

REVIEW PAPER

ROS as key players in plant stress signalling

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Abstract

Reactive oxygen species (ROS) play an integral role as signalling molecules in the regulation of numerous biological processes such as growth, development, and responses to biotic and/or abiotic stimuli in plants. To some extent, various functions of ROS signalling are attributed to differences in the regulatory mechanisms of respiratory burst oxidase homologues (RBOHs) that are involved in a multitude of different signal transduction pathways activated in assorted tissue and cell types under fluctuating environmental conditions. Recent findings revealed that stress responses in plants are mediated by a temporal–spatial coordination between ROS and other signals that rely on production of stress-specific chemicals, compounds, and hormones. In this review we will provide an update of recent findings related to the integration of ROS signals with an array of signalling pathways aimed at regulating different responses in plants. In particular, we will address signals that confer systemic acquired resistance (SAR) or systemic acquired acclimation (SAA) in plants.

Key words: Reactive oxygen species (ROS), respiratory burst oxidase homologue (RBOH), stress response, systemic acquired acclimation (SAA), systemic acquired resistance (SAR), temporal–spatial coordination.

Introduction

The reactive oxygen species (ROS) signalling network is highly conserved among aerobic organisms and controls a broad range of biological processes such as growth, development, and responses to biotic and/or abiotic stimuli (Mittler *et al.*, 2011). Although early research involving ROS metabolism focused on the potential toxicity of ROS and the different ROS-scavenging mechanisms, more recent studies have focused on the role ROS play as signalling molecules. To utilize ROS as signalling molecules, non-toxic levels must be maintained in a delicate balancing act between ROS production, involving ROS-producing enzymes and the unavoidable production of ROS during basic cellular processes, and the metabolic counter-process involving ROS-scavenging pathways (Mittler *et al.*, 2004). In plants, NADPH oxidases, respiratory burst oxidase homologues (RBOHs), play a key

role in the network of ROS production (Torres and Dangl, 2005; Suzuki *et al.*, 2011). In *Arabidopsis*, RBOHs constitute a multigenic family comprised of 10 genes (i.e. AtRBOHA–AtRBOHJ). In recent years, several studies have revealed that plant RBOHs are involved in a multitude of different signalling pathways including root hair growth, stomatal closure, pollen–stigma interactions, plant defence, and acclimation to different abiotic stresses (Torres *et al.*, 2005; McInnis *et al.*, 2006; Monshausen *et al.*, 2007; Jammes *et al.*, 2009; Miller *et al.*, 2009; Nishimura and Dangl, 2010; Suzuki *et al.*, 2011).

Various biological processes that occur in different tissue types, under a multitude of environmental conditions, might be regulated by temporal and spatial coordination between ROS and other signals. In response to stress stimuli, early signalling events in plants include increased flux of Ca²⁺ into

Abbreviations: ABA, abscisic acid; ACC, 1-aminocyclopropane-1-carboxylic acid; AzA, azelaic acid; BR, brassinosteroid; CDPK, calcium-dependent protein kinase; DA, dehydroabietinal; G3P, glycerol-3-phosphate; JA, jasmonic acid; MAPK, mitogen-activated protein kinase; MeSA, methyl salicylate; NPQ, non-photochemical quenching; PA, phosphatidic acid; PAMP, pathogen-associated molecular pattern; Pip, pipercolic acid; PQ, plastoquinone; RBOH, respiratory burst oxidase homologue; ROS, reactive oxygen species; SA, salicylic acid; SAA, systemic acquired acclimation; SAR, systemic acquired resistance.

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the cytosol, activation of mitogen-activated protein kinases (MAPKs), and protein phosphorylation (Benschop *et al.*, 2007). These regulatory mechanisms can all be activated within seconds or minutes (Benschop *et al.*, 2007; Miller *et al.*, 2009; Finka *et al.*, 2012). Following these early signalling events, long-term responses control phenotypic changes such as growth, development, and survival of cells (Torres and Dangl, 2005; Coupe *et al.*, 2006; Muhlenbock *et al.*, 2008; Pesaresi *et al.*, 2009; Dubiella *et al.*, 2013). The contribution of ROS to these rapid and long-term responses is thought to occur as burst of ROS, often occurring as two distinctive peaks, accompanying the different signalling events (Nishimura and Dangl, 2010; Mittler *et al.*, 2011). In addition, the differential co-expression of assorted RBOHs in various tissue and cell types indicates that, to some extent, a high degree of signal specialization is attributable to the spatial coordination of ROS signals (Suzuki *et al.*, 2011).

Recent findings highlight the significance of cell to cell communication to mediating temporal-spatial coordination of signals in plants. Being sessile organisms, plants evolved sophisticated acclimation and defence mechanisms that can be activated in the primary tissue(s) exposed to stress, as well as in distal portions not directly exposed to stress (Fig. 1). The activation of defence or acclimation mechanisms in systemic

or non-challenged tissues is termed systemic acquired resistance (SAR) or systemic acquired acclimation (SAA), respectively, and both play an important role in preventing further infection or damage to the entire plant (Karpinski *et al.*, 1999; Rossel *et al.*, 2007; Carr *et al.*, 2010; Szechynska-Hebda *et al.*, 2010; Dempsey and Klessig, 2012; Spoel and Dong, 2012; Shah and Zeier, 2013). The involvement of ROS in systemic signalling during plant immunity, wound response, and high light acclimation was initially addressed more than a decade ago by Alvarez *et al.* (1998), Orozco-Cardenas and Ryan (1999), and Karpinski *et al.* (1999), respectively. We recently uncovered the existence of an H₂O₂-dependent long-distance signal induced by various abiotic stimuli (Miller *et al.*, 2009). RBOHD was shown to be required for the initiation and self-propagation of a rapid cell to cell systemic signal that is dependent upon H₂O₂ accumulation in the extracellular spaces to generate a 'ROS wave' (Mittler *et al.*, 2011). In addition, we demonstrated the important biological function of the ROS wave in the SAA of plants to heat or high light stresses (Suzuki *et al.*, 2013). Our studies suggested that the ROS wave functions as a general priming signal in plants, alerting systemic tissues to the occurrence of a localized abiotic stress stimulus. Moreover, we found that the SAA of plants to abiotic stress is mediated by temporal-spatial

