WAGENINGEN UR
For quality of life

Traditional plant breeding methods

Clemens van de Wiel, Jan Schaart, Rients Niks & Richard Visser



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Cover photographs: cut-style pollination, courtesy of Jaap van Tuyl, Wageningen UR Plant Breeding.

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Abstract

The aim of this report is to present an overview of 'traditional' plant breeding techniques, that is, 'traditional' in the sense that they do not lead to plants/varieties covered by the EU directive 2001/18/EC on the deliberate release of GMOs into the environment. Therefore, the term 'traditional' as used here is not implying the absence of modern developments or any lack in sophistication in some of the techniques described. The following categories of techniques are discussed:

- Techniques for overcoming incompatibility barriers, both self-incompatibility and incompatibility in wide crosses between different species (also called incongruity), such as bridge crosses, techniques involving various sorts of treatments of male and/or female flower parts, and *in vitro* techniques like ovary/ovule culture and embryo rescue.
- Techniques of chromosome and genome manipulation, such as increasing (polyploidization) or decreasing (haploidization) the number of genomes, various methods of partial genome transfer, including chromosome addition and/or substitution lines, translocation breeding, mutagenesis and cell fusion, involving exchange of nuclear and cytoplasmic genomes (mitochondrial and/or chloroplastic).
- Miscellaneous methods, such as grafting, hybrid cultivar breeding involving a.o. cytoplasmic male sterility (CMS), marker-assisted breeding (MAB), *in vitro* tissue culture, and adaptation of sex expression and induction of apomixis.

Where relevant, relationships/interactions between various methods are mentioned and cross-referenced. As far as possible, an indication is given to which extent the resulting products may arise spontaneously under natural conditions. Also, an indication is given of current use and the expected developments therein of the individual techniques in the near future. Both indications are summarized in Table 1. The report is completed with a glossary.

Samenvatting

Dit rapport beoogt een overzicht te geven van 'traditionele' plantenveredelingstechnieken, d.w.z. 'traditioneel' in de zin dat ze niet leiden tot plantenrassen die vallen ondere de EU richtlijn 2001/18/EG inzake de doelbewuste introductie van genetisch gemodificeerde organismen (GMOs) in het milieu. Aldus impliceert de term 'traditioneel' hier niet zonder meer een gebrek aan nieuwe ontwikkelingen of aan geavanceerdheid van de technieken die hier behandeld worden. De technieken worden besproken onder de volgende categorieën:

- Technieken voor het overwinnen van incompatibiliteitsbarrières, zowel zelf-incompatibiliteit als incompatibiliteit in kruisingen tussen minder nauw verwante plantensoorten (ook wel incongruïteit genaamd), zoals brugkruisingen, verschillende behandelingsmethoden van mannelijke of vrouwelijke bloemdelen en *in vitro* methoden, zoals vruchtbeginsel- of zaadknop-cultuur en 'embryo rescue' (*in vitro* opkweek van het embryo).
- Technieken waarmee chromosomen of genomen gemanipuleerd worden, zoals het vermeerderen (polyploïdisatie) of verminderen (haploïdisatie) van het aantal genomen, verschillende methoden voor het partiëel overbrengen van genomen, inclusief chromosoomadditie- en/of substitutielijnen, translocatieveredeling, mutagenese en celfusie, waarbij uitwisseling van kern- en/of cytoplasma-genomen (mitochondriëel en/of chloroplast-) toegepast wordt.
- Overige technieken, zoals enten, productie van hybride rassen, o.a. met gebruikmaking van cytoplasmatische mannelijke steriliteit (CMS), merkergestuurde veredeling (MAB), weefselkweek, en beïnvloeding van sexe en inductie van apomixie.

Waar van toepassing, worden relaties tussen verschillende methodes besproken. Voor zover mogelijk wordt een indicatie gegeven van de mate waarin de producten van individuele veredelingsmethoden ook onder natuurlijke omstandigheden kunnen ontstaan. Er wordt ook een indruk gegeven van de mate waarin individuele technieken gebruikt worden in de huidige veredelingspraktijk en de verwachtingen daarin voor de nabije toekomst (komende 5-10 jaar). Beide laatste aspecten zijn samengevat in Tabel 1. Het rapport eindigt met een verklarende woordenlijst (glossary).

1. Introduction

In the EU Directive 2001/18/EC on the deliberate release of genetically modified organisms (GMOs) into the environment, organisms obtained by genetic modification are distinguished from organisms obtained by 'traditional[1]' breeding methods. These 'traditional' breeding methods encompass a wide range of different techniques, which already soon after the enactment of the previous Directive, 90/220/EEC, have been tentatively listed in an EU background paper 'Current plant breeding techniques', DOC.XI/464/92. This background paper had only a limited scope and distribution and since its production, developments in breeding methods have also continued at a fast pace. Thus, the aim of this report is to further clarify what is meant by 'traditional' breeding methods, by describing classical techniques already in use at the time before the enactment of EU directive 2001/18/EC and its predecessor, 90/220/EEC, and developments since then, including new techniques.

With regard to the EU Directive 2001/18/EC, the term 'traditional plant breeding methods' is used for those techniques that are not considered genetic modification. This use of the term 'traditional' may be misleading, since in this case, the term does not imply the absence of modern developments or any lack in sophistication. In this sense, 'traditional' may entail both basic and advanced methods and generally, it would perhaps be more appropriate to make reference to all methods not involving recombinant DNA techniques. However, the latter description is actually also not entirely unequivocal or complete. For example, mutagenesis does not involve recombinant DNA techniques, but, in a strict sense, counts as genetic modification according to the definition used in Directive 2001/18/EC: 'an organism, with the exception of human beings, in which the genetic material has been altered in a way that does not occur naturally by mating and/or natural recombination'. In addition, there are also exemptions in the regulations for particular breeding techniques that conform to this definition of a GMO, such as the already mentioned mutagenesis, but which count as 'traditional plant breeding methods' as they have traditionally been used in a number of applications before the introduction of recombinant DNA methods into plant breeding and the development of Directive 2001/18/EC and its predecessor, 90/220/EEC. Therefore, in order to avoid any confusion about the contents of this report, the techniques described here are the ones to which the regulations of the 2001/18/EC directive on the deliberate release of genetically modified organisms (GMOs) into the environment are thought not to apply. Thus, there are two possibilities by which the 2001/18/EC Directive does not apply:

- The plant breeding techniques do not lead to plants falling under the definition of a GMO in article 2(2) of the 2001/18/EC directive.
- The plant breeding techniques lead to plants falling under the 2001/18/EC's definition of genetic modification, but are specifically exempted under article 3(1), as specified in Annex I B of the 2001/18/EC directive:
    - Mutagenesis (section 2.2.6)
    - Cell fusion (including protoplast fusion) of plant cells of organisms which can exchange genetic material through traditional breeding methods (section 2.2.7)

The EU background paper 'Current plant breeding techniques', DOC.XI/464/92, has served as a basis for the techniques to be discussed in the present report. Since the EU background paper is not easily retrievable from public databases, its contents have been reproduced here in Appendix I. In the present report, the range of techniques has been extended with techniques not yet treated in the EU background paper, such as cell-biological methods, like cell fusion (section 2.2.7), and auxiliary molecular-biological methods, like marker-assisted breeding (MAB) that make use of molecular/DNA markers, a.o. for selection purposes (section 2.3.6.1). For each method, a technical description is given and the current application and its relevance are discussed, including the sort of crops in which each method is applied. As far as possible, an indication is given of developments expected for the near future (5-10 years) in the concluding remarks (chapter 3); indications are also summarized in Table 1.

In the GMO definition of directive 2001/18/EC, the term 'natural' is used in relation to making the distinction between products of 'traditional' breeding methods and GMO's. In this report, we attempt to provide some more background for the interpretation of this complex term by discussing the possibilities of the spontaneous occurrence

[1] Directive 2001/18/EC also uses the term 'conventional' in this context, but for consistency throughout the text, only 'traditional' is used here.

of the products of each technique under natural conditions. More advanced plant breeding methods for example make use of sophisticated techniques to overcome incompatibility barriers to create (e.g. interspecific) hybrids (discussed in chapter 2.1). In this case, the likelihood of spontaneous occurrence under natural conditions may be related to the size of the natural (incompatibility) barrier that has to be overcome by the breeding technique. With other advanced methods that are discussed in chapters 2.2. and 2.3, there may be different relationships between the degree of sophistication of the technique and the likelihood of spontaneous occurrence under natural conditions. These points will be discussed separately for each technique at the end of the individual descriptions, as far as is possible on the basis of the available scientific literature. Indications for this occurrence under natural conditions for each technique are summarized in Table 1, together with indications for their current usage, and as far as possible that in the near future, in practical breeding.

2. Traditional plant breeding techniques

2.1 Techniques for overcoming crossability barriers

Sometimes crosses between two different plants are not successful in that they are not leading to viable offspring. This often occurs with crosses where parents are more distantly related (e.g. belonging to different species or even genera); the so-called wide crosses. This type of cross-incompatibility has also been named incongruity (Hogenboom 1973). In outbreeding crops, there is often a self-incompatibility system (SI), so that barriers will have to be overcome when self-fertilization is desired. These barriers are of a morphological nature (relative positions of mature anthers and stigmas in time or space) or of a physiological nature, the latter often encoded by so-called S-factors that inhibit self-pollen tube growth through the stigma and style (e.g. in Solanaceae and Brassicaceae). For an overview of flower parts and terminology, the reader is referred to Fig. 1 and the glossary. Wide crosses become necessary when the desired traits are not found among the crop species' primary gene pool (the set of available varieties of the crop species plus readily crossable ancestral and/or closely related wild species, Harlan & De Wet 1971). Desired traits are often disease resistances, but in ornamental crops, there is also a demand for wide crosses for increasing available variation in esthetic value of the flowers for breeding novel cultivars. Different technologies have been investigated and described which, depending on the species, might result in offspring. Below, general methods are described that have been successfully employed in and between a wide range of species. In crossing barriers, usually a distinction is made in pre- and post fertilization barriers (Stebbins 1958). Pre-fertilization barriers refer to incompatibility between pollen and/or pollen tubes and the stigmatic and/or stylar tissues of the species; techniques for overcoming these barriers are described in sections 2.1.2-2.1.6.2. Post-fertilization barriers refer to incompatibility between the developing zygote/embryo and endosperm and/or maternal tissues of the pistil; techniques for overcoming these are described in sections 2.1.6.2-2.1.6.6.

2.1.1 Bridge cross

Description of the technique

When a direct cross between two species, A and B, is not possible, an intermediate crossing with a third species, C, which is compatible with both species, may bridge the crossing barrier. First, a cross is made between A and C, and the resulting interspecific hybrid is subsequently crossed with species B. So, by indirectly crossing, the genomes or segments thereof from species A and B can be combined.

Examples of application of the technique

Tulipa gesneriana, from which many commercial cultivars are derived, is poorly crossable with *T. kaufmanniana*, which is a source of important resistances. A bridge cross with *T. greigii* as the intermediate was used to transfer genetic material from *T. kaufmanniana* to *T. gesneriana* (Van Eijk *et al.*, 1991). Likewise, with onion (*Allium cepa*) crosses with wild *A. fistulosum* did not lead to fertile progeny, probably due to differences in DNA content and concomitant chromosome length, but crosses through intermediary *A. roylei* proved possible (Khrustaleva & Kik 2000). Bridge crosses are also particularly useful where larger chromosomal differences occur, such as in ploidy levels, in the desired combination of species. For instance, new resistance genes from *Solanum bulbocastanum* against the devastating potato late blight inflicted by *Phytophthora infestans*, have been transferred by 'double-bridge' crosses between four different *Solanum* species (((*S. acaule* x *S. bulbocastanum*) x *S. phureja*) x *S. tuberosum*), the so-called ABPT crosses. In this case, diploid *S. acaule* was crossed with *S. bulbocastanum* and the resulting triploid hybrid was doubled to a hexaploid using the mitotic inhibitor colchicine (see polyploidization in section 2.2.2); the hexaploid was subsequently crossed with diploid *S. phureja*. In this way, a tetraploid hybrid was obtained that could be crossed to the crop species to introgress genes from *S. bulbocastanum* (Hermsen & Ramanna 1973). Thus, bridge crosses make it possible to exploit new sources of traits lacking in directly cross-compatible species, such as disease resistances, but they can sometimes be replaced by more efficient methods. The *T. gesneriana* x *T. kaufmanniana* cross later proved also possible in a more direct manner by the use of embryo

rescue (see 2.1.6.5) or even without the latter technique by careful selection of parental lines/genotypes to be used in the cross (Custers *et al*. 1995).

Occurrence of the products of the technique under natural conditions
In the interspecific hybrid, genomes are combined that are unlikely to become combined under natural conditions, since repeated hybridizations between species and species hybrids are necessary. Nevertheless, in view of successes in bridge crosses even without applying additional techniques, the occurrence under natural conditions is basically possible, particularly when the species' geographic distributions would somewhere overlap. For example, in mixed stands of poplar species from the section *Tacamahaca* (*Populus balsamifera* and *P. angustifolia*) and the section *Aigeiros* (*P. deltoides*) in Canada, individuals intermediate in morphology between all three species are found (Floate 2004). Likewise, in mixed stands of species of the white oak complex (*Quercus robur*, *Q. petraea*, *Q. pubescens* and *Q. pyrenaica*), hybrids between all these species can be detected and individuals with genetic admixtures associated with more than two of these species could be inferred from population-genetic analyses (see Fig. 3 in Lepais *et al*. 2009).

2.1.2 Pollination using sub- or supra-optimal stigma age, or suboptimal conditions

Description of the technique
In order to overcome incompatibility barriers posed by the stigma and/or the style, mature pollen is placed on a stigma that is immature or aged to prevent presence of active factors that inhibit pollen tube growth. Alternatively, pollination is performed at the end of the flowering season, or using plants that have been grown under suboptimal growth conditions.

Examples of application of the technique
Pollinating immature stigmas at the bud stage was shown to be effective for overcoming incongruity in citrus and in pear. Bud pollination is also used for overcoming self-incompatibility (SI), e.g. in multiplying Brassica inbred lines. Delayed pollination is used less often but worked in overcoming SI in apple. Floral aging was also shown to be helpful in improving pollen tube growth with wide crosses in lily. With regard to suboptimal conditions, pollination under high temperature in the greenhouse for instance helps in overcoming incongruity barriers in lily. Low temperature or low light intensity can weaken SI in beets or Brassicas. For a review of such methods, see Van Tuyl & De Jeu (1997).

Occurrence of the products of the technique under natural conditions
Untimely pollination could also occur under natural conditions, e.g. due to particular or suddenly changing weather conditions, or at the beginning or the end of the growing season.

2.1.3 Pollination using application of chemicals

Description of the technique
Plant growth regulators (hormones), such as the classical auxins, cytokinins and gibberellins, but also more recently discovered ones, such as the stress signaling-related salicylic acid, are applied to the plants or flowers to promote successful pollination. They can accelerate pollen tube growth or extend viability of the pistil and promote fruit and seed maturation. Also other chemicals have been used that break incompatibility.

Examples of application of the technique
Classical plant hormones have been used for promotion of successful pollination in Nicotiana, wheat, barley, potato, broad beans, tulip and lily. Also salicylic acid has been used for this purpose in cereals and legumes. Treatment of stigmas with organic solvents, such as hexane and ethyl acetate, was effective in breaking fertilization barriers in poplar (Van Tuyl & De Jeu 1997).

Occurrence of the products of the technique under natural conditions
These treatments are quite artificial and are therefore not likely to occur under natural conditions. In the case of substances that form a normal part of plant metabolism, one could hypothesize phenomena, such as variants in hormone regulation or salicylic acid production that could lead to similar results. In this regard, genetic diversity within crop species for crossability with other species is a common phenomenon (Van Tuyl & De Jeu 1997, see also the discussion about tulip wide crosses in section 2.1.1).

2.1.4 Pollination using treatment of pollen and/or pollen mixtures

Description of the technique
Temperature pretreatment of incompatible pollen can sometimes be effective in overcoming crossing barriers. Alternatively, a mixture of compatible and incompatible pollen can be used for pollination. The compatible pollen is able to germinate and penetrate the style, thereby 'clearing' the way for the incompatible pollen. The compatible pollen also is more likely to fertilize the ovules and for that reason, pre-treatment (by chemicals, using irradiation or mechanically) of the compatible pollen can be performed to reduce its fertilization efficacy. In this manner, the laborious selection of the desired progeny from the incompatible pollen among progeny of the 'assisting' compatible pollen can be made more efficient. With such pretreatment, the 'assisting' compatible pollen is called mentor pollen. Pollination can also be performed in succession, with compatible pollen one or two days ahead of the incompatible pollen. In this case, the 'assisting' compatible pollen is called pioneer pollen.

