

REVIEW

The Roles of ROS and ABA in Systemic Acquired Acclimation ^{OPEN}

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Systemic responses to environmental stimuli are essential for the survival of multicellular organisms. In plants, they are initiated in response to many different signals including pathogens, wounding, and abiotic stresses. Recent studies highlighted the importance of systemic acquired acclimation to abiotic stresses in plants and identified several different signals involved in this response. These included reactive oxygen species (ROS) and calcium waves, hydraulic waves, electric signals, and abscisic acid (ABA). Here, we address the interactions between ROS and ABA at the local and systemic tissues of plants subjected to abiotic stress and attempt to propose a model for the involvement of ROS, ABA, and stomata in systemic signaling leading to systemic acquired acclimation.

INTRODUCTION

Plants are highly complex multicellular organisms with multiple organs and tissues positioned above or below the ground. The different cells and tissues of plants are connected with each other via symplastic, vascular, and/or apoplastic connections enabling short and long distance intercellular communications. Long-distance communication among cells belonging to different tissues or organs is often termed systemic signaling and can be initiated in response to different changes in the biotic or abiotic environment of the plant. A systemic response to biotic stimuli is termed systemic acquired resistance (SAR) and is typically initiated by interactions of certain cells with pathogens (e.g., virus, bacteria, or fungi). A systemic response to abiotic stimuli, such as high light, heat, cold, UV, osmotic stress, or salinity is termed systemic acquired acclimation (SAA), and a systemic response to mechanical stress that could be caused by biotic (e.g., insect feeding) or abiotic stress (e.g., wind or heavy downpour) is termed systemic wound response. The presumed common outcome of all of these systemic responses is to alert all remote and unstressed tissues of the plant to the existence of a biotic or abiotic threat and to trigger the activation of resistance or acclimation pathways in these tissues. These responses typically are measured by enhanced accumulation of defense or acclimation transcripts and proteins and/or by demonstrating an enhanced level of tolerance or resistance to a subsequent abiotic or biotic stress applied to systemic tissues (Karpiński et al., 2013; Suzuki et al., 2013; Gilroy et al., 2014; Shah et al., 2014). Activating resistance or acclimation pathways in remote tissues that were not yet subjected to the stress or pathogen would therefore enable the plant to withstand and survive the upcoming biotic or abiotic challenge. By virtue of increasing the fitness of plants, systemic signaling pathways should be viewed as an evolutionary advantage.

In recent years, a number of chemicals and compounds have been implicated in mediating systemic signaling in plants. These include, among others, salicylic acid and methyl salicylate, jasmonic acid and methyl jasmonate, azelaic acid, auxin, ethylene, abscisic acid (ABA), reactive oxygen species (ROS), different peptides and proteins, electric signals, and RNA molecules (Karpiński et al., 2013; Shah et al., 2014; Wang et al., 2014; Wendehenne et al., 2014). These chemicals and compounds were proposed to function alone or to interact with each other and to mediate or regulate systemic responses. In addition to the increasing number of compounds and pathways implicated in systemic signaling, new studies have begun to focus on the different tissues involved in generating and perceiving systemic signals and on the speed at which these signals are being mediated (Gilroy et al., 2014). In addition, ABA and stomatal functions have been recently implicated in the regulation of systemic responses to abiotic stress (Suzuki et al., 2013). In this review, we will focus on the role of ROS and calcium in SAA and on their interaction with ABA, NO, and stomatal functions.

ABA AND ROS IN STOMATAL REGULATION

ABA plays significant roles in a number of physiological processes, such as development, responses to abiotic stresses, and the regulation of stomatal function. ABA can be synthesized in both roots and shoots, and in particular in veins, seeds, and guard cells (Boursiac et al., 2013). Water deficit and high salinity stress induce the accumulation of ABA levels in plants. ABA accumulation promotes changes in gene expression (Shinozaki and Yamaguchi-Shinozaki, 2007) and the closing of stomata with the concomitant reduction in transpiration and water loss. As a consequence of stomatal closure, there is a decrease in gas exchange resulting in the reduction of photosynthetic activity.

