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SI ABIOTIC STRESS

Reactive oxygen species, abiotic stress and stress combination

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SUMMARY

Reactive oxygen species (ROS) play a key role in the acclimation process of plants to abiotic stress. They primarily function as signal transduction molecules that regulate different pathways during plant acclimation to stress, but are also toxic byproducts of stress metabolism. Because each subcellular compartment in plants contains its own set of ROS-producing and ROS-scavenging pathways, the steady-state level of ROS, as well as the redox state of each compartment, is different at any given time giving rise to a distinct signature of ROS levels at the different compartments of the cell. Here we review recent studies on the role of ROS in abiotic stress in plants, and propose that different abiotic stresses, such as drought, heat, salinity and high light, result in different ROS signatures that determine the specificity of the acclimation response and help tailor it to the exact stress the plant encounters. We further address the role of ROS in the acclimation of plants to stress combination as well as the role of ROS in mediating rapid systemic signaling during abiotic stress. We conclude that as long as cells maintain high enough energy reserves to detoxify ROS, ROS is beneficial to plants during abiotic stress enabling them to adjust their metabolism and mount a proper acclimation response.

Keywords: reactive oxygen species, abiotic stress, stress combination, abscisic acid, systemic signaling.

INTRODUCTION TO REACTIVE OXYGEN SPECIES (ROS) DURING ABIOTIC STRESS

Reactive oxygen species (e.g. $O_2^{\cdot-}$, H_2O_2, $OH^{\cdot}$, $^1O_2$) are partially reduced or activated forms of atmospheric oxygen (O_2). They are considered to be unavoidable byproducts of aerobic metabolism that have accompanied life on Earth ever since the appearance of oxygen-evolving photosynthetic organisms about 2.2–2.7 billion years ago (Mittler *et al.*, 2011). Higher plants have thus evolved in the presence of ROS and have acquired dedicated pathways to protect themselves from ROS toxicity, as well as to use ROS as signaling molecules (Foyer and Noctor, 2013; Vaahtera *et al.*, 2014; Considine *et al.*, 2015; Dietz, 2015; Mignolet-Spruyt *et al.*, 2016). If kept unchecked, ROS concentrations will increase in cells and cause oxidative damage to membranes (lipid peroxidation), proteins, RNA and DNA molecules, and can even lead to the oxidative destruction of the cell in a process termed oxidative stress (Mittler, 2002). However, this process is mitigated in cells by a large number of ROS detoxifying proteins [e.g. superoxide dismutase (SOD), ascorbate peroxidase (APX), catalase (CAT), glutathione peroxidase (GPX), and peroxiredoxin (PRX)], as well as by antioxidants such as ascorbic acid and glutathione (GSH) that are present in almost all subcellular compartments (Mittler *et al.*, 2004). The active process of ROS detoxification in plant cells is also aided by different metabolic adaptations that reduce ROS production, and by maintaining the level of free transient metals such as Fe^{2+} under control, to prevent the formation of the highly toxic hydroxyl radical ($HO^{\cdot}$) via the Fenton reaction (Halliwell and Gutteridge, 2007). On the other hand, plants actively produce ROS that are used as signal transduction molecules.

These are mainly produced at the apoplast by NADPH oxidases (termed respiratory burst oxidase homologs; RBOHs) and some oxidases and peroxidases, and at the chloroplast, mitochondria, peroxisome and possibly other cellular compartments, via different pathways (Suzuki *et al.*, 2011; Vaahtera *et al.*, 2014; Gilroy *et al.*, 2016; Mignolet-Spruyt *et al.*, 2016). This continual process of ROS production (metabolically or for signaling purposes) and ROS scavenging occurs at all cellular compartments of the cells and is controlled by the ROS gene network (Mittler *et al.*, 2004). Because each cellular compartment establishes and controls its own ROS homeostasis, altogether the different ROS levels in the different compartments can be viewed as generating a particular ROS signature. This signature can change depending on the type of cell, its developmental stage, or stress level. Different abiotic stresses and/or different combinations of abiotic stresses (stress combination) are likely to cause the formation of different ROS signatures in plant cells, and decoding these signatures via different ROS sensors can create a stress-specific signal that will tailor the acclimation response to the type of stress/combination affecting the plant.

Decoding ROS signals or signatures by the cell is thought to occur via different redox reactions in which ROS such as hydrogen peroxide (H_2O_2) will oxidize sulfur-containing residues of proteins (e.g. the -SH group of cysteine) and alter protein structure and function (e.g. via the formation of disulfide bonds). Such alterations in protein structure/function can for example regulate the binding of transcription factors (TFs) to DNA and affect transcription (Dietz, 2015, 2016; Dietz *et al.*, 2016). The above-described interface between ROS and redox changes/regulation in cells is generally termed redox biology, and is thought to play a key role in ROS-driven signal transduction and/or metabolic regulation in cells (Foyer and Noctor, 2013, 2016; Dietz, 2015, 2016). Another known effect of ROS on protein structure/function is the interaction between superoxide radicals ($O_2^{\cdot-}$) and iron-sulfur (Fe-S) clusters of certain proteins. Because membranes can function as barriers for redox levels, each subcellular compartment can contain its own redox state that will match its own ROS steady-state level, contributing to the formation of a specific cellular ROS signature during abiotic stress (Noctor and Foyer, 2016).

