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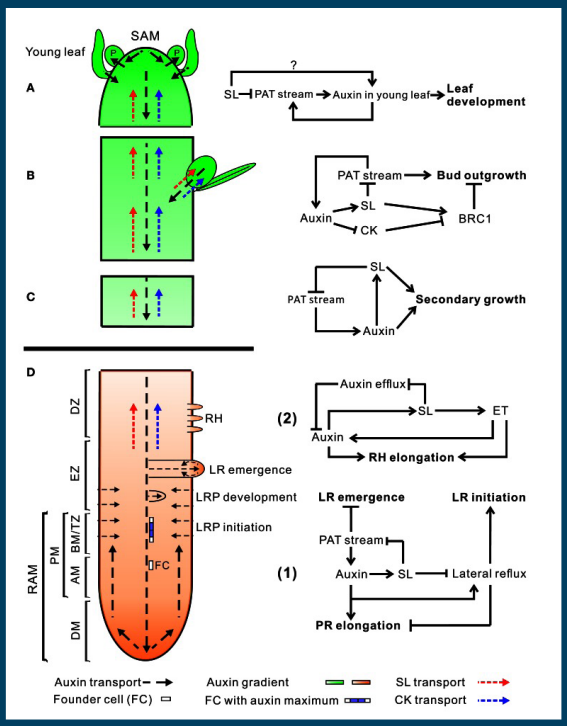
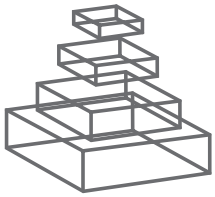


HORMONAL CROSS-TALK IN PLANT DEVELOPMENT AND STRESS RESPONSES

Topic Editors
Sergi Munné-Bosch and Maren Müller

frontiers in PLANT SCIENCE



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HORMONAL CROSS-TALK IN PLANT DEVELOPMENT AND STRESS RESPONSES

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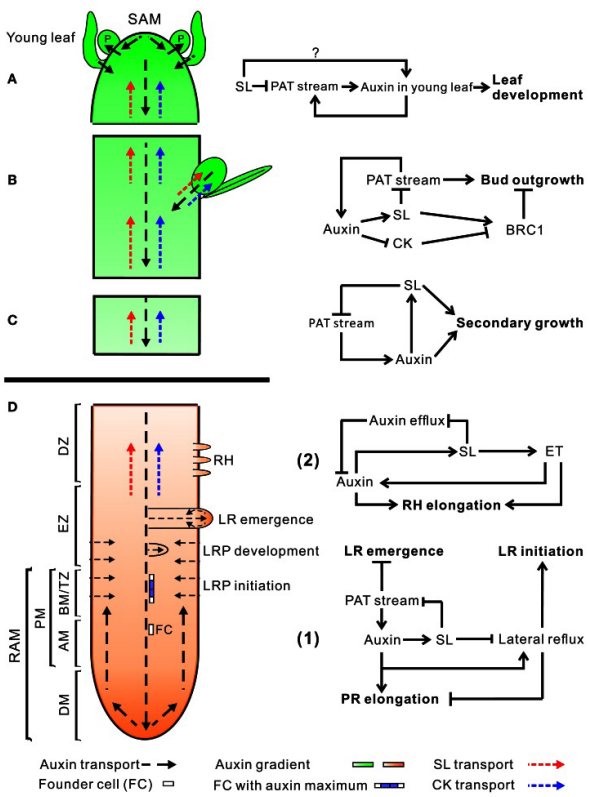

In contrast to animals, plants can continuously cease and resume growth. This flexibility in their architecture and growth patterns is partly achieved by the action of plant hormones. Plant hormones are structurally diverse compounds that act usually at nanomolar concentrations and include five groups of the so-called "classic" hormones, namely auxins, cytokinins (CK), gibberellins (GA), abscisic acid (ABA) and ethylene. Jasmonates (JA), salicylates (SA), strigolactones (SL), brassinosteroids (BR), polyamines and some peptides were recognized as new families of plant hormones. To date, auxin, CK, GA, SL, BR and polyamines are identified as the major developmental growth regulators, whereas ABA, ethylene, SA and JA are often implicated in stress responses. Recent studies support the contention

that hormone actions build a signaling network and mutually regulate several signaling and metabolic systems, which are essential both for plant development and plant responses to biotic and abiotic stresss. Some examples include auxin and GAs in growth regulation; CKs, auxin, ABA and SL in apical dominance; auxin and BR in cell expansion; ethylene and CKs in the inhibition of root and hypocotyl elongation; ethylene, ABA and GAs in some plant stress responses; or SA, JA and auxin in plant responses to pathogens, to name just a few of the reported hormonal interactions. Although earlier work greatly advanced our knowledge of how hormones affect plant growth and development and stress responses focusing on a single compound, it is now evident that physiological processes are regulated in a complex way by the cross-talk of several hormones. In this Research Topic, we aim at collecting a comprehensive set of original research and review papers focused on hormonal crosstalk in plants.