The basic regulatory events involved in the regulation of stomatal movement by ABA are shown in Figure 1. ABA concentrations increase under stress conditions as a result of increased biosynthesis,

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decreased degradation, or release from conjugated forms (Boursiac et al., 2013; Song et al., 2014). These events could occur within the affected cell or in neighboring or remote cells resulting in ABA uptake by nonstressed cells. ABA is sensed in cells by the ABA receptors RCAR/PYR1/PYL (Regulatory Components of ABA-receptor/Pyrabactin resistant Protein/PYR-like proteins; Ma et al., 2009; Park et al., 2009) that bind to ABA and interact with a group of type 2C protein phosphatases (PP2C). PP2Cs interact with SNF1 (Sucrose-Non-Fermenting Kinase 1)-related protein kinase OPEN STOMATA1 (OST1)/SnRK2, resulting in the dephosphorylation of Ser/Thr residues located at the activation loop of the SnRK, leading to its inactivation. The interaction of ABA with the RCAR/PYR1/PYL complex inactivates the inhibitory regulatory function of PP2C, activating SnRK2 protein kinase OST1 (Umezawa et al., 2009). Activated OST1 binds directly to and phosphorylates the anion channel SLOW ANION CHANNEL-ASSOCIATED1, mediating anion release from the guard cells and promoting stomatal closure (Geiger et al., 2009, 2010; Lee et al., 2009; Brandt et al., 2012). OST1 also targets a plasma membrane-bound NADPH oxidase (RBOH) that catalyzes the production of superoxide and, via apoplastic superoxide dismutases, H_2O_2 (Sirichandra et al., 2009). OST1 interacts directly with and phosphorylates the N terminus of respiratory burst oxidase homolog F protein (RBOHF) at the residues Ser-13 and Ser-174, and in the *ost1* knockout mutant, the ABA-induced ROS generation in guard cells is abolished, suggesting a role for OST1 in the regulation of ROS mediated by NADPH oxidase (Wang and Song, 2008). OST1-dependent H_2O_2 formation could provide a signal for an increased release of further active OST1 through the inactivation of PP2C in a positive feedback loop (Raghavendra et al., 2010).

ABA activates hyperpolarization-activated Ca^{2+} channels in the plasma membrane of guard cells (Pei et al., 2000), promoting the elevation of free cytosolic Ca^{2+} concentrations (Schroeder and Hagiwara, 1990). ROS, and H_2O_2 in particular, play a significant role in ABA-mediated activation of the hyperpolarization-activated activated guard cell Ca^{2+} channels (Pei et al., 2000; Kwak et al., 2003). The increase in cytosolic Ca^{2+} concentrations activates plasma membrane-bound anion channels that generate a membrane depolarization leading to the inhibition of inward KAT1 K^+ channels (Osakabe et al., 2014). OST1 phosphorylates the C terminus domain of KAT1, inhibiting its channel activity (Sato et al., 2009). ABA also inhibits the activity of the guard cell plasma membrane H^+ -ATPase, contributing to the depolarization of the guard cell membrane (Hayashi et al., 2011).

CALCINEURIN-B LIKE PROTEINS (CBLs) are proteins that, upon Ca^{2+} binding, interact and regulate the activity of CBL-INTERACTING PROTEIN KINASES (CIPKs) (Luan, 2009). The CBL1/CBL9-CIPK26 complex was shown to interact with RBOHF N terminus and to phosphorylate RBOHF (Drerup et al., 2013). Since RBOHF is located at the plasma membrane, these results suggest that a CIPK26-mediated regulation of RBOHF occurs at the plasma membrane and not via the typical CBL-CIPK-dependent translocation regulatory mechanism (Luan, 2009). The phosphorylation of RBOHF by the Ca^{2+} -CBL-activated kinase CIPK26 resulted in enhanced RBOHF-mediated ROS production (Drerup et al., 2013). Although these results were obtained in a heterologous expression system (HEK293T cells), they suggest a synergistic positive regulation of NADPH oxidase activity, with the