The two major sources of ROS during abiotic stress are shown in Figure 1. They include ROS produced as a consequence of disruptions in metabolic activity (metabolic ROS) and ROS produced for the purpose of signaling as part of the abiotic stress–response signal transduction network (signaling ROS). Metabolic ROS could directly alter the redox status of rate-limiting enzymes and control metabolic fluxes in the cell (flux control), thereby altering different metabolic reactions in order to counter the effect(s) of stress (Miller *et al.*, 2010). In addition it could affect transcription and/or translation by altering the function of key

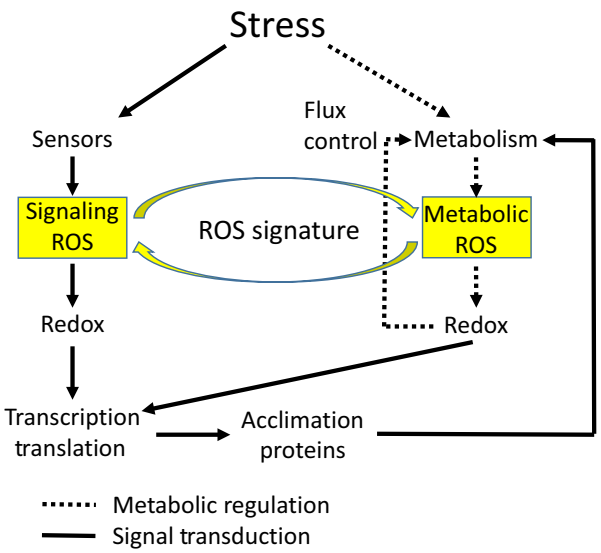

Figure 1. The role of reactive oxygen species (ROS) in abiotic stress acclimation.
The two major sources of ROS during abiotic stress, metabolic and signaling ROS, are shown to interact and form a ROS signature that controls plant acclimation to stress through redox reactions that regulate transcription and translation of stress acclimation proteins and enzymes.

regulatory proteins via ROS-derived redox modifications (Foyer and Noctor, 2013, 2016). In contrast, signaling ROS is generated as a response to stress perception by stress sensors (e.g. cyclic nucleotide-gated channels activated by heat stress; Mittler *et al.*, 2012) and is mediated by calcium- and/or phosphorylation-derived activation of NADPH oxidases (RBOH) at the plasma membrane (PM) (Suzuki *et al.*, 2011; Gilroy *et al.*, 2014). Signaling ROS is also thought to directly alter the redox state of regulatory proteins, and alter transcription and translation resulting in the activation of an acclimation response that would mitigate the effects of stress on metabolism and reduce the level of metabolically produced ROS. Metabolic and signaling ROS could be produced at different subcellular compartments (e.g. metabolic ROS in the chloroplast and signaling ROS at the apoplast). Nevertheless, they can affect the level of each other and even move between compartments (e.g. H_2O_2 that can move across membranes in a regulated process via aquoaporins; Tian *et al.*, 2016).

The different steady-state levels of ROS in the different cellular compartments (apoplast, chloroplast, peroxisome, mitochondria, vacuole, cytosol and nuclei) compile an overall ROS signature that varies in different tissues and cells subjected to different abiotic stresses and/or their combination (ROS signature). A simplified model for this is shown in Figure 2. The regulation of RBOH at the PM by calcium, phosphorylation, hormones such as NO or NADPH availability is shown to generate signaling ROS at the apoplast (Gilroy *et al.*, 2016). This signaling ROS then

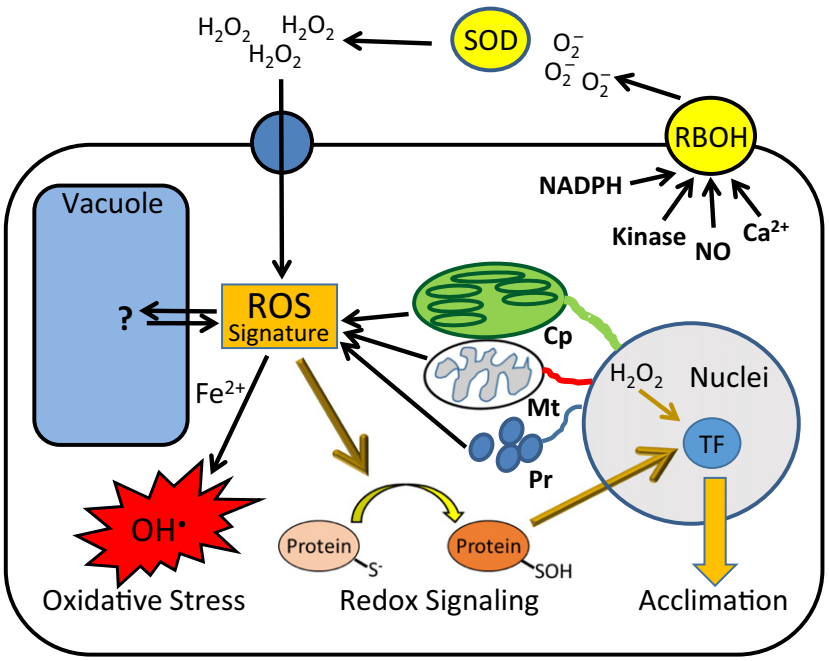

Figure 2. The interaction between reactive oxygen species (ROS) produced in different compartments during abiotic stress, redox signaling that leads to acclimation, and oxidative stress caused by the presence of labile iron in cells.
Metabolic and signaling ROS are shown to accumulate in the different compartments of the cells and generate an abiotic stress-specific ROS signature. This signature is shown to alter protein redox reactions and control plant acclimation. The presence of labile iron in cells is shown to be a primer for the initiation of oxidative stress that could in turn cause oxidative cell injury and death, highlighting the need to maintain the levels of free iron under control during stress. Abbreviations: Cp, chloroplast; Mt, mitochondria; Pr, peroxisome; RBOH, respiratory burst oxidase homolog; TF, transcription factor; SOD, superoxide dismutase.

moves into the cytoplasm via regulated aquaporins (Tian *et al.*, 2016), and together with metabolic and signaling ROS produced in the chloroplast, peroxisome and mitochondria (Dietz *et al.*, 2016; Huang *et al.*, 2016; Kerchev *et al.*, 2016; Rodríguez-Serrano *et al.*, 2016; Takagi *et al.*, 2016) alters the redox status of key regulatory proteins such as TFs affecting gene expression. Recent studies have shown that under stress conditions chloroplast, peroxisomes and mitochondria can extend membrane structures (stromules, peroxules and matrixules, respectively) that will contact the nuclear envelope and could directly alter the ROS status of the nuclei (Noctor and Foyer, 2016). However, if labile Fe^{2+} exist in cells, ROS such as H_2O_2 can react with it to generate the highly toxic hydroxyl radical that would lead to oxidative stress and cell damage. Regulating iron levels in the cell in response to abiotic stress is therefore very important, and was recently highlighted by the interplay between the ROS-response zinc finger protein ZAT12 and iron uptake into cells (Le *et al.*, 2016). Despite the fact that it occupies a relatively large volume of the plant cell and could have a significant buffering capacity of ROS, the role of the vacuole in ROS signaling and metabolism is currently unknown (Figure 2).