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Hormonal cross-talk in plant development and stress responses

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Keywords: hormonal balance, hormone interaction, hormone interplay, plant hormones, phytohormones

In contrast to animals, plants can continuously cease and resume growth. This flexibility in their architecture and growth patterns is partly achieved by the action of plant hormones. Plant hormones are structurally diverse compounds that act usually at nanomolar concentrations and include five groups of the so-called "classic" hormones, namely auxins, cytokinins, gibberellins, abscisic acid, and ethylene. Jasmonates, salicylates, strigolactones, brassinosteroids, polyamines, and some peptides were recognized as new families of plant hormones. Hormones build a signaling network and mutually regulate several signaling and metabolic systems, which are essential both for plant development and plant responses to biotic and abiotic stresses. Although earlier work greatly advanced our knowledge of how hormones affect plant growth and development and stress responses focusing on a single compound, it is now evident that physiological processes are regulated in a complex way by the cross-talk of several hormones. In this Research Topic, we aim at collecting a comprehensive set of original research and review papers focused on hormonal crosstalk in plants.

The goal of this Research Topic is to bring together recent work of experts studying hormonal crosstalk in plant development and stress response. Understanding how hormones and genes interact to coordinate plant growth is a major challenge in developmental biology. The activities of auxin, ethylene, and cytokinin depend on the cellular context and exhibit either synergistic or antagonistic interactions. Liu et al. (2013) use experimentation and network construction to elucidate the role of the interaction of the POLARIS peptide (PLS) and the auxin efflux carrier PIN proteins in the crosstalk of three hormones (auxin, ethylene, and cytokinin) in *Arabidopsis* root development. Naidoo et al. (2013) elegantly describe the transcriptional response of PR genes (*EgrPR2*, *EgrPR3*, *EgrPR4*, *EgrPR5*, *and EgrLOX*) identified in *Eucalyptus grandis* in response to SA and methyl jasmonate (MeJA) treatment. Blanco-Ulate et al. (2013) analyzed a transcriptome study of tomato fruit infected with *Botrytis cinerea* in order to profile the expression of genes for the biosynthesis, modification and signal transduction of ethylene, salicylic acid, jasmonic acid, and abscisic acid, hormones that may be not only involved in ripening, but also in fruit interactions with pathogens. The changes in relative expression of key genes during infection and assays of susceptibility of fruit with impaired synthesis or perception of these hormones were used to formulate hypotheses regarding the involvement of these regulators in the outcome of the tomato fruit–*B. cinerea* interaction.

A series of reviews also add to the current knowledge of hormonal cross-talk in the regulation of plant development and stress responses. Arc et al. (2013) review our current knowledge of ABA crosstalk with ethylene and NO, both volatile compounds that have been shown to counteract ABA action in seeds and to improve dormancy release and germination. McAtee et al. (2013) review current evidence on the topic and elegantly describe the hormonal cross-talk in the developing seed and its surrounding fruit tissue during fruit development. Denancé et al. (2013) address novel insights on the regulatory roles of the ABA, SA, and auxin in plant resistance to pathogens and describe the complex interactions among their signal transduction pathways. The strategies developed by pathogens to evade hormone-mediated defensive responses are also reviewed. Based on these data it is also discussed how hormone signaling could be manipulated to improve the resistance of crops to pathogens. From another perspective, Daszkowska-Golec and Szarejko (2013) review recent findings on phytohormone crosstalk, including changes in signaling pathways and gene expression that impact on modulating stress response through the closing or opening of stomata. da Costa et al. (2013) review current evidence indicating a clear hormonal cross-talk in the regulation of adventitious rooting. Cheng et al. (2013) review current evidence on the recently discovered phytohormone class, strigolactones and their crosstalk with other plant hormones—such as auxin, cytokinin, abscisic acid (ABA), ethylene (ET), and gibberellins (GA)—during different physiological processes. Finally, O'Brian and Benkkova discuss the complex hormonal cross-talk in plant responses to environmental stress, with a focus on cytokinins and other hormones, such as abscisic acid, jasmonates, salicylates, ethylene, and auxin. Of particular interest is the discussion of the impact of this research in the biotechnological industry.