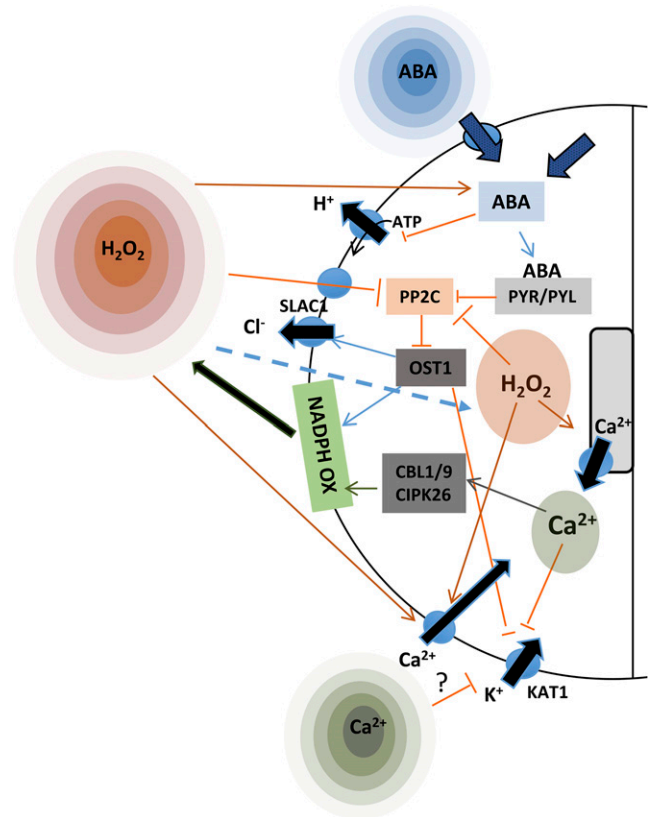


Figure 1. The Interaction of ROS, ABA, and Ca^{2+} in Stomatal Responses.

ABA is shown to enter guard cells via ABA transporters. ABA can also be synthesized in guard cells in response to signals such as elevated ROS. Accumulation of ABA could also result from decreased degradation or release of ABA from conjugated sources. ABA can cause the accumulation of ROS via interaction with the PYR/PYL receptor and the inhibition of PP2C that will result in activation of OST1 and phosphorylation and activation of NADPH oxidase (RBOH). ROS generated by RBOH or arriving at the guard cell with the ROS wave can enter guard cells from the apoplast via aquaporins and result in the opening of ROS-regulated Ca^{2+} channels that in turn will cause the further activation of RBOH proteins by CIPK26. ROS can also directly inhibit PP2C, preventing the inhibition of OST1 and resulting in further biosynthesis of ROS, or accumulate due to enhanced photorespiration.

concomitant ROS production via Ca^{2+} -induced phosphorylation by a CBL-CIPK complex (see also below).

There is evidence that the apoplastic accumulation of ROS is involved in the induction of stomatal closure (An et al., 2008; Khokon et al., 2011). ABA led to decreased GSH concentrations in guard cells (Okuma et al., 2011), and GSH-deficient mutants displayed enhanced ABA-induced stomata closure. The *Arabidopsis thaliana cad2-1* mutant, lacking γ -glutamylcysteine synthase, which catalyzes the first step in GSH biosynthesis, displayed enhanced H_2O_2 activation of the hyperpolarized-activated guard cell plasma membrane Ca^{2+} channel and enhanced H_2O_2 -induced stomatal closure (Munemasa et al., 2013). Since the reduction in guard cell cytosolic GSH was induced by ABA (Okuma et al., 2011)

