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Review

Drought stress and reactive oxygen species

Production, scavenging and signaling

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Abbreviations: ABA, abscisic acid; AOX, alternative oxidase; APX, ascorbate peroxidase; ASC, ascorbate; CAT, catalase; EST, expressed sequence tags; GPX, glutathione peroxidase; GR, glutathione reductase; GSH, reduced glutathione; PEG, polyethyleneglycol; PSI, PSII, photosystems I and II; ROS, reactive oxygen species; RuBP, ribulose-1,5-bisphosphate; rubisco, ribulose-1,5-bisphosphate carboxilase/oxidase; SOD, superoxide dismutase

Key words: drought stress, stomatal closure, oxidative stress, Mehler reaction, water-water cycle, photorespiration, scavenging enzymes, ROS signaling, H_2O_2, ABA

As sessile organisms, plants have evolved mechanisms that allow them to adapt and survive periods of drought stress. One of the inevitable consequences of drought stress is enhanced ROS production in the different cellular compartments, namely in the chloroplasts, the peroxisomes and the mitochondria. This enhanced ROS production is however kept under tight control by a versatile and cooperative antioxidant system that modulates intracellular ROS concentration and sets the redox-status of the cell. Furthermore, ROS enhancement under stress functions as an alarm signal that triggers acclimatory/defense responses by specific signal transduction pathways that involve H_2O_2 as secondary messenger. ROS signaling is linked to ABA, Ca^{2+} fluxes and sugar sensing and is likely to be involved both upstream and downstream of the ABA-dependent signaling pathways under drought stress. Nevertheless, if drought stress is prolonged over to a certain extent, ROS production will overwhelm the scavenging action of the antioxidant system resulting in extensive cellular damage and death.

Introduction

Drought is one of the most important manifestations of abiotic stress in plants. It is the major yield-limiting factor of crop plants and it actively and continuously determines the natural distribution of plant species. Drought exacerbates the effect of the other stresses to which plants are submitted (abiotic or biotic) and several different abiotic stresses result in water stress (like salt and cold stresses). As sessile organisms, plants have to cope with drought stress at least at some point in their life cycle. They have however evolved mechanisms that allow them to adapt and survive periods of water deficit, if not at the whole plant level, at some level or form of plant structure.

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According to the type of strategy adopted, plants are said to escape, avoid or tolerate drought stress,[1] although these are not mutually exclusive. The plant drought response will depend on the species inherent "strategy" but also on the duration and severity of the drought period. If prolonged over to a certain extent drought stress will inevitably result in oxidative damage due to the over production of reactive oxygen species.[2]

Reactive oxygen species (ROS), also called active oxygen species (AOS) or reactive oxygen intermediates (ROI) are the result of the partial reduction of atmospheric O_2. There are basically four forms of cellular ROS, singlet oxygen ($^1O_2$), superoxide radical ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2) and the hydroxyl radical ($HO^{\cdot}$), each with a characteristic half-life and an oxidizing potential. ROS can be extremely reactive, especially singlet oxygen and the hydroxyl radical and, unlike atmospheric oxygen, they can oxidize multiple cellular components like proteins and lipids, DNA and RNA. Unrestricted oxidation of the cellular components will ultimately cause cell death.[3]

Unlike biotic stress where an oxidative burst is part of a defense response that frequently triggers programmed cell death (PCD),[4] the role of ROS production and control during drought stress is yet to be resolved. However, as stated by Dat and collaborators,[5] ROS seem to have a dual effect under abiotic stress conditions that depend on their overall cellular amount. If kept at relatively low levels they are likely to function as components of a stress-signaling pathway, triggering stress defense/acclimation responses.[5,6] However, when reaching a certain level of phytotoxicity ROS become extremely deleterious, initiating uncontrolled oxidative cascades that damage cellular membranes and other cellular components resulting in oxidative stress and eventually cell death.[3,5] This review will focus on ROS production and control during drought stress with highlights on the signaling aspects of ROS generation and action.

Plants are Natural Producers of ROS

Under normal conditions plants are continuously producing ROS. Unlike singlet oxygen and the hydroxyl radical whose production is kept at minimum levels,[7,8] superoxide and H_2O_2 are synthesized at

very high rates under normal conditions.[9] One of the major cellular sites responsible for ROS production is the chloroplast.[10] During photosynthesis, energy from the sunlight is captured and transferred to two light-harvesting complexes (photosystem II and photosystem I) in the chloroplast thylakoidal membranes. A succession of redox reactions occurs within the electron transport chain in the light, until electrons finally reach CO_2, in the dark reactions. However, it is not uncommon that through this path other final acceptors of electrons are used, namely oxygen.[11] Singlet oxygen can be formed by energy transfer from triplet excited state chlorophyll to O_2.[12] On the other hand, the thylakoidal electron transport components on the PSI side such as the Fe-S centers and the reduced thiredoxin are auto-oxydable resulting in the reduction of O_2 (the Mehler reaction) thus forming superoxide and H_2O_2.[12,13] It has been estimated that approximately 10% of the photosynthetic electrons flow to the Mehler reaction.[14] This "leakage" of electrons to O_2 with the generation of ROS is in fact favorable to the electron transport chain since it poises electron carriers thus making them more efficient.[9]

During photosynthesis there is a different pathway, called photorespiration that can also generate ROS (Fig. 1). In fact, rubisco, the enzyme that catalyses the carboxylation of ribulose-1,5-bisphosphate (RuBP) during carbon assimilation, can also use O_2 to oxygenate ribulose-1,5-bisphosphate. This reaction yields glycolate that is then transported from chloroplasts to peroxisomes where they are oxidized by glycolate oxidase and H_2O_2 is generated.[15]