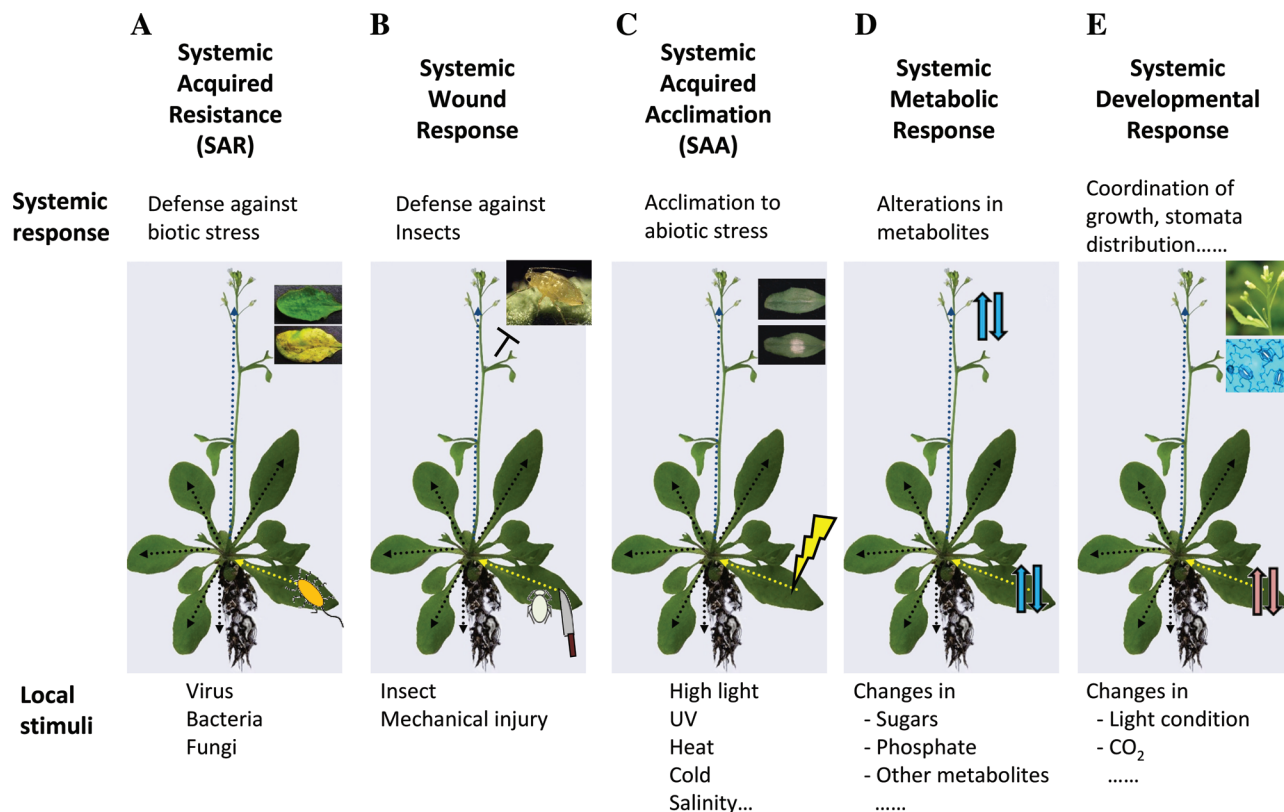


Fig. 1. Different types of systemic signalling in plants. (A) Systemic acquired resistance (SAR) triggered by pathogens (viruses, bacteria, and fungi). The signal enhances resistance of systemic tissues to pathogens. (B) Systemic wound response triggered by insects and mechanical injury. The signal activates defence mechanisms in systemic tissues against insect attack. (C) Systemic acquired acclimation (SAA) triggered by abiotic stimuli such as high light, UV light, heat, cold, or salinity. The signal enhances tolerance of systemic tissues to abiotic stress. (D) Systemic metabolic responses triggered by changes in the level of sugars, phosphate, and other metabolites. The signal alters the metabolic state in systemic tissues. (E) Systemic developmental responses activated by changes in light conditions and atmospheric CO₂. Growth and stomatal distribution are coordinated in new developing leaves.

interactions of the ROS wave with stress-specific hormone or amino acid signals activated in systemic tissues.

In this review, we will provide an update on findings related to the temporal and spatial coordination between ROS and other signals that mediate stress responses in plants, especially SAA and SAR. We will also attempt to address key questions related to the mechanisms that determine signal specificity in response to different stimuli.

Regulatory mechanisms of RBOH proteins

Plant RBOHs have cytosolic FAD- and NADPH-binding domains in the C-terminal region, and six conserved transmembrane-spanning domains that correspond to those in mammalian NADPH oxidases (Kobayashi *et al.*, 2007; Lin *et al.*, 2009; Gyan'ko and Ischenko, 2010; Proels *et al.*, 2010; Kimura *et al.*, 2012). Unlike the mammalian counterpart, plant RBOHs have a cytosolic N-terminal extension comprised of two Ca²⁺-binding EF-hand motifs and phosphorylation target sites that are important for their activity (Kobayashi *et al.*, 2007; Oda *et al.*, 2010; Kimura *et al.*, 2012; Drerup *et al.*, 2013). Once actuated, superoxide (O₂⁻) is produced at the apoplast via the function of RBOH proteins, and dismutates to H₂O₂ spontaneously or catalytically by the action of superoxide dismutase (SOD) (Lin *et al.*, 2009; Wi *et al.*, 2012). Membrane-permeable H₂O₂ can then play a key role as a signalling molecule that regulates cellular metabolism involved in growth, development, and response to environmental stimuli (Sagi *et al.*, 2004; Xia *et al.*, 2009).

Previous studies in *Arabidopsis* have revealed several regulatory mechanisms of RBOH protein homologues (e.g. AtRBOHC, D, and F) (Fig. 2). These mechanisms depend on various signalling components including protein phosphorylation, Ca²⁺, calcium-dependent protein kinases (CDPKs), and phospholipase D α 1 (PLD α 1) (Lin *et al.*, 2009; Monshausen *et al.*, 2009; Zhang *et al.*, 2009; Jakubowicz *et al.*, 2010; Drerup *et al.*, 2013; Dubiella *et al.*, 2013). Mechanical stimulation of plant tissue can initiate an increase in cytosolic Ca²⁺ via an influx from the extracellular space across the plasma membrane (Monshausen *et al.*, 2009). The increased Ca²⁺ then activates RBOHC-dependent ROS production followed by an amplification loop between Ca²⁺ and RBOHC to regulate root hair development (Monshausen *et al.*, 2007, 2009; Takeda *et al.*, 2008). Recent studies uncovered different regulatory mechanisms of RBOHD and RBOHF. Ca²⁺ binding and phosphorylation synergistically activate the ROS-producing activity of RBOHD and RBOHF in *Arabidopsis* (Ogasawara *et al.*, 2008; Kimura *et al.*, 2012). A Ca²⁺ increase in the cytosol was found to be necessary for the activation of RBOHD, and this activation requires conformational changes in EF-hand motifs by Ca²⁺ binding (Ogasawara *et al.*, 2008). PLD α 1 and its lipid product phosphatidic acid (PA) play an integral role in abscisic acid (ABA)-induced production of ROS in guard cells via the function of RBOHD and RBOHF (Zhang *et al.*, 2009). The PA-binding motifs, arginine residues 149, 150, 156, and 157, in RBOHD are required for ROS production and stomatal closure. RBOHF was also