and not H_2O_2 , Munemasa et al. (2013) suggested that the apoplastic ROS signal could modulate the responsiveness of the guard cells to ABA by stimuli other than ABA itself. This notion has not yet been demonstrated experimentally. ROS were proposed to enhance ABA biosynthesis or inhibit ABA degradation, causing an elevation in free ABA levels (Huang et al., 2012; Boursiac et al., 2013; Daszkowska-Golec and Szarejko, 2013; Song et al., 2014). Enhanced ROS levels could therefore result in enhanced ABA accumulation, whereas enhanced ABA levels could result in enhanced ROS production in guard cells, creating a positive feedback loop to mediate stomatal closure. It should also be noted that closer of stomata is likely to induce photorespiration due to limited CO_2 , which in turn would lead to the accumulation of H_2O_2 .

THE ROLE OF THE ROS AND CALCIUM WAVES IN SAA

A burst of ROS production mediated by respiratory burst homolog (RBOH or NOX) proteins is initiated in plant or animal cells in response to many different biotic or abiotic stimuli. In plants, this burst was shown to trigger the production of ROS by neighboring cells initiating a long-distance signal termed the ROS wave (reviewed in Mittler et al., 2011; Gilroy et al., 2014). Each cell along the path of the ROS wave activates its own RBOHD proteins generating a systemic autopropagating wave of ROS production that travels in the apoplast from the initiating tissue to the entire plant at rates of up to 8.4 cm/min (Figure 2). Recent analysis of the ROS wave in *Arabidopsis* demonstrated that it is required for the activation of SAA of plants in response to local stimuli such as high light or heat, as measured by protein and RNA expression as well as enhanced tolerance of systemic

leaves (Suzuki et al., 2013). These findings demonstrated that the ROS wave has a biological function in plants. Although it was required for SAA, it was found that the ROS wave could not convey abiotic stress specificity to this response. This finding prompted the hypothesis that the ROS wave functions to prime the plant for SAA activation and that other stress-specific signals are required to convey specificity to this response. During the SAA response to heat stress, the ROS wave was found to function in coordination with ABA in systemic tissues (Suzuki et al., 2013). This analysis of the ROS wave in *Arabidopsis* also identified a functional link between ROS production by RBOH proteins and electric signals. At least one type of electric signal was found to be suppressed in mutants lacking RBOHD that was required for the ROS wave (Suzuki et al., 2013). The studies described above demonstrated that the ROS wave plays a central role in mediating rapid systemic responses to abiotic stress.

Recent plant-wide dynamic measurements of alterations in cytosolic Ca^{2+} concentrations in response to localized stress application demonstrated the existence in plants of systemic Ca^{2+} waves (Choi et al., 2014). Application of salt stress to the root tip of *Arabidopsis* plants resulted in the initiation of a Ca^{2+} wave that traveled at ~ 2.4 cm/min (400 $\mu\text{m/s}$) and spread through the roots all the way up to the aerial parts of the plant. The Ca^{2+} wave propagated through the root cortex and endodermis layers, demonstrating cell-type specificity. In addition, it was dependent on the function of the vacuolar ion channel TWO PORE CHANNEL1 (TPC1), a cation permeable channel, implicated in calcium-induced calcium release (Choi et al., 2014). Interestingly, TPC1 was shown to be sensitive to H_2O_2 in patch clamp experiments, prompting the hypothesis that the ROS and Ca^{2+} waves are coordinated (Gilroy