Mitochondrial electron transport chain is also responsible for ROS generation under normal conditions, although to a lesser extent than chloroplasts and peroxisomes in the light.[16] It has been estimated that approximately 1–2% of the consumed oxygen, respired by plant mitochondria, will be used to form superoxide.[17] This ROS production is likely to occur mainly in complexes I and III of the mitochondrial electron transport chain.[18]

Plants Keep ROS Under Control by an Efficient and Versatile Scavenging System

In order to cope with continuous ROS production plants have a battery of enzymatic and nonenzymatic antioxidants, which function as an extremely efficient cooperative system. The major scavenging mechanisms include superoxide dismutase (SOD), enzymes and metabolites from the ascorbate-glutathione cycle, and catalase (CAT).[9,19,20] They are located throughout the different compartments of the plant cell, with the exception of catalase that is exclusively located in peroxisomes. SOD is the front-line enzyme in ROS attack since it rapidly scavenges superoxide, one of the first ROS to be produced, dismutating it to oxygen and H_2O_2.[19] However, this reaction only converts one ROS to another, and H_2O_2 also needs to be destroyed since it promptly attacks thiol proteins.[9] The major enzymatic cellular scavengers of H_2O_2 are catalase and ascorbate peroxidase (APX).[9,20] They have however different affinities for this ROS and seem to have different cellular roles in H_2O_2 scavenging. In fact CAT does not need a reductant to scavenge H_2O_2 making it reducing power-free, whereas APX needs a reductant, ascorbate. On the other hand, CAT has a lower affinity for H_2O_2 (mM range) than APX (μM range).[3] All this gathered has led to the hypothesis that APX, an enzyme located in every cellular ROS producing compartment, might function as a fine regulator of intracellular ROS steady-state levels, possibly for signaling purposes, whereas CAT, located exclusively in the peroxisomes, might function as a bulk remover of excess ROS production under stress conditions.[3,9,20]

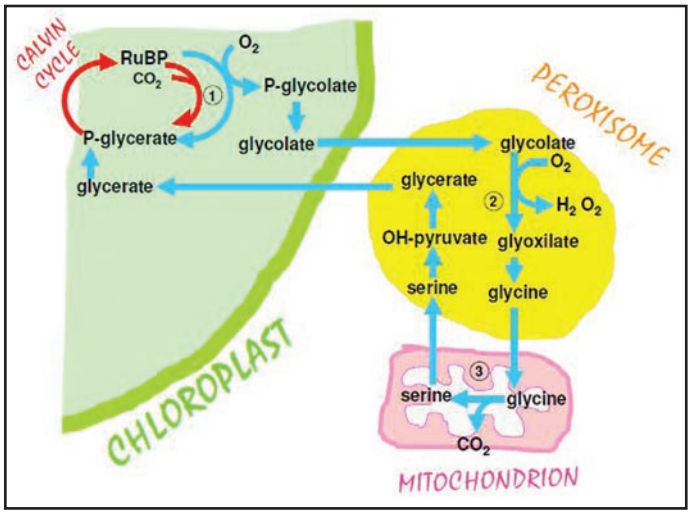

Figure 1. The photorespiratory pathway in photosynthetic plants cells. This cycle involves the oxygenation of RuBP in the chloroplast (1), the formation of H_2O_2 in the peroxisomes (2) and the conversion of glycine to serine in the mitochondria (3). RuBP, ribulose-1,5-bisphosphate.

Glutathione reductase (GR), the last enzyme of the ascorbate/glutathione cycle, has a major role in maintaining the intracellular glutathione pool in the reduced state (GSH).[9] GSH can function as an antioxidant either directly (non enzymatically), like ascorbate, by scavenging singlet oxygen, superoxide or even hydroxyl radicals, or indirectly as a reducing agent that recycles ascorbic acid from its oxidized form to its reduced form by the enzyme dehydroascorbate reductase.[9,21]

In the chloroplast, the Mehler reaction occurring during photosynthesis is an important alternative sink for electrons, but it produces superoxide as side effect. This active oxygen species is however rapidly dismutated by a membrane bound superoxide dismutase (SOD), producing H_2O_2. H_2O_2 is then locally converted to water by ascorbate peroxidase (APX). This is called the Mehler-peroxidase reaction or the water-water cycle.[22] One of the major advantages of the water-water cycle is the scavenging of superoxide and H_2O_2 at their production site, without further damage to the thylakoids or other cellular compartments (Fig. 2). Furthermore, the water-water cycle seems to be autonomous regarding its energy supply since it gets its reducing power directly from the photosynthetic apparatus.[22]

H_2O_2 is a very stable ROS with the longest half-life (~1 ms) and is also the more diffusive[4] so it can readily "escape" from the organelle where it was produced to the cytosol. It can also be directly produced in the cytosol by existing cytosolic SODs. However in the cytosol, H_2O_2 is readily scavenged by ascorbate through the ascorbate/glutathione cycle.