SOURCES OF ROS DURING ABIOTIC STRESS

The major ROS-producing sites during abiotic stress are the chloroplast, mitochondria, peroxisome and apoplast (Dietz *et al.*, 2016; Gilroy *et al.*, 2016; Huang *et al.*, 2016; Kerchev *et al.*, 2016; Rodríguez-Serrano *et al.*, 2016; Takagi *et al.*, 2016). Abiotic stresses that limit CO_2 availability due to stomatal closure enhance the production of ROS such as $O_2^{\cdot-}$ and $^1O_2$ in chloroplasts that, in turn, can initiate retrograde and anterograde signaling (Asada, 2006; Sarvajeet and Narendra, 2010; Baniulis *et al.*, 2013; Kleine and Leister, 2016; Mignolet-Spruyt *et al.*, 2016). ROS production during stress can also balance the energy distribution between PSII and PSI and affect photosystem stoichiometry (Dietzel *et al.*, 2008; Vainonen *et al.*, 2008; Pesaresi *et al.*, 2009). The production of $^1O_2$ in chloroplasts can also cause reprogramming of nuclear gene expression leading to chlorosis and programmed cell death, as well as induce a wide range of responses related to biotic and abiotic stresses through the function of EXECUTER1 (EX1) and EX2, two nuclear-encoded chloroplast proteins associated with thylakoid membranes (Wagner *et al.*, 2004; Lee *et al.*, 2007; Kleine and Leister, 2016). Chloroplastic ROS is mitigated by an array of ROS-scavenging enzymes and pathways such as Fe- and CuZn-SODs and the Asada–Foyer–Halliwell pathway, as well as high concentrations of antioxidants such as ascorbic acid and GSH (Mittler *et al.*, 2004). Mitochondrial ROS accumulation during abiotic stress is typically mediated via electron leakage from complex I and III to produce $O_2^{\cdot-}$, which can be converted to H_2O_2 by Mn-SOD (Quan *et al.*, 2008; Huang *et al.*, 2016). This process can be mitigated by alternative oxidase (AOX), type II NAD(P)H dehydrogenase and uncoupling proteins in the inner mitochondrial membrane (Noctor *et al.*, 2007; Rasmusson and Wallstrom, 2010). ROS can regulate AOX1 expression via WRKY15, which represses AOX1 by binding to its promoter region (Vanderauwera *et al.*, 2012). Alteration in the levels of ROS produced by mitochondria during abiotic stress can induce retrograde signaling between mitochondria and nucleus and control plant acclimation (Woodson and Chory, 2008). Production of ROS in peroxisomes

during abiotic stress is mainly the outcome of enhanced photorespiration resulting in the production of H_2O_2 by glycolate oxidase (Foyer and Noctor, 2009; Sarvajeet and Narendra, 2010; Baishnab and Ralf, 2012; Kerchev *et al.*, 2016). Photorespiratory ROS production is primarily mitigated by CAT, and mutants deficient in peroxisomal CAT have been a major tool in the study of H_2O_2 signaling during stress (e.g. Kerchev *et al.*, 2016). Peroxisomal ROS can impact the cellular redox balance and alter nuclear gene transcription (Vanderauwera *et al.*, 2005).

Production of ROS at the apoplast during abiotic stress is mediated via at least four different mechanisms. The most studied of the four are the PM NADPH oxidase-RBOH proteins that link calcium and ROS signaling during stress and produce superoxide in the apoplast (Gilroy *et al.*, 2014, 2016). RBOHs have been shown to play a key role in signal transduction reactions that mediate plant acclimation to abiotic stress, and mutants deficient in RBOHs such as *rbohD* and *rbohF* have been a valuable tool in the study of ROS-abiotic stress interactions. Apoplastic ROS production during abiotic and biotic stress can also be mediated by peroxidases (O'Brien *et al.*, 2012). Peroxidase-generated ROS was shown, for example, to be involved in regulating root growth and response to potassium deficiency (Kim *et al.*, 2010). Another important ROS-producing protein in the apoplast during abiotic stress is oxalate oxidase. Oxalate oxidase-mediated H_2O_2 production in root cell was, for example, shown to be important for drought stress acclimation (Voothuluru and Sharp, 2013). In addition to these, xanthine dehydrogenase was also recently proposed to play a role in stress signaling (Ma *et al.*, 2016). Countering ROS levels in the apoplast are CuZn-SODs, APXs, cell wall-bound peroxidases, and low levels of ascorbate and GSH. However, these apoplastic ROS-scavenging mechanisms are not as efficient as the intracellular ROS-scavenging systems, and allow the accumulation of ROS to high levels at the apoplast (important for systemic signaling and pathogen defense).

The steady-state level of ROS and the redox state of each of the compartments described above is likely to vary depending on the type of abiotic stress encountered by the plant. Thus, each set of different environmental conditions (e.g. drought, salinity, cold, heat, etc.) will result in a specific subcellular ROS and redox signature that will in turn result in the activation of an acclimation response tailored to it (Figure 2). The activation of acclimation responses by ROS could initially be mediated by interactions of ROS with different proteins and hormones as described below.

ROS-INDUCED PROTEIN MODIFICATIONS AND THEIR ROLE IN STRESS ACCLIMATION

Reactive oxygen species can provoke reversible or irreversible modifications of proteins, causing in turn alterations in the control and regulation of plant metabolism, as well as the activation of transcriptional regulatory networks. The study of ROS-induced protein modifications is, therefore, fundamental to our understanding of how ROS could modify metabolism and gene expression during abiotic stress. Among the most important ROS-induced post-translational modifications are sulfonylation, carbonylation, glutathionylation and S-nitrosylation.