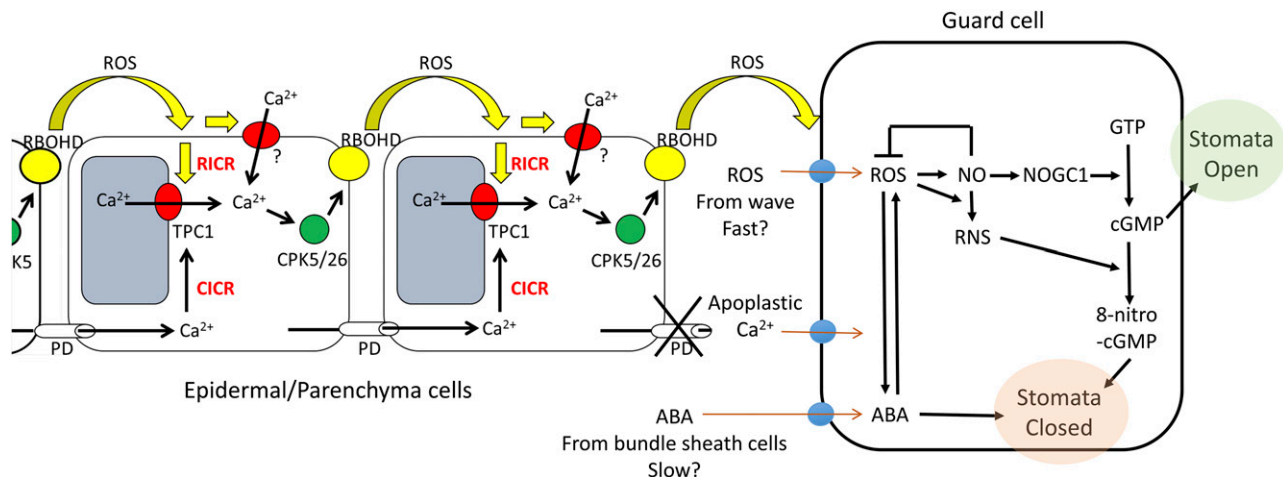


Figure 2. The Interaction of the ROS and Ca^{2+} Waves with Guard Cells.

The ROS wave is shown to be mediated by the RBOHD proteins of each individual cell along its path. This process is integrated with the Ca^{2+} wave through the function of TPC1 or different ROS-regulated Ca^{2+} channels in a process termed ROS-induced Ca^{2+} release (RICR). The elevated Ca^{2+} levels are shown to trigger further ROS production due to phosphorylation of RBOHD by the Ca^{2+} -dependent protein kinase (CPK) 5 and/or 26. A process of Ca^{2+} -induced Ca^{2+} release (CICR) could also be involved in the coupling of the ROS and Ca^{2+} waves. Because mature guard cells are not connected to their neighboring cells by plasmodesmata, they are likely to primarily sense apoplastic changes in ROS and Ca^{2+} originating from the ROS and Ca^{2+} waves or ABA produced by bundle sheath or other neighboring cells. ABA that enters the guard cells via ABA transporters can cause stomatal closure. In contrast, ROS entering the guard cells via aquaporins could cause stomatal opening or closing via NO signaling and cGMP or 8-nitro-cGMP. It is possible that the ROS wave will reach the guard cell before ABA synthesized by bundle sheath cells, resulting in a transient response of stomata (e.g., transient opening that is followed by closing or vice versa).

et al., 2014). In support of this hypothesis is a recent study demonstrating that the calcium dependent protein kinase 5 (CPK5) is important for the propagation of the ROS wave in plants (Romeis and Herde, 2014). ROS generated in the apoplast by an RBOHD protein of a neighboring cell (as part of the ROS wave) could therefore enter a nonactivated cell via aquaporins and trigger the release of Ca^{2+} by channels such as TPC1. The resulting buildup of Ca^{2+} in the cytosol of the nonactivated cell would in turn activate CDPKs that would phosphorylate and activate the RBOHD proteins of this cell, turning on its ROS production and adding it to the activated group of cells participating in the ROS wave (Gilroy et al., 2014; Figure 2). It should be noted that the properties of some of the proteins described above may be different when compared between different plant species, for example, Os-TPC1 and At-TPC1, and that some of the experiments conducted to characterize these properties were performed *in vitro*.