ROS Production is Enhanced Under Drought Stress

The first plant organ to detect a limitation on the water supply is the root system. It has been shown that besides water and minerals, roots also send signals to the leaves through the xylem sap, and the phytohormone abscisic acid is considered to be one of the major root-to-shoot stress signals.[23-26] When the stress signal reaches

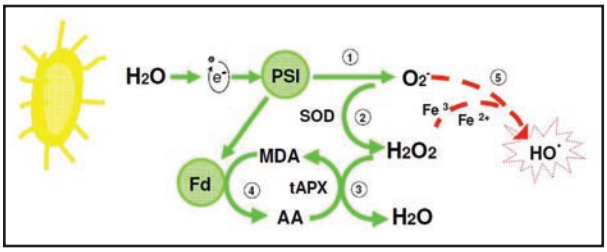

Figure 2. The water-water cycle or the Mehler-peroxidase reaction involves the leakage of electrons from the photosynthetic electron transport chain to oxygen with the generation of superoxide (1), which is further dismutated by SOD forming H_2O_2 (2). H_2O_2 is then scavenged by a thylakoidal APX with the generation of water (3). This cycle gets its reducing power through ascorbate regeneration by ferredoxin (4). Side reactions that can occur in the chloroplasts are the metal catalyzed Haber-weiss/Fenton reactions that result in the formation of the highly reactive hydroxyl radical (5). PSI, photosystem I, Fd, ferredoxin, MDA, monodehydroascorbate, AA, ascorbate, tAPX, thylakoidal ascorbate peroxidase, SOD, superoxide dismutase.

the leaves, it triggers stomatal closure and the plant shifts to a water-saving strategy. Hence, by adjusting stomatal opening, plants are able to control water loss by reducing the transpiration flux, but they are concomitantly limiting the entrance of carbon dioxide (CO_2). This will have direct and indirect effects on the reduction of net photosynthesis and on the overall production of ROS by plants under drought stress.[3] There are many studies that report on increased ROS accumulation and oxidative stress under drought stress.[27-32] In fact, under drought stress, ROS production is enhanced through multiple ways. For instance, the limitation on CO_2 fixation will reduce $NADP^+$ regeneration through the Calvin cycle, hence provoking an over reduction of the photosynthetic electron transport chain. In fact, during photosynthesis and under drought stress there is a higher leakage of electrons to O_2 by the Mehler reaction.[2] It was estimated that in drought stressed wheat, the leakage of photosynthetic electrons to the Mehler reaction was increased by approximately 50% as compared to unstressed wheat.[33] An increase on thylakoid membrane electron leakage to O_2 under drought stress was also seen in sunflower.[34] However, it is quite difficult to assess the part of ROS generated by the Mehler reaction to that generated by photorespiration. In fact, under drought stress the photorespiratory pathway is also enhanced, especially when RuBP oxygenation is maximal due to limitation on CO_2 fixation.[35] The predominance of photorespiration on the oxidative load under drought stress has been recently put forward. Noctor and collaborators[35] have estimated that photorespiration is likely to account for over 70% of total H_2O_2 production under drought stress conditions.

The chloroplast is a quite robust cellular compartment towards ROS because of the different scavenging enzymes and metabolites present.[22,35,36] However, under drought stress one of the real threats towards the chloroplast is the production of the hydroxyl radical in the thylakoids through "iron-catalysed" reduction of H_2O_2 by both SOD and ascorbate. The hydroxyl radical is the ROS which has the shortest half-life (~1 μs) but it also has an extremely strong oxidizing potential reacting with almost every biological molecule.[5] Furthermore, there is no enzymatic reaction known to eliminate the highly reactive hydroxyl radical[6,37] and its accumulation will inevitably lead to deleterious reactions which damage the thylakoidal membranes and the photosynthetic apparatus.

ROS Scavenging and Protection Under Drought

Plants can use the level of steady-state cellular ROS to monitor their intracellular level of stress.[3] However, this steady-state level must be tightly regulated in order to prevent an oxidative burst by over accumulation of ROS, which would ultimately result in extensive cell damage and death.[3,5,12,22] Symptoms of oxidative damage (like lipid peroxidation) have been used to assess the increase in ROS production under drought stress. However, the lack of symptoms does not imply that increased ROS formation is not occurring.[2] Instead, the lack of symptoms is likely to result on the concomitant increase in cellular antioxidant defenses.

Scavenging enzymes. The measure of specific antioxidant enzyme activities and/or expression analysis during water stress treatments has been generally accepted as an approach to assess the involvement of the scavenging system during drought stress. However, contradictory results have been gathered through the years (Table 1). These differences might be related to the plant age and tolerance/strategy towards water stress, but also to the duration and the intensity of the stress treatment. Nevertheless, some authors have detected a direct correlation between the level of the induction of the antioxidant system and the degree of drought tolerance of the plant species (same genus) or the plant cultivar (same species).[31,38-47]

In sunflower seedlings and in grass plants (*Aegilops squarrosa*) a decrease in SOD activity was detected under water stress.[48,49] Opposite to these results were those obtained in wheat,[49] in pea,[50] in common and tepary bean,[46] rice[51] and in olive trees,[52] where water stress increased SOD activity. Lower SOD activity might translate a lower production of superoxide by the Mehler reaction by keeping slight stomatal opening, thus avoiding complete inhibition of CO_2 fixation.[46] Early regulation of stomatal conductance without complete stomatal closure is a characteristic strategy of drought adapted plants such as cowpea[53] and tepary bean.[46] Over expression of SOD in transgenic plants has given quite satisfactory results towards increasing oxidative stress tolerance.[54-59] It could be suggested that enhancement of oxidative stress tolerance is likely to have a positive effect on drought stress tolerance. Confirming this, two independent works have shown that transgenic alfalfa[60] and transgenic rice plants[61] over-expressing a chloroplast targeted MnSOD were more drought tolerant than the wild type plants. Furthermore, the transgenic rice plants over-expressing a pea MnSOD under the control of an oxidative stress inducible promoter, presented an enhanced photosynthetic capacity under a PEG imposed drought treatment.[61] This led the authors to suggest a role for chloroplastic SOD in ROS scavenging during drought stress, eventually through the water-water cycle, hence protecting the photosynthetic apparatus from drought-induced oxidative damage.[61]

