum of salt tolerance among genotypes (Phang et al., 2008). Mechanisms of salt tolerance include maintaining ion homeostasis by withholding toxic ions from sensitive aerial parts, adjusting osmotic potential in cells by accumulating metabolites and restoring oxidative balance to prevent further damage due to excess accumulation of reactive oxygen species (ROS) (Phang et al., 2008).

3.2.2 Screen techniques for Salt Tolerance in Soybean

Appropriate screening techniques are important for successful for screening soybean cultivars with tolerance to salt stress. Three screening techniques have been reported in evaluating soybean genotypes for salt tolerance. Planting in fields with high salt content has been used for evaluation of genotypes for salt tolerance (Parker *et al* 1983; Yang and Blanchar 1993). The most commonly used screening method to evaluate soybean genotypes for salt tolerance has been hydroponics (Pantalone *et al* 1997; An *et al* 2001; Lee *et al* 2004). In the hydroponics method, seedlings of different genotypes are grown in a nutrient solution and NaCl is added to the solution when plants are at the V2 to V4 growth stage. Lee *et al* (2008) proposed a method of exposing V2 to V3 plants growing in a sandy soil in plastic cone-tainers having 100 mM salt solution. After symptoms of leaf scorch showing salt injury are clearly visible, genotypes are rated for tolerance.

3.2.3 Genetic diversity of salt tolerance in soybean

Multiple soybean germplasms including cultivated and wild species have been used to test salt tolerance, and rich genetic variation conferring salt tolerance has been observed in soybean (Table 3).

Table 3: Major salt tolerance germplasm accessions in soybean

Number	Accession name	Type	Phenotype	Reference
1	S-100	<i>Glycine max</i>	Tolerant	Lee <i>et al</i> (2004)
2	Lee	<i>Glycine max</i>	Tolerant	Lee <i>et al</i> (2004)
3	Lee 68	<i>Glycine max</i>	Tolerant	Chen <i>et al</i> (2008)
4	Lee 74	<i>Glycine max</i>	Tolerant	Lee <i>et al</i> (2004)
5	Fengzitianandou	<i>Glycine max</i>	Tolerant	Zhang <i>et al</i> (2014)
6	Baiqiu 1	<i>Glycine max</i>	Tolerant	Zhang <i>et al</i> (2014)
7	FT-Abyara	<i>Glycine max</i>	Tolerant	Xu <i>et al</i> (2016)
8	Jindou 6	<i>Glycine max</i>	Tolerant	Xu <i>et al</i> (2016)
9	Tiefeng 8	<i>Glycine max</i>	Tolerant	Guan <i>et al</i> (2014a)
10	Fiskeby III	<i>Glycine max</i>	Tolerant	Chen <i>et al</i> (2018)
11	PI 468908	<i>Glycine max</i>	Tolerant	Chen <i>et al</i> (2018)
12	PI 561389B	<i>Glycine max</i>	Tolerant	Chen <i>et al</i> (2018)
13	PI 479752	<i>Glycine max</i>	Tolerant	Chen <i>et al</i> (2018)
14	JWS156-1	<i>Glycine soja</i>	Tolerant	Hamwieh and Xu (2008)
15	PI483463	<i>Glycine soja</i>	Tolerant	Lee <i>et al.</i> (2009); Ha <i>et al.</i> (2013)
16	W05	<i>Glycine soja</i>	Tolerant	Qi <i>et al.</i> (2014)
17	PI424107A	<i>Glycine soja</i>	Tolerant	Chen <i>et al</i> (2018)
18	PI597459C	<i>Glycine soja</i>	Tolerant	Chen <i>et al</i> (2018)

3.2.4 Genetics of salt tolerance

Salt tolerance is a heritable quantitative trait, but the phenotypes may be dominated by a few major loci. Results of Abel (1969) suggested that Cl⁻ accumulation in the aerial part of soybean is controlled by a single locus with exclusion being dominant (Ncl) and inclusion being recessive (ncl). Since the occurrence of salt-induced necrosis was associated with high Cl⁻ content in the aerial part, it was implied that Ncl is a major locus determining the salt tolerance capability of soybean. In a separate experiment, a cross was carried out between soybean cultivars with contrasting salt tolerance capability (Shao *et al.* 1994a). The mode of inheritance of salt induced necrosis on leaves (salt sensitivity) followed Mendelian genetics and was controlled by

a major locus with a non-necrotic (salt-tolerant) allele as dominant and a necrotic (salt-sensitive) allele as recessive. The inheritance of salt tolerance in wild soybean was studied using the accession PI483463. The F₂ plants from the cross of PI483463 (R) × Hutcheson (S) segregated into a tolerant to sensitive ratio of 3:1. The F₂:3 lines segregated into a tolerant to segregating to sensitive ratio of 1:2:1. The F₂ plants from the cross of PI483463 (R) × S-100 (R) segregated into a tolerant to sensitive ratio of 15:1, indicating different genes from the two sources. Their results showed that *Glycine soja* line PI483463 had a single dominant gene for salt tolerance, which was different from the gene in *G. max* line S-100 (Lee et al. 2009). Guan *et al* (2014a) also reported single dominant gene for salt tolerance in soybean cultivar Tiefeng 8. However, Chen *et al* (2011) showed that salt tolerance was controlled by three major genes in soybean. It is well known that salt tolerance is a quantitative trait controlled by several genes in soybean which is confirmed by the QTL mapping and GWAS results described below.

The efficiency of traditional breeding is low because of the long breeding cycle, incompatible fertilization, and close linkage between abiotic stress tolerance loci and undesirable characters (e.g. low yield) (Wang et al. 2003). Understanding the mechanisms and identifying the genes involved in salt stress tolerance of soybeans will enable breeders to develop new strategies to enhance salt tolerance. Quantitative trait loci (QTL) analysis was carried out using 106 F₂-derived lines (F_{2:5}) which were originated from a cross between “S-100” (salt-tolerant) and “Tokyo” (salt-sensitive) (Lee et al. 2004). The scoring of salt tolerance phenotype was based on the degree of salt-induced chlorosis in leaves. A major QTL for salt tolerance was found on the linkage group LGN (between simple sequence repeats (SSR) markers Sat_091 and Satt237) and accounted for 41%, 60% and 79% of the total genetic variation for salt tolerance in the field, greenhouse, and combined environments, respectively (Lee et al. 2004). Tracking of this QTL in the descendent USA cultivars revealed that “S100” is the source of the salt tolerance allele in the cultivar “Lee” (the Cl- excluder in Abel’s studies described above). It is believed that this QTL is the Ncl locus (Lee et al. 2004). However, there is also a report suggesting that salt tolerance (scoring by the degree of salt-induced chlorosis in leaves) in soybean is controlled by minor genes (Luo et al. 2004). The discrepancy among different studies is probably due to differences in genetic background of parental germplasms and the variation of scoring parameters.

Hamwieh and Xu (2008) used an F₂ population derived from a cross between Jackson (sensitive cultivar) and JWS156-1 (wild soybean accession) to detect salt tolerance QTL in hydroponic culture conditions. Results confirmed a major QTL associated with salt tolerance with a large dominant effect located on the soybean linkage group N, which accounted for 68.7% of the total phenotypic variance. The major salt tolerance QTL was further validated by three sets of salt-tolerant near-isogenic lines (NILs). Salt tolerance during seed germination is a critical determinant of stable stand establishment under salt stress conditions. Kan *et al* (2016) identified 11 salt tolerance QTLs during the germination stage; these QTLs were located on six chromosomes for the three salt tolerance indices. These QTLs explained 4.49 to 25.94% of the phenotypic variation.

The major salt-tolerance QTL located on chr. 03 (linkage group N) was identified by several research groups. Further attempts were made to clone this major salt tolerance QTL to map salt tolerance QTL in soybean. Guan et al. (2014a) mapped this QTL between the two markers QS1101 and QS100011 and named it GmSALT3. Fine mapping of GmSALT3 was conducted using a new population derived from F₅ heterozygous lines between InDel markers QS1101 and QS100011. GmSALT3 was mapped within a 17.5-kb region that had only one predicted gene: Glyma03g32900. Therefore, Glyma03g32900 was regarded as the candidate gene underlying the salt tolerance QTL GmSALT3 (Guan et al. 2014b). Qi et al (2014) independently identified Glyma03g32900 as a potential candidate gene of salt tolerance. Recent studies have indicated that the dominant gene Ncl described by Abel (1969) is the salt tolerance locus Glyma03g32900. To date, the salt tolerance gene Glyma03g32900 has been given three names (GmSALT3, GmCHX1, and Ncl). Genome-wide association studies (GWAS) were used to detect the associated single nucleotide polymorphism (SNP) markers for salt tolerance in soybean. Most of the SNPs significantly associated with salt tolerance were located within or near the major salt tolerance gene Glyma03g32900 on chr. 03 (Chen *et al* 2018).

3.3 Temperature Stress

Soybean plant growth, development, seed composition and quality of soybean are dependent on many factors including genetic background, growing season, geographical location and environmental stresses (Liu et al. 1995; Piper and Boote 1999). It is known that temperature variation has a significant effect on reserve mobilization and partitioning during seed development, but the molecular and regulatory mechanisms are not well understood. Several studies showed a difference in seed protein and oil composition due to varied temperature regimes from 15/12 to 40/30°C (Carver et al. 1986; Dornbos and Mullen 1992; Rebetzke et al. 1996). In addition to changes in the seed oil concentration, the ratio of fatty acids in soybean oil changes when seeds develop under elevated temperature (Thomas et al. 2003). In soybeans, oleic acid content is most affected by night temperatures rather than day temperatures. As one of the major environmental factors, temperature has a direct role on the protein and oil accumulation during seed development. Wolf et al. (1982) observed the drastic reduction of linolenic acid and stachyose content and increased total oil, methionine and protein content when soybean plants were grown under warm temperature 33/28°C (day/night). In contrast, a negative correlation between oil and protein was reported (Hymowitz et al. 1972; Burton 1987; Watanabe and Nagasawa 1990) where oil concentration tends to decrease as the protein concentration increases. This response was attributed to both environmental and genotypic variations (Watanabe and Nagasawa 1990).

Recent simulation studies showed that in a future warmer climate with increased mean temperatures, heatwaves would become more intense, longer lasting, and/or more frequent (Karland Trenberth 2003; Meehl and Tebaldi 2004) and this certainly will reduce soybean production. Soybean yield reductions of 27% were reported when the plants were exposed to 35°C for 10 hr during the day (Gibson and Mullen 1996). Diers et al. (1992) mapped two major QTL controlling protein and oil concentration with

RFLP markers in a population of F₂-derived lines developed by crossing a *G. max* experimental line and a *G. soja* plant introduction. The QTL mapped were then labeled LG A and K of the soybean map. Chung et al. (2003) reported the mapping of a protein QTL allele to LG I from PI 437088A, a *G. max* accession with a high protein concentration. This protein QTL is designated cqPRO-003 and the oil QTL cqOIL-004 under the category of confirmed QTL at the Soybase website. All these results show that seed component traits can be successfully modified through genetic mapping coupled with marker assisted selection.

Future perspectives

Tremendous progress has been made to identify resistant sources against different biotic and abiotic stresses in soybean. Recent biotechnological techniques have played great role to identify stress resistant genes and study their functions. Next step is introduction of tolerance genes into elite cultivars by molecular marker assisted genomic selection and transgenic technologies. The precise genome modification by the CRISPR/Cas9 System avoids backcrossing, leading to a shorter breeding cycle than conventional breeding methods. In the future, a highly efficient transformation system for soybean will enable CRISPR/Cas9 to be used directly for improving biotic and abiotic stress tolerance in elite soybean varieties.

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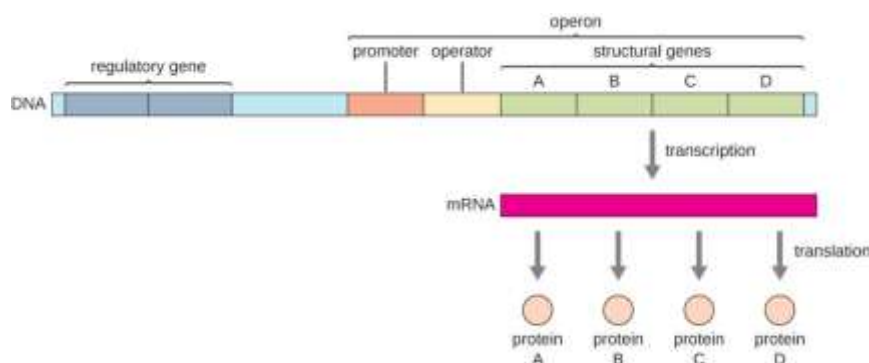
IN SILICO GENE IDENTIFICATION STRATEGIES

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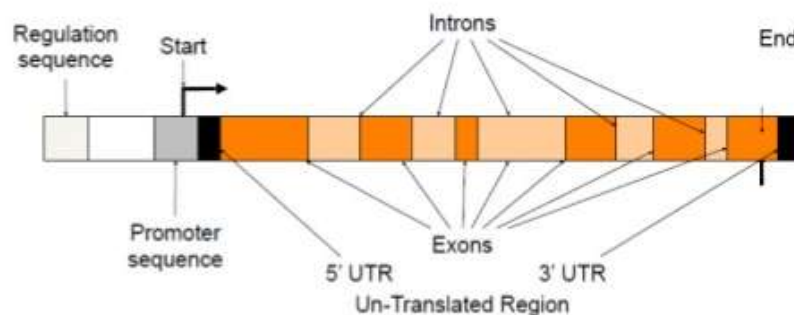
Gene prediction or gene finding refers to the process of identifying the regions of genomic DNA that encode genes. This includes protein-coding genes as well as RNA genes, but may also include prediction of other functional elements such as regulatory regions. Gene finding is one of the first and most important steps in understanding the genome of a species once it has been sequenced.

Prokaryotic and Eukaryotic gene structures

In prokaryotic genomes, DNA sequences that encode proteins are transcribed into mRNA, and then RNA is usually translated directly into proteins without significant modification. The longest ORFs running from the first available Met codon on the mRNA to the next stop codon in the same reading frame generally provide a good, but not assured, prediction of the protein-encoding regions. A reading frame of a genomic sequence that does not encode a protein will have short ORFs due to the presence of many inframe stop codons.



In eukaryotic organisms, transcription of protein-encoding regions initiated at specific promoter sequences is followed by removal of noncoding sequence (introns) from pre-mRNA by a splicing mechanism, leaving the protein-coding exons. As a result of the presence of intron sequences in the genomic DNA sequences of eukaryotes, the ORF corresponding to an encoding gene will be interrupted by the presence of introns that usually generate stop codons.



Two approaches can be used for gene prediction

1. *ab initio* methods
2. Homology based methods

***ab-initio* (Intrinsic):** Predicts genes using only the genomic DNA sequence. It searches for signals and content (specific sequences, codon usage, GC content) of protein coding regions and statistical properties of the given DNA sequence. Example: GeneMarkS, Prodigal, Glimmer.

Homology Based (Extrinsic): The given genome sequence is compared with an extrinsic genome (reference genome datasets) to find coding regions in the given genome.

Tools for Gene Prediction

Prokaryotes

A) ORFfinder: ORFfinder : ORF finder searches for open reading frames (ORFs) in the DNA sequence. The program returns the range of each ORF, along with its protein translation.

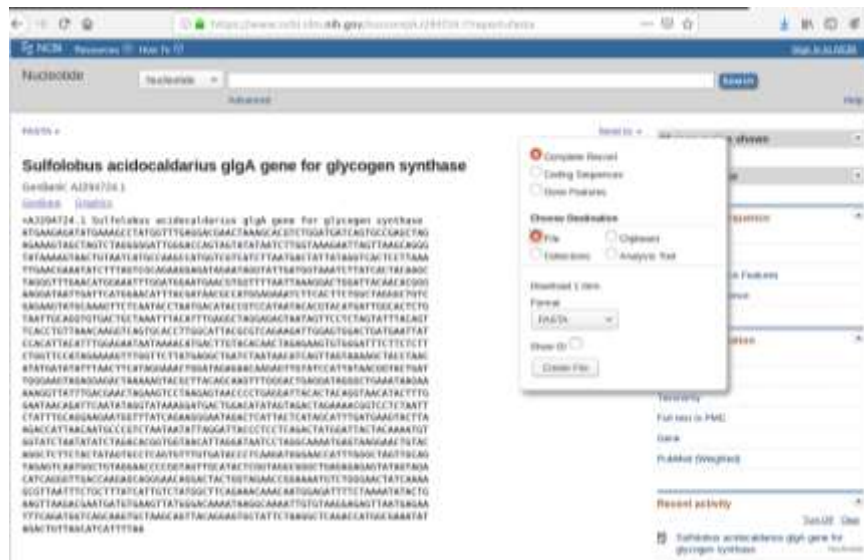
1. Download a nucleotide sequence of accession id **AJ294724.1** It is a *Sulfolobus acidocaldarius* glgA gene for glycogen synthase from NCBI <https://www.ncbi.nlm.nih.gov/nuccore/AJ294724.1>



- 2. Save the sequence in fasta format by clicking the fasta tab and following the instructions**



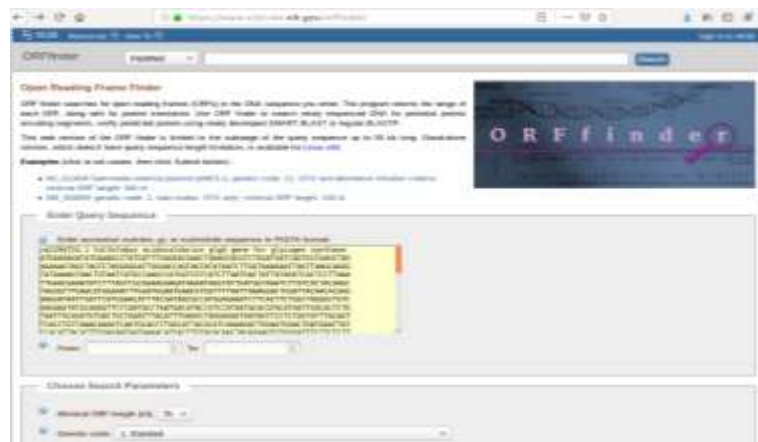
3. Save a sequence in fasta format.



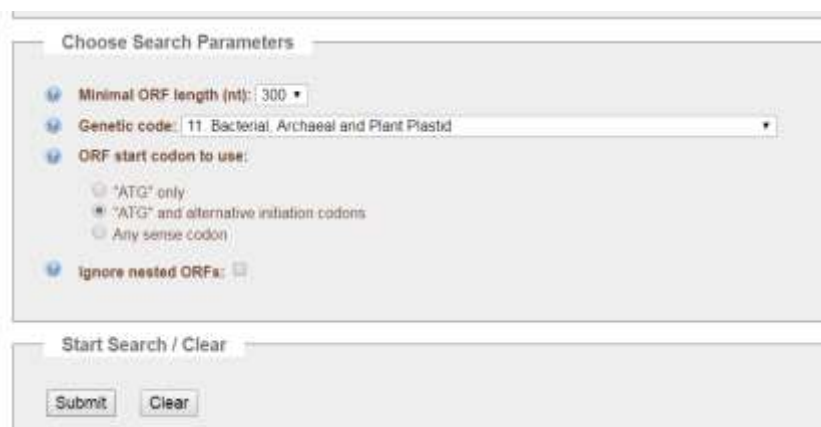
4. Open ORF finder home page by clicking/typing the link

<https://www.ncbi.nlm.nih.gov/orffinder/>

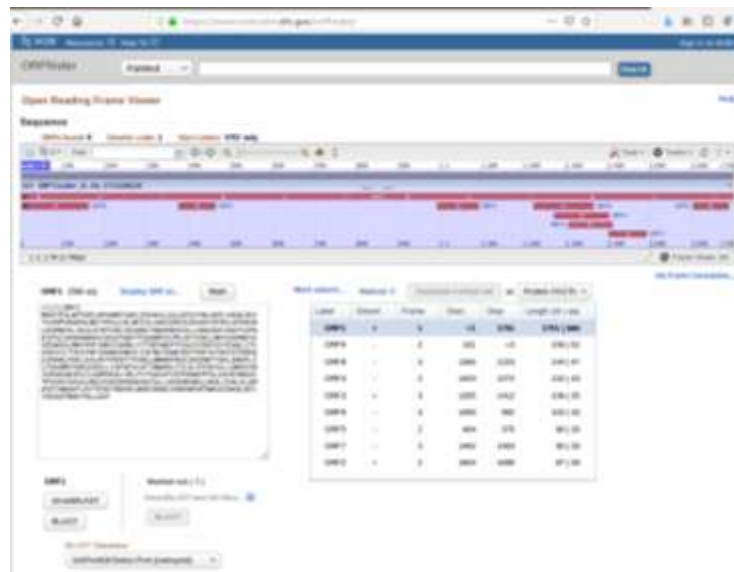
5. Enter accession number, gi, or nucleotide sequence in FASTA format in the Enter Query Sequence box.