Despite work supporting a crucial role for stomata in SAA initiation (Galvez-Valdivieso et al., 2009) and work showing that ABA is important for SAA (Suzuki et al., 2013), questions such as how the ROS wave might affect guard cells of nonstressed plants and how changes in ABA might affect SAA are unknown at present. Below we will attempt to address some of these questions.

INTEGRATING THE ROS-CALCIUM WAVE WITH ABA

Although stomatal responses are important to local and systemic responses of plants to abiotic and biotic stresses, very little is known about how they are involved in these processes. In a key article addressing this question, Galvez-Valdivieso et al. (2009) demonstrated that ABA accumulation in response to high light stress is required for the induction of high light response genes. Interestingly, ABA accumulation in response to high light stress was inhibited under conditions of high relative humidity, suggesting that water loss caused by the opening of stomata during high light stress is required for ABA biosynthesis. Alternatively, high relative humidity could be enhancing the rate of ABA degradation, thereby preventing ABA accumulation (Okamoto et al., 2009). ABA signaling did not appear to be required for ROS production under conditions of high light stress, suggesting that ROS accumulation may function upstream of ABA biosynthesis (Galvez-Valdivieso et al., 2009). The ABA-dependent responses to high light stress were found to occur in detached leaves as well as whole plants, suggesting that they are leaf autonomous and do not involve ABA biosynthesis in roots. Gorecka et al. (2014) have further observed that ABA signaling mutants have an effect on bundle sheath cell responses in terms of *APX2* gene expression and photosynthetic efficiency of these cells, but no effect could be detected at the whole leaf level for any of the signaling mutants in response to high light, despite a series of microarray experiments and extensive physiological analysis. These results suggested that in terms of cell autonomy under high light conditions, systemic responses might be confined to bundle sheath cells and guard cells. One factor that might vary between the different studies described above is the intensity of light used to induce SAA and the physiological condition of the treated plants. These differences could induce different responses in bundle sheath versus mesophyll cells. In a more recent publication, ABA was found to be required for SAA of systemic leaves to heat stress (Suzuki et al., 2013). Interestingly, ABA accumulation in

systemic leaves was found to be transient and dependent on ROS production by RBOHD. These results suggest that ROS accumulation driven by high light stress (Galvez-Valdivieso et al., 2009) or the ROS wave (Suzuki et al., 2013) may be required for the accumulation of ABA that is in turn required for activation of gene expression (Galvez-Valdivieso et al., 2009; Huang et al., 2012) and stomatal responses (Song et al., 2014). The finding that high relative humidity inhibited some of these responses (Galvez-Valdivieso et al., 2009), as well as systemic responses to pathogens (Yoshioka et al., 2001; Gozzo and Faoro, 2013), would suggest that ABA levels and/or alterations in stomatal responses that cause changes in leaf water potential play a key role in systemic responses.

When addressing the role of stomata in systemic signaling, we should consider the conditions that affect the stomata in a systemic nonstressed leaf and the interaction of guard cells with the systemic signal(s). The SAA signal that reaches the systemic leaf most likely involves ROS and Ca^{2+} from the Ca^{2+} and ROS waves as well as electric signals (Christmann et al., 2013; Gilroy et al., 2014). ABA produced by bundle sheath cells in response to these signals may also reach the stomata (Galvez-Valdivieso et al., 2009), but most likely at a slower rate (Figure 2). Mature stomata are thought not to be linked to their neighboring epidermal cells via plasmodesmata. Therefore, they would be able to sense only apoplastic changes such as enhanced ROS, Ca^{2+} , or ABA via aquaporins, Ca^{2+} channels, or ABA transporters, respectively. ROS entering the guard cell could cause the enhanced accumulation of ABA, thereby causing stomatal closure (Song et al., 2014; Figure 1). It could also cause the enhanced accumulation of NO that would in turn facilitate the production of cGMP and result in stomatal opening (Joudoi et al., 2013). ROS interacting with NO could also result in the production of reactive nitrogen species that would convert cGMP into 8-nitro-cGMP and cause stomatal closure (Joudoi et al., 2013; Song et al., 2014). Apoplastic calcium could enter guard cells via channels that could be ROS responsive and trigger the accumulation of more ROS as described in Figure 1. Judging by the transient accumulation of ABA in systemic tissue in response to local heat stress (Suzuki et al., 2013), it is possible that the ROS wave first causes the opening of the stomata that is then followed by its closure.