Regarding the two major enzymes of the ascorbate/glutathione scavenging pathway, it was shown that APX and/or GR activities were enhanced during drought stress in cotton and spurred anoda,[62] wheat seedlings,[63] beans,[46,47] the moss *Tortula ruralis*,[64] rice,[51] alfalfa[65] and in cowpea.[39,40] A time course measure of APX and GR activities under a mild water stress imposed by a PEG treatment (-0,7 MPa) on maize detached leaves also showed a significant increase in both activities.[66] However, in pea plants the enzymes of the ascorbate/glutathione cycle showed a slight decreased activity under moderate drought stress (leaf Ψ_w = -1,30 MPa) followed by a 50% decreased activity under severe drought stress (leaf Ψ_w =

Table 1 **Drought stress responses of the major plant scavenging enzymes***

Drought responses	Plant source	Refs
Scavenging enzyme activity increase		
SOD	Wheat	49,139
	Wheat^T	42
	Pea	50
	Common bean	46
	Tepary bean	46
	Olive tree	52
	Rice	51
	Rice^T	41
	Populus przewalskii	140
	Coffea canephora	141
	Festuca arundinacea Schreb.	75
	Poa pratensis L	75
GR	Cotton	62
	Spurred anoda	62
	Wheat^S	31
	Wheat^T	42
	Cowpea^S	39
	Common bean	46,47
	Tepary bean	46
	Tortula ruralis	64
	Maize	66
	Populus przewalskii	140
	Coffea canephora	45,141
	Spring wheat	142
	Poplar^T	44
APX	Rice	51
	Rice^T	41
	Cotton	62
	Spurred anoda	62
	Cowpea^S	40
	Maize	66
	Pea	67
	Tepary bean	46
	Populus przewalskii	140
	Coffea canephora	45,141
	Wheat^T	42
	Poplar^T	44
	Alfalfa	65
GPX/GST	*Tortula ruralis*	64
	Rice^T	41
CAT	Alfalfa	65
	Pea	50
	Maize	66
	Wheat	73
	Coffea canephora	45,141
	Rice^T	41
Scavenging enzyme activity decrease/unchanged		
SOD	Wheat^S	42
	Sunflower	48
	Aegilops squarrosa	49
	Rice	41
	Alfalfa	65
GR	Wheat^S	42
	Pea	67
	Cowpea^T	39
	Common bean^S	47
APX	Wheat^S	42
	Pea	67
	Cowpea^T	40
	Rice	41
GPX/GST	Spring wheat	142
	Wheat^S	42
CAT	Tepary bean	46
	Common bean	46
	Sunflower	74
	Sorghum	74
	Rice	41,51
	Festuca arundinacea Schreb.	75
	Poa pratensis L	75

* Abbreviations: SOD, superoxide dismutase; GR, glutathione reductase; APX, ascorbate peroxidase; GPX, glutathione peroxidase; GST, glutathione S-transferase; CAT, catalase; T, drought tolerant cultivar; S, drought susceptible cultivar.

-1,93 MPa).[67] The authors argued that this was probably due to the reduction on NADPH regeneration by photosynthesis under severe drought stress (which was shown to decrease by 70%), as well as to substrate depletion (GSH and ASC). Increased transcript accumulation of cytosolic APX was detected under drought stress in pea plants and this was accompanied by a slight enhanced APX protein content and APX activity.[50] Due to the high affinity of APX towards H_2O_2, this "small" increase on APX activity under drought stress was nonetheless suggested to be responsible for the scavenging of the elevated intracellular levels of H_2O_2 produced under this stress condition.[50] The measure of total anti-oxidative enzyme activities does not account for what occurs in the different cellular compartments under drought stress and much information is being missed. In cowpea leaves it was shown that subtle changes occurred in the intracellular distribution of the APX and GR isoenzymes in response to a progressive drought stress that could relate to the tolerance of the cultivar.[39,40]

Recently, in an attempt to raise abiotic stress tolerance, simultaneous over-expression of both APX and CuZnSOD enzymes in transgenic potato and in tall fescue plants has been shown to result in an increased chloroplastic ROS scavenging action.[68,69] These works show that the combination of APX and CuZnSOD expression under the control of an oxidative stress-induced promoter, with simultaneous targeting to chloroplasts, proves to be quite efficient in increasing tolerance to abiotic stress-induced oxidative damage.[68,69]

Besides GR, two other enzymes related to glutathione metabolism, glutathione S-transferase, (GST) and glutathione peroxidase (GPX), have been shown to be induced under drought stress in the moss *Tortula ruralis*[64] and in leafy spurge (*Euphorbia esula)*.[70] Glutathione peroxidases (GPXs) are efficient scavengers of H_2O_2 and lipid hydroperoxides using GSH as a reducing agent but it has been suggest that in plants they preferably use thioredoxin as a reductant.[71,72] Hence, in a tight relationship with thioredoxins, plant GPXs are likely to be effective protectants of biomembranes under oxidative stress conditions.