found to be phosphorylated by OPEN STOMATA 1 (OST1) at Ser174 and Ser13 during ABA-dependent stomatal closure (Sirichandra *et al.*, 2009). Although these findings indicate an integration between RBOHD and RBOHF in the regulation of ABA-dependent stomatal closure, the coordination between PA and OST1 needs to be addressed in future studies. In a recent study, RBOHD was shown to be phosphorylated by calcium-dependent protein kinase 5 (CPK5) during pathogen defence (Dubiella *et al.*, 2013). RBOHD's involvement along the path of the rapid systemic signalling is H₂O₂ dependent and requires CPK5, supporting the hypothesis that Ca²⁺-dependent ROS production is involved in the propagation of the ROS wave over long distances (Miller *et al.*, 2009). In addition, a recent finding demonstrated that the activity of RBOHF is regulated by direct Ca²⁺ binding to its EF-hands and Ca²⁺-dependent phosphorylation by CBL1/9–CIPK26 complexes (Drerup *et al.*, 2013). Taken together, these findings indicate that the diverse functions of RBOH signalling in plants might be, at least partially, attributed to differences in regulatory mechanisms.

Regulatory mechanisms of RBOH proteins were also studied in other plant species and crops. A homologue of mammalian Rac in rice (OsRac1) was shown to be a positive regulator of OsRBOHB involved in pathogen defence (Wong *et al.*, 2007; Oda *et al.*, 2008). OsRac1 activates OsRBOHB by directly interacting with its N-terminal region that includes the EF-hand motifs in a Ca²⁺-dependent manner. The significance of OsRac1 to ROS production was supported by the finding that constitutively active or dominant-negative forms of OsRac1 can stimulate or suppress ROS function, respectively (Ono *et al.*, 2001; Wong *et al.*, 2007). In potato, two Ca²⁺-dependent protein kinases, StCDPK4 and StCDPK5, were found to activate StRBOHB-dependent ROS production (Kobayashi *et al.*, 2007). Two phosphorylation sites, Ser82 and Ser97, were identified in the N-terminal region of StRBOHB, and phosphorylation at Ser82 was shown to be required for the oxidative burst during pathogen defence (Kobayashi *et al.*, 2007). In addition, the N-terminal variable domain of StCDPK5, including the myristoylation and palmitoylation sites, confers subcellular localization which results in interaction and phosphorylation of StRBOHB *in vivo* (Asai *et al.*, 2013). In pepper, *Capsicum annuum*, receptor-like protein kinase 1 (CaRLK1) is induced by pathogen infection and exogenous application of H₂O₂ (Yi *et al.*, 2010). Transgenic plants that constitutively express CaRLK1 exhibit enhanced resistance to pathogen infection and reduced cell death, as well as increased O₂⁻ production and expression of RBOH genes (Yi *et al.*, 2010). As described above, several regulatory mechanisms of RBOH proteins found in different crops seem to be slightly different from those found in *Arabidopsis*. It would be interesting to determine whether RBOH regulatory mechanisms are highly conserved among various plant species or evolutionarily diverse.

RBOH proteins are not the only source for ROS in plant cells. Numerous pathways for ROS production exist in plants, and include photosynthesis (via the electron transport chain and photosystems I and II), respiration (via the electron transport chain), glycolate oxidase, oxalate oxidase, xanthine