Examples of application of the technique
The mentor pollen technique was shown to work in such diverse species as poplar, apple, pear and Cucumis (Van Tuyl *et al.* 1982, Van Tuyl & De Jeu 1997). The pioneer pollen method overcame SI in apple and pear (Visser 1983).

Occurrence of the products of the technique under natural conditions
Particularly the pretreatment of the mentor or pioneer pollen to prevent fertilization makes the situation different from natural conditions. However, a combination of viable incompatible pollen with less viable compatible pollen on the stigma could in principle occur under natural conditions.

2.1.5 Pollination following treatment of the style

Description of the technique
Pollination is performed using mature pollen that is placed on a style treated to overcome the incompatibility barrier. The incompatibility reaction can be reduced or eliminated by heat treatment of the stigma surface, the style or the whole plant before pollination. The same can be achieved by irradiation of the style using ionizing radiation. Another possibility is application of an electric potential difference between pollen and style.

Examples of application of the technique
Irradiation was used successfully in overcoming SI in Nicotiana (Bredemeijer *et al.* 1981). Heating of the style was effective in overcoming crossing barriers in combinations of lily species from different sections of the genus Lilium (Van Tuyl *et al.* 1982).

Occurrence of the products of the technique under natural conditions
Strong ionizing irradiation of the style is unlikely to occur under natural conditions, but unusual temperature conditions during flowering may well occur under natural conditions.

2.1.6 *In vitro* methods for overcoming incompatibility barriers

The techniques in the following subsections 2.1.6.1 – 2.1.6.6 show a high level of sophistication involving *in vitro* methods and often are applied in an integrated and flexible approach to increase the chance of obtaining inter-

specific hybrids with incongruities at several levels (pre- and post-fertilization). The high level of manipulations needed makes occurrence of such hybrids under natural conditions unlikely for all of these techniques, although the fact that these hybrids can be created at all makes it hard to completely exclude their formation in a spontaneous way. At the same time, the very notion of their unlikely occurrence makes it difficult to verify their occurrence, as it necessitates large-scale gene flow research in the field to be able to also detect very rare hybridization events.. This aspect is discussed more fully for all the techniques of subsections 2.1.6.1 – 2.1.6.6 together at the end of this section.

2.1.6.1 Pollination following manipulation of the style

Description of the technique
Removal of the stigma or shortening of the style (cut-style method) may result in removal of inhibiting factors for incompatible pollen tube growth. Cutting a long style short may also help with pollen from short-styled species, which may therefore not be equipped for long pollen tube growth. A refinement is the grafted style method: a style with pollen germinated on a compatible stigma is cut and put on an ovary of the other desired, incompatible parent with some stigmatic exudate applied to connect the cut surfaces.

Examples of application of the technique
The cut-style method (CSM) is widely applied, e.g. in *Datura* (first reported example, from 1945), *Fritillaria*, *Lathyrus*, *Nicotiana*, maize (Van Tuyl *et al*. 1991, Van Tuyl & De Jeu 1997). For instance in lily, several crosses between species from different sections of the genus were successful by cutting the style just above the ovary. The grafted style method (GSM) gave a better seed set, but was successful in only a small percentage of ovaries (Van Tuyl *et al*. 1991, Van Tuyl & De Jeu 1997).

2.1.6.2 *In vitro* pollination

Description of the technique
Pollen is applied to the stigma of pistils that are placed *in vitro*, meaning that the flower bud is first removed from the plant and after dissection of leafy flower parts and anthers (see Fig. 1 for an overview of flower parts), is put on a tissue growth medium. Fertilized ovules can subsequently be isolated and further cultured *in vitro* in order to avoid incompatibility with surrounding maternal tissues (see 2.1.6.4). It is also possible to remove the ovary wall and/or style and stigma, and place pollen on the placenta (placental pollination) in order to completely circumvent incompatibility barriers residing in style and/or stigma. Pollen can even be brought into direct contact with ovules after removal of the ovary wall or after bringing ovules into *in vitro* culture.

Examples of application of the technique
In vitro pollination is often successfully applied in combination with the techniques of the following sections 2.1.6.3 to 2.1.6.5, ovary and/or ovule culture and embryo rescue. Examples were described in *Brassica*, *Petunia*, *Nicotiana*, and maize (Van Tuyl & De Jeu1997).

2.1.6.3 *In vitro* culture of excised ovaries

Description of the technique
After pollination, the ovary is excised from the flower and cultured aseptically on a suitable nutrient medium. When the ovary is large, cutting up into smaller parts may be necessary (called ovary-slice culture), particularly when embryo rescue needs to be performed later on (see section 2.1.6.5). Seeds that develop in the ovary can be germinated in soil. When incompatibility with surrounding maternal tissues of the ovary occurs early on, ovules can be isolated from ovaries and also be grown *in vitro* (see next section, 2.1.6.4).

Examples of the application of the technique
Ovary culture is broadly applied e.g. in *Brassica* and related genera, *Phaseolus*, ornamentals, such as *Nerine* and tulip. Ovary-slice culture was applied in the large *Lilium* flowers and germination of seeds occurred already 30-150 days after pollination (Van Tuyl & De Jeu1997).

2.1.6.4 *In vitro* culture of excised ovules

Description of the technique

Before a certain stage of development, it may not be possible to culture embryos (embryo rescue, see section 2.1.6.5) to circumvent incompatibility with maternal tissues,. In such a case, removing the ovule from the ovary and culturing it *in vitro* may be a successful alternative.

Examples of application of the technique

Ovule culture was successfully applied, after the first report in 1962 for *Papaver* by Kanta *et al.*, in tomato, *Nicotiana*, grape, and ornamentals, such as tulip, *Alstroemeria*, *Nerine*, *Lilium* and *Cyclamen* (Van Tuyl & De Jeu 1997).

2.1.6.5 *In vitro* culture of excised embryos (embryo rescue)

Description of the technique

Embryos can abort after fertilization due to incompatibility with the endosperm and/or surrounding maternal tissues of the ovule and/or ovary. This may be overcome by removing young embryos from developing ovules and growing them aseptically *in vitro* on a culture medium into intact plants. This technique is called embryo rescue. Also cut ovule halves with embryos can be used when embryos are difficult to excise. In case the excised embryo does not develop into a normal plant, the embryo can be induced to form callus, from which plants can be regenerated by organogenesis or secondary embryogenesis.

Examples of application of the technique

The first report on embryo culture stems already from 1904. The technique is very widely used: examples are *Brassica*, *Allium*, *Solanum*, *Phaseolus*, *Trifolium*, cereal species, ornamentals, such as *Rhododendron*, *Alstroemeria*, *Freesia*, *Nerine*, *Hippeastrum*, *Zantedeschia* and *Lilium* (Van Tuyl 1997, Van Tuyl & De Jeu 1997). Raising callus from embryos and subsequently regenerating plants from these calli was the only method working in a cross between tomato and *Solanum* (*Lycopersicon*) *peruvianum* (Poysa 1990).

2.1.6.6 *In vitro* fertilization

Gametes, that is, the egg cell and sperm cell protoplasts, are fused *in vitro* and the resulting zygote is raised *in vitro* to develop in a mature plant. This technique was shown to work with maize as a testing model (Kranz 1997). An alternative where sperm cells are injected into the embryosac was described for *Torenia* (Keijzer *et al.* 1988). Up till now, this technique has a highly experimental character and has apparently not been put to use in breeding practice. It is therefore not discussed further here.

Occurrence of the products of the techniques of 2.1.6.1 – 2.1.6.6 under natural conditions

The high level of sophistication of the techniques of subsections 2.1.6.1-2.1.6.6 needed to overcome incompatibility barriers makes occurrence of such hybrids under natural conditions unlikely. At the same time, the very observation that such hybrids can be produced makes it difficult to totally exclude their occurrence under natural conditions. However, such likely very rare occurrences, if ever, will be in practice hard to ascertain in the field, particularly when hybrids are not easily identifiable morphologically. As an illustration, species of the genus *Brassica* offer an interesting example. Hybrids between *B. napus* and *Sinapis arvensis* can be produced by hand pollination and for a higher efficiency, by embryo rescue, but normally, hybrid formation is extremely rare, e.g. 0 in 6420 from spontaneous progeny in mixed stands of both species and 1 in 1127 progeny from hand pollinations of *S. arvensis* x *B.napus* (Moyes *et al.* 2002). Large surveys in the field enabled by the efficiency of using transgenic herbicide tolerance as marker for hybridization in large-scale progeny screens indeed confirmed the extreme rarity of hybridization between *S. arvensis* and *B.napus* (e.g. none in Bing *et al.* 1996, Moyes *et al.* 2002 and Lefol *et al.* 1996). Nevertheless, one case of spontaneous hybridization was reported in the gene flow studies concurrent with the farm-scale evaluations (FSE) of environmental effects of GM crops in the UK (Daniels *et al.* 2005). Although not optimally documented in this case (no voucher of the plant or progeny has been kept), it implies the remote possibility of even very difficult hybridizations under natural conditions.

2.2 Techniques for chromosome and genome manipulation

Sometimes, it might be advantageous to have either an increased genome size or ploidy level, since such plants may grow larger and in that case, show a higher yield. Polyploidization can also be helpful to avoid problems during meiosis in species hybrids and hence enable their further successful reproduction (see e.g. 2.1.1). In other instances, it is beneficial to have a reduced number of genomes (haploidization). This may be necessary for wide crosses between species of different ploidy level. Haploidization then equalizes the respective numbers of chromosome complements before crossing and when necessary, polyploidization afterwards enables to regain the ploidy level of the crop species. Alternatively, reduction in the number of chromosome complements can result in homozygous plants in a subsequent doubling step, which has obvious advantages during the breeding process, e.g. for finding traits with recessive inheritance or for the production of 'immortalized' mapping populations. Additional methods that may be used for increasing the level of genetic variation, are mutagenesis or chromosomal addition/substitution or translocation methods. Also, alternative methods to combine specific nuclear and cytoplasmic genomes have been developed, e.g. for transfer of cytoplasmic male sterility (CMS), such as cell fusion. In this chapter, techniques are being described which have been successfully employed to achieve or recover fertility in interspecific crosses, to obtain homozygous material, or to tap or create new sources of variation.

2.2.1 Haploidization

Description of the technique

Plants are produced that contain half the number of chromosome complements of normal somatic cells, which are usually diploid. The method mostly involves anther culture as anthers contain a lot of microspores that underwent meiosis for the normal development of haploid pollen. The microspores themselves can also be cultured directly to form (haploid) plantlets. For crops where anther cultures (androgenesis) did not work well, regeneration of haploid plants from embryosacs in ovule or ovary culture was developed. As this usually involves parthenogenic development of the egg cell, this gynogenesis shows some similarities to developmental processes in apomixis, in which case however the egg cell has an unreduced genome (see section 2.3.5). Usually, the haploids are diploidized to obtain homozygous plants, so-called doubled haploids. Doubled haploids can be obtained by direct selection for diploids among plantlets regenerated from *in vitro* culture of anthers or ovaries using flow cytometry (FCM, see 2.3.6.3). When such direct selection does not lead to efficient isolation of doubled haploids, selfing or a mitotic inhibitor, such as colchicine, to double the number of chromosome complements (see also section 2.2.2), can be used with regenerated haploid plants. An alternative use of haploidization is with crosses between species of different ploidy levels, to lower the ploidy level of the crop species for making a wide cross with a species of a lower ploidy level (see also polyploidization, 2.2.2).

Examples of application of the technique

The generation of doubled haploids is a very efficient method for making fully homozygous lines, which otherwise could only be obtained by a far more lengthy series of propagation (selfing) rounds. In the case of strong outbreeders, such as *Brassica oleracea* and *B. rapa*, use of repeated selfing rounds to obtain inbred lines is even less efficient due to inbreeding depression. Inbred (homozygous) lines generally allow more efficient selection of favourable gene combinations than segregating populations and they are used for producing hybrid varieties (see section 2.3.2). Inbred lines can also be used for selection of recessive mutants and for the creation of 'immortalized' (QTL) mapping populations when generated from the progeny of a specific cross. Other examples of crops in which haploids are generated through androgenesis, are barley, wheat, rice, maize, tobacco and *Brassica*'s. In *Brassica*'s, direct selection of doubled haploids from microspore cultures is feasible (Gil-Humanes & Barro 2009). Anther culture was first developed in the 1960s with *Datura*, after spontaneous haploid production was already reported for *Datura stramonium* in 1922 and put to use in maize in the early 1950s by Chase (review Wędzony *et al*. 2009, see for maize also below under 'Occurrence of the products of the technique under natural conditions'). For beet, onion and cucurbits, regeneration through gynogenesis was developed as alternative for the failure of anther culture (Bohanec 2009). An exceptional way by which haploids can be generated is found in certain wide crosses (see chapter 2.1) where the chromosomes from the (wild) parent (pollen donor) are being eliminated during early growth of the hybrid

zygote (using *in vitro* culture for lack of good endosperm development, see embryo rescue 2.1.6.5). Examples are crosses of barley with *Hordeum bulbosum*, and of wheat with maize (Wędzony *et al.* 2009). With potato, so-called prickle pollination using pollen from *Solanum phureja* is applied to induce formation of dihaploids enabling to execute a breeding programme of wide crosses at the diploid level (Hutten *et al.* 1993, see also section 2.2.2).

Occurrence of the products of the technique under natural conditions
The described method of raising homozygous lines through doubled haploids involves a set of highly sophisticated *in vitro* methods. However, haploids do arise spontaneously, albeit rarely. In maize, for example, haploids occur at a rate of 1 in 1,000-2,000 progeny through maternal parthenogenetic development (Chase 1969). Subsequent chromosomal doubling likewise can occur spontaneously, again in maize, for example, in 1 of 10 haploids. Furthermore, similar inbred lines can also be produced by classical inbreeding procedures using repeated selfing, which can also spontaneously occur under natural conditions. For example, in a fine-scale study of a natural population of the selfing species *Medicago truncatula*, Siol *et al.* (2008) showed the occurrence of genotypes resembling recombinant inbred lines from hybridization between the most frequently occurring genotypes in the population.

2.2.2 Genome doubling, polyploidization

Description of the technique
Doubling the number of chromosomes is sometimes necessary to restore the fertility of plants obtained through interspecific crossing, to overcome problems with meiosis in the interspecific hybrid. It is also used with crosses between species with different ploidy levels, for example, to regenerate the ploidy level of the crop species after a wide species cross was made at a lower ploidy level (see haploidization, section 2.2.1). However, polyploidization is also applied in specific cases where plants can be obtained with superior performance. Application of chemicals (mitotic inhibitors), such as colchicine, oryzalin or trifluralin, is performed to induce chromosome doubling.

Examples of application of the technique
Examples of autopolyploids with better performance are in forage grasses, such as ryegrass, and clovers. In sugar beet, triploid hybrid varieties (see section 2.3.2) produced by crossing tetraploid and diploid inbred lines have been popular. Triploids are also popular with crops, such as watermelon, to obtain hybrid varieties with seedless fruits. A well known ancient polyploid (allopolyploid or amphidiploid) species hybrid with doubled chromosome complements is oilseed rape (*Brassica napus*), with ancestral species *B. rapa* and *B. oleracea*. This combination comprises one side of the classical triangle of U, which illustrates the hybrid relationships between the cultivated *Brassica* species (Fig. 2). In order to increase genetic diversity, *B. napus* has also been resynthesized in more recent breeding research (Chen & Heneen 1989). An important example created by more recent breeding efforts is triticale, an intergeneric hybrid of wheat and rye, for which both hexaploid and tetraploid forms of wheat were used, leading to an octoploid and hexaploid species hybrid, respectively. The hexaploid form is favoured from an agronomical point of view (Oettler 2005). Polyploidization also plays a role in species hybridization with potato: these crosses are often made at a diploid level, using dihaploids of the crop, to equalize the parents' ploidy level, and the normal tetraploid crop is recreated by chromosome doubling (see also bridge crosses, 2.1.1). Nevertheless, making such wide crosses is not very popular, because the subsequent problems with sexual reproduction and adapting ploidy level make the breeding process more cumbersome. The wide crosses are more popular in ornamental breeding (a.o. to introduce new traits of esthetic value), particularly so where the further propagation is mostly vegetative: *Alstroemeria*, *Freesia*, *Gladiolus*, *Lilium*, *Iris*, *Tulipa*, *Ornithogalum*, *Nerine*, *Narcissus* and *Chrysanthemum* (Van Tuyl 1997).