Reports on catalase activity under drought stress are also heterogeneous. CAT activity has been shown to increase[50,65,66,73] and also to remain unchanged or even decrease under water stress.[46,74,75] Luna et al[73] have shown that severe drought stress induced an enhancement of CAT activity but this was not related to enhanced transcript accumulation, hence suggesting a rather more complex system of regulation. Transgenic tobacco plants expressing a bacterial catalase were shown to be photosynthetically more tolerant to high irradiance during drought stress than the wild type.[76] Furthermore, these authors have suggested that CAT is a less susceptible scavenging enzyme than APX regarding oxidative stress. Taken together these different reports seem to indicate that CAT activity is only enhanced under severe drought stress whereas under moderate drought stress H_2O_2 scavenging is preferably made by ascorbic acid through the ascorbate/glutathione cycle. CAT has in fact a lower affinity for H_2O_2 than APX which suggest its role in counteracting excessive H_2O_2 production. Furthermore, excess H_2O_2 may attack and inhibit APX, hence CAT activity is likely to be favorable in maintaining APX activity under severe drought stress.

Interesting results have shown that double antisense tobacco plants lacking both APX and CAT activate an alternative/redundant defense mechanism that compensates for the lack of these antioxidant

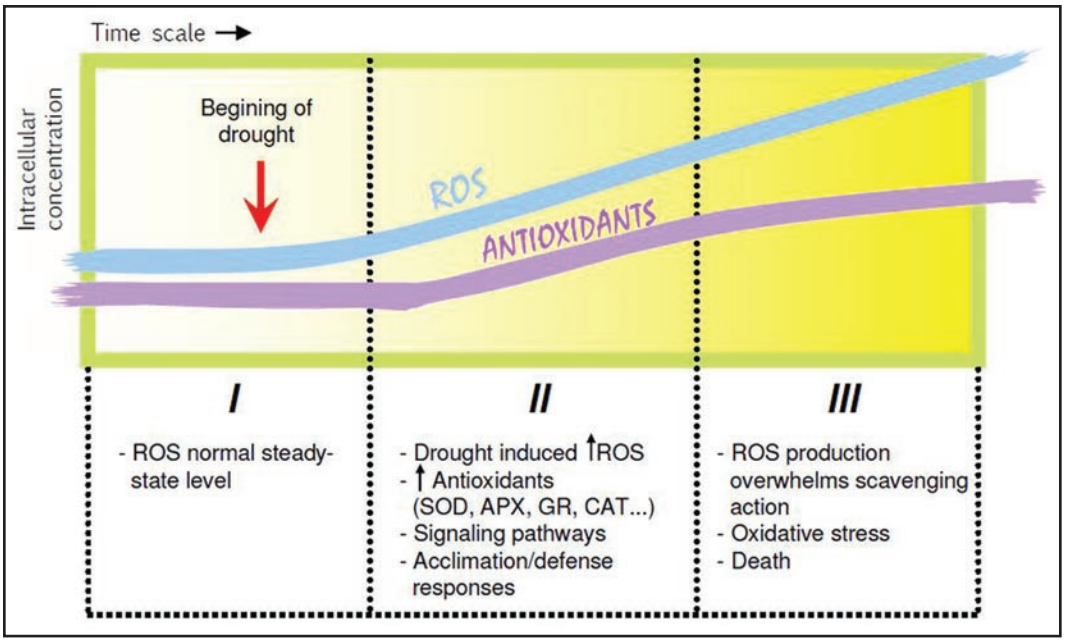

Figure 3. Proposed model of the drought stress response in three successive phases. The normal ROS steady-state level is disturbed by drought stress (I). Enhancement on ROS production due to stomatal closure shifts the equilibrium upwards and this triggers defense signal transduction pathways (II), prolonged drought stress will result in exacerbated ROS production that cannot be counterbalanced by the antioxidant system, leading to deleterious oxidative events which ultimately result in cell death (III).

enzymes.[77] Amongst the mechanisms that correlated to reduced susceptibility to oxidative stress of the double antisense plants was suppressed photosynthetic activity, the induction of metabolic genes of the pentose phosphate pathway, the induction of monodehydroascorbate reductase, and the induction of a chloroplastic homologue of the mitochondrial alternative oxidase (*IMMUTANS* gene).[77] These authors showed that a similar mechanism was not activated in single antisense plants lacking APX or CAT, which intriguingly rendered these plants more sensitive to oxidative stress compared to double antisense plants. This highlights the plasticity of the plant genome which is able to adjust and compensate for the lack of one or more scavenging enzymes under oxidative stress conditions.[77]

Other antioxidants. Superoxide produced by the Mehler reaction can also be directly reduced by ascorbate which is present at high concentrations in the chloroplast.[9] Apart from ascorbate and glutathione, plant cells possess other non enzymatic antioxidants that also participate in ROS scavenging or avoidance under normal and drought stress conditions. Their accumulation under drought stress relates to the drought tolerance of the plant species.[78] Alpha tocopherol is an antioxidant that not only prevents the formation of singlet oxygen and the hydroxyl radicals, but also scavenges lipid peroxyl radicals.[78] Under drought stress, this potent protector of thylakoidal and chloroplastic membranes has been shown to accumulate in several plant species.[63,78,79] On the other hand, the drought tolerant wild watermelon highly accumulates citrulline and CLMT2, a type 2 metallothionein, both with an extremely efficient hydroxyl radical scavenger activity, effectively protecting proteins and DNA from oxidative damage.[37,80,81]