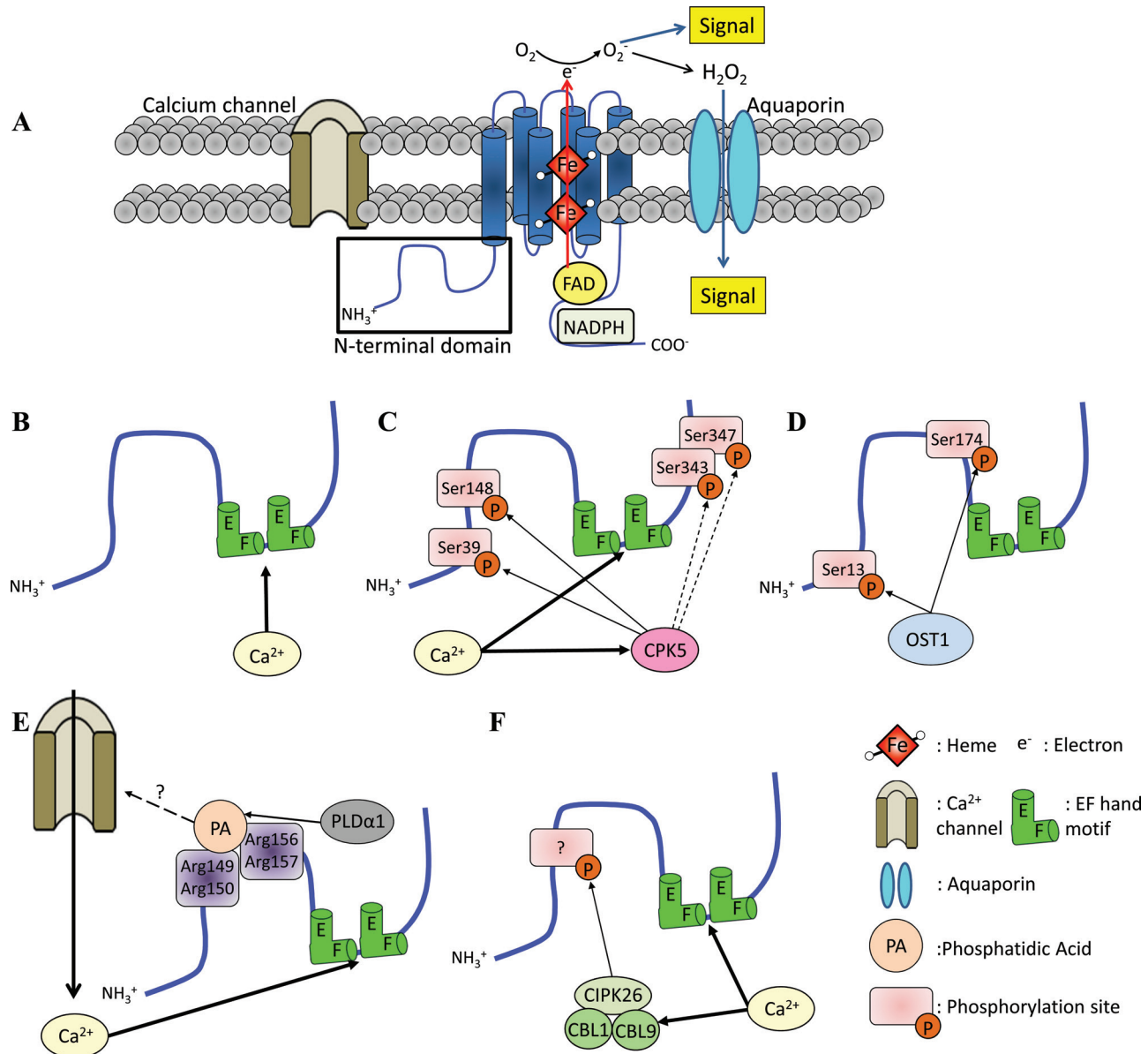


Fig. 2. Regulatory mechanisms of RBOH proteins in *Arabidopsis*. (A) Basic structure of plant RBOH proteins. RBOHs have cytosolic FAD- and NADPH-binding domains in the C-terminal region, and six conserved transmembrane-spanning domains (blue cylinders). The N-terminal domain (box) contains two EF-hand motifs and phosphorylation target sites that are important for activity of RBOHs (B–F). (B) Binding of Ca^{2+} to EF-hand motifs is required for activation of RBOHs (Ogasawara *et al.*, 2008; Drerup *et al.*, 2013). (C) Activation of RBOHD by phosphorylation of Ser39, Ser148, Ser343, and Ser347 residues via the function of CPK5 (Dubiella *et al.*, 2013). (D) Activation of RBOHF by phosphorylation of Ser13 and Ser174 residues via function of OPEN STOMATA 1 (OST1) kinase (Sirichandra *et al.*, 2009). (E) Activation of RBOHD by binding of phosphatidic acids (PAs) produced via the function of phospholipase $\text{D}\alpha 1$ ($\text{PLD}\alpha 1$) to Arg149, Arg150, Arg156, and Arg157 residues (Zhang *et al.*, 2009). PA also activates a Ca^{2+} channel located at the plasma membrane. (F) Activation of RBOHF by phosphorylation of the N-terminal domain via the function of CBL1/9–CIPK26 complexes and direct binding of Ca^{2+} to the EF-hand motifs (Drerup *et al.*, 2013).

oxidase, amine oxidase, excited chlorophyll, fatty acid oxidation, and peroxidases (Mittler, 2002). Different oxidases were found to play a role in the response of plants to various biotic and abiotic stresses. Oxalate oxidase was shown to be involved in ROS production in root cells during drought stress (Voothuluru and Sharp, 2013), whereas glycolate oxidase (GOX) has been shown to play a role in non-host pathogen defence in *Arabidopsis* and tobacco (Rojas and Mysore, 2012; Rojas *et al.*, 2012). Peroxidases also play important

roles in ROS production during defence responses in plants. PEROXIDASE33 (PRX33) and PRX34 have been identified as major contributors to ROS production in *Arabidopsis* during responses to a fungal cell wall elicitor or bacterial pathogens (Daudi *et al.*, 2012; O'Brien *et al.*, 2012b; Wrzaczek *et al.*, 2013). Peroxidase-dependent ROS are also involved in callose deposition and expression of defence genes (Daudi *et al.*, 2012; Wrzaczek *et al.*, 2013). Peroxidases might be responsible for about half of H_2O_2 production that is induced

by bacterial pathogens (O'Brien *et al.*, 2012b). Interestingly, PRX knockdown plants exhibited stronger dysfunction of defence gene expression and callose disposition compared with *rbohD* plants in response to fungal elicitor, suggesting that ROS production by peroxidases might not be functionally equivalent to ROS generated by RBOH proteins (Daudi *et al.*, 2012; Wrzaczek *et al.*, 2013). This hypothesis can be supported by the finding that stomatal closure and ROS burst induced by a yeast elicitor were not inhibited in *rbohD* and *rbohF* mutants in *Arabidopsis* (Khokon *et al.*, 2010). In addition, recent studies uncovered a role for peroxidase-dependent ROS in the regulation of root growth and response to potassium deficiency (Kim *et al.*, 2010; Jia, 2011; Kwasniewski *et al.*, 2013). The integration between RBOH proteins and peroxidases should be addressed in future studies.

Functional differences between RBOH proteins and peroxidases may be partially attributed to differences in the types of ROS generated by these enzymes. Superoxide (O_2^-), generated by RBOH proteins, can activate specific signalling pathways distinct from those activated by H_2O_2 (Suzuki *et al.*, 2011). Another possibility is that diverse functions between these different types of enzymes might be due to differences in their respective reductants. NADPH serves as a reductant for the RBOH-dependent generation of superoxide. In contrast, different chemicals or compounds including phenols, organic acids, and auxin have been suggested as candidate reductants for the peroxidase-dependent generation of H_2O_2 (O'Brien *et al.*, 2012a). Pathways involving these different reductants could be integrated with ROS signals activated via the different functions of these enzymes.

Temporal coordination of ROS signals in plants

Changes in environmental conditions are likely to cause rapid changes in the level, composition, and structure of different metabolites, proteins, and RNA molecules, that precede signal transduction or acclimation events in plants. Early signalling events including ion fluxes across the plasma membrane, increased Ca^{2+} levels in the cytosol, activation of MAPKs, and production of ROS can all be activated within minutes following application of biotic or abiotic stimuli (Benschop *et al.*, 2007; Miller *et al.*, 2009; Finka *et al.*, 2012). For example, ROS production can be triggered in tobacco cells within 3 min following heat stress, and heat stress-induced ROS can be inhibited by an NADPH oxidase inhibitor (Konigshofer *et al.*, 2008). A plasma membrane channel that initiates an inward calcium flux has been identified as one of the heat sensors (Saidi *et al.*, 2009; Finka *et al.*, 2012; Mittler *et al.*, 2012). Ca^{2+} channels transiently open and induce an inward flux of Ca^{2+} into the cytosol within 10 min following heat stress (Saidi *et al.*, 2009; Finka *et al.*, 2012). This Ca^{2+} signal might be linked to regulatory mechanisms of ROS-producing enzymes (Mittler *et al.*, 2012). Using phosphoproteomic approaches, researchers demonstrated that a pathogen elicitor, flg22, activates MAPKs and induces phosphorylation of membrane proteins including ion channels, calmodulins,

protein kinases, protein phosphatases, and proteins associated with auxin signalling, as well as RBOHD in *Arabidopsis* suspension-cultured cells within 5–10 min (Benschop *et al.*, 2007). In addition, CDPKs in plants are biochemically activated within a few minutes following exposure to biotic stimuli and participate in the induction of early defence responses (Ludwig *et al.*, 2005; Kobayashi *et al.*, 2007; Boudsocq and Sheen, 2013). CPK5-dependent *in vivo* phosphorylation of AtRBOHD can be induced within 15 min in leaves not directly challenged with a pathogen elicitor, flg22 (Dubiella *et al.*, 2013).