Occurrence of the products of the technique under natural conditions
Application of mitotic inhibitors clearly represents an artificial method of obtaining polyploid species (hybrids). However, such polyploidized hybrids may also arise spontaneously, e.g. through 2n (diploid) gametes. The allopolyploid *B. napus* described above and the hexaploid wheat are examples of ancient crop hybrid species from long before the era of advanced breeding. Furthermore, species formation through polyploid hybridization is not uncommon in angiosperms in nature, a recent example being *Spartina anglica* (Paun *et al.* 2007, Arnold 1997).

2.2.3 Production of alien addition or substitution lines

Description of the technique
The transfer of a single (pair of) chromosome(s) from one species to another can be useful for the introduction of desirable traits, such as disease resistances. For this, aneuploid plants are produced that contain an extra single chromosome or an extra chromosome pair from a donor plant, called monosomic or disomic addition lines, respectively. Such addition lines can be created by hybridization between donor and recipient plant lines followed by repeated backcrossing with the recipient plant line. Donor and recipient are usually from different species. In substitution lines, the introduced chromosome or chromosome pair has replaced the homoeologous pair from the recipient species.

Examples of the technique
Examples are the addition of chromosomes from *Aegilops* or barley to wheat (Islam *et al.* 1981). Substitution of one chromosome or a complete pair can also be useful, e.g. the introduction of disease resistances by the exchange of chromosome 1B of wheat for 1R of rye (Khush, 1973). As addition lines often show reproductive instability, they are mostly not of direct use in breeding. However, they can also be used for the localization of genes for particular traits to specific chromosomes. For this purpose, also other variants can be used, namely monosomic or trisomic lines within crop species. These are lines lacking one chromosome (2n-1) or having one extra chromosome of a particular pair (2n+1). Such lines can be made by applying the mitotic inhibitor colchicine, radiation (see also sections 2.2.4-2.2.6) or selection in the progeny of triploid plants (Khush 1973). Sets of such lines have been compiled that together represent the complete haploid chromosome complement of a crop species. Such sets are for instance known for oat, barley, wheat, rice, sorghum, cotton, asparagus, pepper, tomato and tobacco. Sets of monosomic or trisomic lines are laborious to maintain, involving complicated cytogenetics (microscopic chromosome counting), and with the advent of molecular marker technology (see section 2.3.6.1), dense genomic linkage maps can now be routinely made and used as a more efficient method for gene localization.

Occurrence of the products of the technique under natural conditions
Aneuploid lines can also sometimes arise spontaneously, often through hybridization under natural conditions. Like with polyploids, aneuploidy is often accompanied with an apomictic mode of reproduction (see 2.3.4).

2.2.4 Translocation breeding

Description of the technique
Chromosome translocation is effected by the interchange of parts between non-homologous chromosomes. The translocation can be induced by irradiation of a trisomic addition line (with one extra homoeologous chromosome from another species) and subsequent recovery of plants with chromosome segments of the added chromosome incorporated into their genome. Translocation can also be induced by introducing 'gametocidal' (inducing chromosome breakage) chromosomes from another species.

Examples of application of the technique
The introgression of the resistance gene against leaf rust from *Aegilops umbellulata* was stably incorporated into the wheat genome by translocation of the alien chromosomal segment harbouring the resistance gene to wheat chromosome 6B in a wheat trisomic alien addition line. The reproductively unstable wheat trisomic alien addition line was originally created by a bridge cross (see section 2.1.1), i.e. by backcrossing the hybrid of *Triticum dicoccoides* and *A. umbellulata* to wheat, which resulted in a plant containing the normal wheat chromosome complement and one *Aegilops* chromosome. Translocation could also be induced in wheat-barley addition lines by the introduction of 'gametocidal' chromosomes from *Aegilops cylindrica* (Jauhar 2006).

Occurrence of the products of the technique under natural conditions
Some of the techniques mentioned here are artificial (e.g. the use of irradiation), but translocations can also happen under natural conditions, albeit less frequently.

2.2.5 Manipulation of chromosome pairing in meiosis

Description of the technique
Reduced chromosome pairing and lack of recombination is an important problem in the production of interspecific hybrids. Pairing of chromosomes that normally show a low extent of pairing (i.e. between the homoeologous chromosomes derived from the different parental species) can be induced by specific mutations. Recombination frequencies can also be increased artificially by the use of various chemical agents, physical stress like a temperature shock or by UV irradiation. In breeding with complex allopolyploids, there is also the possibility of suppression of genes favouring homologous pairing in order to induce exchange of segments between homoeologous chromosomes (so between chromosome complements derived from different parental species). On the other hand, formation of multivalents during meiosis, particularly in newly created autopolyploids, is preferably avoided in normal propagation because of the consequent reproductive instabilities. This may be achieved by selection for improved fertility of progeny and/or induction of chromosomal changes leading to a 'diploid' (or 'allopolyploid') behaviour of the polyploid (diploidization), so that, in practice, mostly bivalents are formed during meiosis. Sometimes occurring supernumerary chromosomes (so-called B chromosomes, versus the normal A chromosomes complement) can also have an effect of favouring recombination between homologous chromosomes over that between closely related homoeologous ones.

Examples of application of the technique
In hexaploid wheat, the *Ph1* gene prevents homoeologous pairing between chromosomes of the A, B and D genomes. This control system of homoeologous pairing can be circumvented using substitution lines (see section 2.2.3) lacking the chromosome harbouring *Ph1* or using a *ph1* mutant, or crossing with wild relatives capable of inactivating *Ph1*, such as *Aegilops speltoides* (Jauhar 2006). Homoeologous pairing was also shown to be repressed in the presence of B chromosomes in an allotetraploid species hybrid between *Lolium perenne* and *L. temulentum* (Jones *et al.* 2008). Induced autopolyploidy is used less in breeding due to the reproductive instability, which is only less of a problem in forage species like *Lolium* and *Trifolium* (already mentioned under polyploidization, section 2.2.2), as discussed by Evans (1981). Diploidization or allopolyploidization of autoployploids by induction of chromosome differentiation (e.g. by structural rearrangements) proved to be highly complex (Sybenga 1973, Gillies 1989).

Occurrence of the products of the technique under natural conditions
Some of the techniques described here are artificial in the use of physical stresses like UV irradiation, but *Ph1* gene systems and B chromosomes will also occur under natural conditions. Chromosomal rearrangements leading to diploidization in polyploids have been shown to occur under natural conditions, e.g. in recent species hybrids such as *Tragopogon miscellus* (Paun *et al.* 2007).

2.2.6 Mutagenesis

Description of the technique
Mutations are induced by treatment of plant parts with ionizing radiation (e.g. X-rays or gamma rays) or chemical mutagens, such as sodium azide or alkylating agents such as EMS (ethyl methyl sulfonate). The consequences can be chromosome breakage, chromosome (segment) translocation (see also section 2.2.4), chromosome (segment) doubling, chromosome elimination (all prevalent with irradiation) and single base-pair changes (substitution, insertion and deletion, all prevalent with chemical mutagens). Seeds can be treated, which will lead to the development of chimeras, necessitating selection for completely mutated plants through germinating and going through one or two rounds of sexual reproduction, producing generations M1 and M2. To avoid chimeras, pollen may be treated and selection performed on the progeny of plants pollinated with the mutagenized pollen, but pollen is more difficult to

handle, a.o. due to short viability. With clonally propagated species, vegetative parts can be treated, preferably buds where meristems capable of regenerating new plants can be targeted. The plants primarily resulting from mutagenesis may show unintended phenotypic traits next to the desired one (whichc could be called 'mutagenic drag', analogous to 'linkage drag' with wide crosses, see section 2.3.6.1). Therefore, further propagation or backcrossing together with selection is often needed to eliminate undesirable traits.

Examples of application of the technique
The technique was already used in the 1930s to produce a tobacco cultivar. Dwarf cultivars that played a crucial role in the 'green revolution', were developed by mutagenesis. For example, the dwarfing habit of Norin 10 developed in Japan in the 1930s was used by Borlaug in the fifties to develop high yielding wheat varieties. 'Canola' types of oilseed rape, that is types low in both erucic acid and glucosinolates, called 'double zero', have also been produced by mutagenesis. Mutagenesis is particularly useful in clonally propagated, highly heterozygous and long-lived crops, such as fruit trees. An example in fruit trees is apple with changes in tree shape and fruit colour. Mutagenesis is popular in ornamental breeding to change flower shapes and colours. Sports encompassing a whole range of colours of a new variant are often routinely produced in this manner. Such colour sports often represent periclinal chimeras, like in *Chrysanthemum*, in which only the outer (LI) tissue layer is changed genetically. The FAO is hosting a database of plant varieties obtained using mutagenesis (http://mvgs.iaea.org/AboutMutantVarieties.aspx).

Occurrence of the products of the technique under natural conditions
Mutations also arise spontaneously and actually comprise the very basis of evolution, but they naturally occur at a much lower rate than with mutagenic treatments.

2.2.7 Cell fusion

Description of the technique
In vitro cell fusion techniques, also called somatic hybridization, can increase the efficiency of making hybrids that can also be produced by sexual crossing, but only with large difficulties. These difficulties are often due to crossing barriers, such as those described for wide crossings in chapter 2.1. Alternatively, somatic hybridization can also be used to combine specific nuclear and cytoplasmic (plastid and mitochondrial) genomes in an efficient manner, that is, avoiding more lengthy backcrossings with further compatible crosses. In order to enable cell fusion to occur, protoplasts are prepared from usually leaf cells or cell suspension cultures by enzymatically or mechanically removing the cell walls. The somatic hybridization itself then involves chemically (e.g. by polyethylene glycol, PEG) or electrically induced fusion of plant protoplasts. After selection for successful combination of the two genomes, fusion products are induced to regenerate cell walls and are allowed to divide to form calli on artificial media. Plants are regenerated from calli by organogenesis or embryogenesis using normal tissue culture techniques. Desirable hybrids can be selected by cell sorting (see section 2.3.6.3) with the aid of flow cytometry (FCM).

Examples of application of the technique
Somatic hybridization has been applied to many species, e.g. carrot, tobacco, cabbage, potato. An example in potato is a polyploid fusion product between tetraploid *Solanum tuberosum* and wild diploid *S. brevidens* to obtain virus resistance. Introgression from *S. brevidens* into potato through sexual crossing is in principle also possible, but was shown to be quite cumbersome. Introgression involved bridge crosses (section 2.1.1) and/or crosses to 'dihaploidized' (section 2.2.1) potato material. In order for these crosses to succeed, both embryo rescue (section 2.1.6.5) and 'rescue' pollination using *S. phureja* was necessary (Watanabe *et al*. 1995). Rescue pollination is a variant of the mentor pollen method used to promote fertilization by incompatible pollen in wide crosses (see 2.1.4), which represents another use of *S. phureja* than for the 'prickle' pollination mentioned in section 2.2.1. Fusion products may be complete hybrids (symmetrical) or may only have parts of the parental genomes (asymmetrical hybrids). In a completely symmetrical hybrid, the cytoplasm (mitochondria and chloroplasts) will be heteroplasmic, but usually during culture, cytoplasmic genomes become homogenized. In cybrids, the cytoplasm of one cell is combined with the nucleus of another cell. Cybrids can be obtained by selecting for specific combinations of nuclei and (recombinations of) cytoplasm among fusion products. Alternatively, protoplasts in which the nucleus has been

removed by irradiation from one parental line can be fused with protoplasts in which the cytoplasm has been inactivated using iodoacetamide from the other parental line (Pelletier *et al*. 1995). Thus, with cybrids, specific cytoplasmic and nuclear traits can be combined in a single step, so without a need for backcrossing as in normal crossing schemes. In this way, somatic hybridization is often used to transfer cytoplasmic male sterility (CMS, see also section 2.3.2), e.g. from *Raphanus sativus* to *Brassica napus* (the so-called Ogura cytoplasm, named after its discoverer (Ogura 1968, reviewed in Kumar *et al*. 2000).

Occurrence of the products of the technique under natural conditions
Somatic cell fusion is not expected to occur under natural conditions (but see for a possible occurrence with grafting under section 2.3.1). In the context of this report (thus only referring to fusion products that could also arise by 'traditional' sexual crossing methods, see Introduction, chapter 1), its products could in principle also be generated through any of the crossing methods described in chapter 2.1. Their likelihood of occurrence under natural conditions is discussed under the respective sections 2.1.1 – 2.1.6.

2.2.8 Partial genome transfer

Several techniques have been tested with more or less success to achieve partial genome transfer. Irradiation can be applied to pollen or female parts of parental flowers, or to protoplasts in cell fusion, followed by pollination and embryo formation or cell fusion, respectively. With irradiated pollen, the method will only lead to plants with chromosome additions from the male when the egg is more or less capable of parthenogenic development (Powell *et al*. 1983), as with apomixis (see section 2.3.5). Irradiation damage can be a disadvantage with this method, leading for example to problems with regeneration after cell fusion. A more direct way was explored in the form of microinjection of chromosomes. Chromosomes are isolated from synchronized metaphase cells and separated by flow cytometry (see section 2.3.6.3). Isolated chromosomes are then injected into the nucleus of a recipient cell (De Laat *et al*. 1989). This method has met with little success as of yet. More promising may be the use of micro-protoplast fusion (Ramulu *et al*. 1995): in cell suspensions, micronuclei, containing only one or a few chromosomes are induced using chemicals inhibiting DNA synthesis and spindle formation for cell division. Subsequently, these microprotoplasts are fused with normal protoplasts of the recipient species. In this way, addition lines with one or more extra chromosomes can be created (see also 2.2.3). Another variant is microinjection of mitochondria into protoplasts to avoid incompatibilities arising from the chloroplasts co-occurring in somatic hybridization. This technique has been shown to produce calluses with the desired combination of nuclear and mitochondrial genomes from *Brassica napus* and *Raphanus sativus*, respectively (Verhoeven *et al*. 1995).

2.3 Other relevant plant breeding-related techniques

This chapter describes miscellaneous techniques used in plant breeding, other than overcoming crossability barriers and genome manipulation described in the two previous chapters.

2.3.1 Interspecific grafting

Description of the technique
A shoot (or bud) is grafted upon a rootstock of another plant, in this case another species, to improve growth characteristics or resistance to soil-borne pathogens. The (developing) shoot is now called a scion.

Examples of application of the technique
Grafting was already known in classical antiquity. Examples of interspecific grafting are found in ornamentals, such as roses, and fruit trees, such as apples, and also in tomato and grapevine it is commonly used in cultivation. In cucurbits, it is applied successfully to obtain resistance against soil-borne pathogens (Davis *et al*. 2008). It can also be used to increase flower formation, for instance, a potato scion on a tomato rootstock will show profuse flowering for lack of tuber formation (Maierhofer 1959). However, nowadays mostly simpler methods are used for this,

involving cultivation in a manner allowing direct removal of tubers (Hutten, pers. comm.). Grafting a hybrid from a wide cross with low vigour on one of the parents can help in regaining good growth, e.g. with oaks, where a hybrid between *Quercus ilex* and *Q. robur* (*Q.* x *turneri*) shows poor root growth, but does well on a rootstock of the *Q. robur* parental species (Hadfield 1961).

Occurrence of the products of the technique under natural conditions
By grafting, an interspecific chimera is created artificially, but metabolites and signalling substances like growth hormones can be exchanged between the rootstock and the scion. Recently, even exchange of cytoplasmic (plastid) genomes between rootstock and scion at the cut site was reported (Stegemann & Bock 2009). Although the authors claim that they may have found a new way of DNA transfer, a more straightforward explanation of fusion of cells (see section 2.2.7) from the scion and the stock at the junction could still not be excluded. Under natural conditions, parts of different plants are also known to grow together, e.g. roots of neighbouring trees.

2.3.2 Production of hybrid varieties

Description of the technique
Hybrid cultivars are produced by crossing two highly inbred lines. In order to assure the mother line is pollinated by the desired father line, emasculation of the mother line was originally performed. Also thermal inactivation or chemicals (e.g. ethanol or more specific gametocides) may be used to induce male sterility, but this is not used in routine seed production. It is more efficient to use genetically based male sterility, if available in the crop species. The sterility can be based in the nuclear genome or in the cytoplasm, i.e. in the mitochondria. In nuclear male sterility, the mother line is propagated by crossing to an isogenic line having the male sterility locus at a heterozygous state with a dominant allele for male fertility. The progeny half having received the recessive male sterility allele is selected for use as maternal line in hybrid production. As this is not very practical, cytoplasmic male sterility (CMS) is the method of choice in hybrid production whenever it has been made available in the crop species. With CMS, propagation of mother lines is accomplished by using isogenic lines with male-fertile cytoplasm as pollen donors: in this way, the male sterility of the maternal line is overcome by pollen from a practically identical genotype thus keeping the inbred line intact, whilst the CMS is kept intact because cytoplasmic genomes are normally not passed on through the pollen. In the hybrids, male fertility is regained by using paternal lines containing nuclear-based fertility-restoring genes (for review of male sterility, Kumar 2000).