6. Click the Submit Button



7. Results show the strznd, Frame, start, end and length of the ORF.



B). Exprasy Translate : Translate is a tool which allows the translation of a nucleotide (DNA/RNA) sequence to a protein sequence.

1. Open <https://web.expasy.org/translate/>



2. Paste DNA or RNA Sequence in the search box.



3 Press Translate button.



4. ORF are shown in red color. You have to select the longest open reading frame coding for the gene.



C) **GeneMarkS** : a self-training method for prediction of gene starts in microbial genomes.

1. Open the home page of the GeneMarkS

<http://exon.gatech.edu/GeneMark/genemarks.cgi>

2. Enter or upload the FASTA sequences in the Input sequence box.

Input sequence

Enter sequence (FASTA or multi FASTA format)

```
>AJ294724.1 Sulfolobus acidocaldarius glgA gene for glycogen synthase
ATGAAGAGATATGAAAGCCTATGTTTGGAGACGAACTAAAGCACGTCTGGATGATCAGTGCCGAGCTAG
AGAAAGTAGCTAGTCTAGGGGGATTGGGACCAGTAGTATATAATCTTGGTAAAGAATTAGTTAAGCAGGG
TATAAAGTAACTGTATCATGCAAGCCATGGTCGTATCTTAATGACTATTATAGGTCACCTCTTAAA
TTGAACGAAATATCTTTAGTCGCAGAGGAGATAGAATAGGTATTGATGGTAAATCTTATCACTACAAGC
TAGGGTTTGAACATGGAAATTTGGATGGAATGAACGTGGTTTAAATTAAGGACTGGATTACAACACGGC
```

or, upload file:

Browse...

No file selected.

3. Select from the output options

Options			
Sequence type	Output format for gene prediction	Output options	Optional: results by E-mail
<input checked="" type="radio"/> Prokaryotic <input type="radio"/> Intronless eukaryotic <input type="radio"/> Virus <input type="radio"/> Phage <input type="radio"/> EST/cDNA	<input checked="" type="radio"/> LST <input type="radio"/> GFF	<input checked="" type="checkbox"/> Protein sequence <input checked="" type="checkbox"/> Gene nucleotide sequence <input type="checkbox"/> Coding potential graph (not for multi FASTA) <input checked="" type="checkbox"/> PDF <input checked="" type="checkbox"/> PostScript	E-mail <input type="text"/> Subject <input type="text"/> GeneMarkS <input type="checkbox"/> Compress files
Advanced options			
<input checked="" type="radio"/> genetic code 11 <input type="radio"/> "TGA" codon as a Tryptophan (not as a stop codon), genetic code 4 <input type="radio"/> "TGA" codon as a Glycine (not as a stop codon), genetic code 25 <input type="checkbox"/> Switch off search for gene start related motif(s)			
Contact Us Home			

4. Press start GeneMarkS.

Action	Start GeneMarkS	Reset
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5. Prediction results.

GeneMarkS	
John Besemer, Alexandre Lomsadze and Mark Borodovsky GeneMarkS: a self-training method for prediction of gene starts in microbial genomes. Implications for finding sequence motifs in regulatory regions. <i>Nucleic Acids Research</i> (2001) 29, pp 2607-2618	
This webpage provides accesses to version 4.28 of gene prediction program GeneMarkS. This version combines the original 2001 prokaryotic GeneMarkS with later development, which extended the unsupervised gene prediction to intron-less eukaryotes, eukaryotic viruses, phages and EST/cDNA sequences.	
Browse GeneMarkS manual	
GeneMarkS is running job ID = genemarks.20190819.062632.2000 ; Estimated run time: 1 second(s)	
Gene Prediction Results	
output	link
Coordinates of predicted genes	gms.out
PostScript	gms.out.ps
PDF	gms.out.pdf
Protein sequences	gms.out.faa
Nucleotide sequence	gms.out.fnn
Contact Us Home	

6. Open the links to view the final output.

a Coordinates of predicted genes link shows

```
GeneMark.hmm PROKARYOTIC (Version 3.26)
Date: Mon Aug 19 06:26:32 2019
Sequence file name: seq.fna
Model file name: GeneMark_hmm_heuristic.mod
RBS: false
Model information: Heuristic_model_for_genetic_code_11_and_GC_39

FASTA definition line: AJ294724.1 Sulfolobus acidocaldarius glgA gene for glycogen synthase
Predicted genes


| Gene # | Strand | LeftEnd | RightEnd | Gene Length | Class |
|--------|--------|---------|----------|-------------|-------|
| 1      | +      | 1       | 1701     | 1701        | 1     |