Early responses of plants to high light have also been described in previous studies (Karpinski *et al.*, 1999; Rossel *et al.*, 2007; Muhlenbock *et al.*, 2008; Szechynska-Hebda *et al.*, 2010; Gordon *et al.*, 2012). Studies utilizing transgenic plants expressing a luciferase reporter gene under the control of an APX1, APX2, or ZAT10 promoter demonstrated the activation of acclimatory responses within 5–20 min following application of high light both in leaves locally exposed to the stimuli and in distal tissues that did not directly encounter the stimuli (Karpinski *et al.*, 1999; Rossel *et al.*, 2007; Szechynska-Hebda *et al.*, 2010). These high light responses are shown to be associated with redox changes in the plastoquinone (PQ) pool, increased production of ROS and ethylene, reduction of maximal photochemical efficiency and non-photochemical quenching (NPQ), and changes in extracellular electric potential (Karpinski *et al.*, 1999; Rossel *et al.*, 2007; Szechynska-Hebda *et al.*, 2010).

Research has also demonstrated a rapid response in plants to mechanical wounding. In *Arabidopsis*, elevated levels of jasmonic acid (JA) accumulate in damaged tissues as well as undamaged systemic leaves within 30 s to 5 min in response to mechanical wounding (Glauser *et al.*, 2009; Koo *et al.*, 2009). The velocity of the long-distance signal leading to *de novo* synthesis of JA in systemic tissues was 3.4–4.5 $cm\ min^{-1}$ (Glauser *et al.*, 2009; Koo *et al.*, 2009). In a recent study, we demonstrated that the NADPH oxidase homologue RBOHD is required for the initiation and amplification of a rapid auto-propagating systemic signal that travels at the rate of $\sim 8.4\ cm\ min^{-1}$, and is induced by various abiotic stimuli including mechanical wounding (Miller *et al.*, 2009). In addition, the potential involvement of electric signals that propagate with similar rates was also implicated in RBOHD-triggered rapid systemic signalling during wounding (Zimmermann *et al.*, 2009; Mittler *et al.*, 2011; Suzuki and Mittler, 2012). These findings implicate the integration of JA and mobile signals such as ROS and electric signals.

We recently revealed that RBOHD-dependent long-distance signals play an important biological role in the SAA response of plants to heat or high light (Suzuki *et al.*, 2013). SAA, which enhances the plant's tolerance to heat stress, was correlated with activation of the ROS wave and occurred as early as 5–10 min following heat stress application. In addition, metabolome analysis revealed the rapid accumulation of glycine, serine, and glycerate in leaves directly exposed to high light within 60 s as well as in systemic tissues of plants at 15 min and 45 min following local high light treatment, suggesting that a portion of the photorespiratory machinery is involved in the early

response of plants to high light. Rapid local responses to high light were altered in the mutant lacking cytosolic APX1, demonstrating the involvement of H₂O₂ scavenging in this process.

Long-term responses to fluctuating environmental conditions regulate phenotypic changes such as growth, development, and survival of cells. For example, defence mechanisms associated with ROS signalling confer protection to plants hours or days following pathogen infection (Torres *et al.*, 2005; Muhlenbock *et al.*, 2008; Dubiella *et al.*, 2013). ROS accumulation mediated by RBOH proteins is also observed hours or days following pathogen infection, and ROS accumulation has been shown to be accompanied by gradual necrotic symptoms (Kobayashi *et al.*, 2007; Wi *et al.*, 2012).

Developing leaves, not directly exposed to changes in light conditions and atmospheric CO₂ experienced by mature leaves, can alter their photosynthetic rate and tolerance to high light, as well as growth and development (Coupe *et al.*, 2006; Araya *et al.*, 2008; Jiang *et al.*, 2012). Although alterations in photosynthetic rate and response to high light implicate ROS and redox signalling in the systemic regulation of long-term responses in new developing leaves, links between ROS signalling and these responses have yet to be conclusively established. ROS signalling can be modulated by the redox state of the PQ pool in chloroplasts and plays a key role in the response of plants to changes in fluctuating light conditions (Muhlenbock *et al.*, 2008; Li *et al.*, 2009; Mittler *et al.*, 2011). Changes in light quality result in imbalances in energy distribution between the photosystems and induce altered thylakoid composition via the function of STN7 kinase within hours and days (Pesaresi *et al.*, 2009).

How are rapid and long-term responses of plants to stress stimuli linked? Previous studies demonstrated that the biphasic production of ROS consists of a primary phase that occurs within minutes and a secondary phase that occurs within hours/days (Soares *et al.*, 2009; Nishimura and Dangl, 2010; Kunihiro *et al.*, 2011; Mittler *et al.*, 2011). Such a biphasic ROS production accompanies several different signalling events in many biological systems (Soares *et al.*, 2009; Nishimura and Dangl, 2010; Mittler *et al.*, 2011). For example, mechanical wounding induced an initial burst of O₂⁻ within 3 min followed by later production of O₂⁻ and H₂O₂ after 6 h (Soares *et al.*, 2009). Inhibition of early ROS accumulation by an NADPH oxidase inhibitor suppresses later production of O₂⁻ and accumulation of wound response proteins. These results indicate that a rapid burst of ROS is required for the later phase of ROS production which regulates downstream pathways and acclimation of plants to stress stimuli. Our recent findings suggest that these two phases of the ROS burst are linked via the ROS wave that communicates the initial ROS burst in the local tissue to the systemic tissue via a cell to cell relay mechanism (Miller *et al.*, 2009; Suzuki *et al.*, 2013).