Examples of application of the technique
The technique of producing hybrid cultivars is very widely used, e.g. in important arable crops, such as maize, sunflower, sugar beet and oilseed rape, and in vegetable crops, such as cabbage, carrot, onion and tomato. Hybrid varieties namely offer the advantage of hybrid vigour, that is, a performance superior to inbred lines, and varietal homogeneity at the same time. For the breeder, they have the additional advantage of innate protection of the cultivar, since further multiplication of seeds leads to severe losses in quality due to segregation of traits. CMS was for the first time reported already more than seventy years ago in onion (Jones & Emsweller 1936). CMS, though, is not yet available for practical breeding in all of these crop species, notably tomato, mainly due to the lack of nuclear restorer genes. Nuclear restorer genes compensating the CMS are particularly important in a crop grown for its fruits, when their development depends on successful pollination and thus restoration of male fertility. CMS is also useful in breeding itself by avoiding the more tedious mechanical emasculation for making specific crosses.

Occurrence of the products of the technique under natural conditions
Hybrids are a normal phenomenon under natural conditions, particularly within outcrossing species. Male sterility is a naturally occurring form of incompatibility in hybridization events.

2.3.3 *In vitro* tissue culture

In vitro micropropagation, meristem culture, somatic embryogenesis and somatic organogenesis are techniques that are applied for the multiplication of plants. As such, they are part of procedures described in other sections,

such as haploidization (2.2.1) and cell fusion (2.2.7), or they can be used to propagate heterozygous progeny of crosses for further analysis and selection while avoiding the segregation inherent to sexual reproduction. *In vitro* tissue culture may induce somaclonal variation through DNA methylation, mutation and polyploidization, and thus may be used to generate variation like with mutagenesis (section 2.2.6). As described in section 2.2.6, it can be enhanced by application of mutagenic agents, which would be particularly effective in combination with cell suspensions because of the lower likelihood of chimera formation. Thus, an application specific for breeding would be selection, e.g. for disease resistances, during tissue culture (*in vitro* selection, e.g. for resistance to *Alternaria* fungal toxins in onion cell lines, Tripathi *et al.* 2008). However, generally, *in vitro* selection did not have much success and it is not much performed anymore. This is related to the often poor relationships between, for instance, resistance *in vitro* and in normal cultivation and also to problems with reproductive instability. Somaclonal variation namely can be due to epigenetic changes, such as in DNA methylation patterns, and will then not be stable over subsequent generations (Phillips *et al.* 1994). Tissue culture can be used to obtain virus-free material for further use in breeding and propagation. Particularly in ornamentals, such as trees and flower bulbs, tissue culture can be important to speed up the multiplication necessary for the commercialization of new cultivars (an important rate-limiting step in the development of new cultivars, e.g. lily or tulip, Kuijpers & Langens-Gerrits 1997).

2.3.4 Sex expression in monoecious or dioecious species

In some plant species, individual flowers have either only male or female organs. In monoecious plants, both male and female flowers occur on the same plant, whereas in dioecious plants, male and female flowers occur on separate individuals. The sex of flowers can be influenced by application of plant hormones or compounds influencing plant hormone metabolism. In cucumber, application of gibberellin, silver nitrate or aminoethoxyvinyl glycine (AVG) promotes formation of male flowers in gynoecious (female) plants; in addition, silver nitrate and AVG also induced hermaphroditic flowers in the same plants (Atsmon & Tabbak 1979). In asparagus, gibberellin, together with or without cytokinin, induced stamen formation in female plants, but the anthers were sterile; treatment of male plants with cytokinin increased the amount of hermaphrodite flowers, but here seedless fruits developed (Lazarte & Garrison 1980). With asparagus, also andromonoecious plants are known and the sex determination system is based on a pair of sex chromosomes: XX female, XY male, the andromonoecious variant also has XY and in addition, there are 'supermales' having YY. 'Supermales' are interesting for the production of hybrid cultivars (see section 2.3.2), since offspring (XX x YY) is exclusively male (XY). Such all-male hybrid cultivars are preferred in cultivation because of their larger vigour as compared to female plants. This system has been further elucidated by the identification of the sex chromosome using a set of trisomic addition lines (see section 2.2.3) (Löptien 1979) and more recently, by the development of molecular markers linked to genes involved in sex determination (see section 2.3.6.1) (Reamon-Büttner *et al.* 1998). Likewise, in *Cucumis*, three genes have been found to be involved in sex determination and markers have been developed (e.g. Li *et al.* 2008). In *Cucumis*, andromonoecious and monoecious types mostly occur, but the monoecious type is preferred in breeding, as there is no need for emasculation of the hermaphrodite flowers occurring in the andromonoecious types, in order to enable crossing and hybrid cultivar development (see section 2.3.2).

2.3.5 Apomixis

Apomixis is essentially seed formation without fertilization. This would be an attractive trait for breeders as it enables to efficiently propagate elite hybrids through seed. Thus, the more cumbersome way of producing seeds of hybrid varieties each time from crossing of inbred lines could be avoided. The advantage to breeding companies of the innate protection of hybrid cultivars would be lost at the same time. A disadvantage of apomictic crop species is the difficulty to intercross genotypes as in normal breeding programmes. Thus, in the apomictic *Poa pratensis*, mutagenic induction of sexual reproduction or heat treatments (Han & Funk 1968) are needed in order to induce sexual reproduction enabling the performance of such crossing programmes.

Apomixis often appears to be determined by a few usually dominant loci that may be closely linked or part of a large linkage block. Nevertheless, there are apparently enough complications to make it difficult to introgress the trait into

a sexual crop species (Ozias-Akins & Van Dijk 2007, Whitton *et al.* 2008). In this regard, the most intensively studied cross involving an important crop species has been that of apomictic *Tripsacum dactyloides* with maize. From this cross, hybrid apomictic lines could be derived and mapping of the apomixis trait could be performed, but introgression into maize was unsuccessful up till now (Leblanc *et al.* 2009). Most recently, even some genes involved in apomixis have been identified in the model plant *Arabidopsis* (d'Erfurth *et al.* 2009). Further experimentation will show whether such genes are useful in introducing apomixis into crops.

2.3.6 Advanced selection methods

The following methods, marker-assisted breeding, TILLING and cell sorting, are used for selection of desirable products of various techniques already described in the previous chapters. Therefore, the likelihood of the occurrence of the plant products under natural conditions will not be discussed under the following sections.

2.3.6.1 Marker-assisted breeding (MAB)

Description of the technique

Molecular markers, such as generated by AFLP (Vos *et al.* 1995), microsatellites (e.g. Schlötterer 2004) and SNPs (Single Nucleotide Polymorphisms, e.g. Schmid *et al.* 2003), are used to introgress specific traits and remove linkage drag, i.e. undesired traits 'hitchhiking' along with a desired trait by their close genetic linkage. After the initial cross with a parent containing a desired trait, such as a disease resistance, the genotype of the recipient line is recovered by repeated backcrossing to it. In order to be able to check efficiently for the presence of the desired trait in the selection during the repeated backcrossing, markers are used that are tightly linked to the trait. This increases efficiency with traits that are expensive to evaluate, e.g. the use of disease tests during introgression of a resistance gene. At the same time, selection against marker alleles of the donor parent on the rest of the genome away from the desired trait gene can also be performed to reach nearly full recipient parent genome identity in an earlier BC generation than when such background selection is not performed. This use of molecular markers is sometimes also called marker-assisted selection (MAS). Markers can also be used to screen diversity in available germplasm and to find loci involved in desired characteristics, so-called Quantitative Trait Loci (QTLs). For detection of QTLs, a dense genetic map is made using markers distributed across the whole genome and QTLs are detected by statistical association of markers with the desired phenotype.

Examples of application of the technique

The technique is widely used and more sophisticated high-throughput marker methods, such as SNPs (Single Nucleotide Polymorphisms), are being developed at a fast rate, but the way that they are used in selection remains essentially the same. One of the early examples was the introgression of a resistance against the aphid *Nasonovia ribis-nigri* into lettuce from its wild relative *Lactuca virosa* (through a bridge cross via the more closely related *L. serriola*, see 2.1.1 for bridge crosses). Removal of linkage drag was particularly difficult because undesirable characteristics (dwarfing and accelerated aging) were tightly linked to the resistance gene. It took five years of crossing before this linkage was broken in specific progeny. Subsequently, using this progeny, AFLP markers closely linked to the aphid resistance gene could be developed so that further efficient introgression of the aphid resistance gene into elite breeding material could be achieved (Van der Arend *et al.* 1999). Other examples are nematode resistance in beet and blast resistance in rice.

2.3.6.2 TILLING

Description of the technique

TILLING represents a novel approach to screening for alleles at specific loci of interest. The technique can be applied to assess allelic variation in germplasm collections (then called Ecotilling, Comai *et al.* 2004) or to screen mutants obtained by mutagenesis. With mutagenesis, the same type of populations as described in section 2.2.6 are screened for mutations in selected candidate genes that are of interest in relation to agronomic performance, product quality, etc. Detection of variation in the sequence of such genes can be performed using efficient molecular marker methods (see also section 2.3.6.1) like SNP detection techniques, such as cleavage at mismatched bases

by endonucleases (CEL1 TILLING) or high-resolution melt analysis (HRM) of heteroduplexes caused by mismatching bases, or using high throughput DNA sequencing. This approach is called 'reverse genetics', as opposed to the term 'forward genetics' for the classical method of screening mutagenized populations for desirable phenotypic traits. An additional advantage over phenotypic screening is that TILLING also allows identification of recessive mutations in a heterozygous state.

Examples of application of the technique
TILLING platforms have been developed for diverse crops, such as barley, wheat, rice, sorghum, oilseed rape, soybean, pea. Examples of traits (genes) targeted are digestibility, oil quality, virus resistance (Parry *et al.* 2009).

2.3.6.3 Cell sorting

Description of the technique
With cell sorting, individual cells are selected by leading them in a liquid flow through an apparatus that can detect certain desired traits by measuring fluorescence or light scattering, the flow cytometer (FCM). Like marker-assisted selection and TILLING (see section 2.3.6.2), cell sorting is a screening technique that is applied in combination with other methods, i.e. cell fusion (section 2.2.7) and manipulation of ploidy level (haploidization, section 2.2.1, and polyploidization, section 2.2.2).

Examples of application of the technique
With cell fusion, desirable hybrids can be selected by cell sorting with the aid of flow cytometry (FCM). For this, the contributions from different parents are distinguished by applying specific fluorescent dyes beforehand to each parental cell line (Ochatt 2008). With ploidy level manipulation, FCM can be used to select pollen (mother cells) with the desired ploidy level, e.g. those containing unreduced gametes for the production of polyploid progeny on a selected mother plant (Eeckhaut *et al.* 2005).

3. Concluding remarks

In this report, a comprehensive overview is given of a highly diverse array of 'traditional' plant breeding techniques that basically have only one common denominator, which is that they are thought not to be subject to regulation according to the EU 2001/18/EC directive on the deliberate release of GMOs into the environment. Therefore, making generalizations will often not be possible and remarks about their usage or spontaneous occurrence of their products can only be made for individual techniques. In addition, the list of 'traditional' breeding methods and supporting techniques can not be seen as definitive, as new plant breeding techniques will be developed or existing ones adapted and modified.

The development of some methods dates back quite a longer time than others, which may already be apparent from the publication years cited with the respective techniques. However, the extent to which the publication zenith of particular techniques dates back, does not need to be representative for their present use in the plant breeding practice at companies and research institutes, because continued more or less routine usage will not lead to new scientific publications about these techniques themselves. An indication of the present and near-future popularity of individual techniques is given in Table 1. It will not be possible to exactly rate the popularity of specific techniques at breeding companies, due to confidentiality for obvious commercial reasons. Nevertheless, in the following, some indications are given based on the knowledge and experiences of experts of the Advisory Committee of this report and of Wageningen UR Plant Breeding, where the authors of this report are based.

In general, techniques are favoured that increase the speed by which new varieties can be developed and brought to market, i.e. efficient multiplication methods, doubled haploids and marker-assisted breeding (MAB). These techniques have progressed enormously by developments in the auxillary science fields of cell biology and molecular biology. The increase in screening efficiency enabled by novel molecular techniques, such as TILLING, has renewed interest in mutagenesis as a tool for increasing variation in companies' germplasm. Thus, companies are again creating new mutagenized populations for their major crops. The novel techniques are extensively used in breeding programmes of larger crops. They are also more often used in smaller crops, such as the ornamentals, but financial resources can remain limiting in the crops with relatively small markets. Particularly in the ornamentals, more laborious techniques like wide crosses will continue to be used for introduction of traits lacking in the crop species. Therefore, techniques enabling wide crosses, such as polyploidization and embryo rescue, will remain in use.in many of these crops. Deficiencies in male and female fertility of complex hybrids are less of a problem in ornamentals than in most field crops, since propagation in ornamentals is often vegetative. Likewise, cell fusion remains in use for transferring cytoplasmic male sterility (CMS) through the creation of cybrids. CMS is much sought after, since it is the most efficient tool in the production of hybrid cultivars. Hybrid cultivars, in turn, are in favour because of their superior yield performance and inherent intellectual property (IP) protection.

Particularly in vegetatively multiplied crops like ornamentals, but also in e.g. potato, speeding up of multiplication for bringing a variety to the market by *in vitro* culture can be highly important. *In vitro* culture also remains important as part of other techniques, such as haploidization, but not anymore for selection purposes, a.o. since much of somaclonal variation is epigenetic and not stable over generations. Likewise, grafting is hardly used for breeding purposes, but can be an important tool in increasing the efficiency and reliability of seed production by the vegetable nursery industry. For that purpose, varieties for seed production can be grafted on superior rootstock varieties. Despite efforts to produce better plants from seeds, the use of grafting is also still widespread in growing crops, such as tomato, apple/pear and rose.

The likelihood of occurrence of products under natural conditions varies between the different techniques discussed. For example, interspecific hybrids created by highly sophisticated techniques used to overcome large incompatibility barriers (discussed in chapter 2.1) may be expected to show a low likelihood of spontaneous occurrence under natural conditions. However, very rare occurrences are by definition hard to assess. With readily identifiable hybrids in the field, this might be less of a problem, but how many testing would be required for more difficult identifications, such as among the yellow crucifer (Brassicaceae) species? This was discussed with the example of the rare finding

in the field of a hybrid between *Brassica napus* and *Sinapis arvensis* enabled by the more efficient detection afforded by a transgenic herbicide resistance marker at the end of chapter 2.1. Moreover, even very rare spontaneous species hybrids could well become established under certain natural conditions, when they have sufficient advantages in fitness (e.g. Arnold 1997). On the other hand, the lack of occurrence of a specific species hybrid needs not be related to the size of the natural (incompatibility) barrier that has to be overcome by the breeding technique, but may for instance simply be due to non-overlapping species distributions (allopatry). Furthermore, a sophisticated technique used to overcome strong incompatibility barriers like somatic hybridization (cell fusion, section 2.2.7), can be used as well to efficiently combine specific nuclear and cytoplasmic genomes that could also be made through repeated backcrossings, albeit with less efficiency. Such combinations can readily occur spontaneously under natural conditions. Likewise, the doubled haploids raised through the sophisticated haploidization techniques (section 2.2.1) can also be obtained through repeated backcrossings and thus can be expected to also arise spontaneously.

In conclusion, the 'traditional' plant breeding methods will continue to remain important and will be developed further. In part, this may depend on developments in the acceptance of genetic modification. Elaborate cell fusion or wide crossing techniques could sometimes also still be used for introducing traits, such as male sterility or disease resistance, because transformation techniques potentially more efficient for these purposes, are less attractive in relation to the more cumbersome placing on the market of varieties. It is more difficult to make predictions about the techniques of a more experimental nature. For more efficient seed production of superior hybrid crops, there is for instance a long standing interest in introducing apomixis in sexual crop species. This has proved a recalcitrant trait for breeding, but in recent years, more insights have been gained in genetics of apomixis and most recently, even some genes involved have been identified in the model species *Arabidopsis thaliana*. However, for lack of real breakthroughs in crop species, it is yet uncertain whether introduction of apomixis will soon be a widespread approach in plant breeding. The particularly fast pace at which DNA sequencing becomes more efficient not only facilitates techniques, such as TILLING, but also MAB, as molecular markers can be more quickly identified and scored with very efficient high-throughput methods. This leads to a future need for higher data analysis capacity for handling the enormously increased amount of data, and in turn, also to a demand for more efficient characterization methods of the plant material itself, i.e. phenotyping efforts are once again a rate-limiting step in plant variety development.