Sulfonylation, one of the main mechanisms that regulates the activity of many enzymes and TFs in plants, is the oxidation of sulfhydryl groups. This oxidation is mainly induced by H_2O_2 generating sulfenic acid (R-SOH) that can lead to the formation of disulfide (S–S) bonds between cysteine residues, which in turn result in conformational changes that alter protein/enzyme activity. The 'recovery' of a protein from this oxidized state is mainly mediated via thioredoxins (Trxs), PRXs and the GSH system, which respond to stress and regulate redox homeostasis. Several enzymes of the Benson–Calvin cycle [e.g. Fru-1,6-bisphosphatase (FBPase) or the malate valve] are regulated, for example, in this manner; reduced and active in the light and oxidized and inactive in the dark (Scheibe *et al.*, 2005). Regulation via reduced Trx therefore prevents a waste of energy by activation of FBPase and seduheptulose-bisphosphatase in the reductive cycle, and a parallel inactivation of the Glc-6-P dehydrogenase in the oxidative cycle. This directs FBP into the reductive cycle in the light. In the dark, Trx becomes oxidized and the opposite situation becomes predominant. The reduction state of Trx creates, therefore, a conditional separation of metabolic fluxes within the same compartment. Another type of sufonylation occurs with methionine (Met) oxidation, which yields Met-sulfoxide. Enzymes affected by this modification can sometimes be reactivated via reduction by methionine sulfoxide reductase using Trx as reductant (Gustavsson *et al.*, 2002), providing yet another example of the importance of the Trx pathway in the control of numerous enzymatic activities under stress conditions.

As indicated above, the first step in the ROS-dependent redox signaling pathway results from the oxidation of cysteine residues to sulfenic acid. Sulfenic acid-containing side-chains are highly reactive and can form covalent bonds with low molecular weight thiols, such as GSH giving rise to S-glutathionylation. This modification can act as a redox-driven regulator of signal transduction cascades and metabolic pathways (Fratelli *et al.*, 2004). Glutathionylation can be reversed via the activity of thiol-disulfide oxidoreductases Grxs (also known as thioltransferases; Gallogly and Mieyal, 2007). S-Glutathionylation may also be of physiological importance in buffering the GSSG/GSH pool as well as having additional regulatory functions (Di Simplicio *et al.*, 1998; Schafer and Buettner, 2001).

Tryptophan (Trp) oxidation to Trp hydroperoxide, which is highly unstable and rapidly decomposes into N-formylkynurenine and kynurenine as major end-products, represents another mode by which ROS can modify proteins (Ronsein *et al.*, 2008). This mode of protein oxidation was

found to play an important role in the regulation of photosynthesis. Thus, oxidation of Trp365 to NFK in the CP43 subunit of PSII correlates with high light stress and increased photoinhibition (Dreaden *et al.*, 2011; Kasson and Barry, 2012). CP43 and D1 Trp oxidation to NFK appears to be linked to D1 degradation and subsequent replacement of the damaged D1 proteins (Kasson and Barry, 2012). In a mass spectrometry study of Arabidopsis mitochondrial cell culture proteins, Trp oxidation was also found in glycine decarboxylase (one of the main enzymes in the photorespiration process catalyzing the oxidative decarboxylation and deamination of glycine; Douce *et al.*, 2001), mitochondrial peptidase from complex III (critical in the oxidative phosphorylation and the biochemical generation of ATP; Crofts, 2004) and in Mn-SOD.

Carbonylation, the oxidation of residues, such as Arg, His, Lys, Pro and Thr, constitutes another form of protein oxidation, which is thought to be irreversible (Shacter, 2000). Carbonylation of proteins can also be mediated by indirect reactions of lipoperoxidation products with Cys and His residues (Madian and Regnier, 2010). Several mitochondrial enzymes such as aconitase, pyruvate dehydrogenase and glycine decarboxylase are sensitive to inactivation by oxidation and carbonylation, and the inhibition of these enzymes by an increase in ROS production may result in slowing down the flow to the TCA cycle and a consequent decrease in the energy status of the cell (Schwarzlander and Finkemeier, 2013; Camejo *et al.*, 2015). Several chloroplastic proteins were also shown to be the target of carbonylation during oxidative stress induced by high light in Arabidopsis. These included Cys synthase, Asp kinase and Rubisco (Davletova *et al.*, 2005).

S-Nitrosylation, the covalent binding of NO to thiol groups of Cys, is another post-translational modification that can regulate the function of some proteins during stress. Camejo *et al.* (2013) showed that different enzymes involved in respiration, antioxidation and photorespiration were S-nitrosylated during salinity stress. In plants subjected to low temperatures, the main S-nitrosylated proteins were those related to C metabolism (Puyaubert *et al.*, 2014). S-Nitrosylation of proteins is essential for metabolic reprogramming that is necessary to keep homeostasis under stress conditions. S-Nitrosylation also induces changes in some TFs, which affect their binding to DNA, as well as inactivate RBOH (Yun *et al.*, 2011). For example, S-nitrosylation can act as a negative regulator of MYB TFs, which are essential regulators of abiotic and stress responses (Tavares *et al.*, 2014).

INTERACTIONS OF ROS WITH STRESS HORMONES

Plant hormones play a key role in shaping the acclimation response of plants to abiotic stress. Recent studies have shown that an intricate interplay exists between plant hormones and ROS during abiotic stress. Thus, in addition to directly affecting proteins and altering metabolic fluxes and transcription, the accumulation of ROS during abiotic stress affects the level and function of different plant hormones, such as abscisic acid (ABA), auxin, brassinosteroids (BRs), gibberellins (GAs) and NO. Below, we highlight some of these interactions.