Spatial coordination of ROS signals in plants

Plant genomes, such as that of *Arabidopsis*, contain a host of RBOH homologues. In recent years, several studies have

revealed that plant RBOHs perform a multitude of signalling functions in assorted tissue and developmental stages (Suzuki *et al.*, 2011), implying that various biological processes are regulated by coordination between different ROS signals activated in individual plant tissue types.

To some extent, signals generated in plants during SAA are similar in local and systemic tissues. Rossel *et al.* (2007) compared the transcriptomes of local leaves, directly exposed to high light, and systemic leaves, not directly challenged by the stimulus. More than 70% of the transcripts up-regulated in local leaves in response to high light were also altered in systemic leaves, suggesting that similar signals exist between local and systemic tissues during high-light-mediated SAA. Similarities between local and systemic responses to high light are demonstrated by findings showing that alterations in ROS and redox signals occurred both in local and in systemic tissues (Muhlenbock *et al.*, 2008; Miller *et al.*, 2009; Szechynska-Hebda *et al.*, 2010). Our recent study demonstrated that amino acids involved in the photorespiratory pathway such as glycine, serine, and glycerate are similarly altered in both local and systemic tissues in response to local application of high light (Suzuki *et al.*, 2013). Local application of heat or cold stimuli can also induce similar stress response proteins or transcripts in both local and systemic tissues (Gorsuch *et al.*, 2010; Suzuki *et al.*, 2013). In particular, induction of heat-responsive proteins in systemic tissue was shown to be ROS wave dependent (Suzuki *et al.*, 2013).

Although signals generated in local and systemic tissues showed considerable overlap, previous studies have demonstrated differences in alterations of transcripts or metabolites between these types of tissues. Ethylene accumulated in both local and systemic tissue in response to local application of high light; nevertheless, the signal mediated by ethylene insensitive 2 (EIN2) was shown to be required for induction of APX2 only in systemic tissues, but not in leaves directly subjected to high light (Muhlenbock *et al.*, 2008). In addition, SID2 delays induction of APX2 in leaves directly exposed to high light but not in systemic leaves. Expression of APX2 in local and systemic tissue, in response to high light, might be regulated by the coordination between ethylene and SA signalling during SAA. A recent study demonstrated spatial diversity in high light responses between different leaves of plants during SAA (Gordon *et al.*, 2012). Thus, local high light treatment resulted in the accumulation of different transcript levels of ZAT10 and Redox Responsive Factor 1 (RRTF1) in systemic leaves, depending on leaf position (Gordon *et al.*, 2012). Plants possess mechanisms to alter spatial distribution of metabolites in response to stress stimuli (Schwachtje and Baldwin, 2008; Simon *et al.*, 2010). Metabolic profiling of pathogen-infected and adjacent uninfected leaf tissues demonstrated a quantitatively different distribution of secondary metabolites between these regions of leaf tissues (Simon *et al.*, 2010). The distribution of secondary metabolites was altered in a mutant lacking CAT2, suggesting a role for ROS-scavenging mechanisms in determining the distribution of secondary metabolites in response to pathogen infection.

How are the signals generated in local and systemic tissues linked? The ROS wave may play a key role in propagating

signals from local tissues to systemic tissues. The initial abiotic stress-induced burst of ROS in a local group of plant cells triggers a cascade of cell to cell communication events that propagates throughout different tissues of the plant and carries a systemic signal over long distances (Miller *et al.*, 2009). Szechynska-Hebda *et al.* (2010) uncovered the pattern of spreading systemic changes in NPQ, H₂O₂ concentration, and APX1 expression during SAA response of plants to high light. Wave-like patterns of APX1 expression in systemic tissue of plants correlate positively with H₂O₂ accumulation, but negatively with NPQ (Szechynska-Hebda *et al.*, 2010; Karpinski *et al.*, 2013). The activation of systemic signals by local application of high light was recently shown to be accompanied by plasma membrane electrical signals in a light wavelength-specific manner (Szechynska-Hebda *et al.*, 2010). Our recent finding that the RBOHD-dependent ROS wave is associated with the generation and/or propagation of systemic potential variations may demonstrate a link between electric signals in plants and ROS production (Suzuki *et al.*, 2013). In addition, recent studies identified a number of different chemicals and compounds involved in pathogen-induced SAR in plants, and these signalling molecules might be transported from local tissue to systemic tissue (Dempsey and Klessig, 2012; Shah and Zeier, 2013). Integration of ROS signals and these metabolic cues could be a promising subject for future studies.

Previous studies have highlighted the signalling links between shoot and root during the defence response following insect attack (Soler *et al.*, 2013; Wondafrash *et al.*, 2013). For example, root herbivory elicits water limitation in the shoot, resulting in induction of ABA accumulation in leaves (Soler *et al.*, 2013). Involvement of ROS signalling in mediating a connection of signals between different tissue types is suggested by the distribution of RBOH proteins from roots to reproductive tissues (Suzuki *et al.*, 2011) and our finding that RBOHD-dependent long-distance signals propagate from leaves to the entire plant (Miller *et al.*, 2009). In our recent study, grafting experiments between wild-type and *rbohD* seedlings demonstrated the significance of RBOHD to propagating a signal from leaves to roots (Suzuki *et al.*, 2013). Local application of heat stress to the cotyledons (local tissue) of control grafted seedlings (wild type–wild type) resulted in the enhanced SAA response to heat stress in root tips (systemic tissue). In contrast, this SAA response was attenuated in all grafting experiments that involved a deficiency in RBOHD in the local or systemic tissues.

Integration of ROS signals with chemicals, compounds, and hormones

Numerous studies uncovered metabolic cues including hormones, amino acids, and chemical compounds activated by stress stimuli. Plants recognize various pathogens by utilizing extracellular surface receptors to decode pathogen-associated molecular patterns (PAMPs), and consequently initiate defence responses (Dempsey and Klessig, 2012). Defence responses in local and systemic tissues are characterized by