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Table 1

Indications for the likelihood of occurrence under natural conditions of the products of specific traditional plant breeding techniques, and the current and near future use of these techniques. The scores can only be taken as indicative for there is variation possible within techniques, e.g. depending on the type of crop species or variants of the technique, and for that reason sometimes a range is given. Also, it is often difficult to give general clues as to natural occurrence for a given technique based on the available literature. Therefore, the reader should also refer to the relevant sections for more detailed explanations.

Legend

a	
Product could arise under natural conditions	●●●
Product could rarely arise under natural conditions	●●
Product unlikely to arise under natural conditions, but it cannot be excluded	●
Experimental	exp.
Selection technique	sel.
b	
Ranges mostly due to variants of the technique; these are mentioned according to the respective scores	
c	
Popular	+++
Routinely in use	++
Becoming obsolete or used only for highly specific purposes	+
Experimental	exp.
d	
Ranges mostly due to differences between crop species in relation to available resources; these are mentioned according to the respective scores	

Continued on pages 30-31

Traditional plant breeding techniques	Section	Occurrence under natural conditions [a]	Explanation of range of scores [b]	Current and near future use [c]	Explanation (of range) of scores [d]
Techniques for overcoming crossability barriers	2.1				
Bridge cross	2.1.1	●●		++ - +	ornamentals - large vegetable & ornamental crops
Pollination using sub- or supra-optimal stigma age, or suboptimal conditions	2.1.2	●●● - ●	pollination of immature or overmature pistils - opening of buds for pollination	+	
Pollination using application of chemicals	2.1.3	●● - ●	application of growth regulators to stigma - application of other chemicals not normally produced by plants	+	
Pollination using treatment of pollen and/or pollen mixtures	2.1.4	●● - ●	Application of mixtures of compatible & incompatible pollen - treatment of compatible pollen in such mixtures by e.g. irradiation	+	
Pollination following treatment of the style	2.1.5	●● - ●	Treatment of stigma/style using: heat - ionizing irradiation	+	
Pollination following manipulation of the style	2.1.6.1	●		++ - +	ornamentals - large vegetable & ornamental crops
In vitro pollination	2.1.6.2	●		++ - +	ornamentals - large vegetable & ornamental crops
In vitro culture of excised ovaries	2.1.6.3	●		++ - +	ornamentals - large vegetable & ornamental crops
In vitro culture of excised ovules	2.1.6.4	●		++ - +	ornamentals - large vegetable & ornamental crops
In vitro culture of excised embryos (embryo rescue)	2.1.6.5	●		+++ - ++	ornamentals - large vegetable & ornamental crops
In vitro fertilization	2.1.6.6	exp.		exp.	
Techniques for chromosome and genome manipulation	2.2				
Haploidization	2.2.1	●●● - ●●	doubled haploids in principle identical to homozygous progeny of the plants used for haploidization - spontaneous occurrence of haploids/doubled haploids	+++	

Traditional plant breeding techniques	Section	Occurrence under natural conditions [a]	Explanation of range of scores [b]	Current and near future use [c]	Explanation (of range) of scores [d]
Genome doubling, polyploidization	2.2.2	●●		++ - +	ornamentals - large vegetable & ornamental crops
Production of alien addition or substitution lines	2.2.3	●●		+	
Translocation breeding	2.2.4	●●		+	
Manipulation of chromosome pairing in meiosis	2.2.5	●●		+	
Mutagenesis	2.2.6	●●		+++	increase due to improved screening by TILLING etc.
Cell fusion	2.2.7	●●● - ●	cybrids of readily crossable species - fusion products of wide crosses	++	mainly for transferring CMS
Partial genome transfer	2.2.8	exp.		exp.	
Other relevant plant breeding-related techniques	2.3				
Interspecific grafting	2.3.1	●● -●	rootstock/scion combinations as such unlikely to arise spontaneously, but comparable growing together of plant parts of different individuals naturally ocurring	++	
Production of hybrid varieties	2.3.2	●		+++	
In vitro tissue culture	2.3.3	●●● - ●	plant propagation - embryo rescue (see 2.1.6.5)	++	
Sex expression in monoecious or dioecious species	2.3.4	●●● - ●	selection of gene (trait) variants for sex - treatment with growth regulators	++	
Introduction of apomixis	2.3.5	exp.		exp.	could become important with breakthroughs in important crops
Marker-assisted breeding (MAB)	2.3.6.1	sel.		+++	
TILLING	2.3.6.2	sel.		+++	
Cell sorting	2.3.6.3	sel.		+++ - ++	ploidy level of cells - cell fusion products

Glossary

addition lines plant lines in which one or more extra chromosomes are present in addition to the normal genome complement, in the case of extra chromosomes being derived from another species, they have the prefix 'alien'
AFLP® molecular marker method using DNA restriction ('cutting') enzyme digestion and PCR with specially designed primers to produce multi-locus patterns of bands that can be scored as present or absent
allele one of several DNA sequence variants occurring at a specific locus on the genome
allopolyploid (alloploid) having more than two sets of the basic chromosome complement, with at least one of these derived from another species
amphidiploid (amphiploid) allopolyploid combining the complete chromosome complements of two species
androgenesis haploid induction from the male germ line
andromonoecious having both male and bisexual flowers on the same plant
aneuploid having a number of chromosomes that is not the exact multiple of the basic complement
apomixis asexual reproduction through seeds
autopolyploid having more than two sets of the basic chromosome complement of the same species
B chromosome supernumerary chromosome differing in several characteristics, among which mitotic behaviour, from the normal A chromosomes of a species
BC back-cross, i.e. a hybrid line crossed to one of the original parents
bivalent a pair of homologous chromosomes associated during meiosis, at which moment cross-over (recombination) can take place
callus a cluster of undifferentiated plant cells in tissue culture
chimera an organism having tissues with different genetic make-ups
CMS cytoplasmic male sterility, i.e. anthers producing sterile pollen due to incompatibility between the cytoplasmic genome (usually mitochondrial) and nuclear genome
cybrid cytoplasmic hybrid, a cell fusion product combining the nucleus from one parental cell with the cytoplasmic genome from the other parental cell
dioecious having male and female flowers on separate plants
dihaploid having half the number of basic chromosome complements (i.e. two) of a tetraploid, which represents the same ploidy level as in normal gametes
diploid having two basic chromosome complements, in the most usual situation with each set derived from one of two (male and female) parents
disomic referring to two homologous chromosomes, e.g. as extra pair in an addition line
dominant referring to an allele overruling phenotypic expression of another (recessive) allele at the same locus
doubled haploid diploid resulting from chromosome doubling of a haploid, which in turn was induced by anther or embryosac culture
embryosac tissue arising in the ovule from the megaspore that in turn is a haploid product of maternal meiosis, containing the egg cell from which the embryo develops after fertilization by one of the sperm cells brought in by a pollen tube from pollen deposited on the stigma (Fig. 1b)
endosperm tissue arising from fusion of polar nuclei in the embryosac with one of the sperm nuclei brought in by a pollen tube from pollen deposited on the stigma (Fig. 1a), usually serving as nourishment for developing embryo
epigenetic somatically or meiotically heritable reversible changes in gene expression that are not based on changes in DNA sequence but on changes in chromosome structure, such as methylation of specific bases
exudate a fluid substance emanating from a tissue or plant part, such as the stigma or style
FCM, flow cytometry measuring fluorescence or light scattering by individual particles in a liquid flow during its passage through the measuring device
gene flow spreading of genes/genome parts between populations/species through pollination or seed dispersal
genome the total genetic onformation carried by the chromsomes in the nucleus or the (single) circular chromosomes in chloroplasts or mitochondria
gynoecious referring to female flower (parts)
gynogenesis haploid induction from the female germ line
haploid having one basic chromosome complement

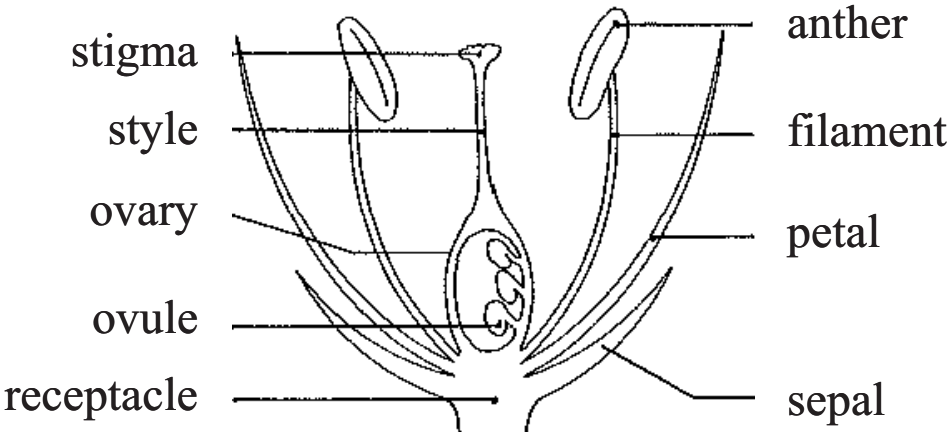
hexaploid having six basic chromosome complements
homoeologous referring to homology between chromosomes from different species
***in vitro* tissue culture** growing plant (parts) outside of the normal plant growth situation in soil on an artificial medium, literally 'in glass'
inbred result of successive rounds of self-fertilization of a plant (line)
inbreeding depression decrease in vigour as a consequence of selfing or mating of very closely related genotypes, usually ascribed to increased likelihood of the occurrence of recessive disadvantageous alleles
incongruity incompatibility in hybridizations between more distantly related parents (species)
isogenic having practically identical genetic make-up
linkage drag genes conferring characteristics unfavourable to a crop that have become introgressed together with a gene encoding a desirable trait through genetic linkage
locus, loci (pl) a site on a chromosome where a specific DNA sequence/gene is located
MAB marker-assisted breeding, application of molecular (DNA) markers in breeding
MAS marker-assisted selection, application of molecular (DNA) markers in selection processes, such as for specific desired genes during backcrossing after initial crosses with e.g. wild species leading to introduction of linkage drag
meiosis cell division in germ cells reducing the number of chromosomes by one half
meristem plant tissue with cells capable of continued cell division, e.g. in growing tips of shoots or roots
metaphase the stage at cell division where chromosomes line up in the equatorial region and where association for crossing-over between homologous chromosomes has taken place in case of meiosis
microspores cells produced by meiosis in the male germ line in the anther that will subsequently develop into mature pollen (Fig. 1a)
microsatellites repeated DNA sequence with repeat units of one to six base pairs in length, due to high potential variability in number of repeat units between individuals often suitable as molecular marker
monoecious having male and female flowers on the same plant
monosomic referring to only one chromosome of a homologous pair being present, e.g. as extra chromosome in an addition line or as a single chromosome in an aneuploid line lacking the other chromosome of the homologous pair
multivalent referring to association of more than two homologous chromosome pairs during meiosis in polyploids or aneuploids (addition lines)
ovary pistil part that contains the ovules and develops into the fruit (Fig. 1a)
ovule part of the ovary that contains the embryo sac en that develops into the seed after fertilization (Fig. 1a and b)
parthenogenic refers to egg development into a new individual without fertilization
PCR polymerase chain reaction, method for amplification of particular DNA sequences by use of specific primers, much used, a.o., in molecular marker and sequencing methods
pistil the female part of a flower consisting of ovary, style and stigma (Fig. 1a)
placenta the part inside the ovary where ovules arise in plants (Fig. 1a)
ploidy referring to the number of chromosome complements in a cell, with a prefix (e.g. di- or tetra-) indicating the actual number
polyploid having more than two basic chromosome sets
protoplast a plant cell without a cell wall
QTL quantitative trait locus, a specific site at a chromosome statistically associated with a quantifiable phenotypic character by genomic mapping analysis of a crossing population
recessive referring to an allele of which the phenotypic expression is overruled by another (dominant) allele at the same locus
scion a plant part (bud or shoot) grafted upon a root stock from another plant
self-incompatibility (SI) having barriers towards fertilization by own pollen
SNP single-nucleotide polymorphism, variation at a single basepair in a DNA sequence between individuals, much used as molecular marker, particularly in recent high-throughput methods
somaclonal variation (epi)genetic variation arising between cells during *in vitro* tissue culture
somatic body cells, as opposed to germ line cells
sport a sudden deviation from the varietal type, usually as a consequence of mutation
stamen..the male parts of a flower each consisting of anther and filament (Fig. 1a)
stigma top part of pistil specialized to catch pollen (Fig. 1a)

style connecting part between ovary and stigma of the pistil, through which the pollen tube grows towards the ovule for fertilization by the sperm cells within the tube
substitution lines plant lines in which one or more chromosomes have been replaced by homoeologous ones from another plant, in the case of the chromosomes being derived from other species, they have the prefix 'alien'
tetraploid having four basic chromosome complements
translocation lines plant lines in which a chromosomal segment has become connected to another non-homologous chromosome, in this way an alien addition line can become genetically stabilized
triangle of U diagram showing the relations ships between three basic *Brassica* species, *B. rapa*, *B. nigra* and *B. oleracea*, and their respective amphidiploid species hybrids, *B. juncea*, *B. carinata* and *B. napus*, as originally inferred by the Japanese scientist U in 1935 (Fig. 2)
triploid having three basic chromosome complements
trisomic having an extra chromosome homologous to one of the pairs of homologous chromosomes of the normal complement
zygote the fusion product of the female and male gametes; in a flowering plant, the egg cell and one of the two sperm nuclei brought in by a pollen tube from pollen deposited on the stigma (Fig. 1a).

Figure 1

a) Diagram of a typical bisexual (hermaphroditic) angiosperm flower. Anther and filament make up the stamen (male part of flower), stigma, style and ovary make up the pistil (female part of flower); b). Diagram of ovule with embryo sac. The egg cell within the embryo sac will develop into the embryo after fertilization by one of the two sperm cells brought in by the pollen tube growing from the pollen deposited on the stigma, the central cell will develop into the endosperm after fusion of the polar nuclei with the nucleus from the other sperm cell.

a



b

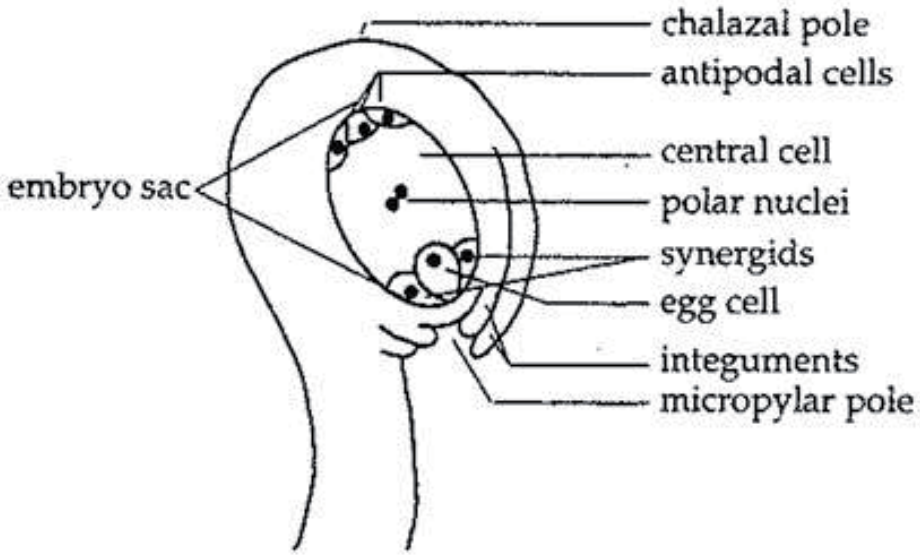
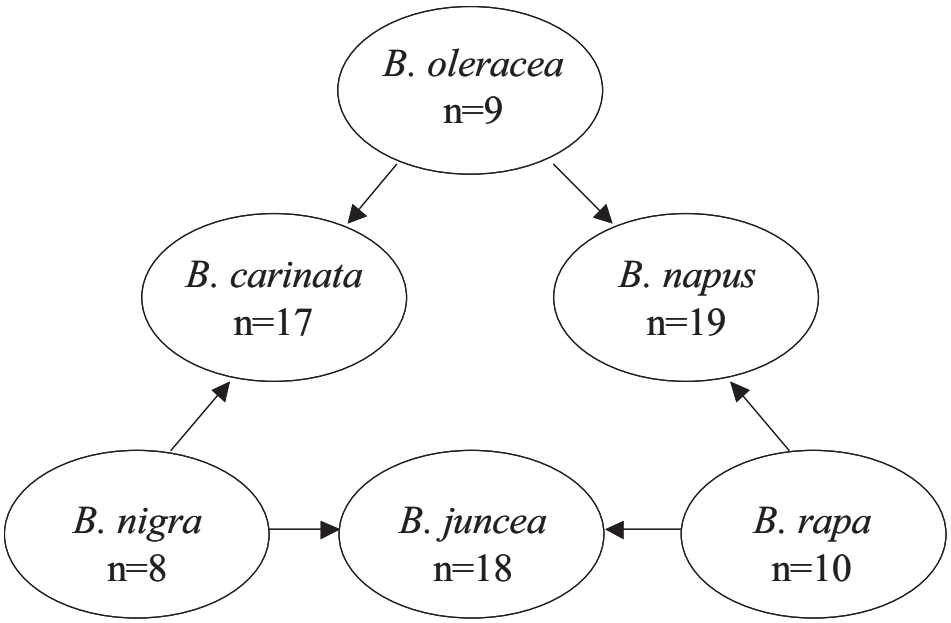


Figure 2

Triangle of U, showing the relationships between three diploid *Brassica* species with haploid chromosome numbers of 8, 9 and 10, respectively, and the allopolyploid (amphidiploid) hybrids between them resulting in haploid chromosome numbers of 17, 18 and 19, respectively.