Plant responses to abiotic stress were recently shown to be influenced by reciprocal interactions between ROS and auxin, affecting auxin balance and resulting in altered growth (Tognetti *et al.*, 2012). Stress-induced ROS production can alter auxin gradients in the plant and also reduce auxin-mediated signaling (Xia *et al.*, 2015). The mechanisms associated with changes in auxin homeostasis and signaling attenuation include: oxidative auxin degradation (Kawano, 2003); auxin conjugation (Tognetti *et al.*, 2010); and auxin distribution through changes in the expression of genes encoding auxin transporters (Grunewald and Friml, 2010). Auxins can induce the production of ROS (Tognetti *et al.*, 2012) and regulate ROS homeostasis (Pasternak *et al.*, 2005), hinting at the relationship between auxin signaling and oxidative stress (Tognetti *et al.*, 2012). For example, auxins activate a Rho-GTPase (RAC/ROP) that interacts with NADPH oxidases, resulting in apoplastic ROS production (Duan *et al.*, 2010). On the other hand, ROS trigger a MAPK cascade that inhibits auxin-dependent signaling while activating oxidative stress signaling (Kovtun *et al.*, 2000). The auxin-dependent increase in apoplastic superoxide ions facilitates cell wall modifications during cell elongation in *Zea mays* (Schopfer *et al.*, 2001). Auxin-induced changes in cellular redox status, brought about by auxin-induced ROS production, regulate plant cell cycle (Vivancos *et al.*, 2011). Although the examples cited above suggest a close association between ROS and auxin-mediated processes, the cellular/molecular mechanisms controlling auxin-induced ROS synthesis remain unknown.

Brassinosteroids are hormones associated with a number of biochemical and physiological processes in plants, and are linked to the response of plants to abiotic stress (Xia *et al.*, 2009). Abiotic stress tolerance in tomato was correlated with BR synthesis. BRs induced *RBOH* transcription and increased NADPH oxidase activity with the concomitant increase in apoplastic H_2O_2 (Nie *et al.*, 2013). Similar effects of ABA on *RBOH* expression and the production of apoplastic H_2O_2 have been reported (Xia *et al.*, 2015) and, although the genetic basis of a crosstalk between BRs and ABA remains to be clarified, the notion of a positive feedback mechanism in which BRs induce, through the activation of *RBOH*, the transient accumulation of H_2O_2 and the induction of ABA biosynthesis has been proposed (Zhou *et al.*, 2014). This mechanism would lead to prolonged H_2O_2 production and the induction of plant stress tolerance.

Gibberellins are involved in the response of plants to abiotic stress, and their action is associated with the

control of growth through the control of cell division and cell elongation (Colebrook *et al.*, 2014). GAs exert their function through the regulation of DELLA proteins, negative regulators of GA signaling (Achard *et al.*, 2006). The binding of GAs to the nuclear receptor GID1 induces conformational changes in the protein, favoring its interaction with DELLA proteins. As a consequence of this interaction, DELLA is ubiquitinated and targeted for degradation via the 26S proteasome (Colebrook *et al.*, 2014). GA signaling regulates stress tolerance through the control of cellular redox homeostasis. Water-deficit reduced leaf GA contents in maize (Wang *et al.*, 2008), leading to an increase in DELLA activity that resulted in increased ROS quenching capacity and improved survival. Arabidopsis quadruple DELLA mutants showed increased expression of genes encoding antioxidant enzymes, with the concomitant reduction in ROS accumulation in plants under high salinity (Achard *et al.*, 2009). Similar results were seen in rice plants expressing *SUB1A* (*SUBMERGENCE 1A*; Fukao *et al.*, 2011). SUB1A restricted the accumulation of ROS and diminished oxidative damage during submergence stress, through the accumulation of negative regulators of GA signaling, the DELLA protein SLR1 and the SLR-like 1 (Fukao *et al.*, 2006).

Abscisic acid plays significant roles in plant development, the regulation of stomata function and the response of plants to abiotic stresses. During the exposure of plants to stress conditions, ABA concentrations in the plant increase as a result of increased biosynthesis, release of active ABA from its conjugated forms or decreased degradation (Boursiac *et al.*, 2013). Water-deficit and high salinity stress promote ABA accumulation that induces changes in gene expression (Shinozaki and Yamaguchi-Shinozaki, 2007) and the closing of stomata (Mittler and Blumwald, 2015). Stomata closure reduces transpiration and water loss, but also promotes decreased gas exchange and a reduction in photosynthetic activity. Upon binding of ABA to the pyrabactin-resistance protein/PYR-like proteins (PYR/PYLs) receptor complex, and the suppression of protein phosphatase 2C (PP2C), the activation of the SnRK2 protein kinase OST1 leads to the activation of PM-bound NADPH oxidase (RBOH) that mediates the production of superoxide and the generation of H_2O_2, via the action of apoplastic CuZn-SODs (Sirichandra *et al.*, 2009). H_2O_2 generated by RBOH-SOD and/or arriving at the guard cells with the ROS wave (Mittler and Blumwald, 2015) resulted in the opening of ROS-regulated Ca^{2+} channels that in turn induced the activation of RBOH by CIPK26 (Drerup *et al.*, 2013), resulting in further biosynthesis of ROS and generating a positive feedback loop for stomata closure. Because ROS can directly inactivate PP2C, ABA and ROS can function in a positive amplification loop that controls stomatal function as well as gene expression during stress (Mittler and Blumwald, 2015).

NO is the most abundant among reactive nitrogen species, and has been associated with numerous plant physiological processes (Niu and Liao, 2016). NO mediates the post-translational modification of target proteins through S-nitrosylation and nitration. Under water-deficit conditions, ABA induces NO and ROS synthesis. Both NO and ROS form 8-nitro-cGMP inducing stomata closure (Joudoi *et al.*, 2013). Similar to ABA, auxin can also induce ROS and NO synthesis, and both can act on auxin-mediated signaling (Yadav *et al.*, 2011; Farnese *et al.*, 2016;). Shi *et al.* (2015) compared auxin signaling and auxin transport in Arabidopsis Col-0 and gsnor1-3 (a mutant defective in protein de-nitrosylation), and showed that auxin signaling and polar auxin transport were reduced, demonstrating the role of S-nitrosylation in auxin signaling.