In conclusion, taken together these original and review articles reflect the explosion of interest and considerable progress that has recently been made in the dynamic field of plant biology, with a particular focus on better understanding hormonal cross-talk in plant development and stress responses. It will be intriguing to see how future work on hormonal cross-talk in plants will continue. We hope that the articles that have been compiled will provide new insights into this topic and shed new light concerning the complex but exciting phenomenon of hormonal cross-talk in plant development and stress responses.

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Interaction of PLS and PIN and hormonal crosstalk in *Arabidopsis* root development

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Understanding how hormones and genes interact to coordinate plant growth is a major challenge in developmental biology. The activities of auxin, ethylene, and cytokinin depend on cellular context and exhibit either synergistic or antagonistic interactions. Here we use experimentation and network construction to elucidate the role of the interaction of the POLARIS peptide (PLS) and the auxin efflux carrier PIN proteins in the crosstalk of three hormones (auxin, ethylene, and cytokinin) in *Arabidopsis* root development. In ethylene hypersignaling mutants such as *polaris* (*pls*), we show experimentally that expression of both PIN1 and PIN2 significantly increases. This relationship is analyzed in the context of the crosstalk between auxin, ethylene, and cytokinin: in *pls*, endogenous auxin, ethylene and cytokinin concentration decreases, approximately remains unchanged and increases, respectively. Experimental data are integrated into a hormonal crosstalk network through combination with information in literature. Network construction reveals that the regulation of both PIN1 and PIN2 is predominantly via ethylene signaling. In addition, it is deduced that the relationship between cytokinin and PIN1 and PIN2 levels implies a regulatory role of cytokinin in addition to its regulation to auxin, ethylene, and PLS levels. We discuss how the network of hormones and genes coordinates plant growth by simultaneously regulating the activities of auxin, ethylene, and cytokinin signaling pathways.

Keywords: hormonal crosstalk, root development, auxin flux, PIN proteins, PLS protein, signaling network

INTRODUCTION

Hormone signaling systems coordinate plant growth and development through a range of complex interactions. The activities of auxin, ethylene, and cytokinin depend on cellular context and exhibit either synergistic or antagonistic interactions. Additionally, auxin is directionally transported through plant tissues, providing positional and vectorial information during development (Vanneste and Friml, 2009). Hormones and the associated regulatory and target genes form a network in which relevant genes regulate hormone activities and hormones regulate gene expression (Chandler, 2009; Depuydt and Hardtke, 2011; Vanstraelen and Benkov, 2012). In addition, hormones also interact with other signals such as glucose to control root growth (Kushwah et al., 2011). An important question for understanding these complex interactions is: what are the mechanisms that regulate the fluxes of plant hormones and levels of the proteins encoded by the regulatory and target genes?

Patterning in *Arabidopsis* root development is coordinated via a localized auxin concentration maximum in the root tip (Sabatini et al., 1999), requiring the regulated expression of specific genes. This auxin gradient has been hypothesized to be sink-driven (Friml et al., 2002) and computational modeling suggests that auxin efflux carrier activity may be sufficient to generate the gradient in the absence of auxin biosynthesis in the root (Grieneisen et al., 2007; Wabnik et al., 2010). However, other experimental studies show that local auxin biosynthesis modulates gradient-directed planar polarity in *Arabidopsis*, and a local source of auxin biosynthesis contributes to auxin gradient homeostasis (Ikeda et al., 2009). Thus genetic studies show that auxin biosynthesis (Ikeda et al., 2009; Normanly, 2010; Zhao, 2010), the AUX1/LAX influx carriers (Swarup et al., 2005, 2008; Jones et al., 2008; Krupinski and Jonsson, 2010), and the PIN auxin efflux carriers (Petrásek et al., 2006; Grieneisen et al., 2007; Krupinski and Jonsson, 2010; Mironova et al., 2010) all play important roles in the formation of auxin gradients. Since auxin concentration is regulated by these and diverse interacting hormones, it cannot change independently of these various components in space and time.

Therefore, a quantitative understanding of the effects of any perturbation experiment on auxin gradients and root development (e.g., genetic perturbations or exogenously applied hormones) must be analyzed in the context of hormonal interactions. For example, ethylene promotes auxin flux in the root, in a process dependent on the POLARIS (PLS) peptide (Ruzicka et al., 2007; Swarup et al., 2007; Liu et al., 2010). Furthermore, PIN levels are positively regulated by ethylene and auxin in *Arabidopsis* roots (Ruzicka et al., 2007). Interestingly, cytokinin can negatively regulate PIN levels (Ruzicka et al., 2009), while repressing auxin biosynthesis and promoting ethylene responses (Nordstrom et al., 2004; Chandler, 2009; Liu et al., 2010). Cytokinin also has the capacity to modulate auxin transport, by transcriptional regulation of the *PIN* genes (Ruzicka et al., 2009).