```

b. Post script



c. Protein Sequences

```
< > ↺ ↻ ⌂ ⓘ exon.gatech.edu/GeneMark/tmp/genemarks.20190819.062632.2000.gms.out.f [
>gene_1|GeneMark.hmm|566_aa|+|1|1701 >AJ294724.1 Sulfolobus acidocaldarius glgA gene for glycogen synthase
MKRYE$LWFEDELKHVMMISAELEKVASLGGGLPVVYNLKGELVKQGIKVTVMPSHGRH
LNDYYRSLKLLNEISLVAEGDRIGIDGKSYHYKLGFEGHNLDMNVVLKGLDYNTGRII
DSWNIYDNAMEKSSLLARAVEKYAKFSIPNDIPSIHVHDWHSVIAGVTAKFTFEARRVI
VPLVFTVHLLNKVSAPWHYASEDSGLMNYPHYIWRICKHLYTTREVWDFSSGSIEKF
GSYEADLITSVSKSYLTYDIFNFIGNWIENKSCIHNGTDWEVEETKKYAYSKFGTEDRA
EIRKRLFDELEVLRTVPEDYTTGNILWNNRNFNIGIKDDWTYSRENGPLILFAGRMVYQK
GIDSLLIADFVLTINNARLILGLPSSDYGLLQNVVSNISRHGGNIRIILGKMSKELY
RLFYYSASVFVIPSREWEPGLVAVESMAVGTPVVAYSVGGRESIVDIRVDQEQGTGLLV
EPENWELSKALISALSMASETNNGDFLKYTEVKTNDVKLWDKIRQNCVRRVNFNFRW
SASAKQLQECYSKAQTMAYRLLASF
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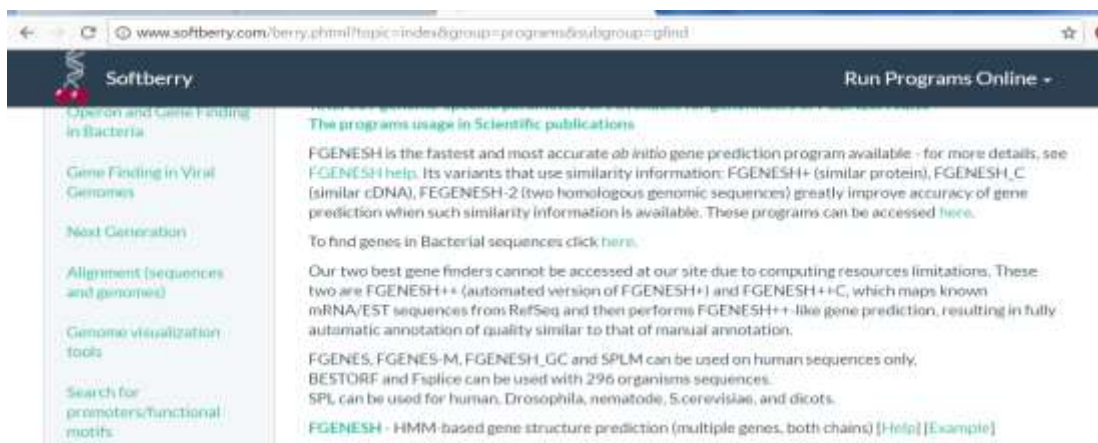

1. Connect to Softberry homepage <http://www.softberry.com/>



2. On the left frame, select GENE FINDING in Eukaryotes under Run programs online

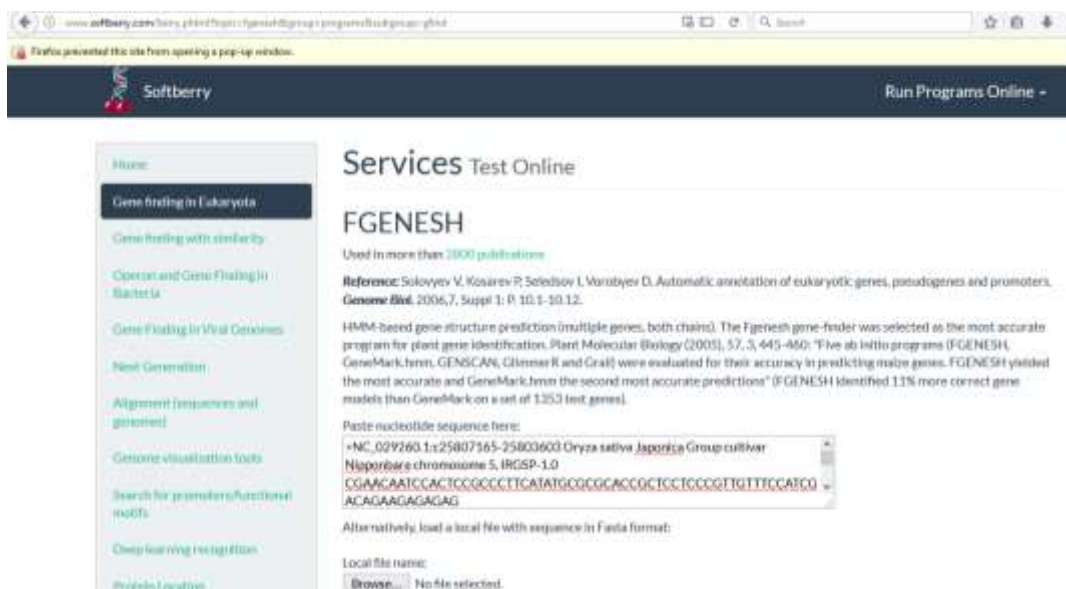


3. Select the program FGENESH.

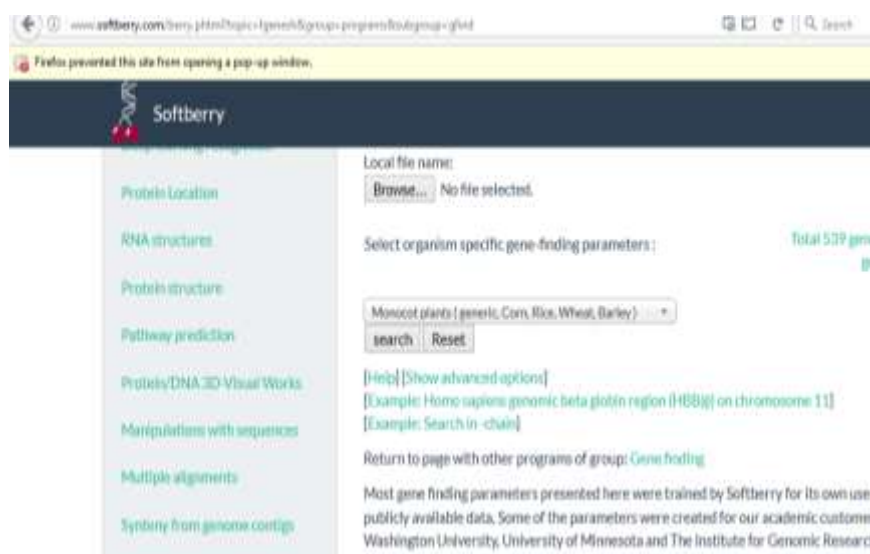




4. Paste DNA sequence.

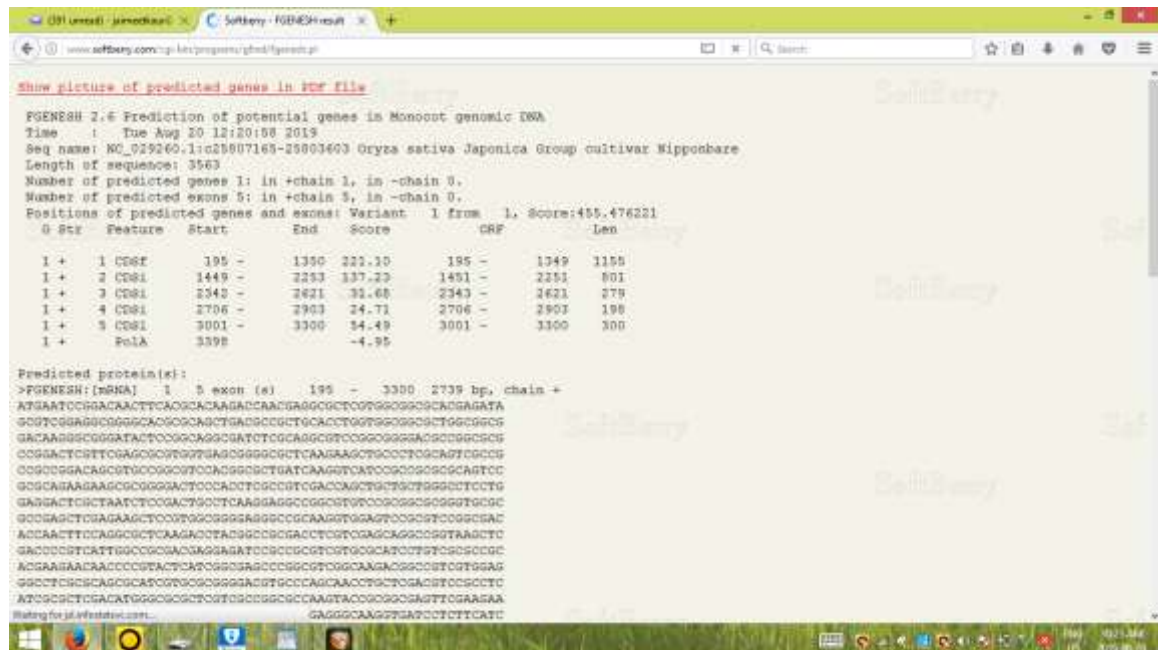


5. Select Organism

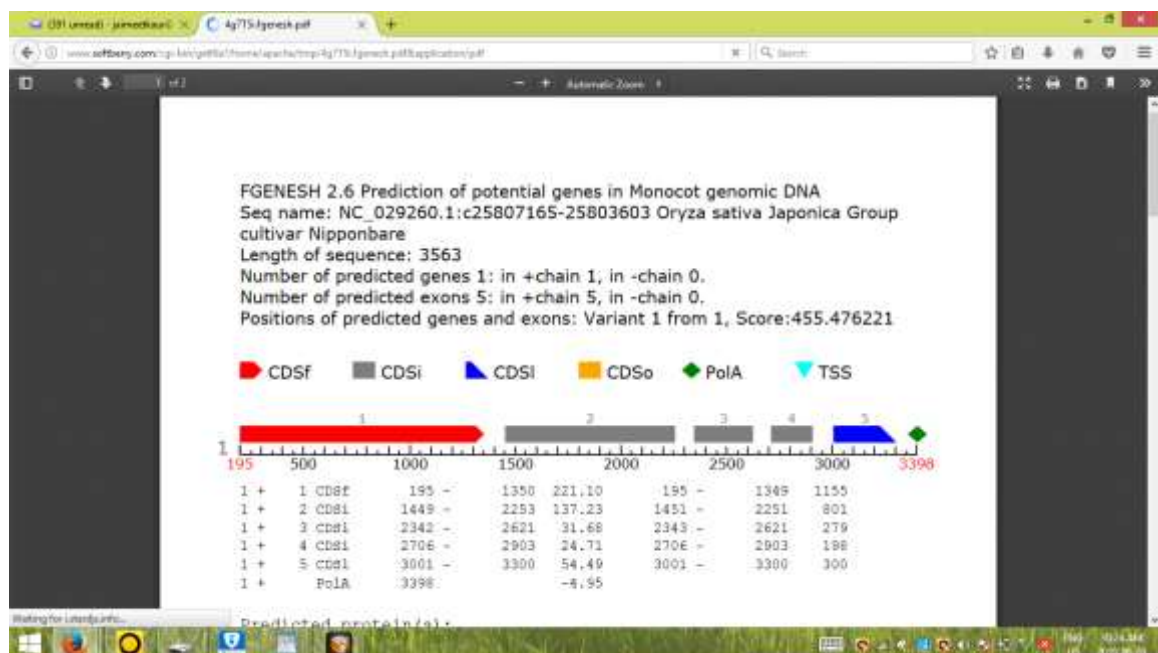


5. Press Search button.

6. View the predictions results



7. Open the PDF file to have a detailed gene structure view



B) GENSCAN: Identify complete exon/intron structures of genes in genomic DNA. Novel features of the program include the capacity to predict multiple genes in a sequence, to deal with partial as well as complete genes, and to predict consistent sets of genes occurring on either or both DNA strands.

1. Connect to the GENSCAN site. <http://hollywood.mit.edu/GENSCAN.html>.




2. Paste DNA sequence or upload the DNA sequence in fasta format.

3. Press Run Genscan button .



4 View Gene prediction results.


RefSeq: [NC_012920.1](#) (h1n1/gene/prog)
Search

GENSCAN Output

View gene model output: [PS-POE](#)

GENSCAN 1.0 Date run: 26-Aug-11 Time: 12:28:01

Sequence /tmp/00_24_18-12:28:01.fasta : 3628 bp : 61.86% C+G : Isom95a 4 (27 - 160 C+G%)

Parameter Matrix: Ambiguity4to.8845

Predicted genes/exons:

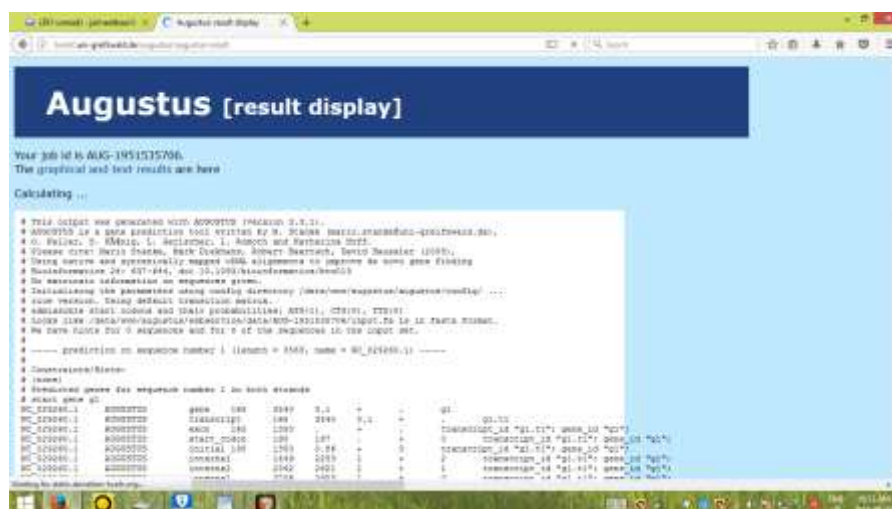
Exon	Type	Start	End	Len	Fr	Ph	3'/Ac	In/T	Code	Fr	Score
1.01	Intr	+	255	1410	1150	2	1	95	106	2405	0.389 282.98
1.02	Intr	+	1408	2319	606	1	1	86	106	1468	0.498 183.83
1.03	Intr	+	2402	3481	250	2	1	-10	97	423	0.384 36.13
1.04	Intr	+	2788	2789	130	2	0	78	92	238	0.448 30.78
1.05	Intr	+	3061	3350	289	0	2	78	86	750	0.375 70.74

Final model shown with probability > 1.00E

C) Augustus: software for gene prediction in eukaryotic genomic sequences that is based on a generalized hidden Markov model, a probabilistic model of a sequence and its gene structure.

1. Open <http://bioinf.uni-greifswald.de/augustus/submission.php>.

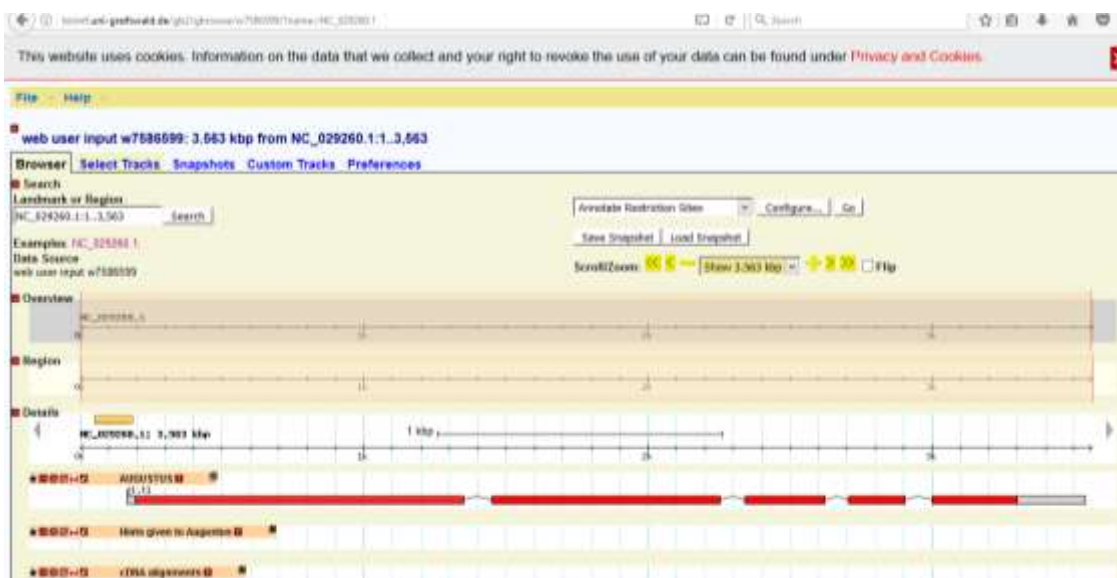
4.Run Augustus.



5. Open Graphical and Text results



6.View the gene prediction results in genome browser.



MOLECULAR DETERMINANTS OF RESISTANCE IN PLANTS AGAINST FUNGAL DISEASES

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Abstract: This presentation was prepared with an objective of making trainees aware of (1) Basics of host-pathogen interaction at molecular level (2) Approaches for identification of candidate resistance genes through forward and reverse genetics tools and their validation through functional genomics tools: here application of these tools was discussed with specific examples on two important diseases namely Verticillium wilt disease in potato and Fusarium head blight disease in barley (3) why phenotyping is so important in identification of resistance genes and (4) what alternate technologies are there for phenotyping.

Keywords: Host-pathogen interaction, Verticillium wilt, Fusarium head blight, Mapping, Metabolomics, transcriptomics, Candidate genes, VIGS

Details of presentation

Plants are often challenged by several biotic and abiotic stresses which lead to yield penalties and even complete losses of crops. Plant response to pathogens is very complex in nature and involves hierarchies of genes from signal perception to signal transduction to downstream defense response. Pathogens are often recognized by plants through pathogen associated molecular patterns (PAMPS) which leads to PAMP triggered immunity also known as pattern triggered immunity (PTI). However, this immunity is suppressed by effectors released by pathogens inside the plant cells. Plants also have evolved mechanisms to recognize these effectors via means of resistance genes called R genes. However, pathogens can evolve and defeat R gene mediated resistance. Host defense is broadly classified into two-broad categories: qualitative and quantitative. Qualitative defense involves one gene whereas quantitative defense is due to action of more than one gene and several mechanisms are involved in the later. Identification of resistance genes can be done by several approaches which are broadly covered under forward and reverse genetics approaches. In forward genetics, we try to fine the gene or genomics region associated with a particular phenotype whereas in case of reverse genetics it is other way round. Here we talk about application of these approaches to find out resistance genes in two different crops, potato and barley against their respective pathogens as two different case studies as discussed below:

Case Study I: Verticillium wilt disease in potato

Despite decades of research to control Verticillium wilt (VW), which is caused by the soil-borne fungal pathogens *Verticillium dahliae* and *V. albo-atrum*, this disease continues to be a recurrent problem for potato production throughout North America. It can result in yield losses of up to 50% and is currently controlled by fumigation. In tomato, resistance to VW is governed by resistance gene *Ve*, which has been exploited in breeding programs from more than 50 years. However, in potato, our studies indicate

that even though *Ve* is a major dominant resistance gene, it is not sufficient for complete resistance (Meier and Halterman, 2016). Two homozygous diploid parents, *S. tuberosum* DM1-3 (susceptible to VW) and *S. chacoense* M6 (resistant to VW) were crossed and an F2 population was created by selfing of one F1 individual. Since M6 is homozygous for the dominant *Ve* gene, we were expecting three quarters of the F2 population to be resistant. However, in field and greenhouse experiments, more than 70% of the F2 clones were susceptible. This suggests that there are other signalling components necessary for proper function of *Ve* and they are segregating in the F2 population. Marker analysis of the *Ve* locus was done using diagnostic primer pairs, but analysis was complicated by gene duplications in the region. We SNP genotyped the population, phenotyped it under greenhouse conditions (Kumar et al., 2017) and mapped a QTL on chromosome 1 and found two candidate genes that might play an important role in imparting resistance against for VW. However, we were not able to replicate the results under field conditions suggesting a need of a better and precise phenotyping protocol. There we explored application of various imaging techniques like thermal imaging, NDVI cameras, and hyperspectral imaging with an idea that we will be able to discriminate disease in a precise way and in future these can be used as precise phenotyping tools.

Case Study II: Fusarium head blight disease in barley

Fusarium head blight (FHB) management is a great challenge in barley and wheat production worldwide. Though barley genome sequence and advanced omics technologies are available, till date none of the resistance mechanisms has been clearly deciphered. Hence, this study was aimed at identifying candidate gene(s) and elucidating resistance mechanisms induced by barley resistant genotype CI9831 based on integrated metabolomics and transcriptomics approach. As a pre-requisite, we developed a Real-time quantitative PCR based method for the quantification of fungal biomass to discriminate quantitative resistance in barley and wheat genotypes to fusarium head blight (Kumar et al., 2017) and employed this to inoculate barley for identification candidate resistance genes in this study. Following *Fusarium graminearum* infection, we identified accumulation of specific set of induced secondary metabolites, belonging to fatty acids, phenylpropanoid, hydroxycinnamic acid (HCAA) and jasmonic acid pathways, and their biosynthetic genes. These metabolites were mapped into satellite metabolic pathways and transcriptomic data was integrated. Using Virus induced gene silencing we proved the function of three genes in regulating resistance in barley against Fusarium head blight. These include a *WAX INDUCER1* (*WIN1*) transcription factor (Kumar et al., 2016), a novel chitin elicitor receptor kinase gene *HvCERK1* (Karre et al., 2016) and a transcription factor *HvWRKY23* (Karre et al., 2019) that confers resistance against to combat Fusarium head blight using different mechanism. In nutshell:

- (a) We identified FHB resistant mechanism in **CI9831** genotype.
- (b) Successfully identified ***HvWIN1***, ***HvCERK1*** and ***HvWRKY23*** as candidate genes for FHB resistance, which can be searched for polymorphism in other FHB susceptible genotypes.

- (c) **Secondary metabolites** reported in study can be used to develop **biomarkers** upon further validation.
- (d) **CRISPR-Cas9 based genome editing** can be employed to replace polymorphic *HvCERK1* and *HvWRKY23* genes in FHB susceptible genotypes.
- (e) Further molecular and biochemical characterization of *HvUDPGT*, *HvLaccase15* could pave to identify new resistant related secondary metabolites.
- (f) Can be **easily translated to wheat** due to high synteny.

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DEVELOPMENT OF ROOTSTOCKS FOR FRUIT CROPS

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The fruit trees usually consist of two parts, the 'scion' (the fruiting variety) and the 'rootstock' provides the roots. Rootstock is the lower portion of the trunk and the scion is usually budded or grafted on the rootstock. The scion variety imparts the fruit characteristics such as size, colour and quality factors. The variety selected as rootstock determines the tree size, precocity; resistance to insect pest and diseases and soil qualities. Like the fruit varieties, rootstock breeding and selection is going on throughout the world. A significant scientific effort has been focussed on developing rootstocks for apple, but significant work has been done in pear, peach, plum and cherry. The significant findings in the development of rootstocks for apple, citrus, peach, pear and guava is discussed here.

Apple

In East Malling UK during early 1900', Ronald Hatton made selections from rootstocks and important rootstocks used in European nurseries. The selections were numbered M.1-M. 25 and were referred as Paradise rootstocks. These selections were dwarfing, precocious and productive.

Description of some important apple rootstocks

I. Malling and Malling Merton series

2. **Malling 27 (M.27):** It was selected from a cross of M.13 x M.9 at East Malling Research station in Maidstone, Kent, England in 1934. It was tested as Malling 3431 and a very dwarfing rootstock. Unless the central leader is supported, the tree will be very small. Little is known about disease or insect susceptibility. To date, most commercial nurseries are using this rootstock only as an intermediate stem piece on MM.106 or MM.111. If handled and spaced properly, it can be a very productive stock for a vertical axe system.
3. **Malling 9 (M.9):** The pedigree of M9 is not known. It was selected in England from a group of French genotypes known collectively as "Juane de Metz" during late 1800s. The traditional and best-known dwarfing rootstock. It should be planted on a well-drained site. Trees on this rootstock always require leader support. The rootstock is very susceptible to fire blight and can develop burr knots. Numerous clones of M.9 are now being sold by nurseries mentioned as under:
 - a) **M.9 NAKB 337:** the current dominant strain. It is a virus-free clone from the Netherlands and appears to be 5–10 percent less vigorous than M.9EMLA.

- b) **M.9EMLA** is a virus-free clone from the East Malling/Long Ashton research stations. It is approximately 25–30 percent more vigorous than M.9.
 - c) **Pajam 1 (Lancep) and Pajam 2 (Cepiland)** are French selections that are relatively new. They are 35 to 40 percent more vigorous than M.9 NAKB 337. M.9 RN 29, selected by Rene Nicolai in Belgium. In plantings at University Park with Gala, it is approximately 30 percent larger than M.9 NAKB 33
 - d) **Nicoli 29 (Nic 29)** and Pajam 2 are more vigorous M9 hence, preferred over M9/ M9 NAKB 337
4. **Malling 7 (M.7):** This rootstock produces a semi-dwarf tree that is freestanding in deep well drained soils. It was selected from unknown parentage at the East Malling Research station in Maidstone, Kent, England in 1912. In rocky, steep, or shallow soils, it tends to lean. High budding and deeper planting will help remedy this problem. The rootstock may sucker profusely and is susceptible to collar rot. M.7a is a clone of the original M.7 that has had some of the inherent viruses removed.
 5. **Malling 2 (M.2):** An older rootstock that is reappearing in nurseries and orchards. It produces a semidwarf to semistandard freestanding tree, depending on scion variety. Trees are strong, crop well, and do not have collar rot problems.
 6. **Malling-Merton 106 (MM 106):** It was selected in 1932 from a cross of Northern Spy × Malling 1 by the John Innes Horticultural Institute and the East Malling Research Station in England. A rootstock, slightly larger than M.7, that produces freestanding, early bearing trees. Trees on MM.106 are susceptible to collar rot when planted in wet soils and are not recommended for poorly drained sites. Delicious on MM.106 is susceptible to apple union necrosis.
 7. **MARK:** Formerly named MAC 9, developed in Michigan. It is an open-pollinated seedling of M.9. Trials in Pennsylvania indicate that this rootstock is not freestanding and is slightly larger than M.9. The central leader tends to lean. In recent years this rootstock has fallen into disfavor due to an abnormal growth proliferation at the soil line. Trees with this growth proliferation cease to grow and become spur bound; therefore, it is not recommended to be planted unless supplemental irrigation is provided. Very drought sensitive.
 8. **MM 111:** Developed from a cross Merton 793 × ‘Northern Spy’ at John Innes Horticultural Institute and the East Malling Research Station in England during 1952. MM.111 is semi vigorous rootstock producing a tree about 85 to 100% the size of seedling. It is resistant to woolly apple aphid and is quite tolerant to fire blight and crown and root rots. It is fairly winter hardy and produces

moderate amounts of burr knots and root suckers. MM.111 EMLA produces a free-standing tree and does not need support, and it is more precocious than seedling and is fairly productive when trees are mature. Now, MM.111 EMLA is being planted less as growers plant higher density orchards that require precocious dwarfing rootstocks.

II. Geneva apple rootstock series: Eleven Geneva rootstocks have been released in the U.S. of which seven are being supported for further commercial application. Many more genotypes are in the process of being tested and commercialized in these countries. The important characters of the Geneva rootstocks are mentioned below.

- **Unique Genetic Sources:** When compared with commercial and experimental rootstocks from other breeding programs, most of the Geneva elite rootstocks stand out from other apple rootstock germplasm. Currently in the Geneva breeding pipeline, thanks to multiple crosses between elite rootstocks and wild germplasm, there is a tremendous amount of genetic variation which can be utilized for breeding, selection and characterization of new traits, including disease resistance, vigor, precocity, and productivity.
- **Resistance to Woolly Apple Aphids (WAA):** Woolly apple aphids, are not just a nuisance to growers but can have a significant negative effect on fruit production in certain climates. Most commercial dwarfing rootstocks including M.9, B.9, M.26, M.7, Ottawa 3 are severely susceptible. Resistant apple rootstocks may contribute in the eradication of this pest in the orchard by preventing the overwintering stage that occurs in the soil while the insects feed on root systems, and the escape from aerial pesticide treatments during the growing season. The Geneva® breeding program has been the first to release several dwarfing apple rootstocks (G.41, G.969, G.214, G.210, G.202) that possess a different and more effective type of resistance than the MM.106, MM.111 type.
- **Resistance to Fire Blight:** Resistance to *E. amylovora* bacteria which causes fire blight has been a major goal since the inception of the Geneva breeding program. All released rootstocks today are tolerant or immune to this disease and allow the survival of susceptible scion varieties even under high disease pressure. Breeding with new sources of fire blight resistance continues in the Geneva® program in an effort to prevent new strains from overcoming the resistance genes that have already been deployed.
- **Tolerance to Apple Replant Disease:** Apple Replant Disease (ARD) is a major problem in the apple production areas of the world as less fumigants are available to sterilize the soil prior to replanting an orchard. Tolerance to ARD is one of the hidden traits that was not on the priority list in the initial stages of the breeding program, but has been discovered in the elite germplasm developed in Geneva. ARD causes stunting of young trees and substantial

- losses in production over the lifetime of the orchard. It is a complex disease and several causative agents have been implicated in the development of ARD symptoms including *Cylindrocarpon destructans*, *Phytophthora cactorum*, *Pythium spp.*, and *Rhizoctonia solani* (Mazzola, 1998). Not all orchard sites may be affected by all these pathogens, and some may have only one or two that are a factor.
2. **Geneva 11** – It was developed from a cross between M.26 x Robusta 5 Crabapple. It is similar in size to B.9 in some trials and similar to M.9 T337 in others. It is very precocious, has very high yield efficiency and reduces bienniality with Honeycrisp. It is fire blight resistant and has good resistance to *Phytophthora* root rot, but it is not resistant to wooly apple aphids or apple replant disease. G.11 has good layerbed and nursery characteristics and produces high quality nursery trees. It is proving to be an excellent replacement for M.9 in North America and Europe.
 3. **Geneva 41:** It was developed from a cross between M.27 and Robusta 5 and introduced by the New York State Agricultural Experiment Station, Geneva, NY. It is similar in size to vigorous clones on M.9 such as Nic29 or Pajam 2. It is usually the most efficient dwarf rootstock and reduces bienniality with Honeycrisp. It has excellent fruit size and induces wide branch angles. It is highly resistant to fire blight and is also resistant to *Phytophthora* and wooly apple aphids. It has some tolerance to apple replant disease and has good winter hardiness. G.41 is a shy rooter and requires specialized rooting techniques including tissue cultured stoolbed mother plants to improve its rooting. It has brittle roots and a brittle graft union similar in strength to M.9 and must be handled with care.
 4. **Geneva 935:** It is similar in size to M.26 in some trials and similar to the vigorous clones of M.9 in other trials. It is highly precocious has very high yield efficiency. It induces wide branch angles, is highly resistant to fire blight and *Phytophthora*, and appears to have some tolerance to apple replant disease. It also appears to be very winter hardy, but it is not resistant to wooly apple aphid. Fruit size has been slightly smaller than with M.9. It is an excellent new rootstock for weak growing cultivars like spur-type 'Delicious', 'Honeycrisp', 'Sweet Tango' or 'Snapdragon
 5. **Geneva 969:** It is similar in size to M.26 in some trials and similar to M.7 in others. It is highly yield efficient and was released in 2010 as a free standing semi-dwarf tree for processing orchards. It is resistant to fire blight, *Phytophthora*, and wooly apple aphid. It has excellent stoolbed propagation characteristics, which may make it easier to be introduced quickly. It performs well in northern climates. It may be an excellent stock for weak growing cultivars in northern climates like Honeycrisp, Sweetango or Snapdragon when planted at high densities. The first commercial stoolbeds of this stock will be planted in 2013.

6. **Geneva 890:** It is similar in size to M.7 or MM.106. It is more yield efficient than either M.7 or MM.106 and was released in 2010 as a free standing semi-dwarf tree for processing orchards. It has excellent stoolbed propagation characteristic and is resistant to fire blight, *Phytophthora* and wooly apple aphid. The first commercial stoolbeds of this stock will be planted in 2013. Four other Geneva rootstocks (G.65, 16, 30 and 210) have also been released but have limited commercial propagation.
7. **G.65:** It is too dwarfing in many locations while G.16 is susceptible latent virus infections when grafted with infected budwood. G.30 very productive in the orchard and is proving to be useful in northern growing areas where it shows wide soil adaptation, good winter hardiness and high yields. However, it is difficult to handle in the stoolbed and nursery due to excessive production of sharp spines. G.210 is newly released with similar characteristics as G.30 but with less spines.