The findings reported by Galvez-Valdivieso et al. (2009) and Suzuki et al. (2013) suggest the existence of a tight sensing cycle that involves stomatal function and ABA levels. Changes in environmental conditions, such as a decrease in water potential, light intensity, or humidity sensed in leaf cells by different hydraulic, osmotic, redox, or temperature sensors (Mittler et al., 2012; Christmann et al., 2013), could therefore lead to the accumulation of ABA, ROS, and/or NO that would in turn cause stomatal closure or opening depending on the type of stress or signal involved (Figure 3A; Joudoi et al., 2013; Kollist et al., 2014; Song et al., 2014). The opening or closing of stomata would in turn affect the micro-environment within the leaf and alter environmental sensors and/or ROS, ABA, and NO levels creating a cycle (Figure 3A). The sensing cycle shown in Figure 3A could function in each leaf of the plant in a "leaf autonomous" fashion (Galvez-Valdivieso et al., 2009), allowing the different parts of the plant to coordinate their response to different environmental stimuli that may affect different parts of the plant, and the plant as a whole, to achieve the best balance suited for acclimation to its environment. Different leaves or parts of

the plant could communicate with each other over long distances using SAA (or SAR) signals via the ROS and Ca^{2+} waves (Gilroy et al., 2014), electric signals (Szechyńska-Hebda et al., 2010), and/or hydraulic waves (Christmann et al., 2013) that could be caused by alterations in water potential in a particular leaf due to stomata opening or closing, mechanical injury, or pathogen infection. Figure 3 illustrates a model proposing how the function of the leaf autonomous sensing cycle in one leaf (Figure 3A) could be connected

to other leaves (Figure 3B). Sensing of changes in temperature or light intensity in a local leaf could result in the accumulation of ROS that in turn will cause stomata to open or close via ROS-NO interactions (Joudoi et al., 2013; Song et al., 2014). This would cause a decrease or increase in leaf water potential, the accumulation or degradation of ABA, and the activation of gene expression regulated by ABA (Galvez-Valdivieso et al., 2009), as well as the triggering of a ROS/ Ca^{2+} /hydraulic/electric wave that will travel from