Photorespiration as a cellular protective route under drought. Although photorespiration produces H_2O_2 which is a potent thiol inhibitor, it is an extremely beneficial pathway for plants during drought stress, when the rate of RuBP carboxilation is reduced due to the limitation of CO_2 fixation by stomatal closure.[15,35] First of all it is an alternative route in energy dissipation which would otherwise provoke photoinhibition of the photosynthetic apparatus.[2] Secondly it shifts H_2O_2 production to peroxisomes rather than chloroplasts where the production of the hydroxyl radical is favored and where thiol enzymes of the Calvin cycle are targets to H_2O_2 inhibition. Furthermore H_2O_2 production in the peroxisomes is counterbalanced by the reductant-independent scavenging action of catalase as well as other peroxisomal APX isoforms which promptly detoxify the organelle. Finally, the photorespiration cycle yields glycine which is the precursor of glutathione, a metabolite of the ascorbate-glutathione pathway, hence providing additional protection against oxidative stress.[15,82] Plant peroxisomes are dynamic organelles that adapt their number in response to environmental changes[83] and have been shown to proliferate in response to H_2O_2 accumulation[84] which suggest that this is also likely to occur during drought stress.

Mitochondria, alternative oxidase and ROS production avoidance. The avoidance of ROS production during drought stress is also an important strategy that enables plants to cope with water shortage without extensive damage. Mitochondria play an important role in the avoidance of ROS production by efficient energy dissipation mechanisms.[18] The alternative oxidase (AOX) pathway is an alternative to the cytochrome pathway in the mitochondria and it diverts electrons flowing through the electron transport chain to produce water by the reduction of O_2.[85,86] In the case of durum wheat mitochondria, a Mediterranean plant well adapted to drought, three active energy-dissipating systems have been shown to coexist: the ATP-sensitive plant mitochondrial potassium channel ($PmitoK_{ATP}$),[87] the plant uncoupling protein (PUCP)[88] and the alternative oxidase (AOX).[89] In fact, the activation of such energy-dissipating systems causes a significant reduction in mitochondrial ROS production.[90,91] Both the $PmitoK_{ATP}$ and the PUCP are modulated by the level of ROS production thus making them a very effective mechanism of ROS steady-state level regulation.[91] AOX protein level and capacity have also been shown to increase in the presence of H_2O_2.[92,93] On the other hand, AOX seems to be under the control of the photorespiratory cycle. In fact in durum wheat mitochondria it has been shown that the intermediate products of the photorespiratory pathway (glyoxylate and hydroxypyruvate) activate AOX,[89,91] hence establishing a cooperative mechanism of energy dissipation and ROS avoidance under drought stress when CO_2 fixation is inhibited and photorespiration is at its maximum.

ROS Signaling Under Drought

There is a frail balance between ROS production and scavenging that defines the normal steady-state level of intracellular ROS. Under drought stress this balance suffers an upward shift, ROS production being enhanced due to stomatal closure and the concomitant limitation on CO_2 fixation (Fig. 3). Instead of having an immediate deleterious effect, this raise in ROS production is likely to be beneficial to the plant if nevertheless kept under tight control. In fact, enhanced cellular ROS production is sensed by the plant as an alarm signal that triggers defense pathways and acclimatory responses, enabling the plant to adapt to the changing environment. Numerous reports highlight a signaling role for ROS production under stress.[3,5,6,57,94-99]

H_2O_2 as a secondary messenger. The candidate ROS that is most likely to act as a secondary messenger in a stress-response signal transduction pathway is H_2O_2. The hydroxyl radical ($HO^{\bullet}$) has a diffusive-limited reaction due to its half-life (~1 μs) and its extremely reactive nature. In fact, it is impossible for this ROS to migrate from its site of production and function as a signal molecule, instead it will react locally with other molecules including itself and other ROS, proteins and lipids.[100] Regarding H_2O_2, it is not only the most stable ROS with the ability to easily diffuse from one cellular compartment to another but it also can be readily metabolized by an efficient cellular antioxidant system. Since it is produced at high rates under drought stress, a decrease in its concentration by the action of the antioxidant system allows for the rapid switch "on" and "off" of the signal, a condition essential for a secondary messenger to be effective. On the other hand, the relative toxicity of this active oxygen species has been put to question,[3,14,16] and its affinity to protein thiol groups suggests its possible role as a modulator of protein conformation and/or biochemical activities.[101] Furthermore, the intracellular thiol status is considered to be one of the mechanisms that enable for ROS sensing.[101] Plants are extremely tolerant of H_2O_2 in comparison to animals and the antioxidant systems appear to function as tight controllers of the cellular redox-state instead of annihilators of all intracellular H_2O_2.[14,101] In this sense, antioxidants are key components on the modulation of the ROS signal since they determine the lifetime and the intensity of this signal.[101,102]

The specificity of the cellular ROS signal can be determined by its site of production, control and transduction.[16] Hence, the different plant cell compartments will influence differently the setting of the cellular redox signal under drought stress. Although the rate of H_2O_2 production is faster in peroxisomes and chloroplasts,[16] mitochondria are the most vulnerable organelles to oxidative damage.[103] This can be explained by a lower antioxidant buffering in mitochondria as compared to peroxisomes and chloroplast. In this sense mitochondria play a crucial role in setting the cellular redox-state and initiating signal transduction cascades under drought stress.