accumulation of free salicylic acid (SA) and its 2-*O*- β -D-glucoside (SAG), as well as elevated expression of pathogenesis-related (PR) genes (Dempsey and Klessig, 2012). These metabolic changes, which may be induced by pathogens, can result in SAR (Dempsey and Klessig, 2012). Signals are initiated in infected tissue, translocated by the vascular tissue to distal portions of the plant, typically by the phloem, and perceived in systemic tissue (Dempsey and Klessig, 2012; Kachroo and Robin, 2013). Research in the area of phloem-mobile SAR signals identified several biologically active molecules in phloem sap, including methyl salicylate (MeSA) (Park *et al.*, 2007), a glycerol-3-phosphate (G3P) derivative (Chanda *et al.*, 2011), a lipid transfer protein (DIR1) (Maldonado *et al.*, 2002), azelaic acid (AzA) (Jung *et al.*, 2009), dehydroabietinal (DA) (Chaturvedi *et al.*, 2012), jasmonic acid (JA) (Truman *et al.*, 2007), and pipercolic acid (Pip) (Dempsey and Klessig, 2012; Shah and Zeier, 2013). Current research has demonstrated that MeSA, AzA, DA, and G3P all induce systemic resistance when applied locally (Kachroo and Robin, 2013). Research has also demonstrated that primary pathogen infection results in rapid accumulation of AzA and G3P (Chanda *et al.*, 2011; Kachroo and Robin, 2013). Neither AzA nor G3P induces SA accumulation; however, AzA is thought to prime the plant for future SA accumulation in response to a secondary infection (Dempsey and Klessig, 2012; Kachroo and Robin, 2013). In *Arabidopsis*, AtBSMT1, up-regulated by JA and NtSAMT1 expression in tobacco, converts a portion of accumulating SA to MeSA (Dempsey and Klessig, 2012; Kachroo and Robin, 2013). This biologically inactive molecule can then travel to systemic leaves via the phloem, and upon arrival is converted to SA by SABP2 (Dempsey and Klessig, 2012). DIR1 (defective in induced resistance1), which gains access to the phloem via the cytosol of companion cells, and AZI1 (AzA INDUCED 1) interact with each other, and are essential for AzA-, DA- (Dempsey and Klessig, 2012), and G3P-mediated SAR (Dempsey and Klessig, 2012; Kachroo and Robin, 2013). Additional putative SAR signalling molecules in the phloem include DA and Pip (Chaturvedi *et al.*, 2012; Dempsey and Klessig, 2012). Pip, a potential mobile SAR signal, is elevated in local and systemic tissues following inoculation with *Pseudomonas syringae* pv. *maculicola*, and is believed to induce SAR by positively regulating its own synthesis and priming SA accumulation in systemic tissues (Dempsey and Klessig, 2012). DA is a rapid mobile SAR signal, which elevates levels of SA and expression of PR-1 and ICS1 (an SA biosynthesis enzyme), and has been shown to induce resistance to virulent pathogens in *Arabidopsis*, tobacco, and tomato (Dempsey and Klessig, 2012). DA requires SA synthesis and signalling to function, and works synergistically with SFD1/GLY1 and AzA to induce SAR (Dempsey and Klessig, 2012).

Various forms of biotic and abiotic stress result in increased formation of ROS which can be linked to signals caused by changes in the regulation of plant hormones (Fujita *et al.*, 2006). Ethylene biosynthesis was found to be modulated by positive regulation via RBOH proteins and negative regulation by CTR1 (constitutive triple response 1) (Jakubowicz *et al.*, 2010). In *Arabidopsis*, CTR1 can be inhibited by

PA which positively enhances activation of RBOHD and RBOHF (Jakubowicz *et al.*, 2010). Previous studies revealed the involvement of ethylene in the regulation of SAR and SAA to high light induced by local high light application (Muhlenbock *et al.*, 2008; Karpinski *et al.*, 2013). In response to high light, alterations in the redox state of the PQ pool can initiate a signal that induces production of 1-aminocyclopropane-1-carboxylate (ACC; the immediate precursor of ethylene), ROS, and the expression of ethylene-regulated genes (Muhlenbock *et al.*, 2008). Increased ROS production results in bleaching of leaves, and programmed cell death that relies on regulation of EIN2 by LESION SIMULATING

DISEASE 1 (LSD1) (Muhlenbock *et al.*, 2008; Karpinski *et al.*, 2013). Recent studies demonstrated the involvement of brassinosteroids (BRs) in SAR and SAA to high light in cucumber (Xia *et al.*, 2009, 2011; Li *et al.*, 2013). Although BRs are not directly involved in long-distance signalling, they affect other signals such as auxins and polyamines (Li *et al.*, 2013). Local applications of 24-epibrassinosteroid (EBR) can induce SAR and SAA to oxidative stress accompanied by local and systemic expression of known defence/acclimatory genes such as APX and catalase (Li *et al.*, 2013). Involvement of BR signalling in ROS-dependent stress responses was also supported by the finding that exogenous BR treatments