Appendix I.

Background paper on 'current plant breeding techniques', DOC.XI/464/92

DOC.XI/464/92

BACKGROUND PAPER

ON

"CURRENT PLANT BREEDING TECHNIQUES"

JK/ml
07.92

Table of contents

I. INTRODUCTION

The purpose of this document is to give an overview of current basic and other plant breeding techniques, mainly applied at organismal level and not of breeding strategies. As far as possible, a technical description of the method, an explanation of the procedure, and the applicability of the technique will be presented. Regarding the answer to the question "what are traditional breeding methods", the document should serve as a basis for further discussion. Therefore, personal opinions of the author have been avoided as far as possible.

A general characteristic of the techniques analysed is that they are applied at organismal level and that they mainly interfere with the pollination process, the fertilisation process, and the very early development from zygote to embryo. Other available approaches to combine desirable traits into plants such as techniques applied at cellular or molecular level (Figure 1) are beyond the scope of this paper and will not be discussed here.

II. GENETIC MODIFICATION IN PLANTS: TECHNIQUES APPLIED AT THE ORGANISMAL LEVEL

1. Basic breeding techniques

Basic breeding techniques comprise all the methods that are employed to hybridize parent plants which can also cross in nature. Basically, they involve (i) planned self-pollination and (ii) cross-pollinations with cross-compatible plants which are deliberately selected by the breeder.

With the only purpose of illustrating how breeders use self- and cross-pollinations to direct their experiments in a very defined way, a description of the commonly used back-cross procedure follows. Back-crossing is a technique used in plant breeding to introduce a desirable trait (e.g. a disease resistance) from a donor parent X into the genomic background of the recipient parent Y. To do so, parent X is crossed with parent Y. The progeny from this cross, which contains 50% of the donor's genetic material, are screened for the desired character. The offsprings possessing the desired trait are then crossed back to the recipient parent B. The progeny of this cross (the first back-cross generation B_1) now contains 25% of the genetic material of parent X. The plants are again screened for the desired characteristic, for instance the resistant plants, again back-crossed with parent Y; this process is repeated until about the seventh or eighth back-cross generation. At this stage, less than 0.25% of the donor's genetic material remains and the plants of the B_7 or B_8 generation are self-fertilized (crossed with each other) to produce plants homozygous for the desirable trait.

The process described above assumes the allele for the desirable trait is dominant. If it were recessive, then it is necessary to alternate back-crossing with self-fertilization of the back-cross generations.

Obviously, the main consequence of the human intervention is that the creation of a particular combination of genetic material is (drastically) accelerated. Stated differently, all plants (genotypes, cultivars) produced by using basic breeding techniques could, in principle, also originate in nature, but it would take much more time.

To obtain progeny of the desired male and female parentage, it is, nevertheless, necessary to master techniques for manipulating the hybridization process (e.g. to be able to cross-pollinate a self-pollinating plant or vice versa). Some typical technical interventions are:

(1) emasculation of flowers to prevent self-pollination and to allow the breeder to fertilize the plants with pollen of his own choice. Emasculation is generally obtained by manual removal of the anthers. Chemical emasculation treatments applied at the time of pollen meiosis have been employed by breeders of some crops (e.g. spraying with ethephon reduces the male fertility of eucalyptus [Su and Wu, 1984]; fenridazon induces 100% male sterility in wheat [Mizelle *et al.*, 1989].

(2) Isolation of female flowers to promote self-pollination and/or to prevent unwant pollination by wind or insects. Isolation, generally obtained by bagging the flowers, must be done before anthesis.

(3) Artificial application of viable pollen at the stage of optimal receptivity of the female sex organs.

In particular situations, the human intervention goes somewhat further.

1.1. Overcoming spatial barriers

(1) In nature
In general, the larger the spatial distance between parent A and parent B, the lower the chance that A and B will cross. The possibility that a successful cross will take place is reduced further if there is an extended physical barrier (e.g. ocean, chain of mountains, etc.). It should be noted that spatial barriers are considered as one of the factors with a strong indirect influence on the divergence of species during evolution.

(2) Breeders
Spatial barriers are never a limitation for breeding (e.g. worldwide transport of seeds, plants, and pollen is possible).

1.2. Overcoming chronological barriers

(1) In nature
The shorter the overlap between the period of fertility of parent A and parent B, the less chance A and B will cross. If there is no overlap at all, there will be no successful crosses.

(2) Breeders
Temporal barriers can easily be circumvented: the use of greenhouses with climatical conditions adapted in such a way that the (geographical) races A and B flower at the same time; long-term storage of pollen makes germplasm available at any time of the year. For instance, pollen from pearl millet (*Pennisetum glaucum*) has been successfully stored up to 8 years at -73 °C and continues to be viable. On the contrary, pollen from field-grown pearl millet plants lose viability after one day at 27°C and after three weeks at 4°C (Hanna, 1990; for a review on pollen storage in tree crops, see Sedgley and Griffin, 1989).

2. Techniques for overcoming crossability barriers

In nature, several barriers are known to limit the probability that two parent plants will cross with each other. The goal of this chapter is to present an overview of the possible barriers and to describe techniques which breeders use (or might use) to overcome these barriers. In general, the techniques for overcoming barriers are ranked in increasing order of complexity.

Throughout the text, the terms "compatibility", "incompatibility", and "self-incompatibility" will be used frequently. To avoid confusion, the definitions used are given below.

(1) Two plants are said to be (cross-)compatible in nature if their respective gametes are able to fuse and if the resulting zygote is able to develop into fertile progeny.

(2) Two plants are incompatible in nature if their gametes are unable to fuse and/or the resulting zygote is unable to develop into fertile progeny because of physiological barriers.

(3) Self-incompatible plants are unable to self-fertilize in nature because of physiological barriers.

As a general rule, one can say that plants belonging to the same species are cross-compatible and plants belonging to different species are cross-incompatible. However, exceptions in both directions do exist. A well known example of such an exception, is modern hexaploid wheat. Nowadays, it is generally accepted that this species originated several times over the past 10,000 years after rare successful crosses between a wild diploid wheat-like plant and a primitively cultivated tetraploid *durum* wheat.

In regard with the scope of this document, it is worthwhile to mention that several of the more complex techniques for overcoming incompatibility barriers bear the potential to generate hybrids which can hardly or not at all be created by nature.

2.1. Barriers caused by spatial and chronological separation of sex organs (so-called morphological incompatibility)

(1) In nature

In the course of evolution, nature has developed several mechanisms to prevent inbreeding*[1] and to promote outbreeding* in flowering plants. The main outbreeding mechanisms are:

- ♦ the presence of incompatibility factors (self-incompatibility); this is a barrier at the physiological level and will be discussed in section 2.2.2.1.
- ♦ spatial separation of sex organs
  - dioecy: male and female flowers on different plans (e.g. red campion)
  - monoecy: separate male and female flowers or inflorescences on the same plant (e.g. maize, hazels).
  - herkogamy: reduced efficiency of self-fertilization by structure or positioning of pistils* and stamens* (e.g. *Primula*)
- ♦ chronological separation of sex organs
  - protandry: release of pollen from the anthers* before the stigma* in the same flower is receptive (e.g. many members of the *Compositae* and *Leguminosae*);
  - protogyny: the stigma becomes receptive before the anthers in the same flower release their pollen (e.g. members of the *Rosaceae* and the *Cruciferae*).
- ♦ Remark: in many cases (ii) or (iii) go along with (i).

(2) Breeders

Spatial and chronological separation of sex organs can be considered as special cases of spatial and chronological barriers. Therefore, breeders are capable of creating any planned mating (at least if the mentioned barriers are not complemented with physiological ones) using similar approaches as mentioned in sections 2.1.1 and 2.1.2.

[1] Asterisk refers to Annex 2.

2.2. Physiological barriers resulting in incompatibility

2.2.1 Self-incompatibility (SI)

Based on the calculations of different authors, a minimum of 3,000 self-incompatible species would exist, divided over 415 genera within the angiosperms (East, 1940; Charlesworth, 1985). The traditional explanation for the evolution of self-incompatibility is that it arose as a mechanism to minimize inbreeding and promote outcrossing in flowering plant populations (Darwin, 1876; de Nettancourt, 1977). However, other scientists state that there is not necessarily a relationship between SI and the level of inbreeding and out-crossing in populations (Olmsted, 1989).

In most self-incompatible species, the incompatibility reaction takes place in the phase between pollination and fertilization. For a short description of the different pollination events, see annex 1. Inhibition of pollination occurs at different levels:

(1) Inhibition on the stigmatic surface

Pollen grains are unable to germinate or form short pollen tubes that do not penetrate the stigma. This type of incompatibility is common in *Compositae, Cruciferae*, and *Gramineae.*

(2) Growth barrier in the style*

Pollen tubes germinate, penetrate the stigma but the tube growth is impeded after some time. In this way, pollen tubes are prevented from reaching the ovary*. This type of incompatibility is common in *Solanaceae, Leguminosae,* and *Scrophulariaceae.*

(3) Growth barrier in the ovary

Inhibition of the pollen tube takes place only in the ovary or in the embryo sac (e.g. *Beta vulgaris, Freesia* spp.). In cacao, even the generative gametes are discharged but gametic fusion does not occur.

Although self-incompatibility is known to be genetically controlled (in many families self-incompatibility is controlled by one locus, the *S* locus at which multiple alleles have been described) and despite significant progression in the elucidation of the molecular biology of this process, the exact method by which the genetic information manifests itself is still not clearly understood. To date, the molecular analysis of pollen/pistil interactions has been focused on genes encoding glycoproteins thought to be involved in the recognition of self-pollen in two plant families, the *Brassicaceae* and the *Solanaceae.*

In *Brassica* the 60 known alleles at the *S* locus have been associated with a class of stigmatic glycoproteins, the so-called *S*-locus-specific glycoproteins (SLSGs). The concentration of SLSG increases as the pistil matures and maximal synthesis rates are

attained at the onset of self-incompatibility in the developing stigma (Nasrallah *et al.*, 1985). Furthermore, SLSGs are secreted into the papillar cell walls where they accumulate (Kandasamy *et al.*, 1989). This localization is consistent with inhibition of pollen on the stigmatic surface of *Brassica* and suggests that the secreted SLSGs coat the surfaces of the papillae and might diffuse onto the pollen grains soon after pollination (Nasrallah *et al.*, 1991).

In *Nicotiana alata,* the alleles at the *S* locus seem to be associated with a class of carpel* glycoproteins, designated the *S*-associated glycoproteins (SAGPs). These proteins are localized primarily in the intercellular matrix of the stylar transmitting tissue and at lower levels, in the papillar cells of the stigma and the placental epidermis of the ovary (Cornish *et al.*, 1987). This localization is consistent with inhibition of pollen tube growth in the style of the *Solanaceae.* Recently, several of the genes encoding the *Brassica* SLSGs and the *Nicotiana* SAGPs have been isolated and sequenced. Since detailed description goes beyond the scope of this document, I refer to the following publications: Nasrallah *et al.* (1985, 1991), Anderson *et al.* (1986), Lalonde *et al.* (1989), Anderson *et al.* (1986), Kheyr-Pour *et al.* (1990), McCormick (1991).

Two important remarks regarding the subject of this document are:

(i) the self-incompatibility response is regulated during the development of the flower and is typically acquired at 1-2 days before anthesis* (Roberts *et al.*, 1979);

(ii) even in nature self-incompatibility is not always total.

2.2.2 Interspecific incompatibility

Interspecific*, intergeneric*, and intertribal* hybridizations offer plant breeders a method for increasing the range of variation within the cultivated plants. In nature, only a limited number of species and very few genera and tribes undergo natural hybridizations.

Several hypotheses have been forwarded to explain the interspecific incompatibility:

- interspecific incompatibility is governed by the same locus as self-incompatibility (de Nettancourt, 1977);
- interaction between the *S* gene and other loci play a substantial role in the interspecific incompatibility response to major genes or polygenes acting either as rejectors or as regulators. Major genes that act as rejectors in the pistil reject certain pollen phenotypes, whereas regulators control the *S* gene and are hypothesized to switch *S* activity on or off in certain genotypes or to affect the strength of the incompatibility reaction (Abdalla and Hermsen, 1972).
- Interspecific incompatibility is completely distinct from self-incompatibility. Interspecific incompatibility as interpreted by Hoogenboom (1984) is termed "incongruity": "the non-functioning of an intimate partner relationship resulting from a lack of genetic information in one partner about some relevant characters of the other".

The barriers preventing hybridization between two different species can be divided into two classes.

2.2.2.1 Pre-zygotic (pre-fertilization) barriers

This includes all incompatibility reactions which take place in the phase between pollination and fertilization. The following sites of expression can be distinguished (see also section 2.2.2.1):

(1) inhibition on the stigmatic surface: in the large majority of crosses between unrelated species, the pollen fail to germinate;
(2) growth barrier in the style
(3) growth barrier in the ovary

2.2.2.2 Post-zygotic (post-fertilization) barriers

Post-fertilization barriers hinder or retard development of the zygote after fertilization and interfere with the normal development of the seed. In a large sense, post-zygotic barriers also include reproductive abnormalities in F1 hybrids and their later generation progenies.

Unsuccessful port-zygotic development can be caused by:

(1) Hybrid inviability or weakness
    - In many interspecific crosses between related species, fertilization occurs but the growth of the embryo is stopped after a few cell divisions or at any stage before formation of viable seeds. In other interspecific crosses, abnormalities of endosperm result in non-viable hybrid seeds.
    - Some examples:
        - intergeneric cross: sugarcane x maize (Vijendra Das, 1970); result: fertilization of the egg and the polar nuclei takes place, the fertilized egg and the endosperm nuclei undergo a few further divisions and then the embryo degenerates.
        - intertribal cross: rye x maize (Zenkteler & Nitzsche, 1984); result: fertilization takes place , but the globular embryos degenerate six to ten days after pollination.
        - production of trispecific hybrid: bread wheat x amphiploid of *Triticum durum* x *Aegilops squarrosa* (Siddiqui & Jones, 1969); result: hybrid grows for two months, produces tillers, but then develops necrosis and dies before maturity.
    - Causes for hybrid inviability or weakness
        - Action of specific genes that are know to induce lethality, chlorosis, or weakness of F1 hybrids.
          Example: viability of the F1 hybrids between *T. monococcum* and *Ae. umbellulata* is affected by a gene with multiple alleles: allele L^e causes lethality at early stage, allele L^l at late stage, and allele 1 does not affect the hybrid development.
        - Disharmonious interaction between nucleus of one species and cytoplasm of the other (this can explain reciprocal differences in interspecific crosses).
          Example: wheat x barley hybrids having barley cytoplasm and wheat nucleus are associated with pistilloidy (the reciprocals are normal).
        - Disharmonious interaction of the two genomes within the hybrid nucleus; differences in chromosome number, cell cycle rhythm, genomic ratio, and presence of telomeric heterochromatin may contribute singly or

collectively toward disharmony of the two genomes. *Example: Triticale*, a man-made wheat x rye hybrid, suffers in several ways from the disharmony between the rye and wheat genome:

+ univalency is of common occurrence, probably because of differences in cell cycle rhythm of the wheat and rye parent.;
+ nuclear instability affects the seed fertility and endosperm development (grain shriveling), probably because:
  – the late-replicating DNA (mostly telomeric heterochromatin) in rye chromosomes causes bridge formation at anaphase*;
  – such bridges cause the production of abnormally polyploid endosperm nuclei;
  – these aberrant nuclei cause sterility or shriveled grain (Beckett, 1981).