Up to now, the known downstream events modulated by H_2O_2 are calcium mobilisation, protein phosphorylation and gene expression.[97] Changes in cytosolic free calcium ($[Ca^{2+}]_{cyt}$) have been reported in numerous abiotic and biotic signal transduction pathways.[104] It has been shown that ROS induces an increase in $[Ca^{2+}]_{cyt}$ by the activation of hyperpolarization-dependent Ca^{2+}-permeable channels in the plasma membrane of *Arabidopsis* guard cells.[105] This ROS induced increase in $[Ca^{2+}]_{cyt}$ has also been detected in other cell types which suggests that the activation of Ca^{2+} channels could be a key step in many ROS-mediated processes.[100] On the other hand, several reports have shown that H_2O_2 induces mitogen-activated protein kinases (MAPKs), which are in turn implicated in several signal transduction cascades that modulate gene expression.[106-109]

H_2O_2 stress response signaling pathways promote the accumulation of several cellular protectants that may act directly or indirectly in the regulation of the cellular redox-status, and consequently control the extent of the signal itself. For instance H_2O_2 has been shown to induce the expression of the nuclear gene encoding for the mitochondrial alternative oxidase (AOX), Aox1.[110] In *Arabidopsis thaliana* cellular suspension culture four genes have been shown to be induced by H_2O_2 by an RNA differential display approach.[111] Amongst the induced transcripts detected was a clone with sequence homology to a DNA damage repair protein (DRT112), one identical to serine/threonine protein kinase gene (APK2b), and one similar to an *Arabidopsis* late embryogenesis-abundant (LEA) protein homologue senescence-associated, SAG21 (the fourth gene presented no sequence homology to any known gene).[111] It is interesting to find that the detected H_2O_2-induced genes seem to be involved in cellular repair/protection mechanisms (DNA damage repair protein and LEA protein) or in the H_2O_2 stress response signal transduction pathway (the serine/threonine protein kinase). Regarding the LEA proteins, these are characteristically expressed during the acquisition of desiccation tolerance in seeds but they have also been extensively associated to drought tolerance in many plant species.[112] Although their cellular functions are still not clearly understood, it has been shown that a citrus dehydrin CuCOR9 (LEA family D11) protects catalase activity under a freeze-thaw process,[113] prevents lipid peroxidation under cold stress[114] and was later shown to have radical scavenging properties,[115] making it a potent antioxidant that enables plants to cope with several abiotic stresses. A wider transcriptomics analysis of H_2O_2 regulated genes in *Arabidopsis* by cDNA microarray technology was further undertaken and a total of 175 genes were identified has being H_2O_2 responsive, 113 up-regulated and 62 down-regulated (the chip representing ~30% of the whole genome).[116] Amongst the up-regulated genes, 14 ESTs with known function were selected and assessed for expression studies by RNA blots. Wilting treatment by rapid desiccation induced the expression of several of the selected ESTs and the authors showed that this effect was partially mediated by H_2O_2 in the case of the ESTs encoding calmoduline, a calcium-binding protein, the DREB2A transcription factor, the *Arabidopsis* MAP kinase ATMPK3, and a zinc finger protein.[116] This suggests that H_2O_2 is likely to be a key component in the orchestration of plant drought stress responses, modulating Ca^{2+} signaling, MAPK cascades and gene expression.

H_2O_2 has also been recently shown to promote NO mediated activation of the proteasome complex in mammal endothelial cells which is involved in the degradation of oxidatively damaged proteins.[117] This H_2O_2 triggered activation of proteases could be suggested to also occur in plants since it has been previously shown that drought stress induces a raise in cellular endoproteolytic activity.[118] Plants submitted to drought stress are also subjected to heat stress because of the reduced transpiration flux due to stomatal closure. Heat shock proteins which are molecular chaperones involved in the heat stress response have been shown to be induced by H_2O_2, which suggests its involvement in the heat stress signaling pathway.[119]

ABA and ROS signaling under drought. There seems to be an intricate relation between the hormone ABA and ROS. Drought stressed plants show enhanced accumulation of ABA and this triggers downstream responses that adapt the plant to the stress condition in an ABA-dependent manner.[120-123] However, several studies have shown that some ABA-dependent water stress responses cannot be elicited by ABA alone.[99] For instances, ABA accumulation is needed to induce proline accumulation in *Arabidopsis thaliana* at low water potentials (Ψ_w) but exogenous application of ABA at high Ψ_w does not reproduce low Ψ_w induction of proline accumulation.[124] This suggests that other factors besides ABA are required to modulate the ABA response under water stress. One hypothesis that could explain this is the metabolic status of the plant.[99] In fact drought stress induces many physiological and biochemical changes that alter the metabolic status of the plant which could influence the cellular susceptibility to ABA accumulation. One important modification induced by drought stress is in the cellular redox-status by enhanced ROS production.[101] Hence ROS production under drought stress has been suggested to be the link between the metabolic status and ABA signaling that acts downstream of ABA and modulates the ABA signal transduction pathway.[99] ABA-dependent proline accumulation under drought stress highlights further the intricate and complex relation between ABA and ROS since the proposed functions of proline under stress are ROS scavenging[125] and regulation of the redox-status.[126]