We previously developed a hormonal interaction network for a single *Arabidopsis* cell by iteratively combining modeling with experimental analysis (Liu et al., 2010). We described how such a network regulates auxin concentration in the *Arabidopsis* root, by controling the relative contribution of auxin influx, biosynthesis and efflux; and by integrating auxin, ethylene, and cytokinin signaling. Here we integrate PIN-mediated auxin flux with interacting hormone signaling modules. Specifically, we build on the hormonal crosstalk model (Liu et al., 2010) and construct a network to describe interaction of PLS and PIN proteins and hormonal crosstalk in *Arabidopsis* root development, using experimental data in the literature and our measurements.

RESULTS

RELATIONSHIP BETWEEN AUXIN, ETHYLENE, CYTOKININ, AND PLS

Our previous experimental measurements have shown the following response of auxin, ethylene, cytokinin to *PLS* expression. In the *polaris* (*pls*) mutant, auxin concentration decreases, cytokinin concentration increases and ethylene concentration remains approximately unchanged (Casson et al., 2002; Chilley et al., 2006; Liu et al., 2010). In the *PLS* overexpressing transgenic *PLOSox*, auxin concentration increases, and ethylene concentration remains approximately unchanged. In the *pls etr1* double mutant, auxin concentration is approximately recovered to the same level as that in wild-type seedlings.

In addition, the exogenous application of indole acetic acid (IAA) to wild-type seedlings increases both endogenous auxin concentration and *PLS* expression, while exogenous application of cytokinin to wild-type seedlings decreases both endogenous auxin concentration and PLS expression. Moreover, when 1-aminocyclopropane-1-carboxylic acid (ACC) is exogenously applied to wild-type seedlings, auxin concentration increases, but *PLS* expression decreases. However, in *pls*, although endogenous auxin concentration is lower than that in wild-type, the exogenous application of ACC further decreases auxin concentration (Chilley et al., 2006; Liu et al., 2010).

Therefore, PLS has a role in the crosstalk between auxin, ethylene, and cytokinin. By iteratively combining modeling with experimental analysis (Liu et al., 2010), we developed a hormonal crosstalk network. We described how such a network regulates auxin concentration in the *Arabidopsis* root, by controling the relative contribution of auxin influx, biosynthesis and efflux; and by integrating auxin, ethylene, and cytokinin signaling.

EXPERIMENTAL MEASUREMENTS OF THE RELATIONSHIP BETWEEN PINs AND PLS

Here we experimentally determined PIN1 and PIN2 protein levels in the seedling root of wild-type, *pls* mutant, *PLSox*, *etr1*mutant, and *pls etr1* double mutant (**Figure 1**). Immunolocalization studies revealed that both PIN1 and PIN2protein levels increase in the *pls* mutant, and decrease in *PLSox*. In the ethylene-insensitive *etr1* mutant, PIN1 and PIN2 levels are lower than in wild-type. In addition, the double mutant *pls etr1* exhibits reduced PIN1 and PIN2 levels compared to *pls* and slightly lower PIN1 and PIN2 levels compared to wild-type.

These experimental data show that PLS and PIN1/PIN2 form an interaction network, which regulates hormonal crosstalk between auxin, ethylene, and cytokinin. Previously, we were able to model interactions between auxin, ethylene, and cytokinin (Liu et al., 2010). Here we describe an expanded network that integrates the interactions between these hormones and PIN auxin transporters, based on the newly identified relationship between PLS and PINs (**Figures 1A,B**) and previous experimental data on ethylene effects on auxin transport (Ruzicka et al., 2007; Swarup et al., 2007) and PLS effects on ethylene responses (Chilley et al., 2006). All the analysis in this work is applicable to both PIN1 and PIN2, and we use the term PIN generically. We do not consider other forms of PINs, as our experiments and modeling focus on the auxin fluxes through the plasma membrane in this work.