- Incompatibility between embryo and endosperm (and maternal tissue). In these crosses the endosperm starts to disintegrate soon after fertilization, the embryo being deprived of its initial food supply and growth regulators.

(2) Hybrid sterility
In hybrids where the genome is composed of two different sets of chromosomes the difference in structure and number of chromosomes, the lack of chromosome homology resulting in a variable number of univalents, and the production of unbalanced gametes frequently results in hybrid sterility.
Example: the intergeneric cross cabbage x radish yields viable but sterile hybrids. This can be explained as follows: radish has 18 chromosomes ($2n_1 = 18$, $n_1 = 9$) and cabbage has 18 chromosomes ($2n_2 = 18$, $n_2 = 9$); the cabbage x radish hybrid has 18 chromosomes, $n_1 + n_2 = 18$; however, the homology among the 9 chromosomes from cabbage and the 9 chromosomes from radish was insufficient for normal synapsis and disjunction so that the hybrids were sterile.

(3) Elimination of chromosomes
In some interspecific crosses a variable number of chromosomes of one or both parents is eliminated.
Example #1: the interspecific cross between *Hordeum vulgare* and *H. bulbosum* results in a complete elimination of all *bulbosum* chromosomes during development of the hybrid embryos (Kasha and Kao, 1970; Ho and Kasha, 1975).
Example #2: the hybrids produced in an intergeneric hybridization between common wheat cultivars and cultivated barley frequently lack the barley chromosomes 1 and 5 (Koba *et al.*, 1991).

2.2.3 Techniques for overcoming incompatibility barriers

The array of techniques described in this section have been used to obtain seed from otherwise self-incompatible and/or interspecifically incompatible hybridizations. In some hybridizations, where self-incompatibility or interspecific incompatibility is not total (i.e. a very low seed set is possible in nature), the techniques only enhance a natural process. In other crosses, however, researchers claim that application of the techniques described below resulted in the production of hybrids which could not arise in nature because of absolute self-incompatibility or interspecific incompatibility.

2.2.3.1 Techniques for overcoming pre-zygotic barriers

(1) Bud pollination
- ♦ Description of the technique
  Mature pollen is placed on the immature stigma of still unopened flowers (= flower buds). The optimal stage for bud pollination is genotype dependent and varies between two to four days prior to anthesis. The effect of bud pollination is usually attributed to the absence or incomplete activity of the pollen tube growth inhibiting substances in the immature stigma.
- ♦ Application
  This technique is, for instance, widely employed in the production and maintenance of self-incompatible inbred lines for commercial hybrid seed production in cruciferous and other crops. In citrus and pear pollination at the bud stage results in full seed set between partners which are normally totally cross-sterile (Soost and Cameron, 1975; Yamashita and Iwanaga, 1984; Hiratsuka *et al.*, 1985a, 1985b).

(2) Modified bud pollination (stigma complementation method)
- ♦ Description of the technique
  Immature stigmas are first immersed briefly in an aqueous medium containing H_3BO_3 and $Ca(NO_3)_2.4H_2O$ in a glass capillary tube having an inside diameter twice that of the stigma. Subsequently, the stigma is similarly immersed in a small volume of light mineral oil containing a suspension of incompatible pollen. This kind of bud pollination offers the extra advantage that the applied medium mimics the mature stigma exudate functions in encouraging adhesion, preventing desiccation, and providing moisture at the proper potential gradient for pollen hydration.
- ♦ Example
  The technique has been used to overcome self-incompatibility in several members of the *Solanaceae* (Gradziel and Robinson, 1989a, 1989b).

(3) Delayed pollination
- ♦ Description of the technique
  Mature pollen is placed on aged stigmas three or more days after anthesis. The effect of delayed pollination may result from a rapid loss of the inhibiting substances in the pistils.
- ♦ Application
  The technique is less generally applied than bed pollination.
  Example: delayed bud pollination has been used to produce hybrids in the naturally self-incompatible apple Cox's Orange Pippin (Williams and Maier, 1977).

(4) End-of-season compatibility
- ♦ Description of the technique
  Pollination is performed at the end of the flowering season or by placing pollen on flowers developing towards the end of the life cycle of a plant. Physiological ageing of the plant and worsening growth conditions at the end of the season are thought to result in a decreased capacity to produce incompatibility substances. The loss of incompatibility in old flowers and the end of the season seems to be genetically determined.

(5) Sub-optimal growth conditions
- ♦ Description of the technique

Mature pollen is placed on the stigma of plants cultivated under suboptimal growth conditions. Low temperature in the flowering stage appears to be most effective. Low light intensity, high relative, humidity, and poor soil conditions have also been used successfully. The sub-optimal growth conditions seem to weaken the incompatibility reaction.

- Application
  The method has been used to overcome self-incompatibility in sugar beet, red beet, primrose, *Brassica*, and some others (de Nettancourt, 1977).

(6) Use of exogenous growth substances

- Description of the technique
  Growth substances are applied to the plants on the pistils one or two days before or after pollination. Regularly used growth substances are gibberellic acid (GA) and natural and artificial auxins such as indole-3-acetic acid (IAA), α-naphthalene acetic acid (α-NAA), and 2,4-dichlorophenoxyacetic acid (2,4-D). One possible explanation of the auxin effect is that the delay in floral abscission enables slow-growing incompatible pollen tubes to reach the ovary before flower drop (Van Marrewijk, 1989). Another explanation is that auxins exert their effect on ovary and embryo development (Inagaki, 1986). GA application seems to promote pollen tube growth and ovary development (Larter and Chanbey, 1965).
- Application
  Application of GA (75 ppm) or 2,4-D (100mg/l) one or two days before or after pollination has become a routine procedure for interspecific and intergeneric crosses in several cereal crops like *Triticum* and *Hordeum* (Brar and Khush, 1989).

(7) Use of other chemicals

- Description of the technique
  Application of a variety of chemicals to the plant, the pollen, or the stigma has been tested for its effect on incompatibility. The mechanism of action is unknown.
- Examples
  - Injection of the lysine analog, ε-aminocaproic acid into the *Triticum turgidum* parent one day after pollination with rye pollen significantly enhanced the embryo production in the cross *T. turgidum* x *Secale cereale* (Taira and Larter, 1976).
  - Treatment of pollen with sugars or of the stigma with lectins before pollination leads to considerably improved seed set in incompatible petunia crosses (Shivanna and Johri, 1985).
  - Other chemicals that have been used with varying degrees of success are RNA- and protein-synthesis inhibitors such as 6-methylpurine, pyromycine, and cycloheximide; chloramphenicol, acriflavin, naringenin, paraffin, and salicylic acid.

(8) Use of mentor pollen

- Description of the method
  Pollination performed with a mixture of incompatible pollen and another source of compatible (mentor) pollen results in the pistil accepting the incompatible pollen. A problem of this method is how to separate the seeds originating from compatible and incompatible combinations. The generally followed approach to circumvent this problem is to use pollen mixtures in which the compatible pollen have been pretreated in order to stop it from

fertilizing. The only function of the mentor pollen is to open the door for the incompatible pollen tubes. So, they must still be able to germinate and to penetrate the style but tube growth rate is reduced to such an extent that the incompatible pollen grains are competitive and occupy all (or most) the egg cell nuclei. Several procedures are followed to sexually kill the mentor pollen: radiation (^{60}Co γ-rays), methanol treatment, repeated freezing/thawing cycles, etc.

- Application
  The technique has been applied with quite some success in several crops (Shivanna and Johri, 1985).

(9) Use of pioneer pollen

- Description of the method
  A first pollination with compatible pollen (the pioneer pollen, eventually irradiated) is succeeded by a second pollination with incompatible pollen one or two days later.
- Example
  Self-fertilized seed of self-incompatible apple and pear could be obtained by pollinating with compatible pollen followed by a second pollination with self-pollen one or two days later (Visser and Oost, 1982; Visser, 1983).

(10) Use of pollen coat extracts

- Description of the method
  Extracts of compatible pollen coats are smeared on the stigma surface before pollination with incompatible pollen. The presence of specific substances in the extract should be able to "mislead" the incompatibility reaction.
- Example
  Coating foreign pollen with proteins extracted from the pollen wall of compatible grains of poplar enhances the success of interspecific crossing (Knox *et al.*, 1972; Willing and Prior, 1976).

(11) Irradiation of the style

- Description of the method
  In a first approach the style is exposed to a short but high dose rate of X-, UV-, or γ-rays immediately before pollination. In a second approach the complete plant is chronically exposed to a low dose rate of X-, UV-, or γ-rays during the entire flowering season. Irradiating the style with a high dose rate would temporarily eliminate the incompatibility mechanism; chronic irradiation would reduce the incompatibility reaction.
- Examples
  Acute treatment of the style with 2 Krad X-rays applied immediately before pollination induced half of the treated flowers to set seed in petunia (Linskens *et al.*, 1960). Much higher doses up to 200 Krad γ-rays are effective to overcome self-incompatibility in *Nicotiana alata* (Bredemeyer *et al.*, 1981).

(12) Mutagenesis

- Description of the method
  Pollen are irradiated with UV- or γ-rays or treated with mutagenic agents (e.g. nitrosourea and sodiumazide) prior to pollination.
- Example
  This procedure has, for instance, been used to produce hybrid seeds between *Pinus nigra* and *P. sylvestris* (Kormutak, 1985).

(13) Heat shock and high temperature

- Description of the method
  In a first approach, styles are immersed in hot (50°C) water before pollination. In a second approach, the whole plant is exposed to high temperatures (32-40°C) around the flowering stage. The nature of the temperature effect is as yet not fully understood. A first possible explanation is that the incompatibility breakdown results from inactivation or denaturation of one or more incompatibility-determining enzyme systems (SLSGs or SAGPs). Alternative explanations are turning-off of the synthesis of the incompatibility proteins or stimulation of pollen tube growth-promoting exudates in the stylar conductive tissue (de Nettancourt, 1977).
- Example
  In *Lilium longiflorum* a 1-a 2-minute immersion of the style in hot (50°C) water is enough to set aside the self-incompatibility reaction (Campbell and Linskens, 1984).

(14) Changing the atmospheric composition

- Description of the method
  Soon after pollination the flowers are exposed to CO_2 concentrations ranging from 3% to 10% in air. Potential explanations for the action of CO_2 in the blocking of incompatibility include (i) the enhancement of pollen activity during germination and tube growth (Dhaliwal *et al.*, 1981), (ii) blocking of the callose accumulation in the stigma papillae (O'Neill *et al.*, 1984), (iii) an increase in the rate of pollen adhesion, which is an initial event of the pollen/stigma interaction (Palloix *et al.*, 1985).
- Application
  This method has, for instance, been applied to overcome self-incompatibility in cruciferous species (Nakanishi and Sawano, 1989).

(15) Electric-aided pollination (EAP)

- Description of the method
  In a first approach an electrical potential difference of 100 Volt is applied between pollen and the stigma for 2 to 3 seconds. In a second method the pollinated stigma is approached with electrode tips charged with 600 to 1000 Volt. There is no solid explanation for the EAP effect. A first possible explanation is that a forced sticking of the pollen to the stigmatic papillae provokes a normal germination. Another explanation is that the treatment mutilates the stigmatic surface by melting away the cuticle or the proteinaceous pellicle which covers it. As a consequence, the pollen grain would be able to escape the site of inhibition.
- Application
  This procedure has been used to overcome self-incompatibility in *Brassica* (Roggen *et al.*, 1972; Roggen, 1982).

(16) Thermally aided pollination (TAP)

- Description of the technique
  At pollination, the stigma surface is heated (70-80°C) for about 2 seconds by means of a mini soldering-iron. It is assumed that the heat application changes the surface of the stigma papillae by melting or softening of the cuticle/pellicle thereby disturbing the recognition mechanism. Another explanation could be that the heat denatures the S-specific proteins.

- Application
  This technique has been used to overcome the self-incompatibility in Brussels sprouts, white cabbage, and early spring cabbage (Roggen and Van Dijk, 1976).

(17) Steel-brush pollination

- Description of the method
  The method consists in collecting mature pollen by touching ripe anthers with a miniature steel-wire brush and subsequently gently pricking the steel hairs with the attached pollen grains into the mature stigma surface. The exact mechanism of action is unknown.
- Example
  Steel-brush pollination with 12-hour-old pollen was able to overcome tube growth inhibition in sunflower (Bhaumik *et al.*, 1982).

(18) Surgical techniques

- Description of the method
  In a first approach the mature pollen are injected in the ovary. In a second approach pollination is performed after complete removal of the stigma and/or upper style parts (the cut-style technique). Shortening of the style can be combined with stimulation of pollen germination by placement of agar/gelatin/sugar compositions on the cutting-plane. Since the substances (proteins) responsible for the incompatibility are localized on the stigma or in the style, surgical removal of these structures results in removal of the inhibiting substances.
- Example
  Direct injection of pollen in the ovary resulted in self-fertilization in self-incompatible *Petunia axillaris* (Rangaswamy and Shivanna, 1967). The cut-style technique has been used to overcome self-incompatibility in *Lilium longiflorum* (Janson *et al.*, 1988).

(19) In the (near) future, the elucidation of the exact nature and action of the recognition factors (SLSGs, SAGPs, others?) will quite probably result in new and very specific methods to circumvent prezygotic incompatibility barriers. It is, for instance, not unlikely to imagine that chemical substances that specifically block the action of the incompatibility factors will become available.

2.2.3.2 Techniques for overcoming post-zygotic barriers

(1) Bridging species technique (bridge cross)

- Description of the technique
  When direct crosses between species A and B (with the same or different ploidy levels) are impossible to accomplish, the bridging species technique offers sometimes a possibility to combine the genomes of A and B. For instance, many diploid species of related genera of wheat do not cross with hexaploid wheats. Under such conditions, tetraploid species serve as a bridge for transferring genes from the diploid wild species to the cultivated hexaploid wheat.
- Examples
  - Diploid *Haynaldia villosa* is difficult to cross with hexaploid wheat *Triticum aestivum*. However, tetraploid *T. dicoccoides* is compatible with *H. villosa* and *T. aestivum* and can serve as a bridging species for

transferring genetic information of *H. villosa* into *T. aestivum* (Brar and Khush, 1989).

- *Tulipa gesneriana* is not crossable with *Tulipa kaufmanniana*. A bridge cross with *Tulipa greigii* as the intermediate was used to transfer genetic material from *T. gesneriana* to *T. kaufmanniana* (Van Eijk *et al.*, 1991).

(2) Embryo rescue technique

- ♦ Description of the technique
  The technique involves excising the young hybrid embryos aseptically and growing them in a simple, solid nutrient medium. Usually, 10- to 15-day-old embryos are excised. In cases where embryo abortion starts at earlier stages of development, it may be necessary to use more complex culture media. Once the embryos develop shoots they are generally transferred to the greenhouse where they develop into intact plants.
- ♦ Application
  The technique (developed by Laibach, 1925) is widely used to circumvent hybrid inviability or weakness (see 2.2.2.2) (e.g. when endosperm breakdown prevents embryo development). Embryo rescue has, for instance, been successfully used to produce hybrids involving interspecific and intergeneric crosses in cereals (e.g. Sharma and Gill, 1983; Li and Dong, 1991).

(3) *In vivo/vitro* embryo culture

- ♦ Description of the method
  In vivo or *in vitro* embryo culture is a modification of the embryo rescue technique. Hybrid embryos are excised and put on naked endosperm of the female parent which is itself placed on a normal growth medium.
- ♦ Application
  The technique is particularly useful where young embryos are to be cultured. Such embryos are difficult to excise and also require complex media which could be laborious to devise for a large number of cross-combinations.

(4) Ovary* culture

- ♦ Description of the technique
  In this technique ovaries excised soon (3 to 7 days) after pollination are cultures aseptically in a suitable nutrient medium. Seeds obtained from ovary culture are sown directly in the soil or the hybrid embryos that develop in ovaries are transferred to another adapted nutrient medium.
- ♦ Application
  Ovary culture is easier than embryo and ovule (see further) culture because there is no need to remove small structures such as embryos or ovules. The procedure has, for instance, been successfully applied to obtain intergeneric hybrids between *Diplotaxis siifolia* (a wild species) and cultivated *Brassicaceae* (Batra *et al.*, 1990) and to create intersubtribal hybrids between *Moricanda arvensis* and *Brassica* A and B genome species (Takahata and Takeda, 1990). According to these authors it was impossible to obtain this type of hybrids by using basic hybridization techniques.