One of the best characterized ABA-induced physiological responses under drought stress is leaf stomatal closure. This response operates more or less precociously on the onset of the drought period, depending on the plants' inherent strategy (and acclimation) towards drought stress. In the recent years major findings have shown that ABA activates the synthesis of H_2O_2 in guard cells by a membrane bound NADPH oxidase and that H_2O_2 mediates stomatal closure by activating (through hyperpolarization) plasma membrane Ca^{2+} channels.[98,105,127] Furthermore, the use of several *Arabidopsis* ABA mutants have enabled the dissection of some sequencing events in the pathways involving ABA and ROS signaling in guard cells. For instances, ABI1, and ABI2, two protein phosphatase 2C (PP2C)-like enzymes which are negative regulators of the ABA signal in guard cells, were suggested to act respectively up-stream and down-stream of ABA/ROS-mediated stomatal closure.[127-129] The sequencing events proposed in guard cells early ABA signal transduction were as follows: ABA, *abi1-1*, NAD(P)H-dependent ROS production, *abi2-1*, hyperpolarization-activated (I_{Ca}) Ca^{2+} channel activation followed by stomatal closing.[127] A different Arabidopsis mutant, *ost1* was like *abi1-1* impaired in ABA-induced ROS production and stomatal closure.[130] However, if external H_2O_2 or calcium was applied, the mutant stomata responded like the wild-type. The protein OST1 is a Ser/Thr protein kinase which has a positive effect on ABA-mediated stomatal closure, acting upstream of ABA-induced ROS production.[130] Recent data have shown that *Arabidopsis thaliana* glutathione peroxidase 6 (ATGPX6) is also involved in ABA mediated guard cell H_2O_2 signal transduction through a negative effect in ABI2 activity.[131] These authors also showed that transgenic plants overexpressing ATGPX6 were more resistant to drought stress and recovered better than the wild type. ATGPX6 was suggested to have a dual role, being not only an important scavenger of H_2O_2, but also an essential element of the ABA signaling pathway mediating stomatal regulation in response to drought stress.[131]

All this gathered places ROS in a central role in the guard cell ABA signaling network.[132] Furthermore, ROS signaling under drought stress acts not only downstream of stomatal closure but also upstream in the ABA signaling network. Interestingly, using wheat seedling root tips it has been shown that ROS (and NO) production plays a role in ABA synthesis under drought stress.[133]

Sugars and ROS signaling. Sugars, and more precisely soluble sugars such as glucose and sucrose, seem to play a dual role with respect to ROS, either promoting ROS production or participating indirectly in ROS scavenging mechanisms through NADPH generating pathways, such as the oxidative pentose-phosphate pathway.[134] Photosynthesis activity leads to the accumulation of ROS, by the Mehler reaction and also sugars. Furthermore, sugars have been shown to be involved in the regulation of the expression of several photosynthetic related genes as well as some ROS related genes such as superoxide dismutase.[134,135] Hence it has been suggested that soluble sugars could function as signals, useful for the plant in sensing and controlling not only the photosynthetic activity but also the cellular redox balance.[134] The connection between sugar sensing and ROS signaling is however extremely complex and seems to also involve ABA signaling, at least in some specific pathways. This can be illustrated by the ABA-induced proline accumulation under water deficit. Proline accumulation under water deficit is produced in an ABA-dependent manner. In a recent work, the use of several *Arabidopsis* ABA-insensitive mutants, (*abi1*, *abi2*, *abi3*, *abi4* and *abi5*) revealed that *abi4* had an increased proline accumulation under water deficit but presented a decreased sensitivity to exogenously applied ABA.[124] This response was altered by the supply of sucrose indicating that ABI4 has a role in connecting ABA and sugar in regulating proline accumulation.[124]

Concluding Remarks and Future Challenges

For the last 15 years much information has been gathered regarding the involvement of a dynamic ROS equilibrium in the plant drought response. Although ROS can reach phytotoxic levels if drought stress is prolonged over to a certain extent (Fig. 3), the early signaling role of ROS under drought stress has been unequivocally established. The ROS signaling role is under tight modulation of the scavenging system and slight changes in ROS production or scavenging action under drought stress are likely to have immediate effects on the signal transduction. However, in order to have a clear view of the chronology of the events triggered by drought stress, there is a great need to homogenize drought treatments amongst researchers. In fact, it is quite difficult to compare the response of a plant submitted to a progressive drought stress, imposed by water withdrawal over several days, to the response of a plant submitted to a more immediate drought stress, imposed by watering with an osmotically active agent such as PEG for several hours. Furthermore, the response will also be quite different if the drought treatment is imposed on the intact plant, where the different organs cooperate as a whole or if it is applied to cut leaves. The terms "moderate" and "severe" drought stresses are also quite subjective and vary from one group to another. Recently, Verslues et al[136] have proposed several protocol-systems to study and quantify resistance to drought which could be extremely useful in a future standardization of water stress treatments.

Although the relationship between ROS, ABA, Ca^{2+} and sugars has been revealed, it is not a straightforward one and the molecular nature of the interconnecting pathways remains to be solved. ABA, ROS and Ca^{2+} are common players that are involved in cross-tolerance to many types of abiotic and biotic stress.[95,137,138] Nevertheless the information gathered shows only a glimpse on the intricate, complex and intriguing relation between ABA, Ca^{2+} and ROS signaling that is still far from being completely resolved. Some fundamental questions remain unanswered: is ROS production enhancement under drought due mainly to "side effects" of stomatal closure or are there other oxidative bursts generated by positive feedback loops (like guard cell NADPH oxidases)? Are there cellular ROS receptors? How toxic really are ROS to plant cells? Does their toxicity change with the plants physiological status and/or acclimation? The use of transgenic plants overexpressing or expressing antisense constructs resulting in inhibition of specific scavenging enzymes, or the use of mutants with impaired H_2O_2 generation will be extremely useful and are likely to help further in the study of the antioxidative mechanisms, and in disclosing the functions and biological roles of H_2O_2 in response to drought stress.

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