NETWORK FOR INTERACTION OF PIN AND PLS AND HORMONAL CROSSTALK

Experimentally measured data (**Figure 1A**) are applicable for tissues rather than for a single cell. PIN1 and PIN2 levels in **Figure 1B** are the overall levels of the whole tissues. However, the interaction of PIN and PLS is at the cellular level. In order to use experimental data to analyze the interaction of PIN and PLS at a cellular level, the data for tissues have to be linked to the interactions in each cell (**Figure 2**). To do this, the following assumptions are made. First, all measured data are at steady states. Second, all fluxes or concentrations are relative to the respective counterparts in wild-type. If the auxin flux from shoot to root is increased or reduced, the influx in a single cell is considered to be correspondingly increased or reduced. This is because, at a steady state, the sum of total auxin influx from all neighboring cells and auxin biosynthesis rate in the cell must be equal to the total auxin efflux from the cell (**Figure 2**). Therefore, for all connecting cells in a tissue, the auxin flux from shoot to root affect the influx of all cells. A third assumption is that, when the level of PIN is compared, we assume the location of PIN does not change. For example, in the *pls* mutant, both PIN1 and PIN2protein levels increase (**Figure 1**). We consider this occurs at the original location of PIN1 and PIN2.

At a cellular level, PIN and PLS interact and a hormonal crosstalk network forms (**Figure 2**). Auxin fluxes and biosynthesis rates are regulated by all components in the network. At a tissue level, multiple cells interact and auxin gradients emerge (**Figure 2**). The current analysis concentrates on the study of the regulatory network for hormonal crosstalk: namely how PIN and PLS interacts at a cellular level and how hormonal crosstalk occurs. The spatial distribution of auxin in the root is due to spatial setting of PIN in multiple interacting cells, as modeled by Grieneisen et al. (2007).

In order to analyze the interaction of PIN and PLS and crosstalk with other hormonal signaling systems, we integrate the newly identified relationship between PLS and PIN (**Figure 1**) with the experimental data in the literature. When these data are incorporated into the network (Liu et al., 2010), two regulatory relationships emerge: first, that ethylene signaling promotes PIN levels; and second, that a decrease in PIN levels occurs following exogenous application of cytokinin (Ruzicka et al., 2009). Network construction for the interactions between hormonal pathways and PIN protein levels is described as follows.

First, an increase in PIN level (**Figure 1**) and the observed simultaneous decrease in auxin concentration in the *pls* mutant