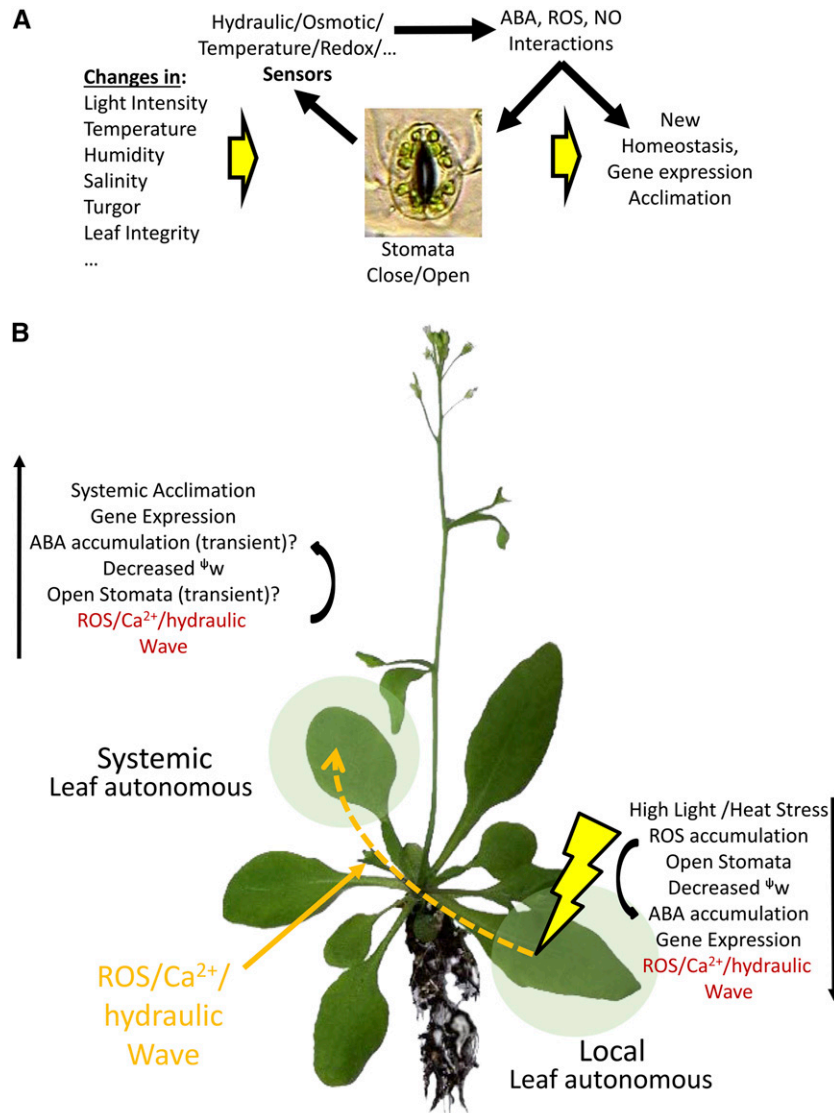


Figure 3. Leaf Autonomous and Systemic Regulation of Stomata in Response to Stress.

(A) Leaf autonomous regulation of stomatal responses. Sensing of environmental stimuli by receptors is shown to trigger ABA, ROS, and NO signaling and regulate stomatal aperture and gene expression. The new micro-climate achieved in the leaf airspaces is balanced with new patterns of gene expression to achieve a new state of leaf homeostasis and acclimation. This process that occurs in a leaf autonomous manner can trigger systemic signals such as ROS, Ca^{2+} , hydraulic, and/or electric signals that will propagate to the entire plant and induce SAA.

(B) A proposed model for systemic responses to high light or heat stresses. High light or heat stress is shown to induce ROS accumulation in a local leaf and to cause the opening of stomata. This process will result in a decrease in leaf water potential and the accumulation of ABA that in turn will trigger gene expression and the initiation of a systemic wave. The triggering of ABA biosynthesis and gene expression could also be mediated directly by ROS. The systemic ROS/ Ca^{2+} /hydraulic/electric wave will reach a systemic leaf causing the transient opening of stomata and the transient accumulation of ABA that will turn on gene expression and induce SAA. Triggering of ABA accumulation in systemic tissues could also be directly mediated by ROS.

the local leaf to the entire plant (Szechyńska-Hebda et al., 2010; Suzuki et al., 2013; Gilroy et al., 2014). The sensing of this wave in the systemic leaf could cause the transient opening or closing of stomata (Figure 2), inducing a transient decrease or increase in systemic leaf water potential, as well as a transient biosynthesis or degradation of ABA. The transient alteration in ABA levels in the systemic leaf would in turn trigger ABA-induced gene expression and acclimation of the systemic tissue (Galvez-Valdivieso et al., 2009; Suzuki et al., 2013). Transient stomatal responses and sensing in the different leaves (Figure 3A) driven by environmental stimuli or systemic waves would therefore orchestrate the whole plant response to stress and link environmental sensing to ABA responses and acclimation (Figure 3B). Further studies to dissect the role of stomata and ABA in SAA and SAR could be facilitated by the recent developments of advanced imaging tools to measure systemic signals as well as omics and modeling tools.

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AUTHOR CONTRIBUTIONS

The authors contributed equally to preparing and writing this article.

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