8. **Geneva 202:** It produces a tree slightly larger than M.26. It has high yield efficiency and is precocious. It is resistant to fire blight, *Phytophthora*, apple replant disease and to wooly apple aphid. It is a useful with weak growing cultivars and as an alternative to M.26 in climates that have problems with woolly apple aphid. It has become a popular dwarfing rootstock in New Zealand.
9. **Geneva 214:** It is similar in size to M.9 in some trials and similar in size to the vigorous clones of M.9 in other trials. It was released in 2010. It is slightly less yield efficient than G.41 but has better stoolbed propagation characteristics, which may make it easier to be introduced quickly.

III. Budagovski apple rootstock series

1. **Bud-9.** It was developed from a cross of M.8 × 'Red Standard' (Krasnij Standard) from Russia. Smaller than M.9, very cold hardy, some resistance to fireblight, excellent branch angle, and tree vigor, very resistant to *Phytophthora*, some resistance to powdery mildew, very productive in first 6-8 years of tree life, but yield and fruit size declines afterwards, susceptible to burrknots. Do not combine with Honeycrisp or similar low vigor varieties.
2. **Bud-118.** It was developed from a cross between Moscow pear × mixture of M8 and M9 pollen at Michurinsk College of Agriculture, former Soviet Union. Very large tree, slightly smaller than seedling. Very cold hardy, and resistant to *Phytophthora* and scab, but susceptible to powdery mildew, and crown gall. Does not need support and had good productivity. Good for weak varieties grown in poor soils.
3. **Bud-490.** It originated from a cross of Red-Leaved Paradise (B.9) x Bud. 13-14 at the Michurinsk College of Agriculture, former Soviet Union. Similar in size to MM.106, does not need support, very cold hardy, earlier production than M.106, develops few suckers, moderate resistance to *Phytophthora*, slightly susceptible to fireblight. Good For honeycrisp and other weak varieties.

- 4. Bud-491:** Very dwarfing rootstock, similar to M.27. Good for testing new varieties on a limited bases. Smaller fruit size, susceptible to collar rot.

Citrus

In the entire world, citrus fruits are grown over an area of 9.09 million ha with 137.4 million tons production. The major producers of citrus in the world are China (33.73 m tons), Brazil (19.51 m tons), India (11.65 m tons), Mexico (7.67 m tons), USA (6.78 m tons), Spain (6.95 m tons) and Egypt (4.80 m tons). The major problems in citrus are *Phytophthora* root rot, citrus decline, fruit drop, poor nutrition and non-availability of quality planting material. Most of these factors are influenced by rootstock which is the major contributor to tree performance and longevity as it determines tolerance to various biotic and abiotic stresses. To develop rootstock with dwarf stature, imparting high yield and good quality to the fruits on scion variety. Develop a rootstock resistant to biotic (*Phytophthora*, citrus nematode, tristeza and exocortis).

Rough lemon is a well-suited rootstock for lemon, orange and pummelo varieties as it is high yielding, resistant to tristeza virus, suitable for high pH soils. However its susceptibility to *Phytophthora* fungus has become a major cause of citrus decline. Among citrus rootstocks, it holds most prominent place in India, as around 80 per cent of citrus orchards are on rough lemon rootstock, whereas in Punjab state this figure is about 90 per cent. In Central India, where a million plants are propagated every year, more than 20 per cent plants die due to *Phytophthora* infection in the nurseries. It has caused enormous losses in citrus cultivation in whole Punjab state because of which several Kinnow mandarin orchards in the state have been abandoned. Naqvi (2002) observed the prevalence of *Phytophthora* in the districts of Abohar (10-100%), Bathinda (10-50%) and Hoshiarpur (10-50%). Moreover, it is vigorous in nature which limits its use in high density planting and imparts poor quality to fruits of scion varieties. Hence, there is an urgent need to develop *Phytophthora* tolerant rootstock of citrus suitable for Kinnow mandarin under Punjab conditions.

It has been observed that in citrus more than twenty characters are influenced by rootstock like tree vigour and size, depth of rooting, freeze tolerance, adaptation to certain soil conditions, resistance to diseases and pests, fruit yield, size, texture, internal quality and maturity date, etc. Utilizing rootstocks resistant to *Phytophthora* foot rot may prove to be the best strategy for sustainable citrus industry. Rootstocks like sour orange (*Citrus aurantium* L.) and its strain like Gou Tou imparts traits like high quality fruits, higher yield and tolerance against *Phytophthora*, xyloporosis, citrus blight and exocortis (Castle *et al* 1993). Several hybrids of trifoliate orange (*Poncirus trifoliata* L. Raf) viz. X 639 citrandarin (Cleopatra mandarin × trifoliate orange), Citranges (Sour orange × trifoliate orange) and Swingle citrumelo (Duncan grapefruit × trifoliate orange) are also tolerant to *Phytophthora* and are widely used as rootstocks for citrus varieties in the entire world (Cheng and Roose, 1995). Rangpur lime (*Citrus limonia* Osbeck.) is productive and tolerant to *Phytophthora* foot rot, scab, exocortis viroid, and

drought (Castle, 2010). These citrus rootstocks have been reported to be resistant or tolerant to *Phytophthora*. But, none of these rootstocks are an ideal rootstock for Kinnow mandarin, the major citrus cultivar of Punjab. Despite of having so many good traits these rootstocks are unsuitable for Indian conditions, however; these can be utilized in breeding programme to develop new suitable rootstocks.

Sexual hybridization is complicated in most citrus species because of its complex reproductive biology. Mostly, citrus cultivars are heterozygous, polyembryonic and important traits show single-gene inheritance patterns. Polyembryony interferes with zygotic embryo recovery and impairs creation of large segregating populations for selection; therefore, achievement of desired characteristics is complicated even when using complementary parents. Nucellar embryos are more vigorous and inhibit the full development of the zygotic embryo and cause its degeneration prior to seed maturation (Soost and Roose, 1996). Thus, nucellar embryos limit the range of genetic variability that can be observed in the progeny of a cross and the possibility of finding new genotypes. Embryo rescue is an important tool for recovery of hybrid embryos in sexual breeding of citrus (Shen *et al* 2011). It has been successfully used to recover diploid hybrids from crosses between polyembryonic cultivars. The success of embryo culture is largely determined by the stage of embryo rescue, the ingredients of medium and the genotype of the plant. The germination capacity of hybrid embryos can be affected by the embryo's genetic structure and stage of development (Viloria *et al* 2005). The stage of embryo rescue has been reported to vary with the genotype of the parents.

Rootstock Breeding

In Spain

Rootstock hybrid	Parentage	Important traits
Forner Alcaide 5	Cleopatra mandarin x Trifoliate orange	Semidwarfing, Resistant to <i>tristeza</i> , citrus nematode, Tolerant to lime induced chlorosis, CI excluder, excellent tolerance to wet and flooded soils, good fruit quality (can replace Carrizo in Spain). It is an interspecific hybrid.
Forner-Alcaide 13	Cleopatra mandarin × Trifoliate orange	Trees deciduous, CTV resistant, phytophthora resistance, susceptible to citrus nematode but salinity tolerant.

Source: Forner *et al* (2003)

USA- Rootstock released in recent years:

Rootstock hybrid	Parentage	Important traits
US-812	Sunki × Benecke	It is highly productive of good quality fruit on a moderate sized tree and exhibits tolerance or resistance to citrus tristeza virus. Released in May 2001. Resistant to Huanglongbing (HLB)
US-897	Trifoliolate orange Mandarin × trifoliolate orange	

Source: Bowman and Rouse (2006)

Molecular markers association with traits in Citrus

Species	Markers	Trait linked	Reference
<i>P. trifoliata</i> × <i>C. sunki</i>	RAPD	<i>Phytophthora</i> QTL	Siviero <i>et al</i> 2006
Trifoliolate orange	RAPD	Citrus tristeza virus	Fang <i>et al</i> 1998

Zygotic embryo development within seeds has been divided into globular, heart, torpedo, and cotyledonary for dicots. This classification not only indicates the change in embryo shape but, also a progression towards independence from surrounding tissues for nutrients. An embryo is easier to isolate at a latter developmental stage and has a greater chance for *in vitro* survival. The late heart-stage embryo coincides with the change from heterotrophic to autotrophic growth of the embryo. Nucellar embryos are smaller in comparison to zygotic embryos at young age. The production of large numbers of polyembryonic seeds with high germination rate and percentage of nucellar progenies is one of the essential attributes that a rootstock should possess (Broadbent and Gollnow 1993). The apomitic seedlings are derived from somatic cells during the early stage of seed development and, are genetically true to type of their mother plant thus, ensure the uniformity of the nursery seedlings. Therefore, citrus rootstock breeding programmes should usually use at least one polyembryonic parent to ensure the production of polyembryonic hybrids. However, the citrus zygotic embryos are usually weaker than nucellar ones and, have lesser chance to succeed in the competition for nutrients during their development and germination (Soost and Roose 1996). Embryo rescue, offers a solution to this problem. It was also successfully used to obtain diploid hybrids from crosses between polyembryonic cultivars. Singh *et al.*, 2016 also developed hybrid rootstocks tolerant to *Phytophthora* root rot by using Rough Lemon as seed parent and trifoliolate orange and its hybrids as a pollen parent through embryo rescue

Table 1. Resistance source for rootstock breeding in citrus (Krueger and Navarro, 2007, Sharma *et al.*, 2016)

Stress	Source
Iron Chlorosis	Rough lemon (<i>C. jambhiri</i> Lush), <i>C. macrophylla</i> Wester, Volkamer lemon (<i>Citrus limonia</i> Osbeck), and <i>C. amblycarpa</i> (Hassk.) Ochse
Salinity	Rangpur lime (<i>C. limonia</i> Osb.) and Cleopatra mandarin both are Cl ⁻ excluder and <i>Poncirus trifoliata</i> is Na ⁺ excluder , Attani-1 (<i>C. rugulosa</i>), RLC-6 and sour orange
Cold	Satsuma mandarins, the Kumquats and <i>P. trifoliata</i>
Drought	Rangpur lime and Erimocitrus
<i>Phytophthora</i> spp.	Certain pummelos and mandarins, sour orange, Volkamer lemon, <i>P. trifoliata</i> and its hybrids, Microcitrus, and <i>C. amblycarpa</i>
CTV	<i>P. trifoliata</i> , partial resistance to CTV of some pummelos and kumquats and tolerance of mandarins, lemons, and several rootstocks such as Cleopatra mandarin, <i>C. amblycarpa</i> , Rangpur lime, Rough lemon, and Volkamer Lemon
Citrus Canker	<i>C. junos</i> Siebold ex Tanaka and some mandarins (Satsuma, Dancy)
Nematode	<i>Tylenchulus semipenetrans</i> Cobb of <i>P. trifoliata</i>

Table 2. Reaction of citrus rootstocks to biotic and abiotic stresses

Rootstock	<i>Phytophthora</i> Tolerance	Tristeza tolerance	Exocortis tolerance	Citrus nematode	Salinity
Trifoliate orange	1	1	5	1	5
Swingle Citrumelo	2	1	2	1	2
Troyer citrange	2	1	4	3	3
Carrizo citrange	2	1	4	3	2
Benton	2	1	4	3	4
Cleopatra mandarin	3	1	1	4	1
Rough lemon	4	2	1	4	4
Key: 1 = best of rootstocks listed; 5 = worst of rootstocks listed					

Table 3. Inheritance pattern (Cheng and Roose, 1995)

Character	Pattern
Trifoliate leaf character	Complete dominance over the monofoliate leaf governed by two principal genes
Polyembryony	A recessive character, controlled by a series of multiple genes, which may regulate the synthesis of a potent inhibitor of embryogenesis in nucellar cells of monoembryonic species.
Monoembryonic x Polyembryonic	Give both types of hybrids in 1:1 ratio.
Nucellar embryony	Controlled by one or two dominant genes.
Dwarfness in Flying Dragon	Single dominant gene causes curved thorns and twisted trunk growth.
Thorniness, pubescence and presence of oil glands in the juice sacs or in the rind or leaf	Dominant character
CTV resistance	Dominant allele at single locus (Ctr). Resistant genotypes are designated as Ctr-Rr and Ctr-RR and susceptible scion as Ctr-rr. Ctr is not the only locus responsible for resistance to CTV in poncirus, but at least one another gene is involved.
Nematode resistance	Polygenic control

Somatic hybridization

Somatic hybridization leads to generation of symmetric allotetraploid hybrids that contain the complete nuclear genomes of both parents. Somatic hybridization has been used to broaden the germplasm base available for rootstock improvement by producing hybrids with sexually incompatible or difficult to hybridize citrus relatives that possess valuable attributes. Combine citrus with sexually incompatible or difficult to hybridize genera that possess traits of interest for germplasm expansion. The greatest level of success in Citrus - due to the highly successful model of fusing embryogenic suspension derived protoplasts with leaf derived protoplasts (Grosser, 2014). Citrus rootstock improvement is challenging as many traits are needed in a successful hybrid

rootstock. In somatic hybridization, the primary strategy was to produce allotetraploid somatic hybrids that combine diploid rootstocks having complementary traits. The second strategy was to combine citrus with sexually incompatible or difficult to combine related species exhibiting important traits. Somatic hybrid rootstocks have excellent potential for use in high density plantings due to polyploidy (Grosser and Chandler, 2003). Cybridization in citrus can be a way to induce phenotypical variations in highly heterozygous secondary species or genotypes (Garcia-Lor *et al.*, 2013), without breaking their very complex multilocus structure by sexual recombination. Very good success has been achieved in symmetric citrus somatic hybridization but, not much advancement has been done in the production of citrus cybrid plants by asymmetric somatic hybridization (Xu *et al.*, 2007; Bona *et al.*, 2009). Male sterility and tolerance traits with cytoplasmic inheritance have been common objective (Varotto *et al.*, 2001). The rootstock improvement programme through hybridization has been focused on the resynthesis of an improved sour orange by combining selected superior mandarin and pummelo parents, and production of more widely-adapted hybrids with a diluted trifoliate orange contribution. Fertile somatic hybrid trees are also providing new opportunities for citrus production rootstock improvement by breeding and selection at the tetraploid level (Grosser and Chandler, 2003).

University of Florida used two somatic hybrids, Nova mandarin + Hirado buntan pummelo (zygotic) and sour orange + rangpur, that are performing well in field trials and produce high percentages of zygotic seed, as females in such crosses at the tetraploid level. Selected high performance somatic hybrids including sour orange + Carrizo, Cleopatra + trifoliate orange and sour orange + Palestine sweet lime are being used as pollen parents. Grosser *et al.* (2003) have coined the term 'tetrazygs' to identify tetraploid progeny from such crosses. Dambier *et al.* (2015) focused on the creation of new cybrids through three different approaches of protoplast fusion: (i) symmetric hybridization, (ii) donor-recipient protoplast fusion by UV irradiation of donor protoplasts, and (iii) cytoplasts + protoplast fusion. Sixteen combinations of symmetric protoplast fusion have produced cybrids. No cybrid was obtained after UV irradiation of donor protoplasts, while three combinations of cytoplasts + protoplast fusion produced cybrids. Therefore, cytoplast fusion appears much more promising than the UV approach for targeted cybridization.

New somatic hybrid combinations rarely produce fruit with adequate quality as necessary to be released as a new cultivar, but it is possible that a few new somatic hybrids may be released as improved cultivars. Citrus rootstock improvement include the production of allotetraploid hybrids that combine complementary diploid rootstocks. Somatic allotetraploid hybrids produced were smaller than their diploid parents, indicating their potential for tree size control and their use in high tree densities. Seed trees of most of these somatic hybrid rootstocks are producing adequate nucellar seed for standard propagation.

Table 1: Somatic hybrids in citrus

Parental combination	Trait	Reference
<ul style="list-style-type: none"> Caipira Sweet orange+ Cleopatra, Caipira + Rough lemon Caipira + Rangpur lime 	Blight tolerance from Sweet orange	Mendes et al. (2001)
<ul style="list-style-type: none"> Cleopatra + Sour orange Rangpur lime + Sour orange 	Blight, <i>Phytophthora</i> from Sour orange and CTV, Exocortis virus and salinity /alkalinity from Cleopatra/ Rangpur lime	Mendes et al. (2001)
<ul style="list-style-type: none"> Shamouti + C-35 citrange, Cleopatra + C-35 citrange Chios mandarin + C-35 citrange Shamouti + Citrumelo 	Soil salinity and alkalinity + <i>P. trifoliata</i> features, high fruit quality	Dambier et al. (2011)
<ul style="list-style-type: none"> Poncirus trifoliata+ C. reticulata cv. Hognju 	Trifoliolate orange with improved Exocortis resistance	Guo and Deng 2001
<ul style="list-style-type: none"> Succari sweet orange + Microcitrus papuana 	Salinity/ Alkalinity + features of Microcitrus	Grosser et al. 1996
<ul style="list-style-type: none"> <i>P. trifoliata</i> (2X) + Fortunella hindsii (4X) 	Cold + improved features of Poncirus	Miranda et al. 1997
<ul style="list-style-type: none"> Sour orange + Carrizo citrange Cleopatra mandarin + Argentina trifoliolate orange 	Polyembryonic, Tree size control, Suitable for commercial plantings	Grosser, et al., 1994
<ul style="list-style-type: none"> Sour orange + Flying dragon Cleopatra mandarin + Flying dragon 	Polyembryonic, highly dwarfing and suitable for backyard planting	Grosser, et al., 1994
<ul style="list-style-type: none"> Willow leaf + Pomeroy Poncirus 	Dwarfing, good fruit quality and yields higher than Carrizo citrange	Mouhaya et al., 2008

Description of some important rootstocks of citrus

- 1. Rough lemon:** Rough lemon (*Citrus jambhiri*) is native to northeastern India. It is adapted to deep, infertile sandy soils. Scions grown on RL are extremely

large and vigorous, especially in sandy soils. RL has a tendency to sucker, and can be identified because of this trait. Yields are high, but fruit quality is poor. RL is particularly poor for mandarins, since fruit quality is low. Root system is extensive, and mature trees on this rootstock are drought tolerant. Scions on RL are not particularly freeze tolerant, due to their vigorous growth habit, although where soil temperatures are low, trees are moderately freeze hardy. RL susceptible to Citrus blight (Citrus decline) is tolerant of **tristeza, exocortis, and highly susceptible to *Phytophthora***. 'Milam' is a RL type that is resistant to burrowing nematodes common in Florida, 'Schaub' is a newly selected vigorous cultivar.

2. **Volkamer lemon:** Volkamer lemon (*Citrus volkameriana*) also produces large, vigorous trees. Yields are heavy, and fruit quality is poor compared with Sour Orange, but is better than Rough Lemon or Macrophylla. South African studies indicate that navel orange production in semi-arid areas is particularly good on Volkamer lemon. Lemons and 'Minneola' tangelo are typically grown on VK in Arizona. Freeze tolerance is slightly more than RL, and the rootstock is tolerant of CTV, exocortis and xyloporosis. Trees are susceptible to -blight, and nematodes. VK trees are more tolerant of *Phytophthora* than Rough Lemon, but less than *C. macrophylla* (**Alemow**).
3. **Alemow or Macrophylla:** Macrophylla (*Citrus macrophylla*) is tolerant of high pH and sandy soils, and has a deep root system lends drought tolerance to the tree. Fruits to scions on MC are large, and fruit quality is poor. MC is susceptible to citrus blight (decline) and citrus tristeza virus, and nematodes, but is highly resistant to *Phytophthora*.
4. **Rangpur lime:** Rangpur lime is a *C. reticulata* hybrid. Recommended for nagpur mandarin Rangpur lime rootstock. It is a common rootstock in Brazil, where it is valued for its resistance to drought and tolerance to CTV. Budding on Rangpur leads to trees of medium vigor, less than Rough lemon but, more than the citranges. Fruit quality and freeze tolerance is also intermediate. Trees are susceptible to CEV (exocortis), *Phytophthora* and blight.
5. **Sour orange:** Sour orange is the most widely planted rootstock planted in the world today. But, it is highly susceptible to CTV that limits its usefulness in Australia, Argentina, Brazil, California, Caribbean Countries, Central America, Florida, Spain, South Africa and Venezuela. Sour orange is an excellent rootstock for fresh fruit production. It produces trees of medium vigor and medium to large size fruit. Yields are adequate, but less than that of the lemon-type rootstocks on sandy soils. The root system is deep and moderately branched. The rootstock is adapted to areas with high pH and highly saline soils. SO does not take up P, K and B as well as other rootstocks. It is moderately tolerant of *Phytophthora*. SO is resistant to CEV and cachexia, but may be damaged by citrus nematode. Trees are freeze hardy, when compared

with those on RL, but are less freeze hardy than trees on trifoliate orange. The real strength of the rootstock lies in the fruit quality it can produce. Fruit size is smaller than RL, but soluble solids and acids are high. High solids will lead to early maturing fruit in some cultivars, such as 'Hamlin' compared to trees on RL. Peel of fruit on SO is thin, but splitting may be a problem. In areas without CTV, the excellent fruit quality and moderate *Phytophthora* resistance of SO, make it the most popular rootstock for fresh orange, mandarin and grapefruit production.