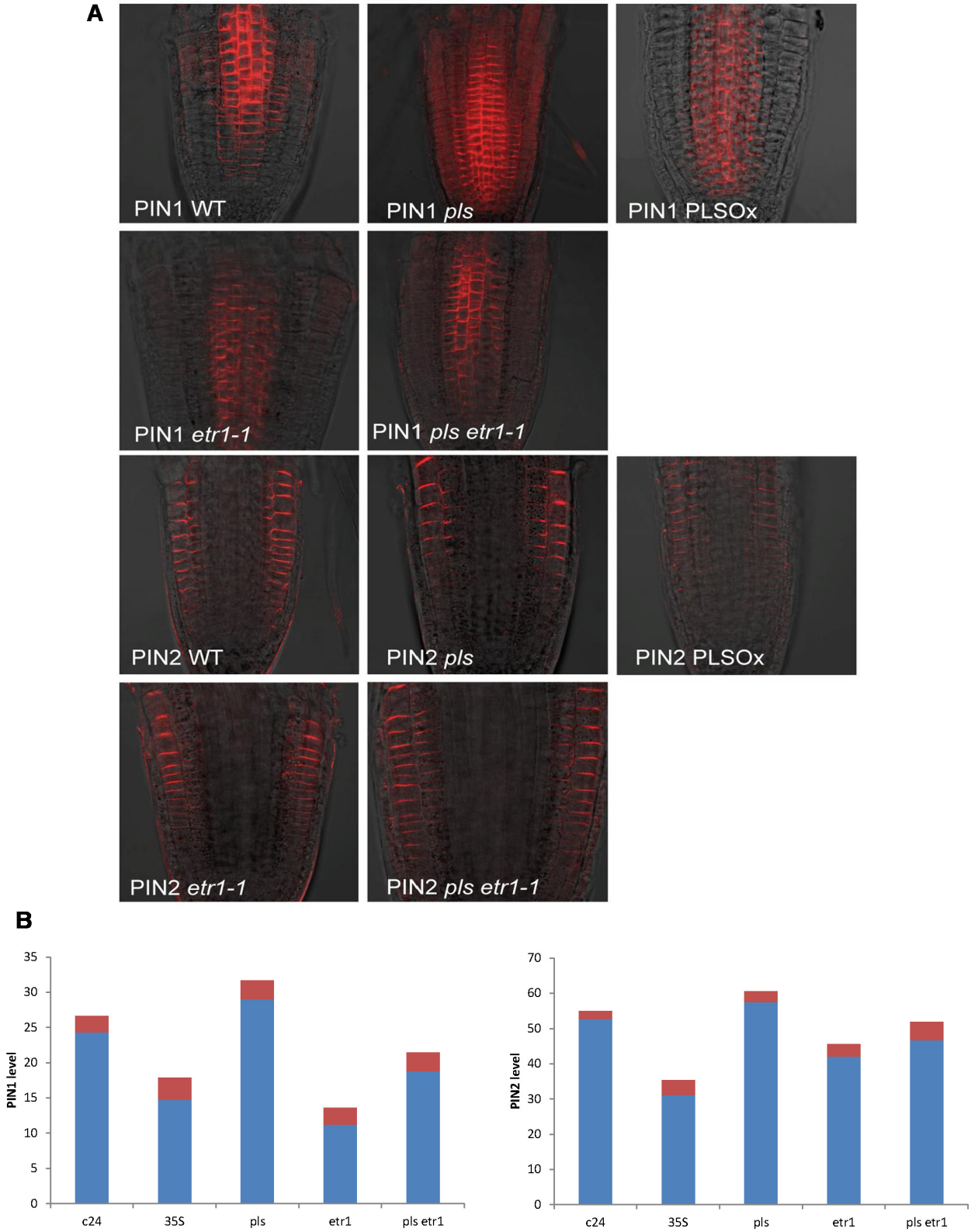

FIGURE 1 | (A) PIN1 and PIN2 immunolocalization in wild-type, *pls*, *PLSox*, *etr1*, and *etr1 pls* double mutants of *Arabidopsis*, showing differences in PIN protein levels. **(B)** Quantification of PIN1 and PIN2 levels in wild-type, *pls*, *PLSox*, *etr1* and *etr1 pls* double mutants in *Arabidopsis*. The red colored bars represent the standard errors of the mean ($n = 10$).

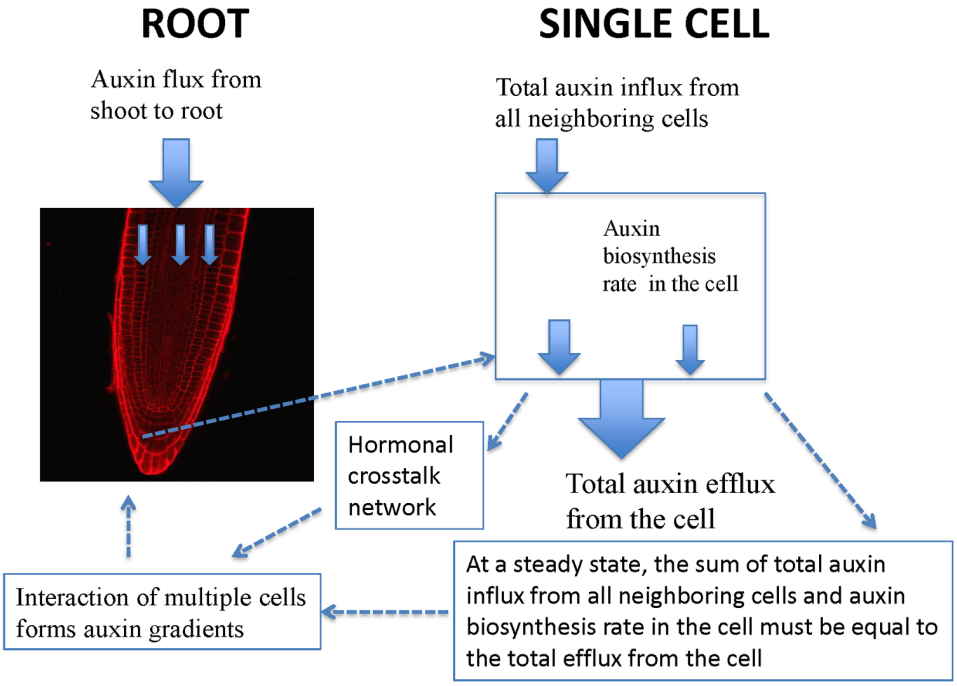

FIGURE 2 | Schematic description of the relationship between auxin spatial gradients and interaction of PIN and PLS as well as hormonal crosstalk at a cellular level. In a single cell, PIN and PLS interact and hormonal crosstalk occurs. At a steady state, the sum of total auxin influx from all neighboring cells and auxin biosynthesis rate in the cell must be equal to the total efflux from the cell. Moreover, when multiple cells interact, auxin gradients emerge.