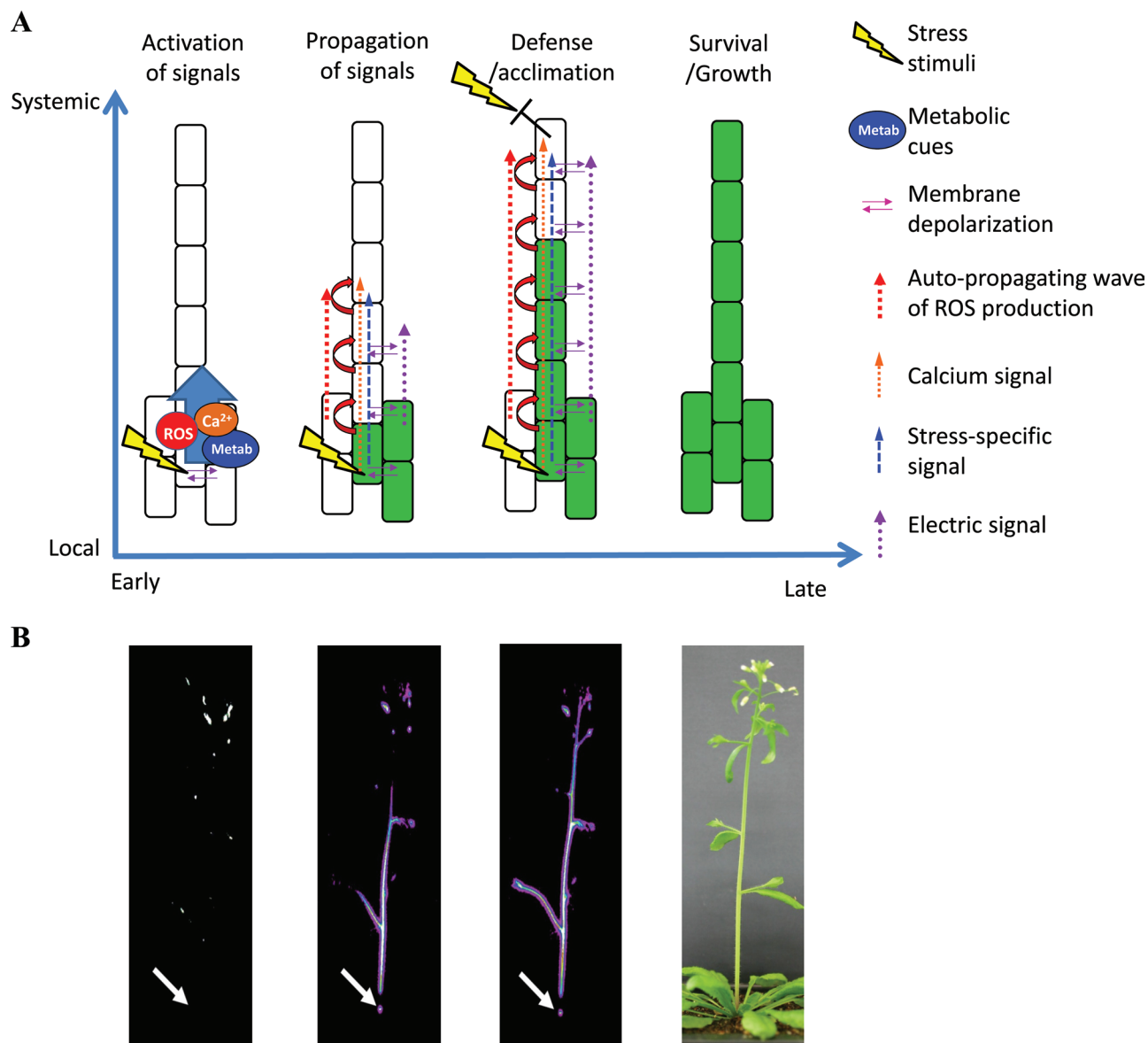


Fig. 3. Temporal-spatial coordination of the ROS wave in plants. (A) A hypothetical model for local and systemic stress response signal transduction in plants. Local stress stimuli can initiate rapid signalling events such as an increase in the cytosolic Ca^{2+} level, production of ROS, and activation of stress-specific metabolic cues within seconds or minutes. These rapid signals that are propagated through the entire plant can activate defence or acclimatory mechanisms in systemic tissues. Long-term responses to fluctuating environments regulate phenotypic changes such as growth, development, and survival of cells. (B) Time-lapse imaging of rapid systemic signal transduction in *Arabidopsis* using a luciferase reporter gene expressed under the control of the *Zat12* promoter (*Zat12:Luc*) in response to wounding (Miller *et al.*, 2009). The wounding site is indicated with a white arrow.

resulted in enhanced tolerance to oxidative stress accompanied by induction of H₂O₂ production in the apoplast and expression of RBOH, MAPK1, and MAPK3 (Xia *et al.*, 2009). A recent study indicated the involvement of auxin in the SAA response of plants to high light. Large portions of the transcripts that exhibited significant changes in the distal leaves overlap with auxin-responsive transcripts (Gordon *et al.*, 2012), indicating a connection between SAA and developmental processes mediated by auxin. Integration between ethylene and BRs during SAR or SAA response to high light need to be elucidated in future studies.

Amino acids involved in the photorespiratory pathway such as glycine, serine, and glycerate were shown to be accumulated in systemic tissues at 15 min following local application of high light, but not heat stress or wounding (Suzuki *et al.*, 2013). Signals regulated by these high-light-specific amino acids also need to be linked with hormone signals.

ABA is involved in a broad range of biological functions, and its integration with ROS has been revealed (Kwak *et al.*, 2003; Sagi *et al.*, 2004; Ma *et al.*, 2012; Drerup *et al.*, 2013). For example, RBOHD and RBOHF function synergistically in signalling cascades to regulate stomatal closure, seed germination, root elongation, and Na⁺/K⁺ homeostasis under salt stress (Kwak *et al.*, 2003; Ma *et al.*, 2012). In addition, ABA and SA treatment have been shown to result in transient increases in H₂O₂ production which induces tolerance to salt, high light, heat, and oxidative stress (Xia *et al.*, 2009). Our recent study demonstrated that SAA of plants to heat stress was correlated with activation of the ROS wave and transient accumulation of ABA in systemic tissues, and these responses were suppressed in a mutant lacking RBOHD (Suzuki *et al.*, 2013). The SAA response to heat stress was also attenuated in mutants deficient in ABA signalling. These results indicate that temporal-spatial interactions between RBOHD-dependent ROS and ABA mediate SAA to heat stress (Suzuki *et al.*, 2013).

Cross-talk between response pathways to different stress stimuli

Cross-talk exists between complex signalling networks that regulate different stress response pathways. There is an extensive overlap between pathogen defence and response to high light (Mullineaux and Baker, 2010; Straus *et al.*, 2010). Accordingly, local application of high light induces tolerance of plants to pathogen infection as well as SAA to oxidative stress in systemic tissues, indicating a cross-talk between high light acclimation and pathogen responses (Rossel *et al.*, 2007; Muhlenbock *et al.*, 2008; Karpinski *et al.*, 2013). This process is accompanied by alterations in ROS and redox signals, and induction of glutathione and SA in both local and systemic leaves (Muhlenbock *et al.*, 2008). During acclimation, plants express LESION SIMULATING DISEASE 1 (LSD1) that suppresses the ROS-/ethylene-dependent programmed cell death pathway by acting as a negative regulator of pathogen response genes, EDS1 and PAD4 (Karpinski *et al.*, 2013). Another example of cross-talk between biotic and abiotic stress responses is insect

attack and wounding. Systemic signalling induced by insect attack and wounding relies on biosynthesis of JA at the site of wounding and the ability to perceive a jasmonate signal in the systemic tissues (Suzuki and Mittler, 2012). JA could play a key role in the regulation of cell death associated with H₂O₂ and SA signalling during insect attack and wounding (Pasqualini *et al.*, 2003; Zhou *et al.*, 2009; Lin *et al.*, 2011).

Conclusions

ROS play a pivotal role in regulating numerous responses to biotic and abiotic stresses in plants. The complexity in ROS responses to various environmental stimuli might be, at least partially, attributed to different regulatory mechanisms of ROS production via NADPH oxidases (RBOHs) that function in an array of tissue types and developmental stages under various environmental conditions. Plants have evolved different mechanisms that control temporal and spatial coordination between ROS and other signals activated in separate parts of the plant at different times (Fig. 3). A key mechanism in coordinating these complex spatial and temporal responses in plants is the cascade of cell to cell communication events that result in the formation of a wave of ROS production that rapidly propagates throughout the different tissues of the plant. The significance of this rapid systemic signalling to SAR and SAA in plants has been revealed by recent studies. How the ROS wave is integrated with different stress-specific signals is, however, an open question that requires further research.

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