6. **Cleopatra mandarin:** Cleopatra mandarin is of minor importance world-wide. Trees on CL are large and moderately vigorous. Root systems are deep, and the tree is moderately drought tolerant. Trees on CL are freeze hardy, but re-growth following a freeze is slower than RL. CL is tolerant of CTV, CEV, and cachexia, but is susceptible to nematodes and citrus blight. CL trees are only partially tolerant of *Phytophthora*. CL trees are tolerant of a wide range of soil types. Fruit size of trees on CL is small, with a high juice quality and intermediate solids. Peel is thin, and this leads to some characteristic splitting. Yields are moderate, less than RL, but more than SO. The rootstock is not precocious, yields reach their maximum 10 years after planting. CL is incompatible with lemon scions. CL is not used on trees that have small-fruit problems, such as oranges or grapefruit, but may be used with some mandarins.
7. **Citranges:** Citranges are hybrids of sweet orange and trifoliate orange, both of which are used themselves as rootstocks. Several have been produced, including Rusk, 'Morton', 'Benton', 'Carrizo', 'Troyer' and 'C-35'. Trees budded to citranges are moderately vigorous, smaller than trees on RL, but larger than those on SO. Citrange fruit are seedy, and have a high percentage of nucellar embryony. Trees will grow well on sandy soils, but often poorly on high pH soils. Citranges are prone to Zn and Mn deficiencies. They often produce a rootstock overgrowth, known as a bench. Carrizo citrange is a hybrid of Washington navel and trifoliate orange. The leaves of trifoliate orange and of other rootstocks with trifoliate orange parentage have "trifoliate" or compound leaves divided into three leaflets or sections. Scions budded to citranges are not tolerant of exocortis or nematodes, but are tolerant of tristeza and cachexia. Citranges are moderately tolerant to *Phytophthora*, although C-35 is said to be more tolerant.
8. **Trifoliate orange:** Trifoliate orange is deciduous, shedding its leaves in the winter. Most citrus varieties propagated on trifoliate orange rootstock are also reduced in size, especially when Flying Dragon, a selection of trifoliate orange. Large number of factors influence tree size, but for most commercial citrus cultivars, research has shown that tree size can be greatly modified by selecting an appropriate rootstock. Trifoliate orange rootstock selections have been generally divided into two groups based on flower size.

9. **Citrumelos:** Citrumelos are hybrids of grapefruit and trifoliolate orange. 'Swingle' is the most widely planted. Orange trees on 'Swingle' are dwarfed, while grapefruit trees on it are vigorous. Swingle citrumelo is a hybrid of Duncan grapefruit and trifoliolate orange. Lemons do not grow well on 'Swingle' rootstock. Trees on 'Swingle' grow well on sandy soils, but not on high pH soils. Yields are moderate, although there are conflicting reports out of Florida and Texas. Yield on a per unit basis is high, suggesting that 'Swingle' would be productive at high densities. 'Swingle' are tolerant of tristeza, exocortis and cachexia, and immune to citrus nematode. Fruit size is moderate, and quality is good. As a seedling, it is highly nucellar, moderately seedy, and vigorous in the nursery. Most common trifoliolate orange grown in the US is 'Rubidoux' trifoliolate, and 'Flying Dragon' Trifoliolate. Trifoliolates are best for colder areas. They are slow growing, and not particularly vigorous. They are cold hardy and susceptible to micronutrient deficiencies, and do not take up nitrogen well. They do not like calcareous orange. Trifoliolate orange is incompatible with Eureka lemon. Flying Dragon is dwarfing.

Peach

Stone fruit crops like peach, almond, plum, apricot and cherry which are grown in Mediterranean regions are mainly localized where root-knot nematodes (RKN), species *Meloidogyne arenaria* (Ma), *M. incognita* (Mi) and *M. javanica* (Mj), are widely distributed. These nematodes are major pests in *Prunus* nurseries and orchards and up-to-now were mainly controlled by pre-planting fumigations with methyl bromide or other active ingredient of high toxicity, some of which will be withdrawn from the market in the forthcoming years. The objectives of our *Prunus* rootstock breeding program is to provide an efficient alternative to the use of these highly toxic fumigants by developing a new generation of *Prunus* rootstocks bearing high resistance to RKN and good adaptation to chlorosis and drought (from almond), tolerance to water logging (from plum), together with graft compatibility with peach (from peach) and good rooting ability (from plum). Interspecific hybrids between Myrobalan plum and peach, almond or almond peach were produced Salesses *et al* (1998) in order to combine their complementary soil adaptative traits and to generate high performance rootstocks for Mediterranean environments. The Myrobalan plum clones P.2175 and P.2980 were chosen as parents for their high-level and wide-spectrum of RKN resistance and their graft compatibility with peach. These clones are heterozygous for the *Ma1* and *Ma3* resistant dominant alleles, respectively Esmenjaud *et al* (1996) and Lecouls *et al* (1997). Amongst material from the *Amygdalus* subgenus, three other sources of RKN resistance were used: the peach Nemared (homozygous for resistance to *M. incognita* (MI), different almond cultivars of the Alnem series and the almond-peach hybrid GF 557, produced from the RKN resistant peach Shalil.

In India, peach is the third most important temperate tree fruit species after apples and pears. Most of the peach cultivars require 500 to 1000 or more chilling hours below 7.2° C for normal bloom and fruiting. High chilling varieties are cultivated

in the hills of Uttarakhand, Himachal Pradesh and Jammu and Kashmir. At the moment, In India, stone fruits (peach and plums) are grown on as 83, 630 ha with an annual production of 4, 71, 380 MT. The low chill (300 cu) peaches introduced by Punjab Agricultural University, Ludhiana during 1968 to 2001 served as an option to diversify to a crop with high economic returns due to shorter juvenile period (2-3 years), high productivity, regular bearing and early maturity (April- May) which made this fruit quite advantageous over citrus, mango, pear etc. Local and exotic low chill peach and nectarine cultivars (100-300 chilling hours) viz. Shan-i-Punjab, Prabhat, Partap, Sharbati, Florida Prince, Earli Grande and Punjab Nectarine recommended by PAU, Ludhiana are also performing very well in lower hills of Himachal Pradesh (Singh *et al.*, 2015), Jammu Region (Singh *et al.*, 2005) and North Eastern Hill Region (Babu *et al.*, 2011).

The peach varieties are commercially grafted on peach rootstocks viz. Sharbati and wild peach which are susceptible to peach tree short life (PTSL). PTSL results in early death of trees and decline of the orchards which is a complex disease related to rootstock and not to variety. It is caused by multiple factor like nematode, bacterial canker, iron chlorosis and variety of non-specific secondary pathogens (Liu *et al.*, 2012), however, nematode is the most important factor for PTSL. The nematode build up also leads to replant problems and it is difficult to establish a new peach orchard in old peach site. Besides, iron chlorosis is also a widespread nutritional disorder associated with cultivation of peach in subtropics due to alkaline soils. The high carbonate and bicarbonate ions in the subtropical soils decrease the iron availability to plants which leads to lime-induced iron chlorosis (Pestana *et al.* 2005) and results in delayed ripening and poor fruit quality (Sanz *et al.* 1997). In peach, variation has been recorded among genotypes for lime induced chlorosis (Chakraborty *et al.*, 2013). Hsieh and Waters (2016) found differences in the plant responses to iron deficiency and high pH induced iron deficiency. Alkaline pH and low iron supply resulted in increased expression of iron uptake genes, but riboflavin synthesis genes responded to iron deficiency but, not alkalinity. Iron deficiency increased expression of some common genes in roots and leaves, but alkaline stress blocked up-regulation of these genes in iron-deficient leaves. The short tree life of peach and nectarines and absence of option of getting improved varieties and rootstocks, peach growers resort to other options which have led to around 49 per cent decrease in present acreage in comparison to average acreage of last few years in India though the area is increasing now from the last two years.

Rootstock breeding programs using interspecific hybridization among *Prunus* species has been initiated in the developed nations. The breeding programs worldwide are working to provide efficient alternative rootstocks for peach bearing high resistance to root-knot nematode, canker and PTSL and better adaptation to chlorosis, drought and water logging, along with good graft compatibility and rooting ability. However, most of them are in private domain and the new improved rootstocks are patent protected and hence, can't be commercially exploited in India. The use of nematicides has been

banned due to environmental issues hence, indigenous breeding programs to breed *Prunus* rootstocks with durable resistance are the need of the hour. The PAU Ludhiana is working on peach varietal and rootstock improvement programme. The rootstock improvement program is focusing on development of candidate rootstocks with better nursery characters and; resistance to nematodes and adaptation to high pH soils.

Meloidogyne incognita is the major root-knot nematode (RKN) which causes severe crop loss in economically important *Prunus* species like peach (*P. persica*), plum (*P. salicina*), almond (*P. communis*) and apricot (*P. armeniaca*). Hence, breeding new *Prunus* rootstocks with root-knot nematode resistance with good adaptation to alkaline soils with graft compatibility and rooting ability will be crucial for sustainable stone fruit (peach, nectarine and plum) production in the subtropics of North West India. Seedlings of Sharbati variety of peach are used as peach rootstock in sub-tropical regions of India, because of its wide adaptability to warm climatic conditions and alkaline soils, but its susceptibility to root-knot nematode leads to PTLS. 'Flordaguard' is a sixth generation interspecific hybrid having different varieties of *Prunus persica* and *Prunus davidiana* parents which provides it resistance to four main *Meloidogyne* species even in warm areas (Rubio Cabetas, 2012). In Indian conditions, 'Flordaguard' has been found to be resistant against the root-knot nematodes (Singh *et al* 2010) but, showing very poor response to propagation by stooling, cutting, seed germination and micropropagation which is the major problem in its large scale multiplication for commercial purposes. Root-knot nematode resistant genes have been identified *Mi-1* from tomato and *Prunus spp* (Williamson and Kumar, 2006). Root-knot nematode resistant gene 'Ma' has been mapped from the Myrobalan plum (*Prunus cerasifera*). *Ma* gene confers complete-spectrum, heat stable and high level resistance against root-knot nematode and used directly or interspecific breeding for rootstock breeding in stone fruits using marker assisted selection (Direlewanger *et al* 2004a). Cao *et al* (2011) identified one gene (PkMi) involved in resistance to root knot nematode and mapped it to linkage group 2 in wild peach. Liu *et al* (2012) identified nine SSR loci which correlate with a response to peach tree short life syndrome and are distributed on four linkage groups. Further, they identified a quantitative locus (QTL) on LG2 in peach controlling PTLS tolerance.

Microsatellite (SSR) DNA markers are highly reproducible, detectable, informative (codominant marker), and are well developed in *Prunus*. All genes described so far to determine disease resistance to root knot nematodes coming from the peach were placed on linkage group 2 of *Prunus* based on common markers with the TxE map (Arús *et al.* 2004). Simple sequence repeat markers (SSRs) have proved to be a very efficient way to evaluate genetic relationships between individuals, marker-assisted selections and for population genetics studies in *Prunus* species (Aranzana *et al.*, 2010; Xie *et al.*, 2010; Cao *et al.*, 2012; Li *et al.*, 2013; Dettori *et al.*, 2015). SNPs have also been used for development of linkage maps to determine the inheritance of many qualitative and quantitative traits in peach (Da Silva Linge *et al.*, 2015; Micheletti *et al.*, 2015; Cao *et al.*, 2016; and Zeballos *et al.*, 2016). SNPs are

powerful for fine mapping, however, mapping technologies using SNP markers are still rather expensive and not applicable in every laboratory (Dettori *et al.*, 2015). The high quality peach reference genome (Verde *et al.*, 2013) empowers to design new SSR markers in a target region and find candidate genes.

In India, most of the improvement work has been limited to introduction of improved varieties and rootstocks of peach from advanced countries. No rootstock breeding work is being done in India except, at Punjab Agricultural University, Ludhiana where breeding programme in peach and nectarine is being undertaken for developing better varieties and rootstocks. *Prunus mira* a natural hybrid between peach and almond and traditionally used as rootstock for peach and almond is self-fertile, tolerant to cold, drought and powdery mildew and has potential for use in breeding (Thakur and Rana, 2017). In low chilling peach the hybrid seeds germinate poorly due to their immature embryos even when the fruits are ripe. *In vitro* embryo rescue is necessary to recover viable hybrid seeds from low chill peach hybrids, which is an important objective in many stone fruit breeding programmes. The effect of stratification on embryo germination in different parents and their hybrids indicated that parent show high germination than their hybrids. The maximum embryo germination in all parents and their respective hybrids were observed when the embryos were stratified for 45 days at 4°C. The study concluded that embryos rescued at 75 days after crossing gave maximum germination in rescued embryos when stratified at 4°C for 45 days (Sandhoury *et al.*, 2014). The peach embryos rescued at 75 days after hybridization resulted in maximum germination when stratified at 4°C for 45 days (Indira Devi *et al.*, 2018). Thakur *et al.* (2014) found that seed stratification at 4-5°C and germination of peach hybrids immediately after fruit harvest during summers is possible under controlled conditions which can save around 7-8 months of a breeding cycle in a year. Seed germination was positively correlated with fruit development period (FDP) and negatively correlated with chilling requirement of seed parent. Rosetting was negatively correlated with FDP and chilling requirement of the seed parent. The cultivars with higher FDP showed higher seed germination. Stratification media of cocopeat + vermiculite + perlite (2:1:1) resulted in highest seed germination. Highest germination (81.5%) was recorded in the cross Florda Glo x Tropic Sweet which did not differ significantly from Tropic Beauty x Florda Grand. Under high density nursery system, the hybrid seedlings of Florda Glo x Tropic Sweet showed highest growth (160 cm) and branches (13). Hence, controlled climate stratification and germination immediately harvest can be a good option for higher recovery of hybrid seedlings and reducing the breeding cycle (Thakur *et al.*, 2015). Irrespective to the genotype combinations, higher embryo germination was recorded from the embryos subjected to *in vitro* embryo rescue at ripe stage in comparison to embryos rescued at harvest maturity. The stratification period varied with the cross combination with time taken for radicle emergence varied from 36.3 to 44.3 days. There was segregation in the stratification period of the seeds in some cross combinations with the high chilling parent as male parent (Thakur *et al.*, 2016). Direct seed germination of peach hybrids is

possible in some cultivars following stratification and growth of seedlings under controlled conditions immediately after fruit harvest in summers which can save around 7-8 months of a breeding cycle in a year (Singh *et al.*, 2017). The high density nursery system has a potential in effectively advancing the breeding programme by promoting short generation time, reducing labour and space and allowing for rating of some characters within two years. In peach rootstock breeding program at PAU Ludhiana, two year old progenies have been developed by crossing Sharbati × Flordaguard and Flordaguard × Sharbati showing better nursery characteristics and very less penetration of juveniles in the roots (Rajput, 2018). The number of juveniles penetrated the root system was negatively correlated with plant height, internodal length, leaf number, petiole length, spad values, ‘a’ value and positively correlated with shoot dry weight, ‘L’ value, ‘b’ value, chroma and hue angle. The characters like plant height, internodal length, leaf number, ‘L’ values, ‘a’ value, chroma, and hue angle were significantly correlated with nematode penetration in root system. These characters can be used to distinguish the seedlings with lower nematode penetration from the seedlings with higher nematode penetration in the roots.

Description of some important rootstocks of peach

1. **Lovell’ and ‘Halford’** are old drying and processing peach cultivars that were selected as seedlings from ‘Lovell’ has high seed germination of uniform seedlings that are compatible with all peach and nectarine cultivars. Scion vigour is slightly less than on ‘Nemaguard’. ‘Lovell’ does not sucker and is susceptible to root-knot and root-lesion nematodes, but has better tolerance to ring nematodes, bacterial canker and PTSL than ‘Nemaguard’. ‘Lovell’ is susceptible to waterlogging, crown gall, *Phytophthora* spp. and *Armillaria* spp. ‘Halford’ is similar to ‘Lovell’ in nursery and field characteristics and is even thought to be a ‘Lovell’ seedling. Since ‘Lovell’ is no longer used as a drying cultivar, seed can only be obtained from nursery seed orchards. Thus, ‘Halford’ has gradually supplanted ‘Lovell’ in the nursery trade. However, since ‘Halford’ is usually sold in bulk as cannery pits, questions about whether it is true to type occasionally arise.
2. **‘Rubira’**: It was selected from peach seedlings grown at INRA from a Californian seed lot imported in 1960 and owes its name to its red coloured leaves. ‘Rubira’ seedlings have uniform and vigorous growth in the nursery, but are slightly susceptible to powdery mildew and resistant to the green peach aphid, *Myzus persicae*(Sulzer). ‘Rubira®’ induces 15–20% lower vigour than ‘GF 677’ and is more precocious with good productivity. It is therefore recommended for vigorous cultivars that are slow to enter into production and for higher planting densities. It is also less susceptible to crown gall (*A. tumefaciens*) and *P. vulnus* than other peach seedlings, but is susceptible to *M. incognita* and *M. arenaria* nematodes (Loreti, 1984).

3. **‘Nemaguard’**: ‘Nemaguard’ rootstock was selected from seedlings from a seed lot received in 1949 by the US Department of Agriculture (USDA), which was labelled *P. davidiana* and was eventually released as ‘FV 234-1’ in 1959. Thought to be a putative hybrid of *P. persica* × *P. davidiana*, field observations and molecular studies indicate that it is primarily *P. persica*. ‘Nemaguard’ seedlings are uniform and vigorous, compatible with peach and nectarine cultivars, and impart excellent scion vigour and productivity. It has good resistance to *M. incognita*, *M. javanica* and *M. arenaria*. However, research has confirmed that a new root-knot species (*M. floridensis*) can reproduce in the roots of ‘Nemaguard’. ‘Nemaguard’ is fairly tolerant of crown gall, but is sensitive to *P. vulnus*, fungal root rots, *Verticillium*, iron chlorosis and root waterlogging and may reduce winter hardiness of scion cultivars in cold climates. ‘Nemaguard’ suckers extensively and is very sensitive to *ring nematode* (*M. xenoplax*) feeding, which leads to tree injury and death from bacterial canker (*Pseudomonas syringae* pv. *syringae* van Hall) and PTSL (Zehr et al., 1976; Nyczepir et al., 1983). Despite these limitations, ‘Nemaguard’ is one of the most widely planted stone fruit rootstocks in California and South America.
4. **‘Nemared’** is a redleaf selection released in 1983 by the USDA that has ‘Nemaguard’ in its lineage. ‘Nemared’ in the nursery is similar to ‘Nemaguard’ but produces seedlings with less lateral branches thus facilitating budding. Field characteristics of ‘Nemared’ are also similar to ‘Nemaguard’, except ‘Nemared’ produces a slightly more vigorous tree with equal or better root-knot resistance, but has increased susceptibility to bacterial canker.
5. **GF 677**: Peach × almond hybrids ‘GF 677’ (‘Paramount®’ in the USA) is a natural hybrid selected by INRA. It may be propagated by softwood and hardwood cuttings, but now is exclusively propagated *in vitro* (micropropagation). It is a very vigorous rootstock (10–15% more vigorous than peach seedlings with a well-developed root system that ensures good anchorage. It is adapted to infertile and droughty soils as long as they are permeable and well-drained. Its resistance to iron chlorosis is high, having good productivity even in soils with 10–12% limestone. In addition, replant problems are ameliorated because of its vigour. ‘GF 677’ often induces excessive scion vigour, resulting in delayed precocity, low yields in the first few years, smaller fruit size and poor fruit colour. However, these defects disappear when the trees achieve a vegetative–reproductive balance and enter into full production. Still, this rootstock is not recommended for very fertile soils or high planting densities. ‘GF 677’ also adapts poorly to heavy and waterlogged soils since it is sensitive to root water logging. Furthermore, it is susceptible to *A. mellea*, *M. incognita*, *A. tumefaciens*, *Phytophthora cactorum* and *S. purpureum*, and has a lesser degree of susceptibility to *Verticillium albo-atrum*, somewhere between that of almond and peach.