(Chilley et al., 2006; Liu et al., 2010) imply that ethylene signaling also regulates PIN levels. This regulatory relationship is derived as follows. Experimentally, it has been shown that exogenous application of IAA and ACC can each increase PIN transcription and protein levels at the plasma membrane (Paciorek et al., 2005; Vanneste and Friml, 2009). However, exogenous application of cytokinin reduces PIN levels (Ruzicka et al., 2009). Moreover, exogenous application of IAA or ACC increases endogenous auxin concentration, as shown by experimental data (Stepanova et al., 2007; Ruzicka et al., 2009) and as analyzed by modeling analysis (Liu et al., 2010). Furthermore, exogenous application of cytokinin decreases endogenous auxin concentration (Eklof et al., 1997; Nordstrom et al., 2004). In contrast, a recent report shows that exogenous application of cytokinin promotes auxin biosynthesis in young, developing tissues (Jones et al., 2010). We construct networks for both effects of cytokinin, based on biological knowledge and our own experimental observations. **Figure 3** shows the case that cytokinin decreases endogenous auxin concentration. For the case in which cytokinin promotes auxin biosynthesis, the network is exactly the same as in **Figure 3** except for the positive regulation of cytokinin to auxin biosynthesis.

As exogenous application of ACC increases both PIN levels and endogenous auxin concentration (Ruzicka et al., 2007), and as exogenous application of cytokinin decreases both PIN levels (Ruzicka et al., 2009) and endogenous auxin concentration (Nordstrom et al., 2004), one possibility is that exogenous ACC and cytokinin exert their effects on PINs by affecting endogenous auxin concentration. However, in *pls*, an increase in PIN levels (**Figures 1A,B**) corresponds to a decrease in auxin concentration (Figure 4C in Chilley et al., 2006). This indicates that auxin is not the only regulator of PIN levels, as otherwise PIN levels should decrease in *pls*. Therefore, ethylene signaling also regulates auxin efflux and this is realized by its regulation of PIN levels (Liu et al., 2010). The decrease in auxin concentration (Figure 4C in Chilley et al., 2006) and the increase in ethylene signaling in *pls* have opposite effects on PIN levels: the reduced auxin concentration that decreases PLS expression in turn reduces PIN levels, while the increase in ethylene signaling increases PIN levels (Chilley et al., 2006; Liu et al., 2010). The net effect is an increase in PIN levels. Therefore, when *PLS* expression changes, effects of ethylene signaling on PINs are more dominant than the effects of auxin. Experimental work (Chilley et al., 2006) and modeling (Liu et al., 2010) show that, in *pls*, endogenous ethylene concentration (evolution) is the same as in wild-type. Therefore, PLS regulates ethylene signaling rather than its synthesis, possibly due to interaction between PLS protein and ETHYLENE RESISTANT 1 (ETR1) (Chilley et al., 2006; Liu et al., 2010). In addition, the relationship between PIN levels and *pls*, *etr1*, and *pls etr1* double mutants supports the (at least genetic, if not physical) interaction between PLS and ETR1: In *pls* and *etr1*, PIN protein levels increase and decrease, respectively. Moreover, the double mutant *pls etr1* exhibits reduced PIN levels compared to *pls*, but increased PIN levels compared to *etr1* (**Figure 1**).

Therefore, the positive regulation of *PIN* expression by ethylene signaling is included in the network (**Figure 3**). The inclusion