As indicated above, ROS can function on many different levels to affect or mediate the acclimation of plants to abiotic stresses. Below we will address the involvement of ROS in two emerging fields of plant abiotic stress research: stress combination and systemic acclimation.

ROS AND STRESS COMBINATION

Stress combination is a term used to describe a situation in which a plant is simultaneously subjected to two or more abiotic stresses (Mittler, 2006). Although stress combination has been acknowledged as a major cause of crop loss worldwide (reviewed in Mittler, 2006; Mittler and Blumwald, 2010; Suzuki *et al.*, 2014), it has only recently been addressed in laboratory studies at the molecular level (Rizhsky *et al.*, 2002, 2004). In general, the combination of two or more abiotic stresses has a negative impact on plants that is greater than that of each of the different stresses applied individually. In addition, stress combinations such as drought and heat, or salinity and heat result in the activation of unique transcriptome responses that involve hundreds of transcripts not altered by each of the different stresses applied individually (Rizhsky *et al.*, 2004; Suzuki *et al.*, 2016). In contrast to examples such as drought and heat, some abiotic stresses (e.g. ozone) could actually enhance the tolerance of plants to another abiotic (e.g. drought) or biotic (e.g. bacterial infection) stress when the two stresses are combined (Mittler, 2006; Mittler and Blumwald, 2010; Suzuki *et al.*, 2014; Foyer *et al.*, 2016). From the standpoint of ROS involvement, a number of studies have shown that ROS levels, the expression of different ROS-scavenging enzymes and the level of different antioxidants display a unique pattern during stress combination that is different than that found to be induced by each of the different stresses applied separately. These changes were reflected in levels of $O_2^{\cdot-}$, H_2O_2, byproducts of lipid peroxidation, expression of enzymes such as SOD, APX, CAT, AOX, peroxidases, glutathione-S-transferase, glutathione reductase and GPX, accumulation of antioxidants such as ascorbate, GSH, flavonols, phenolic

compounds, alkaloids, tocopherol and carotenoids, and accumulation of osmoprotectants such as proline, glycine betaine, trehalose and sucrose (Keleş and Öncel, 2002; Rizhsky *et al.*, 2002; Rizhsky *et al.*, 2004; Giraud *et al.*, 2008; Vile *et al.*, 2012; Prasch and Sonnewald, 2013; Rasmussen *et al.*, 2013; Li *et al.*, 2014; Rivero *et al.*, 2014; Suzuki *et al.*, 2014; Vuleta *et al.*, 2015; Jin *et al.*, 2015; Pandey *et al.*, 2015; Carvalho *et al.*, 2016; Martinez *et al.*, 2016). Because the combination of two different stresses imposes on plants a unique set of physiological restrains, it is likely that the ROS signature generated under conditions of stress combination is unique (Figure 3). For example, a combination of drought and heat stress imposes two opposing demands on the plant: open stomata to cool the leaves off – a typical response of plants to heat a stress; but at the same time close stomata to avoid water loss – a typical response to drought (Rizhsky *et al.*, 2002, 2004). Interestingly, Arabidopsis mutants deficient in cytosolic APX1 (*apx1*), but not chloroplastic thylakoid APX, were found to be highly sensitive to this stress combination suggesting that cytosolic and not chloroplastic H_2O_2 levels are important for acclimation to this particular stress combination (Koussevitzky *et al.*, 2008). In addition, mutants impaired in the function of the ABA and ROS-regulated protein PP2Cs (*abi-1*) were found to be highly sensitive to a combination of drought and heat, as well as salinity and heat, further highlighting the importance of ROS–ABA interactions for plant acclimation to stress combination (Suzuki *et al.*, 2016; Zandalinas *et al.*, 2016). The importance of ROS for plant acclimation to stress combination is also underscored by the large number of studies that found elevated ROS-response transcripts, as a key component of the stress combination-acclimation response pathway (reviewed in Suzuki *et al.*, 2014). Future studies conducted in this emerging and important field of plant stress research will likely highlight additional roles for ROS in plant acclimation to stress combination. For now, however, ROS and ABA appear to be two of the key regulators that mediate the acclimation of plants to stress combination.

ROS IN SYSTEMIC SIGNALING

Reactive oxygen species were recently shown to mediate rapid systemic signaling in plants in response to abiotic stress in a process that is coupled to calcium signaling and perhaps even electric waves (Miller *et al.*, 2009; Mittler *et al.*, 2011; Gilroy *et al.*, 2014, 2016; Figure 4). In order for a plant to achieve maximal fitness in the field, the response of all of its organs and leaves to abiotic stress needs to be coordinated. This coordination is thought to be achieved by an auto-propagating wave of ROS production (Figure 4a; the ROS wave) that is initiated in a group of cells that first senses the stress and spreads to the entire plant at a rate of up to 8.4 cm min^{-1} (Miller *et al.*, 2009). The basic mechanistic model describing the ROS wave postulates that abiotic stress affecting local cells results in