6. **Cadaman:** Selected in Hungary from an interspecific hybrid of *P. persica* × *P. davidiana*, and then introduced in France by INRA (Edin and Garcin, 1996). ‘Cadaman’ can be propagated by softwood or semi-hardwood cuttings. The vigour induced in the scion is initially comparable to that of ‘GF 677’, but vigour tends to decrease 4 or 5 years after orchard establishment. Furthermore, ‘Cadaman’ induces earlier precocity, comparable productivity and larger fruit size compared with ‘GF 677’. ‘Cadaman’ shows good resistance to waterlogging, unlike peach–almond hybrids, and is tolerant to iron chlorosis and replant sickness. It is also resistant to *M. incognita* and *M. javanica*, but is as good a host to *M. xenoplax* as ‘Nemaguard’. Owing to its high vigour, ‘Cadaman’ is not an appropriate root stock for high-density planting systems.
7. **Barrier 1:** ‘Barrier 1’ (‘Primo’ or ‘Empyrean 1’ in the USA) is another peach × *P. davidiana* hybrid selected at the former Institute for the Propagation of Woody Species, Florence, Italy. The vigour induced by ‘Barrier 1’ is similar to or greater than that by ‘GF 677’, and ‘Barrier 1’ has an extensive, deep root system that ensures good anchorage. It propagates easily by hardwood cuttings or micropropagation. It is well adapted to different pedologic environments such as calcareous soil, waterlogging or replant sickness, and has good resistance to root-knot nematodes. Furthermore, it induces higher productivity and larger fruit size than does ‘GF 677’. Therefore, this rootstock is adapted to replant soils that have waterlogging problems where peach–almond hybrids cannot be used
8. **Ishtara:** Ishtara is a complex interspecific hybrid of ‘Belsiana’ plum (probably *P. cerasifera* × *P. salicina*) and a natural hybrid of *P. cerasifera* × *P. persica* selected by INRA. It is a multiple species-compatible rootstock, which induces medium vigour and can be used for peach, apricot and plum cultivars. ‘Ishtara’ has good graft compatibility with peach, rarely suckers, and has shown good resistance to the peach tree borer (*Synanthedon exitiosa*) in the USA (Reighard et al., 2004). It adapts well to different soil and climatic
9. **Guardian’ rootstock** traces its lineage back four generations to a ‘Nemaguard’ cross in 1954 and was released in 1993 jointly by USDA and Clemson University. ‘Guardian’ has many traits similar to ‘Nemaguard’. The primary differences are that ‘Guardian’ has lower seed germination, slightly less root-knot nematode resistance supports fewer *M. xenoplax*, and exhibits significantly higher tolerance to ring nematode, bacterial canker and PTSL. The latter trait has made it the most popular rootstock, especially for nematode-infested replant sites in the south-eastern USA, even though it is susceptible to *Armillaria* root rot.
10. **Siberian C:** was selected by the Agriculture Canada Research Station at Harrow, Ontario (Canada) in 1967. The seedlings are uniform with good cold hardiness. Cultivars grafted to this rootstock show medium or medium–low

vigour and good precocity. Productivity and crop efficiency are satisfactory. ‘Siberian C’ has the additional advantage of inducing **slightly earlier ripening time**, as well as having a lower susceptibility to *Leucostoma* or Valsa canker (*Leucostoma cincta* (Sacc.) Hohn.), but it is not tolerant of root waterlogging, nematodes, bacterial canker or crown gall. ‘Siberian C’ tends to de-harden the scion cultivar before spring in moderate climates, and therefore is mostly planted in colder regions.

- 11. Krymsk 86:** A relatively new peach-plum hybrid rootstock (*Prunus persica* x *P. cerasifera*) that originated in the Krasnodar region of Russia. When grafted to peach, plum, or apricot it is thought to be tolerant of cold temperatures, drought, and excessive moisture. It is also considered to be resistant to *Phytophthora*, and somewhat resistant to lesion nematodes.
- 12. Krymsk 1:** It is a cross between (Chinese Bush Cherry x Myrobalan Plum). This rootstock is considered to be **a dwarfing rootstock and is proposed to control vigour and improve fruit size**. Preliminary research in other regions with peaches and nectarines have reported **50 to 60 % the vigour** of peach seedling with enhanced fruit size. Incompatibility is an issue with some peaches and nectarines. More research needs to be conducted to obtain more information on the fit of this new rootstock in Ontario's tender fruit industry.
- 13. Flordaguard:** The University of Florida released ‘Flordaguard’, a red-leaved peach rootstock, in 1991. Seedlings of ‘Flordaguard’ are recommended as a rootstock for low-chill peach, nectarine, and plum production in non-alkaline soils infested with peach root-knot nematodes as well as other species of root-knot nematodes. Flordaguard’ rootstock’s red-leaf trait allows for easy detection and removal of rootstock suckers. Flordaguard has a chilling requirement of around 300 chill units blooms in early February.

Table 2: Rootstocks based on hybrids among sections *Peach* and *P. davidiana* and *Peach* x *Almond* hybrids

Rootstock	Species	Origin	Calcareous soil tolerance	Waterlogging tolerance	Rootknot nematode resistance	Other characters	Reference
Flordaguard	<i>P. persica</i> x <i>P. davidiana</i>	Univ. of Florida, The USA	Highly Susceptible	Susceptible	Resistant (Mj, Mi)	Red leaf, high vigor	Pinochet et al. (2002)
Nemaguard	<i>P. persica</i> x <i>P. davidiana</i>	USDA-ARS, The USA	Highly susceptible	Susceptible	Resistant (Ma, Mi, Mj)	Easy propagation, high vigor	Layne (1987)
Nemared	(<i>P. persica</i> x <i>P. davidiana</i>) x	USDA-ARS, The USA	Highly susceptible	Susceptible	Resistant (Ma,	Red leaf, easy	Ramming and Tanner

Rootstock	Species	Origin	Calcareous soil tolerance	Waterlogging tolerance	Rootknot nematode resistance	Other characters	Reference
	<i>P. persica</i>				Mi, Mj)	propagation	(1983)
Adafuel	<i>P. dulcis</i> × <i>P. persica</i>	CSIC, Spain	Highly tolerant	Susceptible	Susceptible (Mj)	High vigor, easy propagation	Cambra (1990)
Adarcias	<i>P. dulcis</i> × <i>P. persica</i>	CSIC, Spain	Tolerant	Moderately tolerant	Susceptible	Control of tree vigor, higher fruit quality	Moreno et al. (1994)
Felinem	<i>P. dulcis</i> × <i>P. persica</i>	CITA, Spain	Highly tolerant	Susceptible	Resistant (Ma, Mi, Mj, Mhi)	Red leaf, high vigor	Felipe (2009)
Garnem	<i>P. dulcis</i> × <i>P. persica</i>	CITA, Spain	Tolerant	Susceptible	Resistant (Ma, Mi, Mj, Mhi)	Red leaf, high vigor	Felipe (2009)
Monegro	<i>P. dulcis</i> × <i>P. persica</i>	CITA, Spain	Highly tolerant	Susceptible	Resistant (Ma, Mi, Mj, Mhi)	Red leaf, high vigor	Felipe (2009)
GF 677	<i>P. dulcis</i> × <i>P. persica</i>	INRA, France	Highly tolerant	Susceptible	Susceptible (Ma, Mi, Mj, Mh)	High vigor, easy propagation	Bernhard and Grasselly (1981)
GF 557	<i>P. dulcis</i> × <i>P. persica</i>	INRA, France	Highly tolerant	Susceptible	Resistant (Mi) Susceptible (Mj)	High vigor	Bernhard and Grasselly (1981)
Barrier 1	<i>P. persica</i> × <i>P. davidiana</i>	CNR, Italy	Moderately tolerant	Moderately tolerant	Resistant	High vigor	De Salvador et al. (1991)
Cadaman™	<i>P. persica</i> × <i>P. davidiana</i>	France-Hungary	Moderately tolerant	Moderately tolerant	Resistant (Mj)	Yield efficiency, high vigor	Edin and Garcin (1994)
Castore, Polluce and Sirio	<i>P. dulcis</i> × <i>P. persica</i>	U Pisa, Italy	Tolerant	Susceptible	Unknown	Control of vigor, high fruit quality	Loreti and Massai (2006)
Jaspi™	<i>P. salicina</i> × <i>P. spinosa</i>	INRA, France	Tolerant	Tolerant	Resistant	Peach compatibility?	Bernhard and Renaud (1990)

Rootstock	Species	Origin	Calcareous soil tolerance	Waterlogging tolerance	Rootknot nematode resistance	Other characters	Reference
Ishtara™	(<i>P. cerasifera</i> × <i>P. salicina</i>) × (<i>P. cerasifera</i> × <i>P. persica</i>)	INRA, France	Susceptible	Moderately tolerant	Immune (Mj)	Tolerance to Armillaria? Highly susceptible to bacterial canker	Grasselly (1988)
Hiawatha	<i>P. besseyi</i> × <i>P. salicina</i>	Unknown, Canada	Susceptible?	Unknown	Resistant (Mj)	Peach graft compatibility	Pinochet et al. (2002)
Controller 5™	<i>P. salicina</i> × <i>P. persica</i>	UC, The USA	Susceptible	Susceptible	Susceptible	Control of peach vigor	DeJong et al. (2004)
Evrica	(<i>P. besseyi</i> × <i>P. salicina</i>) × <i>P. cerasifera</i>	KEBS, Russia	Susceptible	Moderately tolerant	Resistant (Mj)	Peach graft incompatibility	Zarrouk et al. (2006)
Bruce	<i>P. salicina</i> × <i>P. angustifolia</i>	Texas A&M, The USA	Unknown	Unknown	Immune (Mj)	Peach graft incompatibility	Pinochet et al. (1999)
Krymsk-86™	<i>P. cerasifera</i> × <i>P. persica</i>	KEBS, Russia	Tolerant	Moderately tolerant	Susceptible (Mj)	Cold hardiness, tolerance to Pv	Jiménez et al. (2008)

Pear

In India, Kainth (*Pyrus pashia* Buch.&Ham.) is the most common pear rootstock. The trees of *Pyrus pashia* are found in the mid-hill region in India between 700 and 2,000 metres amsl. The trees are spiny and used as a rootstock for pear.

In the developed nations, only one *Pyrus* species (*Pyrus communis*) is used extensively as a rootstock in commercial production. Unfortunately, most types of *Pyrus communis* rootstock induce little or no dwarfing of scions propagated upon them and induce poor yield precocity. In the USA and a few areas of Europe, the OHF (Old Home × Farmingdale) clonal selections of *Pyrus communis* are used quite widely, although they are difficult to propagate (except by micropropagation) and provide almost no control of scion tree vigour. New clones produced from the same parents,

The Horner Series, some of which are reported to be more dwarfing than the original OHF selections. A clonal selection of *Pyrus communis* from South Africa, BP1, has induced vigour similar to Quince A or BA29 in some trials. However, trees on BP1 show great sensitivity to infections by the phytoplasma causing Pear Decline and tree death is common following infection. *Pyrus communis* selections made in Italy-Fox 11 and Fox 16. -Early results indicate that they are not fully dwarfing and can only be propagated successfully using micropropagation. The programme of rootstock breeding at HRI East Malling has also produced several new clones of *Pyrus communis*

that are currently under trial as potential pear rootstocks. One of these selections, QR708-36 is easy to propagate by semi-hardwood cuttings, induces vigour similar to BA29 and good yield productivity.

Until very recently, the only commercially viable dwarfing rootstocks for European pears were clones of quince. Pear growers in many countries in Western Europe use trees propagated on Malling (EM) Quince C, Adams Quince, Malling (EM) Quince A, or BA29 clonal quince rootstocks. Quince C is the most dwarfing of these, Adams and Quince A, slightly less dwarfing and BA 29 is classed as semi-dwarfing. These quince clones are sufficiently graft compatible with the scion cultivars Doyenne du Comice and Conference and the trees crop precociously and efficiently, bearing fruits of medium to large size. Unfortunately, many other European pear cultivars and most of the Japanese/Chinese pear cultivars (*Pyrus pyrifolia*) are graft incompatible with these quince rootstocks. Interstocks of varieties such as Beurre Hardy or Comice are sometimes used to overcome this incompatibility and achieve the benefits of dwarfed trees. None of the above quince clones are as fully dwarfing as the apple rootstocks M.9 and M.27. Attempts are being made to produce more dwarfing clones. One selection, C.132, from the HRI-East Malling programme, is slightly more dwarfing than Malling (EM) Quince C in trials conducted in the UK and the Netherlands. Increased fruit size of pears is a frequent demand by the markets and trees on rootstocks such as Quince C often produce smaller fruits than trees on the more invigorating BA29 rootstock. New dwarfing quinces inducing large fruit size are required - one quince selection from the HRI-East Malling programme, QR193-16, produces trees of similar vigour to trees on Adams Quince and shows some potential for increasing the size of fruits in scions propagated upon it. Tolerance to winter cold damage is essential in new clones of quince rootstock, as this prevents their use in many regions of the world. Dwarfing quince clones that are tolerant to alkaline soils are also needed exhibiting better graft compatibility with commercial pear cultivars would also be a major benefit to pear producers. One clone selected in Pisa, Italy, -Cts 212 - shows some improvement in this respect.

I. US breeding program:

A number of *P. communis* rootstocks have become available in recent years. Depending on the objectives of the breeding programs from where they originated, each has different characteristics that need to be carefully considered when selecting them.

Old Home X Farmingdale Series (OHF): The OHF series is one of the more recent overseas pear rootstock breeding programs that have produced a range of rootstocks for different climate and soil types. Professor F. E. Reimer, Oregon State College, searched for a fire blight resistant *P. communis* rootstock. He found this selection and named it Old Home. It had no blight in a bad year, but was not self-fruitful. He found another seedling also resistant and named it Farmingdale 18 (after its town of origin). The cross pollination of these two produced the fireblight resistant OH x F series selected by L. Brooks, Oregon, USA in 1960.

1. **OHF 51:** L Brooks, Oregon, 1960, **Most dwarfing, similar to Quince A and C. Resistant** to fireblight. Uses. Moderately good production, poor yield efficiency, moderately precocious, good anchorage, less tendency to sucker. Propagation with hardwood cuttings is difficult. A weak stool.
2. **OHF 69:** selection by L Brooks, Oregon, 1960. **Vigour. Semi-dwarfing, similar** to BP 1. Resistant to fire blight. Good production, high yield efficiency, moderately precocious, good anchorage, less tendency to sucker. Propagation with hardwood cuttings is difficult.
3. **OHF 97: Very vigorous**, similar to *P. calleryana*, 130% Quince A. Moderately good production, moderately precocious, good anchorage, less tendency to sucker. Propagation with hardwood cuttings is difficult. European, Asian and flowering pears. **Vigorous, widely adapted, disease-resistant, winter hardy, & tolerant of wet soils.**
4. **OHF 217: Semi-vigorous**, similar to BP 3 and WBC seedling. Good production, high yield efficiency, moderately precocious, good anchorage, less tendency to sucker. Equal or superior to BA 29 in size and production, (poor yield efficiency?) depending on soil, good production, moderately precocious, good anchorage, less tendency to sucker. Compared with **OHF 97** it has produced smaller fruit and yield of d'Anjou. Propagation with hardwood cuttings is difficult and a weak stool.
5. **OHF 333 : Semi-dwarfing**, equal or superior to BA 29, similar to BP 1. Propagation with hardwood cuttings is difficult. European and Asian pears are dwarfed to about 2/3 the size of standard, or about 12-15 ft. Widely adapted & disease-resistant.

II. South African Program

BP Series: Seedling selections were made in 1928 from seed of a wild pear tree with a strong chance of Kieffer being involved, at Ellsenberg, South Africa. These were selected and evaluated by A.F. de Wet, Fruit and Fruit Technology Research Institute, Stellenbosch (BP from Bion Donne Research Station).

- **BP 1:** BP 1 was released 1974. Susceptible to fireblight, resistant to collar rot and latent viruses. **Semi-dwarfing**, similar to OHF 333 and OHF 69, 110% Quince A.
- **BP 3:** BP 3 released in 1979. **More vigorous** than Williams' seedling, standard.

III. German breeding program:

1. **Pyrodwarf:** Pyrodwarf originated from a cross between Old Home (fireblight resistance) and Bonne Louise d'Avranches (ability to strike hardwood cuttings) made by Helmut Jacob, at the Research Institute and College, Geisenheim, Germany. Pyrodwarf produce trees smaller than trees on Quince A, which crop precociously and efficiently. Easily grown from cuttings. High precocity in 2–3

years, yield efficiency, uniform fruit size, good anchorage, winter hardiness, no sucker development, no lime-induced chlorosis on high pH soils, more precocious and dwarfing than OHF 97, suitable for high density planting.

IV. East Malling programme, UK

Quince has been used for some time as a rootstock for pears to produce a smaller tree that is more precocious, especially in Europe. Most quinces have some incompatibility with several pear varieties so an interstem of Beurré Hardy is necessary. Thirty selections of quince were collected from Caucasia, via John Innes to East Malling Research Station, U.K., in 1936. H. M. Tydeman tested 15 of these against Quince A, B, and C as possible dwarfing rootstocks for pears. All were more dwarfing than Quince A.

1. **QuinceA (Angers Quince):** Selection by East Malling Research Station for bush and smaller trees and high density plantings. Medium vigour, high tolerance to woolly aphid, resistant to root lesion nematode, mildew and crown gall, medium resistance to pear blast, susceptible to quince fleck and fire blight. Incompatibility. Not compatible with Beurré Bosc, Packham's Triumph, Williams' (except Williams' Compatible), Winter Cole, Josephine or nashi.
2. **Quince C:** Selections by East Malling Research Station for bush and smaller trees and high density plantings. Easily as hardwood cuttings (June), and is highly productive on stools but less productive than Quince A. High tolerance to woolly aphid, resistant to root lesion nematode, mildew and crown gall, medium resistance to pear blast, susceptible to quince fleck and fireblight. Incompatibility. Not compatible with Beurré Bosc, Packham's Triumph, Williams' (except Williams' Compatible), Winter Cole, Josephine or nashi. Quince C medium vigour is similar to A until it crops then falls behind in vigour. Quince C is useful for shy croppers and garden.
3. **BA 29 (BAC 29):** Selection made at the Fruit Breeding Station at Beaucouzé-Angers, France, 1963, named from the initials of the farm at Beaucouzé, Bois l'Abbé. Description. Leaf: large. Shape: ovate roundish with acuminate tip. It is slightly more vigorous than Quince A and OHF 333. Very productive on stools, roots from cuttings (June), not as easily as A and C. Some incompatibility, but fairly good with Williams', better than others with Beurré Bosc and Dr Jules Guyot, but imperfect.
4. **Quince -CQ 127, CQ 129, CQ 130, CQ 131, and CQ 133:** Semi-dwarf. Easily from stools or as cuttings
5. **Quince -CQ 132:** Dwarfing, Propagation easy from stools or as cuttings. Smallest of this group of quinces that have been tested at Orange but yield almost comparable to Quince A at closer plantings.
6. **Quince -CQ 134:** Dwarfing, Easily from stools or as cuttings. Incompatible with major varieties.

Guava

Guava an important fruit cultivated in tropical and subtropical countries of the world have problems associated with rootstocks like wilt disease. Interspecific hybridization between *Psidium molle* and *P. guajava* has led to the development of hybrids which are resistant to wilt and are graft compatible with commercial varieties of *P. guajava* (Negi and Rajan, 2007). Seedless varieties of guava have been found to be autotriploids ($2n = 33$). Crosses made between Seedless triploid and seeded diploid variety Allahabad Safeda produced several aneuploids. Three tetrasomic plants had dwarf growth habit and normal shape and size of fruits with less number of seeds. Aneuploid No. 82, a tetrasomic, has been found to have a strong potentiality of its use as dwarfing rootstock. Biotechniques for in vitro propagation of guava germplasm and rapid multiplication of guava seedlings have been developed. Future line of work has been suggested. Tetrasomic plants had dwarf habit and normal shape and size of fruits with less number of seeds. Trisomics, had promising qualities and may be useful in developing plants with reduced seediness and possibly in providing dwarfing rootstocks. Aneuploid No. 82 through selection was identified at IARI, New Delhi. It has a wider adaptability, dwarfness and field tolerance to guava wilt. It produced fruits of better quality in terms of flesh thickness, vitamin C content, softness of seeds and sweetness compared to Allahabad Safeda on its own roots. A complete set of trisomics was identified in the progeny of triploid guava for the first time in fruit crops. It has helped in the development of trisomics and tetrasomics in guava. Out of many promising aneuploids, Aneuploid No.82 was found the best in imparting dwarfness in commercial cv. Allahabad Safeda and released as Pusa Srijan in 2004. It is a potentially dwarf rootstock for guava and has been recommended for Allahabad Safeda variety for establishing high density orchard. At CISH, Lucknow, interspecific hybrid between *P. molle* and *P. guajava* found resistant to guava wilt and graft compatible with commercial varieties of *P. guajava* (Anon., 2003-04)

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BREEDING INTERVENTIONS IN *BRASSICA CARINATA* - A UPCOMING CROP FOR BIOTIC AND ABIOTIC STRESS TOLERANCE