(5) *In ovulo* embryo culture (ovule* culture)

- ♦ Description of the technique
  In this procedure fertilized ovules are excised soon (4 days) after pollination and transferred to a solid nutrient medium. The early time point of excision

and the isolation of the complete ovule instead of the embryo are the main differences with the embryo rescue technique.

- ♦ Application
  The technique is particularly useful if the postzygotic incompatibility reaction takes place at a very early developmental stage. According to Ahmad and Comeau (1991) *in ovulo* embryo culture is the only successful method to obtain hybrids from the intergeneric hybridization between *Triticum aestivum* (L.) Thell and *Elymus scrabus* (R.Br.) Love. Again, no hybrids could be obtained by only using basic hybridization techniques.

(6) Embryo callus culture technique

- ♦ Description of the technique
  In this technique aseptically excised, immature embryos are initially cultured on a callus induction medium. Subsequently, the callus tissue is used as the source to produce hybrid plants via organogenesis or embryogenesis.
- ♦ Application
  The technique is particularly useful if the postzygotic incompatibility reaction prevents normal embryo development both *in vivo* and *in vitro* (embryo rescue technique).
  Example: the interspecific *Lycopersicon esculentum* x *L. peruvianum* hybrid embryos begin to deteriorate 10 days after pollination. They do not make the transition from heterotrophy to autotrophy. While five techniques (embryo culture, ovule culture, use of chemical agents, use of hormonal treatments, and use of a "high crossability" *L. peruvianum* line) were evaluated for their ability to overcome the incompatibility barriers, no viable interspecific hybrids were obtained. Application of the embryo callus culture technique, however, did result in the production of fertile hybrids (Poysa, 1990).

(7) *In vitro* pollination, fertilization, and ovule culture

- ♦ Description of the technique
  In vitro pollination comprises the pollination of *in vitro*-cultured (i) complete pistils*, (ii) pistils with part of the ovary wall removed, (iii) placental* segments, and (iv) placenta with attached ovules. After a successful fertilization event, the fertilized ovule(s) is(are) isolated and cultured *in vitro*, and finally grown to adult plants.
- ♦ Application
  In vitro fertilization combined with the culturing of fertilized ovules is an important technique for overcoming the barriers inhibiting the pollen tube growth and the very early stage embryo abortion at the same time. The method has been used to produce hybrid embryos in many otherwise incompatible hybridizations (both self-incompatible and interspecifically incompatible), and may be viable alternative to parasexual or somatic cell hybridization (e.g. Dhaliwal and King, 1978; Zenkteler, 1988).

(8) *In vitro* fertilization by electrofusion

- ♦ Description of the technique
  The following steps can be distinguished in the *in vitro* fertilization by electrofusion (see Figure 2): (i) isolation of viable sperm cells from pollen grains (rupture of the pollen grains by osmotic shock), (ii) isolation of viable egg cells from ovule tissue (by enzymatic treatment followed by mechanical isolation), (iii) electrofusion with single pairs of gametes (under microscopic observation), and (iv) cultivation of the fusion product.

- Application
  Until now, this very recently developed procedure of *in vitro* fertilization by electrofusion (Kranz *et al.*, 1991a) has only been used to combine genetic material from cross-compatible maize plants. However, it seems very likely that the technique will also be applicable to fuse the gametes from cross-incompatible plants.
- Remarks
  - The fusion product of a sperm cell and egg cell from maize started to divide within 2.5 to 3 days and multicellular structures (microcalli) developed with high frequencies. Until now, no hybrid plants have been reported.
  - Sperm cell can be fused with the egg cell without any need for cell wall-degrading enzyme treatment prior to fusion (at least in maize, sperm cell in mature pollen grains seem to represent true protoplasts).
  - Recently, the electrofusion method for single sperm and egg cells has been adopted and slightly modified (Kranz *et al.*, 1991b) to fuse (i) single sperm cells with single synergids* and central cells, (ii) single egg cells with single sperm cells in the presence of adhering synergids and the central cell, and (iii) one or two sperm cells with single, enucleated (= without nucleus) protoplasts, thus creating a haploid or diploid cell.
  - An alternative *in vitro* fertilization method in which sperm cells are injected into the embryo sac has also been reported (Keyzer *et al.*, 1988).

3. Techniques for chromosome manipulation and alien gene transfer at organismal level

Several methods have been developed for incorporating complete alien genomes, single chromosomes, small chromosomal fragments, or a few alien genes from a donor plant into a recipient plant.

3.1. Creation of amphiploids

- Description of the method
  Interspecific hybrids regularly show a high degree of sterility. In some cases fertility can be restored by applying colchicine to induce a chromosome doubling of the sterile hybrid. The new hybrids are called amphiploids, which means the hybrid contains an even number of the basic sets of chromosomes from both parent species.
- Application
  The technique has been widely used to create hybrids between cultivated cereal species and a wild relative. However, many of the amphiploids lack genomic harmony and show meiotic instability.
- Remark
  Based on Annex IA part 2(3) in directive 90/220/EEC polyploidy induction is a technique which is not considered to result in genetic modification.

3.2. Creation of alien addition lines

- Description of the technique
  The procedure involves hybridization of two species followed by back-crossing (see 2.1) the hybrid or the amphiploid to the recipient species and selecting a "descendant" which has all chromosomes from the recipient plant and a single pair of chromosomes from the donor plant.
- Application
  The method is used when the addition of a complete genome is accompanied by introduction of many undesirable features. Using this technique alien addition lines have, for instance, been produced in wheat with single added chromosome pairs of *Aegilops*, *Secale,* and *Hordeum* (Lacadena, 1977; Islam *et al.*, 1981).
- Remarks
  - A similar approach can also be used to synthesize alien substitution lines. Alien chromosome substitution refers to the replacement of one or more pairs of chromosomes of the recipient plant with an equal number of pairs of chromosomes of the donor plant (Khush, 1973; Hu *et al.*, 1988).
  - Both alien addition and alien substitution lines sometimes develop spontaneously in nature.

3.3. *Inducing homoeologous pairing*

- Description of the technique
  An important problem in the production of interspecific hybrids is the reduced pairing and lack of recombination between the genomes in the hybrid nucleus. The extent of recombination depends upon the genetic homoeology of the two genomes which may be different for different chromosomes or chromosome segments. In some species, like wheat, it is known that the pairing suppression is controlled by a specific genetic locus (the pairing homoeologous locus, or *Ph* locus in wheat). Several procedures have been established to manipulate this locus and to increase the pairing frequency and the recombination frequency between the two sets of chromosome:
  - wild species can sometimes be used to induce homoeologous pairing and recombination in cultivated species;
  - inactivation of the pairing homoeologous locus by mutation.
- Example
  In wheat, a *ph* mutant with enhanced homoeologous pairing has been isolated after ethyl methane sulfonate (EMS) treatment (Wall *et al.*, 1971) and after X-raying normal pollen (Sears, 1977).

3.4. *Irradiation-induced translocation of alien chromosome segments*

- Description of the technique
  The technique consists in irradiating the pollen or seed of a monosomic* alien addition line followed by recovery of the translocation* of alien chromosome segments in the successive progenies.
- Application
  The technique has been used for translocating a small segment of an alien chromosome to the recipient genome. Search (1956) used the technique for transferring a small chromosome segment carrying leaf rust resistance from *Aegilops umbellulata* to chromosome 6B of common wheat.
- Remark
  Based on Annex IB(1) in directive 90/220/EEC mutagenesis is excluded from the directive.

3.5. *Undirected mutagenesis*

- Description of the technique
  Seeds of existing cultivars are exposed to mutagens, such as ionizing radiation or EMS, and progeny with the desired phenotypes are selected.
- Remark
  Based on Annex IB(1) in directive 90/220/EEC mutagenesis is excluded from the directive.

3.6. *Limited alien gene transfer by pollen irradiation*

- Description of the technique
  Pollen irradiated with high doses of X-rays are used for pollination. The irradiated pollen with inactivated chromosomes take part in fertilization and pulverized chromosome fragments are delivered into the egg nucleus. This pseudofertilization can induce the egg to develop parthenogenetically* and the DNA of the male nucleus can be incorporated into the female chromosome complement during replication. The diploidy is restored as a result of chromosome doubling leading to a zygote with some selected traits of the male parent.
- Application
  The method may be used to transfer small DNA segments (i) among cultivars from the same species and (ii) across crossability barriers, thus overcoming the problems of hybrid inviability or sterility. In this way, it may serve as an alternative to somatic cell hybridization. The method is only applicable to those species where the maternal parent develops parthenogenetic seeds when crossed with killed, irradiated pollen (e.g. Pandey, 1975; Powell *et al.*, 1983).

III. SUMMARY

(i) All techniques are employed to combine genetic material between species belonging to the kingdom of plants. Intraspecific, intergeneric, and intertribal crosses have been reported.

In principle, the techniques can be split into a set of interventions "in planta" level (1 to 22 and 29 to 35) and a set of techniques that make use of in vitro culture (23 to 28).

(ii) Many of the techniques are only used to produce hybrids between plants that are cross-compatible in nature (1, 2, 3, 22, 29-34)[2]

(iii) According to the scientific literature, some of the techniques have or could be used to generate hybrids between plants that are cross-sterile in nature (4, 5, 23-28, and 35). However, it has to be admitted that it is difficult to prove whether a certain hybridization is absolutely impossible by using only basic breeding techniques.

POINTS FOR FURTHER CONSIDERATION

(iv) How many crosses have to be carried out in order to draw the conclusion that two species are 100% cross-sterile in nature?

(v) According to Annex 1A, part 2(1) in Directive 90/220/EEC, *in vitro* fertilization is a technique not considered to result in genetic modification. How does this relate to techniques 27 and 28?

(vi) Techniques 26-28 and 35 have been proposed as alternatives to somatic hybridization. How do they relate to Annex 1A, part 1(3) and Annex 1B(2) of Directive 90/220/EEC?

[2] The numbering refers to that used in Table 1

Table 1. Compilation of techniques applied at the organismal level to genetically modify plants

I. Basic hybridization techniques

1. Selection (choice of parent plants)
2. Self-pollination of cross-compatible plants
3. Cross-pollination of cross-compatible plants

II. Techniques for overcoming pre-zygotic incompatibility barriers

4. Pollination of buds
5. Stigma complementation
6. Delayed pollination
7. End-of-season pollination
8. Pollination under sub-optimal growth conditions
9. Pollination after treatments with exogenous growth substances
10. Pollination after treatments with chemical agents
11. Use of mentor pollen
12. Use of pioneer pollen
13. Use of pollen coat extract from compatible pollen
14. Irradiation of the style
15. Mutagenesis of pollen
16. Application of a heat shock to the style
17. Change of atmospheric composition
18. Electrically aided pollination
19. Thermally aided pollination
20. Steel-brush pollination
21. Surgical removal of stigma and style

III. Techniques for overcoming post-zygotic incompatibility barriers

22. Bridging species techniques
23. Embryo rescue technique
24. *In vitro*/*in vivo* embryo culture
25. Ovary culture
26. Ovule culture
27. *In vitro* pollination, fertilization, and ovule culture
28. *in vitro* fertilization by electrofusion

IV Techniques for chromosome manipulation and alien gene transfer at organismal level

29. Creation of amphiploids (and polyploidization in general)
30. Creation of alien addition lines
31. Creation of alien substitution lines
32. Induction of homoeologous pairing
33. Irradiation-induced translocation of alien chromosome segments
34. Undirected mutagenesis
35. Limited alien gene transfer by pollen irradiation.

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Annex 1. Pollination events

Pollination involves (i) pollen capture, (ii) hydration and germination, and (iii) pollen tube growth. The pollen grain produces a tube that emerges from one of the pores. For instance, in crucifers the pollen tube invades the stigmatic papillae through the action of digestive enzymes and grows within the secondary papillar cell wall. At the basis of the papillar cell, the pollen tube grows intercellularly in the transmitting tissue of the stigma, style, and ovary.

In the ovary, the tube grows over the surface of the septum, penetrates the funiculus, and enters the unfertilized ovule through the micropyle to effect fertilization (see Figure 3, for clarification of anatomical terms). For extended reviews on the pollination events, see Dickinson *et al.* (1989) and Mascarenhas (1989).

Annex 2. Glossary

Anaphase: an early stage in nuclear division, during which separation of either chromatids or homologous chromosomes commences.

Anther: the apical portion of a stamen which produces the microspores or pollen grains.

Anthesis: the period from flower opening to fruit set.

Carpel: the structure that bears and encloses the ovules in flowering plants; it normally comprises the ovary, style, and stigma.

Inbreeding: production of offspring by the fusion of genetically closely related gametes; self-fertilization is the most intense form of inbreeding.

Intergeneric cross: cross between two species belonging to different genera.

Inter(sub)tribal cross: cross between two species belonging to different (sub)tribes.

Interspecific cross: cross between two different species.

Intraspecific cross: cross between two parents from the same species.

Monosomic: an organism deficient in one chromosome from an otherwise diploid set (2n - 1).

Outbreeding: production of offspring by the fusion of distantly related gametes.

Ovary: the swollen, basal part of the carpel in angiosperms, which contains the ovules.

Ovule: the female gamete and its protective and nutritive tissue; in angiosperms, the ovule comprises a central embryo sac containing the gamete and other haploid nuclei, the surrounding nucellus, and one or two protective integuments interrupted by a small opening, the micropyle; the ovule is attached to the placental tissue by means of the funiculus.

Parthenogenesis: the development of an egg cell into an embryo without fertilization.

Pistil: a single carpel or a group of carpels.

Placenta: the tissue by which ovules are attached to the maternal tissue.

Pollination: the transfer of pollen from the male reproductive organs to the female sex organs.

Stamen: the male reproduction organ of the flowering plant.

Stigma: the receptive tip of the carpel, which receives pollen at pollination and on which the pollen grains germinate.

Style: the sterile portion of the carpel between the ovary and the stigma.

Synapsis: the pairing of homologous chromosomes during the early stage of the first division of meiosis.

Translocation: a chromosome mutation in which a chromosome segment has become detached and reattached to a different (nonhomologous) chromosome.

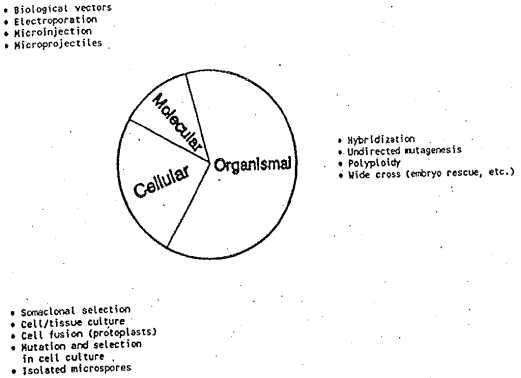

Figure 1. Types of genetic modification in plants.
(Adapted from OECD Group of National Experts in Safety of Biotechnology, A Discussion Paper on Performance Trials for the Development of Plant Cultivars, 1991).

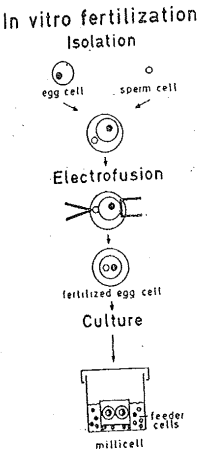

Figure 2. Electrofusion-mediated *in vitro* fertilization.
Single, isolated sperm and egg cells are transferred into the fusion droplets, and pairs of gametes were fused electrically after dielectrophoretical alignment of one of the electrodes. For culture, the fusion products are transferred individually into transparent semi-permeable membranes (millicell) surrounded by feeder cells (adapted from Kranz *et al.*, 1991a).

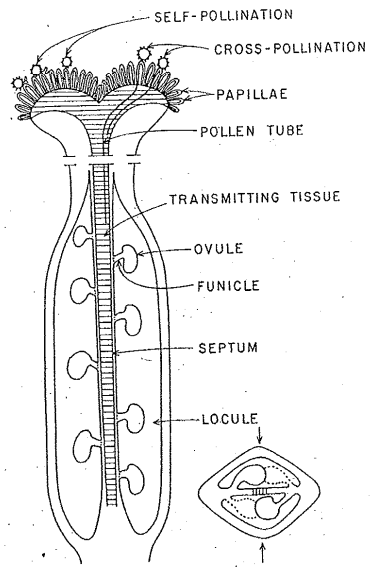

Figure 3. Schematic diagram of a *Brassica* pistil.
The transverse view is at the ovary level (adapted from Nasrallah *et al.*, 1991).