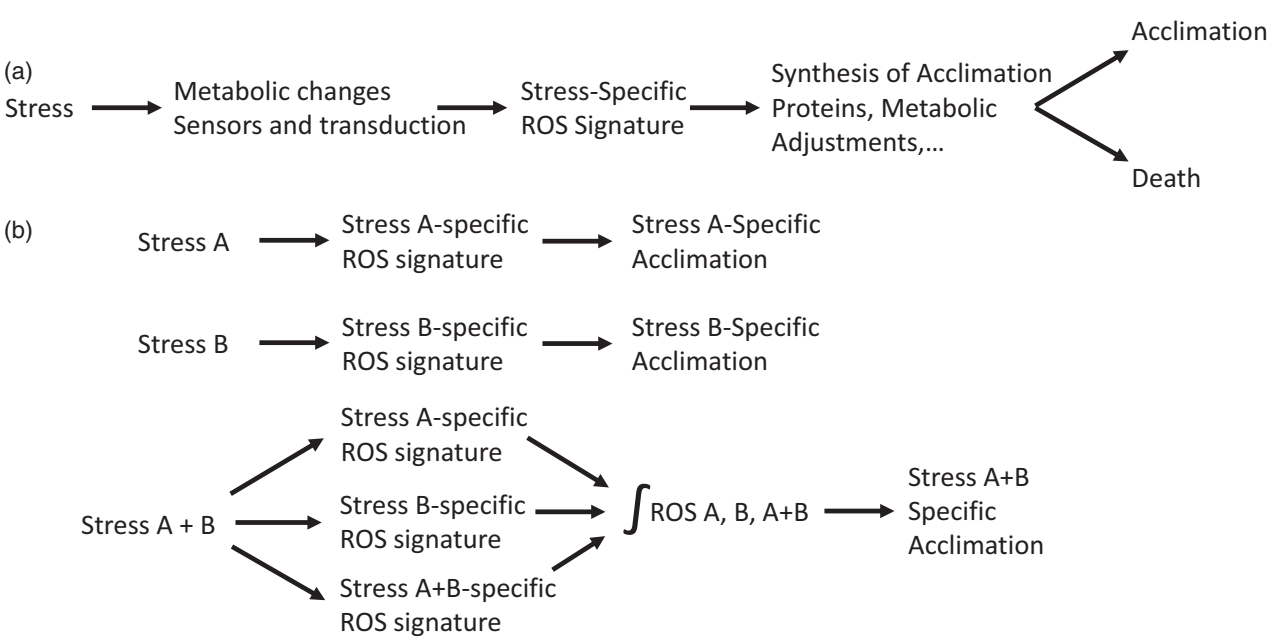

Figure 3. Reactive oxygen species (ROS) signatures during abiotic stress combination.
(a) Abiotic stress is shown to result in the formation of a ROS signature that mediates plant acclimation and cell death.
(b) A combination of two different stresses (Stress A and Stress B) is shown to generate a ROS signature that is unique to the stress combination and is the result of combining three different ROS signatures (ROS signature for Stress A, ROS signature for Stress B, and ROS signature generated from the combination of the two different stresses, i.e. A + B).