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Rapeseed and mustard are the main oilseed crops in India but their average yield is only 0.5-0.6 t per ha although varieties have been developed which yield 2.0-2.5 t per ha under irrigated conditions. The gap between yield potential and average yield is quite large and yields are unstable, partly due to the incidence of pests and diseases and partly because the crops are mostly cultivated in rainfed areas where the level of production is greatly dependent on unreliable supplies of moisture at the time of sowing and at advanced stages of crop growth. Presently, Indian mustard occupies almost 90% of total acreage under rapeseed-mustard cultivation in India. Indian mustard is highly vulnerable to biotic and abiotic stresses that cause severe yield losses every year and is responsible for unstable production trends. Nearly 30.7% area under rapeseed mustard is under rainfed farming, resulting in its low productivity. In rainfed growing areas, moisture stress is one of the main constraints for sustainable and efficient mustard productivity during the crop growth and development. The *B. juncea* genotypes evolved so far have not been tolerable to go with the varied agro-climatic situations so as to maximize the production thereof. Improvements on crop water stress resistance are therefore sought through plant breeding. Brassica species with better ability to access soil moisture and improved water use efficiency could increase yields in an economic and environmentally sustainable way (Sahoo et al. 2015). *Brassica carinata* A. Braun (Ethiopian mustard) is a natural amphidiploid (2n=34, BBCC) originated from diploid progenitors viz. *Brassica nigra* (L.) Koch (2n=16, BB) and *Brassica oleracea* L. (2n=18, CC). It is considered to have originated in Ethiopia (Gómez-Campo and Prakash 1999), where it is used as both an oilseed as well as a leafy vegetable with little differentiation into various crop types (Labana and Gupta 1993). Earlier its cultivation was limited to the Ethiopian plateau and adjacent areas of east Africa but from last two decades, this crop is under evaluation and shows promise agronomically in many other parts of the world. *B. carinata* has been evaluated as a potential oilseed crop in the United States (Knowles et al. 1981; Cohen and Knowles 1984), Canada (Getinet et al. 1996; Rakow and Getinet 1998), India (Katiyar et al. 1986; Singh et al. 1988; Malik 1990; Raj et al. 1998), Italy (Mazzoncini et al. 1993) and Spain (De Haro et al. 1998; Velasco et al. 1999). Under current climatic change scenario, it is also emerging as a resilient crop and has been documented as highly heat and drought tolerant (Knowles et al. 1981; Singh et al. 1988; Malik 1990). This species also has good resistance to blackleg, *Leptosphaeria maculans*. The ability to adapt in arid and semi-arid conditions, resistance to abiotic and biotic stresses and high yield levels make this crop appreciated in terms of agronomic and energy balances as stated by Copani et al. (2009). Most of the studies have highlighted the prospect of introducing this crop into cropping systems, as an alternative to *Brassica napus*, mainly for less seed shattering at harvest with respect to winter rapeseed (Cardone et al. 2003; Copani et al. 2009). It is a low fiber, high protein source for the livestock industry. The

level of crude protein, at an average of 45%, is higher than that of the typical protein content of canola meal. The amino acid profile of *carinata* meal is very close to that of canola meal, with similar levels of sulphur containing amino acid and lysine. Moreover *B. carinata* is more productive than *Brassica napus* both under low-input conditions and adverse environmental conditions (Cardone et al. 2003). Seed oil from *B. carinata* has industrial applications wherever oils with high erucic or linolenic acid contents are required. Its use as a biodiesel is only now being explored. Preliminary agronomic evaluation in Saskatchewan, Canada (Getinet et al. 1996) suggested that *B. carinata* has good potential to become a new oilseed or protein crop for western Canada if adapted, early maturing strains can be developed. It has a relatively large seed size (Getinet et al. 1996) and contains from 26% to 40% oil, depending on the cultivar/genotype and environmental conditions.

It was recognized as a potential oilseed crop for Indian subtropical conditions too. However, the early introductions were not suitable for cultivation due to long maturity duration, tall stature and poor harvest index. In spite of these certain agronomic limitations, *Brassica carinata* has inherent advantages of resistance to major diseases; tolerance to abiotic (heat and drought) stresses and hence, being considered as an alternate crop in the regions of low rainfall. In spite of its potential as an oilseed crop, this is also being considered as Holy Grail for aviation fuel. *Brassica carinata* did not outyield *B. juncea* under irrigated conditions in the experiments described here, but higher yielding lines might be selected by eliminating undesirable plant characteristics through mutation or conventional methods of plant breeding. Poor seed germination is a serious problem in rainfed and saline fields but the high primary and secondary branching capacity of *B. carinata* may compensate for poor plant establishment. Primary and secondary branch development has been found to be strongly associated with seed yield in rapeseed (Campbell and Kondra, 1978). The good performance of *B. carinata* under late sowing conditions appears to be due to its photo-insensitivity, as a result of which late sowing caused less reduction in yield-contributing characters, particularly the number of branches. It also has a longer vegetative phase providing sufficient time for the growth and development of the plant. Being a crop of tropical origin (Ethiopia), *B. carinata* might also have tolerated high temperatures during the reproductive phase better than the control species where high temperatures resulted in forced maturity which severely affected seed and oil yield. These studies indicate that the cultivation of *B. carinata*, a species tolerant of aphids and white rust, should increase the productivity of oilseeds in India under conditions of late sowing and on rainfed and saline soils. The plants and seeds of all brassica oilseeds contain glucosinolates, which are secondary metabolites that serve as chemical protectants (Mitten, 1992). Ranges of glucosinolate content in these species have been reported to be 20 to more than 200 $\mu\text{mol g}^{-1}$ seed total glucosinolates for *B. napus*, *B. oleracea*, and *B. rapa*; 75 to more than 150 $\mu\text{mol g}^{-1}$ seed total glucosinolates for *B. carinata* and *B. nigra*; and 100 to more than 200 $\mu\text{mol g}^{-1}$ seed total glucosinolates for *B. carinata*. Seed oil from *B. carinata* has industrial applications wherever oils with high erucic or linolenic acid contents are required. Its use as a biodiesel is only now being explored. The oil could be used for human consumption now that zero erucic (Getinet et al. 1994) and zero erucic/high

oleic (Velasco et al. 2003) acid forms have been developed. Meal protein from this species could also be used as a feed if low glucosinolate forms were developed or if procedures were developed to remove these compounds; conversely, high glucosinolate forms may have potential as a biofumigant. Furthermore, *B. carinata* is very amenable to transformation technologies (W. Keller, pers. comm.), suggesting that it may be well-suited as a host plant for molecular farming of various products including lipids, novel carbohydrates, bio-plastics, bio-pharmaceutical proteins and feed enzymes. Molecular markers, including⁻¹ seed total glucosinolates for *B. juncea* (Röbbelen & Theis, 1980). The seed oil of *B. carinata* contains 40% erucic acid, which makes it unsuitable for human consumption, but a zero erucic acid line was developed in Saskatoon (Getinet et al. 1994). Carinata meal is a low fiber, high protein source for the livestock industry. The level of crude protein, at an average of 45%, is higher than that of the typical protein content of canola meal, wheat distiller's grains with solubles and similar to that of soybean meal. The amino acid profile of carinata meal is very close to that of canola meal, with similar levels of sulphur containing amino acid and lysine. Carinata meal is relatively low in fibre and an excellent source of crude protein that is readily degradable by rumen bacteria. As such, this meal can be used effectively to meet the rumen degradable protein needs of growing cattle. The unique characteristics of this meal provide new opportunities as feedstocks for plastics and antigen delivery systems; the utility of both oil and meal is essential for complete utilization of the seed products, providing greater sustainability.

B. carinata has a very low level of saturated fat – lower even than canola oil, an oil that provides one of the best biodiesel fuels. In conventional biodiesel, saturated fats are responsible for crystal formation that leads to clouding and filter plugging in cold temperatures, making carinata oil a preferred feedstock for biodiesel in colder climates. The oil contains over 40% erucic acid, a C22 monounsaturated fatty acid. Traditional aviation fuel is composed of C9 through C13 molecules and the longer carbon chain length of carinata oil allows for more efficient conversion into aviation fuel with reduced amounts of low-value secondary products such as liquefied petroleum gas (LPG) and naphtha when compared to other feedstocks used. Furthermore, carinata's long-chain fatty acids are used in multiple industrial applications that include high performance lubricants, coatings and use as a feedstock to create chemical intermediates. Carinata oil is believed to be a sustainable source of these fatty acids for the emerging sustainable or 'green' chemistry industry.

Oil composition comparison of Brassica species revealed that oleic acid content varied from 57.85 to 64.63% in *B. napus*, 12.46 to 16.69% in *B. juncea*, 8.42 to 10.69% in *B. carinata*, 14.21 to 17.81% in *B. oleracea*, 12.47 to 14.05% in *B. nigra*, and 13.78 to 26.72% in *B. rapa*. Oleic acid is found to be one of the main unsaturated fatty acids playing fundamental role in human nutrition. In the present study, *B. napus* showed higher oleic acid content but the most remarkable variation for oleic acid was found in *B. rapa* (Table 4). Other species showed no clear differences among accessions. High oleic acid contained oils are greatly resistant to heating and oxidation and suitable for wide variety of uses. Linoleic acid content in the studied germplasms varied 11.25 to 22.39%. Linoleic acid content varied from 15.71 to 18.41%, 17.46 to 22.39%, 15.03 to

18.86%, 11.44 to 13.30%, 15.99 to 18.38%, and 11.25 to 17.69% in the *B. napus*, *B. juncea*, *B. carinata*, *B. oleracea*, *B. nigra*, and *B. rapa*, respectively. Linolenic acid content were varied from 4.87 to 8.42% in *B. napus*, 18.35 to 22.80% in *B. juncea*, 18.20 to 18.53% in *B. carinata*, 14.76 to 15.82% in *B. oleracea*, 17.66 to 20.03% in *B. nigra*, and 14.95 to 17.74% in *B. rapa*. The highest within variation for palmitic acid content was observed in *B. oleracea* (2.17 to 4.13%). Erucic acid content varied from 0 to 3.30%, 23.75 to 38.19%, 38.89 to 45.23%, 46.25 to 46.38%, 34.76 to 35.50%, and 24.89 to 48.15% in *B. napus*, *B. juncea*, *B. carinata*, *B. oleracea*, *B. nigra*, and *B. rapa*, respectively. Erucic acid is one of the most important fatty acids in mostly found within Brassica genus but this 22-carbon fatty acid is harmful to the human health.

For biofuel manufacturers, *B. carinata* provides a lower-cost, uniform, long-chain monounsaturated fatty acid profile that enables additional efficiencies and returns from biofuel manufacturing processes. Resonance carinata oil has been used for advanced biofuel manufacturing as well as traditional biodiesel. It makes an excellent biodiesel and provides a very useful blend stock for improving the quality of biodiesel made from lower quality feedstocks. In addition to its performance as a biofuel and aviation fuel, using carinata has further benefits. Carinata fuels need only a minimal amount of refining once the oilseeds are crushed and filter. Additionally, it fits well within different crop rotations, enabling efficient and sustainable use of land resources. The agronomic performance and the energetic balance that *B. carinata* adapted better and was more productive both in adverse conditions (clay- and sandy-type soils and in semi-arid temperate climate) and under low cropping system when compared with *B. napus*. The biodiesel, produced by trans-esterification of the oil extracted from the *B. carinata* seeds, displayed physical–chemical properties suitable for the use as diesel car fuel. A comparison of the performance of *B. carinata* oil-derived biodiesel with a commercial biodiesel and petroleum diesel fuel was conducted as regards engine performance, regulated and unregulated exhaust emissions.

Genetic enhancement in *B. carinata* for agronomic limitations

B. carinata possesses many desirable characteristics that are rare in the other Brassica oil crops: heat and drought tolerance, tolerance to various biotic and abiotic stresses and availability of yellow-seeded germplasm (Jiang et al. 2007). In spite of these positive attributes, the crop suffers from many agronomic limitations like longer crop duration, poor harvest index, low oil content. This species is associated with some inherent undesirable attributes like long maturity duration, tall stature, poor harvest index, small seed size and low oil content. To harness the potential of this crop, hence, there is a need for genetic enhancement for desired traits including short maturity duration, short plant height, seed size and oil content.

Interspecific and wide hybridizations

The seed of *B. carinata* has a high 2-propenyl glucosinolate content, which is undesirable. The studies have been done to introgress, through interspecific crosses, genes for low 2-propenyl glucosinolate content from the B genome of *B. juncea* and C genome of *B. napus* into the *B. carinata* B and C genomes and thus develop low glucosinolate *B. carinata*. The cross [(*B. carinata* × *B. juncea*) × *B. carinata*] yielded

plants that contained only ~ 20 µmoles of 2-propenyl glucosinolate, which was an 85% reduction compared with levels in *B. carinata* seed. Plants of the [(*B. carinata* × *B. napus*) × *B. carinata*] cross had normal high concentrations of 2-propenyl glucosinolate. Backcross plants of both interspecific crosses also contained 3-butenyl and 2-hydroxy-3-butenyl glucosinolates. The results of these crosses suggested that genes for glucosinolate synthesis were located on B genome chromosomes of *B. carinata* because *B. napus* C genome introgressions did not result in reductions of total glucosinolate contents. The crossability between *Brassica carinata* (BBCC, 2n=34) and *B. rapa* (AA, 2n=20), and the cytomorphology of their F₁ hybrids were studied. Hybrids between these two species were only obtained when *B. carinata* was used as the female parent. The hybrid plants exhibited intermediate leaf and flower morphology, and were found to be free from white rust and *Alternaria* blight diseases. One of the four F₁ plants was completely male sterile, while the remaining plants had 4.8, 8.6, and 10.9% stainable pollen, respectively. No seed was produced on hybrid plants under self pollination or in backcrosses; but seed was obtained from open pollination.

B. tournefortii (2n=20), known variously as wild turnip or Sahara mustard or Asian mustard or Tournefort's mustard or Tournefort's Birdrape, grown sporadically in a few pockets of arid and semi-arid areas, has a strategy of early and quick growth because this effectively captures available soil moisture, enables it to build a canopy, reproduce and mature before neighbouring species (Minnich and Sanders 2000). It has also been reported as having a good source of tolerance against aphids (Singh et al. 1965) and drought (West and Nabhan 2002). However, lower test weight (2–3 g/1000 seed) and susceptibility to downy mildew (Rajpurohit and Choudhary 1995) are the major constraints in the spread of this species. The hybrid from cross *B. carinata* × *B. tournefortii* was obtained only when *B. carinata* was used as a female parent. This was not anticipated as in the majority of interspecific crosses reported in Brassica, there was greater success when the species with the higher basic chromosome number was used as a female (Quazi 1988, Choudhary and Joshi 1999). Nishiyama et al. (1991) reported that crosses between tetraploid and diploid species were partly successful, while the reciprocal, i.e. diploid × tetraploid, usually failed. The high level of pairing observed in the hybrid might be due to auto/allosyndesis pairing within and among the genomes. This might be attributed to structural similarities and duplications of some of the chromosomes (Röbbelen 1960). However, the heteromorphic appearance of bivalents in some of the PMCs observed and the presence of only 5 univalents, instead of the 27 expected, indicated the extent of homology among B, C and T genomes. Bridges at anaphase I observed in the present material might have resulted from pairing between partially chromosomes of the parent genomes. Such inter-genomic pairing in Brassica hybrid was also observed by Howard (1938). The occurrence of multivalent association at a high frequency in the hybrid studied indicated that parts of the chromosomes of these genomes possess sufficient affinity to allow multivalent association (Newell et al. 1984). This showed that some of chromosomes of these 3 genomes had duplicate archetypes and confirmed the hypothesis of the secondary polyploidy origin of the B, C and T genomes. Secondly, the lower average chromosome association observed in the

present BCT hybrid, compared to that reported in an ABC hybrid by Choudhary et al. (2000b), suggested that the T genome was more distantly related than A to both the B and C genomes. Inter-genomic chromosome associations noticed in the hybrid indicated the possibility of transferring genes across the species through interspecific hybridization.

Currently, the development of genotypes with very high erucic content is a priority in Brassica breeding for industrial purposes

Erucic acid (*cis*-docosa-13-enoic acid, 22:1 Δ 13) is the major VLCFA in the seed oil from HEAR (high erucic acid rapeseed) *B. napus* cultivars, accounting for 45–55% of the total fatty acids. HEAR cultivars are of high interest since 22:1 is a valuable feedstock with more than 1,000 patented industrial applications. Currently the major derivative of erucic acid is erucamide, which is used as a surface-active additive in coatings and in the production of plastic films as an anti-block or slip-promoting agent. Many other applications are foreseen for erucic acid and its hydrogenated derivative behenic acid, for example, in lubricants, detergents, photographic film-processing agents, coatings, cosmetics, and pharmaceuticals. Studies have confirmed that HEAR oil and its derivatives have a higher energy potential than low erucic acid oil. HEAR oils are more suitable for biodiesel production than low erucic *Brassica* oils, because the iodine value is lower in the former (i.e., lower proportion of polyunsaturated fatty acids in the oil) and within the European Union specifications. US industry uses 18 million kg of HEAR oil annually, mostly from imports, but supplies are historically limited. Therefore, a large overall market potential exists for expansion and development of new, annually renewable domestic sources of erucic acid oil.

B. carinata is an attractive host for genetic manipulations to produce triacylglycerols high in strategic VLCFAs (very long carbon chain fatty acids) such as erucic acid for several reasons: (1) It is naturally high in erucic acid (about 35–40%) depending on the breeding line; (2) The proportions of [18:2+18:3], 30–37% in *B. carinata*, are unusually high among HEA Brassicaceae and therefore are amenable to desaturase gene silencing approaches, allowing one to reduce the proportions of 18:2 and 18:3 and re-direct the 18:1 to VLCFA production as was shown in the present study; (3) the rate of out-crossing with other HEA species is usually low (Warwick and Black, 1993) and therefore does not require growth in isolation from HEA or canola quality *B. napus*, *B. rapa* or *B. juncea*; (4) *B. carinata* can be grown in more arid conditions and on more marginal agricultural land; this would allow specialty oilseed crop acreage to spread out along areas approaching desertification.

The oleate desaturase FAD2 (EC 1.3.1.35; 1-acyl-2-oleoyl-sn-glycero-3-phosphocholine desaturase) is one of the most important enzymes for the production of polyunsaturated fatty acids in plants (Okuley et al., 1994). By altering the level of expression of the microsomal oleate desaturase encoded by the FAD2 gene in a seed-specific manner, it is theoretically possible to increase the pool of 18:1 which is available for chain elongation by the fatty acid elongase complex to produce more erucic acid. Silencing of endogenous FAD2 gene in *B. carinata* by co-suppression. When using *B. carinata* for genetic transformation, the co-suppression approach is

attractive, because the high transformation rate in this species affords the opportunity to generate the high numbers of transgenic lines necessary to observe a co-suppression event (Babicet al., 1998).

B. carinata is well suited for genetic engineering, and the generation of transgenics will play a major role in designing this crop for the delivery of VLCFA-enhanced oil feedstocks for bio-products. To enhance the 22:1 or 24:1 acid content of *B. carinata* oil, we selected two nontraditional sources of genes encoding ketoacyl-CoA synthases (KCS), the KCS being the first of four enzymes in an elongase complex that ultimately converts 18:1 Δ^9 (oleoyl)-CoA to erucoyl-CoA and then nervonoyl-CoA by successive cycling of the complex, with malonyl-CoA providing the two carbons needed for each extension cycle. These gene sources were *Crambe abyssinica* and *Cardamine graeca*.

It was proposed that oleate availability may be a rate-limiting factor in the biosynthesis of erucic acid. We have tried to address this question by manipulating the expression of the endogenous FAD2 gene in *B. carinata* using co-suppression and antisense approaches. Both methods resulted in transgenic lines exhibiting decreased proportions of polyunsaturated C18 fatty acids (18:2+18:3) and concomitant and significantly increased proportions of 18:1, 22:1 and total VLCFAs. Co-suppressed FAD2 *B. carinata* lines exhibited 3-18% decreases in 18:2, 22-49% decreases in 18:3 and significantly increased proportions of 18:1 (36-99%), 22:1 (12-27%) and VLCFAs (6-15%). Transgenic *B. carinata* lines developed using an antisense FAD2 approach exhibited decreased proportions of 18:2 and 18:3 (9-39% and 33-48%, respectively) and significantly increased proportions of 18:1 (54-130%), 22:1 (5-19%) and VLCFAs (6-21%).

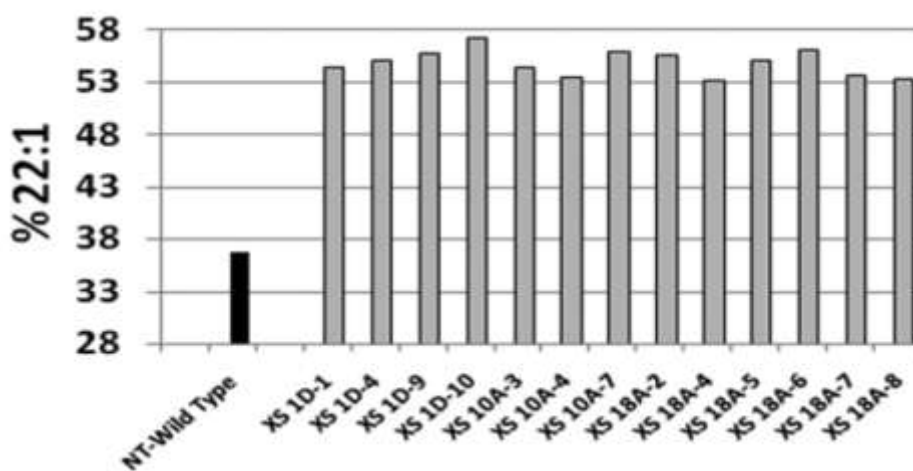
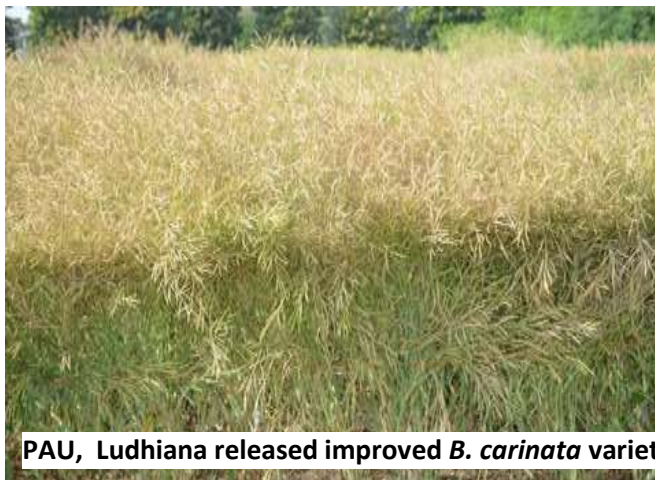


FIG. 2. Erucic acid (22:1) proportions (% wt/wt) in mature seeds of non-transformed *B. carinata* wild type control (NT-Wild Type, black bar) and T_2 seeds of *B. carinata* transgenic lines carrying the Crambe KCS (lines XS 1D-1 through XS 18A-8; grey bars) under control of the seed-specific napin promoter.

At Punjab Agricultural University, the work on genetic improvement of *B. carinata* has been taken up in 1990s. Under the mentoring of ICAR National Professor Dr SS Banga, PAU has commercially released determinate, short stature variety PC-6. It is



PAU, Ludhiana released improved *B. carinata* variety PC

world's first determinate oilseed Brassica variety. PC 10 showed yield superiority of 8.5 % (770kg/acre) over the check variety PBR 357 (710kg/acre) on the basis of research and adaptive trials conducted in the state. Due to its determinate plant growth habit and medium stature, the variety is suitable for combine harvesting. The oil content is 40.1% as against 36.9% of PBR 357. PC 6 remains free of

white rust and is better than mustard checks for its reaction to aphid infestation

Under ICAR sponsored Prebreeding project on genetic enhancement of *B. carinata*, two approaches, Interspecific hybridization (*B. carinata* x *B. juncea*) and population improvement were initiated in 2009-10 at DRMR, Bharatpur to enrich *B. carinata* gene pool for desired traits including short maturity duration, short plant height, seed size and oil content. This research presented the development of derived stable (at F₅ generation) short stature, determinate plant type (making amenable to mechanical harvest), early maturing, 109 improved *B. carinata* lines. The agronomic and biochemical evaluation of these lines with commercially released *B. carinata* variety PC-6 led to the documentation of potential lines which can be further exploited for commercial breeding. The oil content in these lines varied from 31.26 to 41.66 percent and saturated fatty acids (SFA) less than five per cent. Low levels of SFA in Ethiopian mustard lines make them sustainable and green energy source of aviation fuel. In conventional biodiesel, saturated fats are responsible for crystal formation and hence, unsuitable for colder climates, leading to problems of clouding and filter plugging. In developed lines, erucic acid (C_{22:1}; monounsaturated fatty acid) varied from 40 to 48%. Though, high levels of erucic acid is undesired as edible oil for human consumption but the longer carbon chains allow more efficient conversion into aviation fuels and emit fewer particulates than conventional oil. The developed lines infused with desired agronomic traits presented genetic stock for environmentally sustainable source of renewable fuel.



Derived *B. carinata* lines with improved agronomic attributes

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ALIEN INTROGRESSION FOR ENHANCING RESISTANCE TO BIOTIC AND ABIOTIC STRESSES IN *BRASSICA JUNCEA*

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Abstract

Wild *Brassicaceae* species constitute vast reservoirs of genetic diversity for resistance to many biotic and abiotic stresses. Crops Brassicas can tolerate alien introgressions due to polyploid nature of their genomes. Development of wide hybrids has been facilitated by the availability of embryo rescue and protoplast fusion techniques that has allowed bypassing sexual constraints to wide hybridization. Developed synthetic amphidiploid lines, alien gene introgression lines, alloplasmic lines have been produced and characterized at phenotypic and genotypic levels using next generation sequencing techniques. Linkage drag remains a problem for commercial exploitation of available introgressed genetic variations. Practical examples are discussed.

Era of the cytogenetics research in *Brassica* was kickstarted by the determination of chromosome number for *Brassica rapa* (syn. *B. campestris*) by a Japanese researcher Takamine in 1916. This is in spite of the fact that reports regarding interspecific and intergeneric hybrids appeared much earlier (*Rapahanus sativus* x *B. oleracea*, *B. napus* x *B. rapa*). Hybridization between different species and study of their meiotic behavior helped Morinaga (1928-1934) to unravel the genetic architecture of crop *Brassicas*. The high chromosome species *B. carinata*, *B. juncea* and *B. napus* are chromosomally balanced with a regular bivalent forming regime devoid of any homologous pairing. Their allopolyploid nature has been confirmed and there exist many reports on their artificial synthesis, nuclear DNA content, RNA genes (Quiros *et al* 1987), nuclear DNA markers and genomic *in situ* hybridization. Research involving Fraction-1 protein, chloroplast and mitochondrial DNA analysis also revealed the directionality of natural hybridizations. These studies established *B. nigra* and *B. rapa* as the cytoplasmic donors of *B. carinata* and *B. juncea* respectively. Chloroplast DNA restriction patterns of *B. napus* were different from both *B. rapa* and *B. oleracea* but were more similar to *B. oleracea*. A detailed study by Song and Osborn (1992) based on the RFLP pattern of chloroplast and mitochondrial DNA provided strong evidence for multiple origins of *B. napus* and suggested that a closely related ancestral species of *B. rapa* and *B. oleracea* identical to *B. montana* was the cytoplasmic donor of most of the *B. napus* accessions. Studies based on chloroplast specific markers have also shown that *B. juncea* originated several times with both *B. rapa* and *B. nigra* as cytoplasmic donors in separate hybridization events (Kaur *et al* 2014).

Use of interspecific or intergeneric hybridization

During the 1970s, wild germplasm of *Brassica* and related genera was extensively collected and cytogenetical studies were initiated. The theme of research during initial years centered around the polyploidy breeding. The priorities were subsequently shifted to exploitation of wild allies for introgression of nuclear genes for desirable agronomic

traits, cytoplasmic substitutions and construction of chromosome maps, thus, taking advantage of development in the areas of somatic cell and molecular genetics. This was aided by the discovery of *in vitro* techniques such as ovary and embryo culture, and somatic hybridization. Somatic hybridization not only overcomes reproductive barriers but also generates cytoplasmic variability which is not possible through the conventional method of sexual hybridization. This strongly helped in gearing up of the research on alien introgressions.

The *Brassica* coenospecies

The *Brassica* coenospecies encompasses those genera and species which are genetically related to crop *Brassica* and are capable of exchanging genetic material with them (Harberd 1976). He classified this germplasm biologically into cytodemes or crossing groups taking into consideration chromosome, number and pairing, and extent of fertility in the hybrids. He included 91 species belonging to 9 genera of the sub-tribe *Brassicae* viz. *Brassica*, *Diplotaxis*, *Erucastrum*, *Eruca*, *Sinapis*, *Coincya* (syn. *Hutera*), *Hirschfeldia*, *Trachystoma* and *Sinapidendron* but excluded the genus *Reboudia*. Two more genera from the related subtribe *Raphaninae* viz. *Raphanus* and *Enarthrocarpus* were also included. Harberd (1976) recognized 44 cytodemes including the crop species. Six cytodemes have two species each and a large number contain only one single species. Two cytodemes include more than six species each, these are *Coincya* (syn. *Hutera* and *Rhynchosinapis*) and *B.oleracea*.

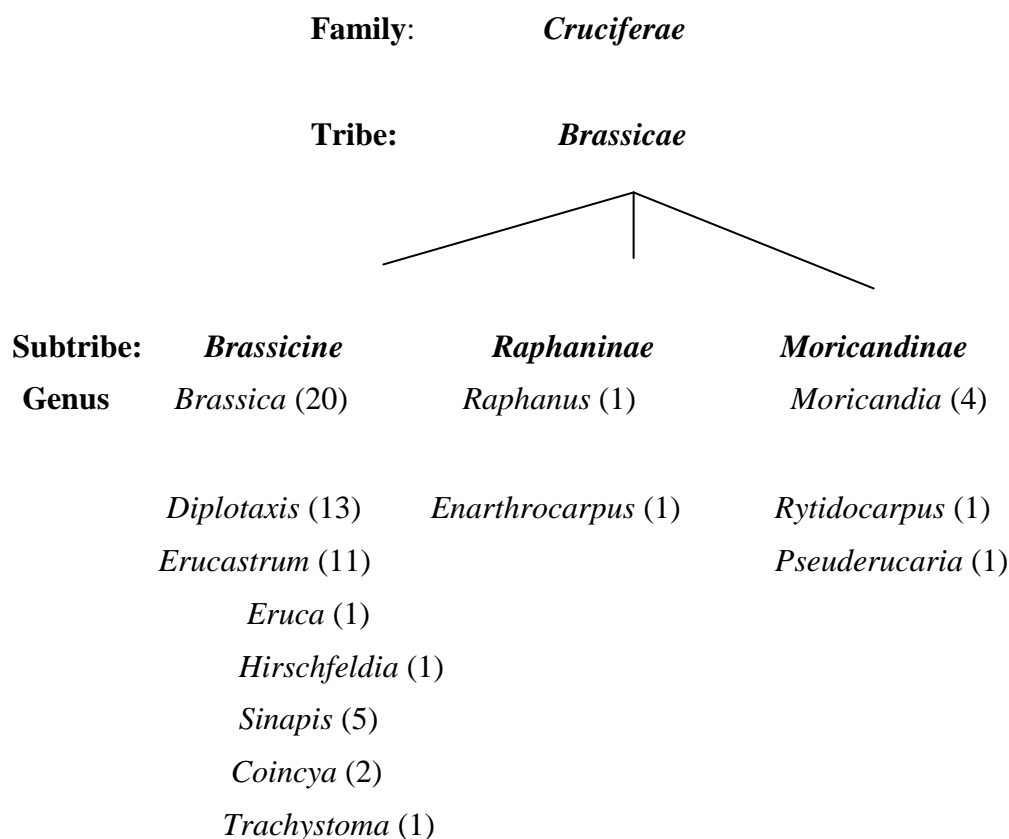


Figure 1: Architecture of *Brassica* coenospecies (number of cytodemes in paranthesis)

Takahata and Hinata (1983) further extended this study and added a few more cytodesmes in recent years. The lowest gametic chromosome number in the group is $n=7$ and is characteristic of seven cytodesmes. Harberd (1976) was of the view that all cytodesmes of $n=13$ or less were diploids (only one, that of *Diploaxis harra* is $n=13$), while cytodesmes are diploids with $n=14$ or higher chromosomes are polyploids. A total of 43 cytodesmes are diploids where every number from $n=7$ to $n=13$ is represented. Around 50% of these cytodesmes have gametic chromosome number $n=9$ and $n=10$. Polyploidy is not uncommon and is characteristic of 20 additional cytodesmes comprising both auto and allopolyploids. Level of ploidy is known to exceed tetraploidy only in *Moricandia* (6x, 8x) and *Brassica repanda*.

Wide hybridization

Genetic enrichment of crop species from their wild relatives is currently one of the major approaches in crop improvement programs. Extensive genetic diversity occurs in the wild and weedy germplasm of *Brassica* allies for nuclear and cytoplasmic genes. This germplasm occurs in nature in a vast stretch from the western Mediterranean area to the eastern end of the Sahara desert in the north-west of India. It occupies very diverse ecological landscapes such as coastal dunes, slopes of coastal volcanos, stony pastures and arid to semi-arid regions (Tsunoda 1980). Wild allies possess number of agronomically useful traits which include nuclear gene encoded resistance to diseases and insects, intermediate C3-C4 photosynthetic activity, tolerance to cold, salt and heat.

Brassica crops host an array of pathogens and pests and since wild taxa are potentially capable of exchanging genetic material with them, breeders are turning more and more towards this variability for introducing desirable genes into existing crops. Hybridization between wild and crop *Brassica* is a pre-requisite for their exploitation in any breeding program. As the wild germplasm belong to the secondary and tertiary gene pool, several kinds of hybridization barriers operate. Barriers operate either at the time of fertilization or after fertilization depending upon the extent of reproductive isolation - more diverse the species, earlier is the stage of operation of barriers. Pre-fertilization barriers include pollen germination, pollen tube entry in the stigma and growth of the pollen tube through the style. Several crosses show unilateral incompatibility i.e. pollination is effective only in one direction while the reciprocal crosses show strong pre-fertilization barriers (Shivanna 1996). In general, success is greater when wild species are used as female parents. Post-fertilization barriers cause embryo abortion leading to formation of shriveled or rudimentary seeds, without the embryo. In recent years *in vitro* fertilization has effectively been used to raise several intergeneric hybrids (Zenkeller 1990). Embryo rescue has been the most effective technique, pioneered by Japanese scientists to overcome post-fertilization barriers and is very widely used to raise hybrids. This phenomenon covers all the techniques which are used to promote the growth of hybrid embryo and includes ovary culture, ovule culture and sequential culture. As a result, a large number of hybrids have been obtained and extensively studied for their meiotic chromosome behaviour.

Many of the wild species are genetically very distant and sexually incompatible with the crop species, thus making the genes of wild taxa inaccessible. Somatic

hybridization overcomes the incompatibility barriers in such situations. Cell fusion also allows the generation of extensive cytoplasmic heterogeneity through organelle reorganization and recombination. Many somatic hybrids have been obtained combining wild and crop species representing interspecific, intergeneric and intertribe combinations. Though the initial synthesis was for demonstration, subsequently the objective shifted towards their practical utilization to introduce nuclear and cytoplasmic genes. The desirable traits for introgression include the C3-C4 photosynthetic system (*Moricaandia arvensis*), high nervonic acid content (*Thlaspi perfoliatum*), club root resistance (*Raphanus sativus*), *Alternaria* leaf spot resistance (*Sinapis alba*, *Camelina sativa*), nematode resistance (*Sinapis alba*) etc.

Introgression of genes

Several fungal diseases cause heavy losses to seed yield in *Brassica* crops. The major ones are white rust caused by *Albugo candida*, *Alternaria* leaf spot (*Alternaria* spp.) blackleg or stem canker (*Leptosphaeria maculans*) and *Sclerotinia* stem rot (*Sclerotinia sclerotiorum*). Nuclear genes conferring resistance to these diseases and for other desirable agronomic traits have been incorporated from related sources exploiting non-homologous recombination following sexual/ somatic hybridization and also by generating alien chromosome addition lines. Successful introgression of genes following sexual hybridization are blackleg resistance from *B. juncea* to *B. napus* (Roy 1984) and from *B. nigra* to *B. napus* (Chevre *et al* 1996, 1997); club root resistance from *B. napus* to cabbage (Chiang *et al* 1977); resistance against *Phoma lingam* from *B. juncea* to *B. napus* (Sacristan and Gerdemann 1986) and from *B. nigra* to *B. napus* (Struss *et al* 1996). Examples of introgression through somatic hybridization include beet cyst nematode resistance (*Heterodera schachtii*) from *Sinapis alba* and *Raphanus sativus* to *B. napus* (Lelivelt *et al* 1993; Lelivelt and Krens 1992); *Alternaria* leaf spot resistance from *S. alba* to *B. napus* (Primard 1988); club root resistance (*Plasmodiophora brassicae*) from *Raphanus sativus* to *B. oleracea* var. *botrytis* (Hagimori *et al* 1992) and blackleg resistance from *B. nigra* to *B. napus* (Gerdemann *et al* 1994).

Case studies from Indian mustard (*B.juncea* L.Coss.)

Indian mustard is highly susceptible to *Sclerotinia* stem rot and mustard aphid. Our group utilized wild *Brassicaceae* species with excellent resistance against stem rot to develop sets of *B.juncea* - *B. fruticulosa* and *B. juncea* - *Erucastrum cardaminoides* introgression lines (ILs). These were assessed for cytogenetic stability and resistance responses to *Sclerotinia* following stem inoculations under disease conducive conditions. *B. juncea* - *B. fruticulosa*. ILs showing normal fertility and genome size were analysed for introgressions using florescent genomic *in situ* hybridization (Fl-GISH). *B. fruticulosa* segment substitutions were indicated in 28 ILs. These were predominantly terminal and located on B-genome chromosomes. In contrast, *E. cardaminoides*, were primarily located on A-genome chromosomes. A final set of 93 highly fertile and euploid (2n=36) *B. juncea* - *B. fruticulosa* ILs were assessed for resistance using stem inoculation technique against a virulent pathogen isolate. Many ILs showed significant levels of resistance, with IL-43, IL-175, IL-215, IL-223 and IL-

277 being best over three crop seasons. These were first genotyped with 202 transferable and 60 candidate gene based markers. Initial association mapping allowed detection of ten significant marker trait associations (MTAs). Predicted genes were: *CNU-m157-2*, *RA2G05*, *CNU-m353-3*, *ACMP00454-2*, *EIN2-3-1*, *M641-1*, *Na10D09-1* and *Na10D11-1*. To further refine genotyping, we resequenced (21x) four ILs and mapped these to *B. juncea* genome v1.5. Sequences were then used to create *B. juncea* pseudomolecules, by replacing SNPs of reference *B. juncea* with those of re-sequenced ILs. Genotyping by sequencing (GBS) was carried out for remaining 88 ILs. Resultant sequence tags were then mapped on to the developed *B. juncea* pseudomolecules and SNP genotypes prepared for each IL. Genome wide association studies helped to map resistance responses to *Sclerotinia* stem rot. After adopting the threshold $-\log_{10}(p)$ value (>3) and clumping of closely located SNPs, a total of 13 significant loci were located on seven *B. juncea* chromosomes (A01, A03, A04, A05, A08, A09 and B05). Annotation of the genomic region around identified SNPs facilitated prediction of candidate genes encoding disease resistance protein families, including TIR-NBS-LRR class, chitinase, malectin/receptor-like protein kinase, defensin-like (DEFL), desulfoglucosinolate sulfotransferase protein and lipoxygenase.

Next, we explored a set of *Brassica juncea* – *Erucastrum cardaminoides* introgression lines (ILs) to identify the genomic regions associated with *Sclerotinia* stem rot (*Sclerotinia sclerotiorum*) resistance from a wild *Brassicaceous* species *E. cardaminoides*. ILs were assessed for their resistance responses to stem inoculation with *S. sclerotiorum*, over three crop seasons (season I, 2011/2012; II, 2014/2015; III, 2016-17). Initially, ILs were genotyped with transferable SSR markers and subsequently through genotyping by sequencing. SSR based association mapping identified six marker loci associated to resistance in both A and B genomes. Subsequent genome-wide association analysis (GWAS) of 84 ILs recognized a large number of SNPs associated to resistance, in chromosomes A03, A06 and B03. Chromosomes A03 and A06 harbored the maximum number of resistance related SNPs. Annotation of linked genomic regions highlighted an array of resistance mechanisms in terms of signal transduction pathways, hypersensitive responses and production of anti-fungal proteins and metabolites. Of major importance was the clustering of SNPs, encoding multiple resistance genes on small regions spanning approximately 885 kb region on chromosome A03 and 74 kb on B03. Five SNPs on chromosome A03 (6,390,210-381) were associated with LRR-RLK (receptor like kinases) genes that encode LRR-protein kinase family proteins. Genetic factors associated with pathogen-associated molecular patterns (PAMPs) and effector-triggered immunity (ETI) were predicted on chromosome A03, exhibiting 11 SNPs (6,274,763-994). These belonged to three R-Genes encoding TIR-NBS-LRR proteins. Marker trait associations (MTAs) identified will facilitate marker assisted introgression of these critical resistances, into new cultivars of *B. juncea* initially and, subsequently, into other crop *Brassica* species.

Mustard aphid is a major pest of *Brassica juncea*, an important edible oilseed crop. We examined *Brassica juncea*- *B. fruticulosa* introgression lines (ILs), developed through hybridization of susceptible *B. juncea* with aphid resistant *B. fruticulosa* for their

resistance responses. These varied for aphid population per plant, percent plant infestation and aphid infestation index under aided epiphytotic conditions. Genome wide association studies (GWAS) allowed identification of significant association of the aphid resistance indicators with the regions on chromosomes A02, A03, A06, A09, B001, B03, B07 and B08. These explained 11.49 to 16.04 percent of the phenotypic variation. Functional annotation predicted 19 genes in the genomic proximity (0.897-27.33 kb) to the significant SNPs. Most of these genes were associated with sugar signalling pathways, cellular responses to sulphur deprivation and toxic substances, wounding responses, besides role in the production of defensive metabolites or cellular regulatory components of signal transduction to herbivory. We also identified catalase (CAT), guaiacol peroxidase (POD), NAD(P)H oxidase and total phenols as determinants of resistance. POD and total phenols were negatively correlated with aphid population per plant. Genome-wide association studies allowed identification of seven resistance associated SNPs on different *B.juncea* chromosomes. These were predicted close to peroxidase genes, *PER65* and *MUA22.11*. Ferric reductase oxidase (*FRO7*) gene was envisaged for NAD(P)H oxidase on chromosome B05. SNPs significant for H₂O₂ were located on chromosomes A02, A03, A08, A09 and B01. Pertinent gene encoding phenolic constituent, phenylalanine ammonia lyase 3 (*PAL3*) was predicted on B05 at 13.8 kb from associated SNPs. Transcriptome analysis indicated higher relative expression for NADH kinase 3 (*NADK3*), respiratory oxidative burst homolog D (*RbohD*), peroxidase (*PER65*, *MUA22.11*), glyceraldehyde-3-phosphate dehydrogenase (*GAPCI*) and *PAL3* in the resistant ILs. CAT, POD and total phenols imparted resistance, while NAD(P)H oxidase was associated with susceptibility. Excellent results for resistance to high temperature and frost have been obtained following screening of *B.juncea* x *Erucastrum abyssinicum*//*B.juncea*(JA) and *B.juncea* x *Diplotaxis tenuisiliqua*//*B.juncea* (JT) introgression lines.

Conclusions

Brassica and allied genera from a gene pool which can be easily manipulated genetically and chromosomally. As these are very amenable to *in vitro* techniques it is comparatively easier to develop introgression conduits. Availability of rapid sequencing techniques has also opened up the possibilities of identifying the sites of introgression and its genomic content. This will permit the use of introgressed variation for crop improvement.

Suggested references

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