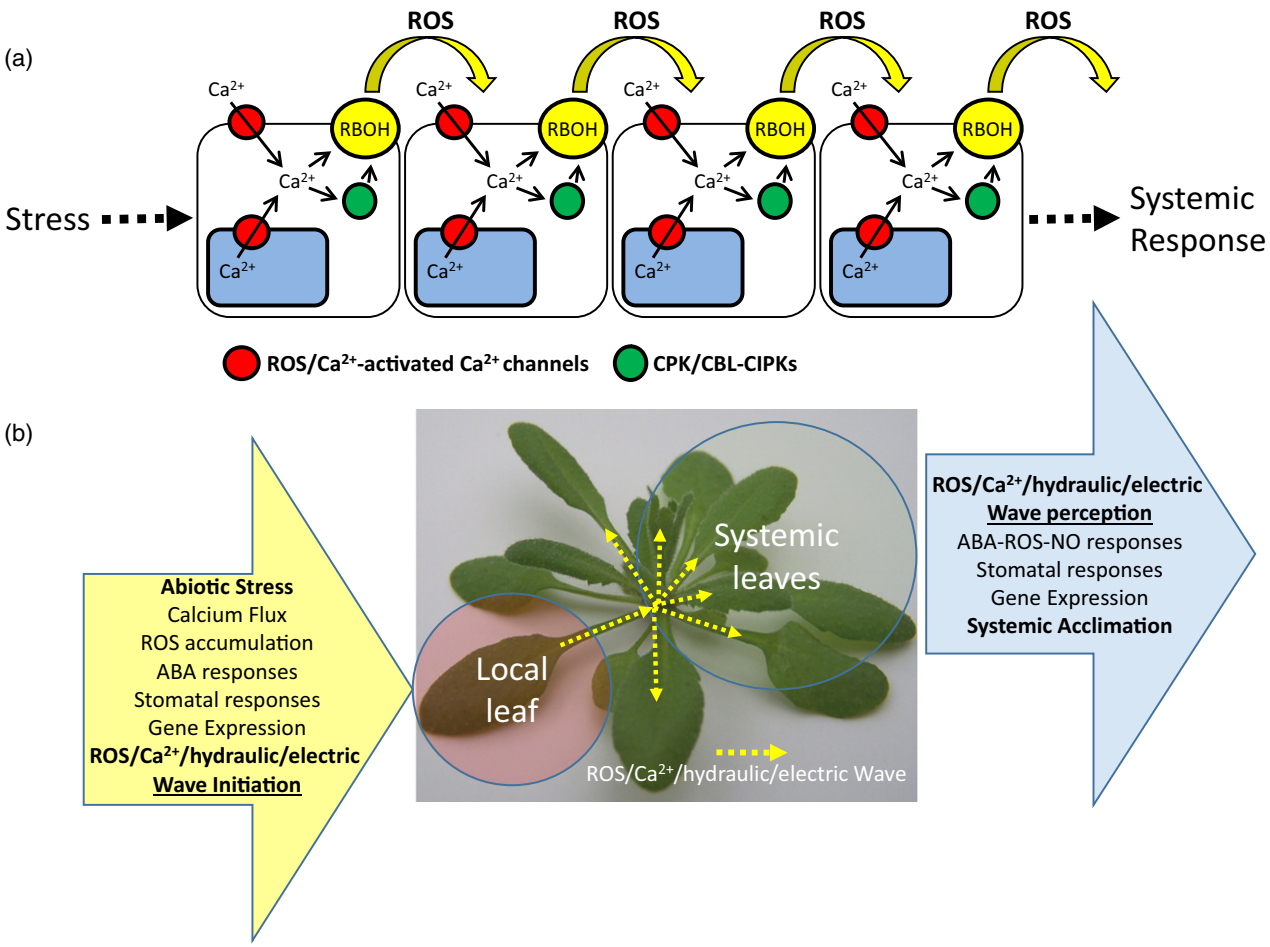

Figure 4. The reactive oxygen species (ROS) wave.
(a) A simplified model for the propagation of the ROS wave. Abiotic stress is shown to cause calcium fluxes in a cell that activates ROS production via respiratory burst oxidase homolog (RBOH). The accumulation of the RBOH-produced ROS in the apoplast is then sensed by a neighboring cell and triggers its ROS/calcium-activated calcium channels, resulting in the formation of calcium fluxes that in turn activate ROS production via RBOH in the neighboring cell. This state of ROS/calcium-activated calcium flux causing RBOH activation and apoplastic ROS production via RBOH (the ROS wave) is then auto-propagated throughout the plant resulting in the activation of systemic acquired acclimation (SAA) in systemic tissues. For a more detailed model, see Gilroy *et al.* (2016).
(b) The leaf-autonomous systemic signaling hypothesis. A local leaf subjected to abiotic stress is shown to activate the combined ROS/calcium/hydraulic/electric wave via the coordinated function of calcium fluxes, ROS, abscisic acid (ABA) and stomatal responses. Upon perception of the combined wave in systemic leaves, each leaf is shown to activate acclimation mechanisms through ABA-ROS-NO and stomata interactions that lead to alteration in gene expression and acclimation. For a more detailed model, see Mittler and Blumwald (2015). Abbreviation: CPK/CBL-CIPKs, Ca^{2+}-dependent protein kinases.

a flux of calcium into the cytosol. This flux directly activates RBOHs, and/or triggers a cascade of events that activates calcium-dependent protein kinases that phosphorylate and activate RBOHs (Miller *et al.*, 2009; Mittler *et al.*, 2011; Dubiella *et al.*, 2013; Gilroy *et al.*, 2014; Figure 4a). The activated RBOHs generate ROS at the apoplast that is sensed by neighboring cells triggering a calcium flux in these cells that will activate their own RBOHs. This state of ROS-derived calcium flux – coupled with calcium-derived activation of RBOHs – is then auto-propagated from cell to neighboring cell throughout the entire plant, and triggers systemic responses to abiotic stress (Miller *et al.*, 2009; Figure 4a). Recent studies have shown that the ROS wave is mediated by RBOHD in Arabidopsis and that it is coordinated with a systemic calcium wave (Gilroy *et al.*, 2016). In addition, the ROS wave was shown to be required for the generation of some electric signals during abiotic stress (Suzuki *et al.*, 2013). Furthermore, the ROS wave was shown to be required to induce a systemic acclimation response to light or heat stress (Suzuki *et al.*, 2013). At least when it comes to heat stress, the ROS wave was also shown to be coordinated with ABA function in systemic leaves (Suzuki *et al.*, 2013). Further to the discovery of the ROS wave (Miller *et al.*, 2009) and its interaction with the calcium wave (Choi *et al.*, 2014; Gilroy *et al.*, 2014), it was proposed that abiotic stress responses in each leaf are controlled in a leaf autonomous way and linked to stomata function, and that the response of each leaf is communicated to all other leaves via the combined function of the ROS, calcium, hydraulic (León *et al.*, 2001) and

electric waves (Mittler and Blumwald, 2010; Figure 4b). This model explains many of the previous observations regarding systemic plant responses to stress, and proposes a key role for ROS, ABA and stomatal responses in the systemic acclimation response of plants to abiotic stress (Mittler and Blumwald, 2010). Although the ROS wave is thought to be primarily regulated by RBOHs, recent studies have highlighted other types of ROS as well as other types of ROS producers as potential contributors to rapid systemic signaling. Singlet oxygen produced in the chloroplast was, for example, shown to be required to initiate the RBOH-derived ROS wave in response to high light stress (Carmody *et al.*, 2016). In addition, a possible role for glutamate receptor-like channels and NO was proposed in integrating the ROS, calcium and electric waves during systemic acquired acclimation (SAA; Gilroy *et al.*, 2016). The studies described above point to a key role for the ROS wave in priming the entire plant for the induction of SAA state. Although it does not convey abiotic stress specificity to the systemic response, the ROS wave is absolutely required for it (Suzuki *et al.*, 2013).

ARE ROS GOOD OR BAD FOR ABIOTIC STRESS?

Reactive oxygen species have recently been shown to be beneficial to animal cells promoting cellular proliferation and overall health (Schieber and Chandel, 2014). During abiotic stress in plants ROS could have a few important beneficial roles. For example, ROS production in the chloroplast could divert electrons from the photosynthetic apparatus preventing overload of the antenna and subsequent damage. A similar sink function could also be mediated by ROS in the mitochondria. Diverting electrons and preventing overload of different systems in the cell during stress via ROS production is, of course, only possible because plant cells contain multiple levels of ROS detoxification pathways and mechanisms (Asada, 2006). ROS could also be mediating the regulation of metabolic fluxes during stress to prevent damage or over-accumulation of certain intermediates toxic to cells. Of course, the most beneficial role of ROS during abiotic stress is likely their function in signal transduction reactions mediating the activation of acclimation pathways (Figures 1–3; Foyer and Noctor, 2013; Vaahtera *et al.*, 2014; Considine *et al.*, 2015; Dietz, 2015; Mignolet-Spruyt *et al.*, 2016; Mittler, 2016), and the application of ROS was for example shown to prime plant defenses to abiotic stress (Hossain *et al.*, 2015). Accordingly, mutants impaired in ROS production or ROS scavenging were found to be more sensitive to abiotic stresses as well as unable to mediate systemic signaling during abiotic stress (Davletova *et al.*, 2005; Suzuki *et al.*, 2013; reviewed in Mittler *et al.*, 2004; Suzuki *et al.*, 2011). Among the negative aspects of ROS function during abiotic stress are their potential toxicity and the energetic costs associated with their detoxification. Thus, pathways such as the Asada–Foyer–Halliwell pathway require energy in the form of NAD(P)H and, once this energy is depleted, these pathways would be unable to prevent ROS toxicity (Mittler *et al.*, 2004). Overall, and as long as the cell maintains sufficient energy reserves to detoxify ROS, ROS appear to be beneficial to plants during abiotic stress, enabling them to adjust their metabolism and mount a proper acclimation response.

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AUTHOR CONTRIBUTION

FKC, RMR, EB and RM wrote the paper.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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