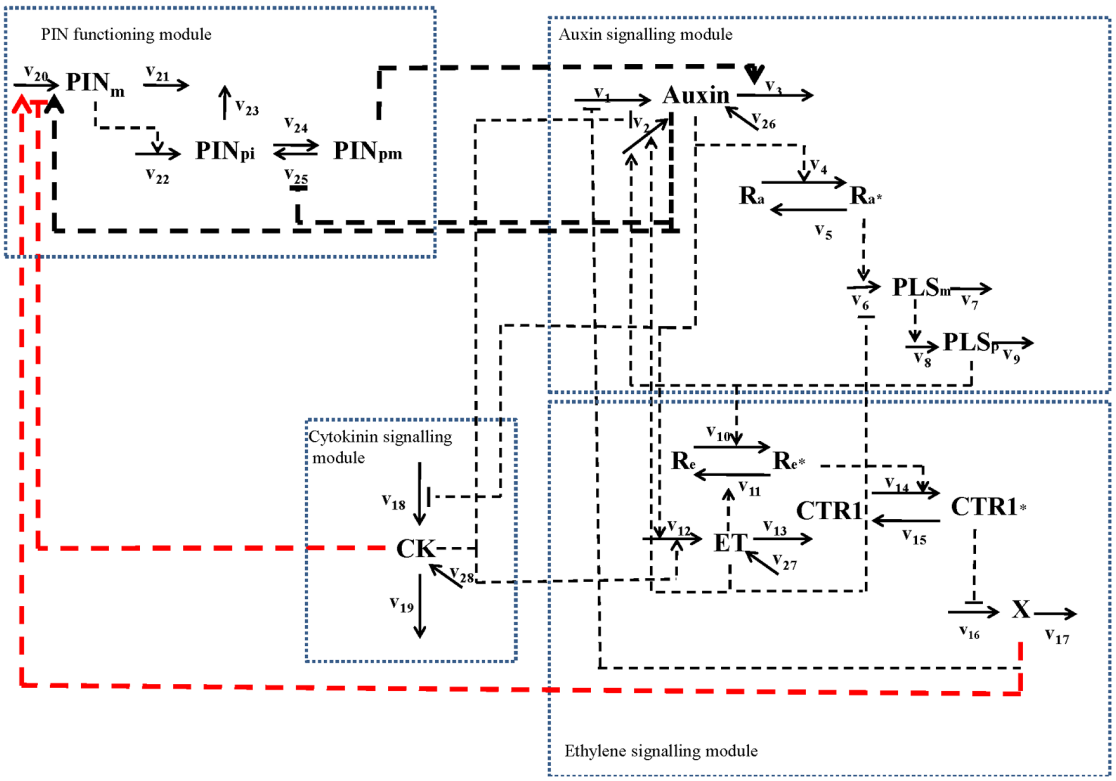

FIGURE 3 | Network for the interaction of PIN and PLS and hormonal crosstalk in the situation in which cytokinin decreases endogenous auxin concentration (Eklof et al., 1997; Nordstrom et al., 2004). The network includes four modules: an auxin signaling module; an ethylene signaling module; a cytokinin signaling module; and a PIN function module. In a previous study (Liu et al., 2010), the first three modules were described in detail. In this work, we integrate the PIN function module with the three hormone signaling modules. The three thick black dashed lines represent the regulatory relationships directly supported by experimental evidence: auxin positively regulates PIN (e.g., PIN1) levels and transcriptional effects associated with auxin regulation were identified; PIN internalization is inhibited by auxin; auxin efflux carrier activity (PIN1 and PIN2) positively regulates auxin transport. The two thick red dashed lines represent the regulatory relationships derived by the combined analysis of experimental evidence and the hormonal crosstalk network described previously (Liu et al., 2010). The reaction rates are: v1: total auxin influx from all neighboring (**Figure 2** and text for details); v2: auxin biosynthesis rate in the cell; v3: total auxin efflux from the cell; v4: rate for conversion of the inactive form of the auxin receptor to its active form; v5: rate for conversion of the active form of the auxin receptor to its inactive form; v6: transcription rate of the *POLARIS* (*PLS*) gene; v7: decay rate of *PLS* mRNA; v8: translation rate of the PLS protein; v9: decay rate of PLS protein; v10: rate for conversion of the inactive form of the ethylene receptor to its active form by PLS protein (PLSp); v11: rate for conversion of the active form of ethylene receptor to its inactive form; v12: ethylene biosynthesis rate; v13: rate for removal of ethylene; v14: rate for conversion of the inactive form of the CONSTITUTIVE TRIPLE RESPONSE 1 (CTR1) protein to its active form; v15: rate for conversion of the active form of CTR1 protein to its inactive form; v16: rate for activation of the ethylene signaling response; v17: rate for removal of the unknown ethylene signaling component, X; v18: rate for cytokinin biosynthesis; v19: rate for removal of cytokinin; v20: transcription rate of the *PIN* gene; v21: rare for the decay of *PIN* mRNA; v22: translation rate of PIN protein; v23: rate for decay of PIN protein in cytosol; v24: rate for transport of PIN protein from cytosol to plasma membrane; v25: rate for internalization of PIN protein. When exogenous hormones are applied: v26: rate for uptake of IAA when exogenous IAA is applied; v27: rate for uptake of ACC when exogenous ACC is applied; v28: rate for uptake of cytokinin when exogenous cytokinin is applied.

of this regulation is consistent with the experimental observations following exogenous application of IAA and ACC. When IAA is exogenously applied, both *PLS* expression and ethylene responses increase (Casson et al., 2002; Stepanova et al., 2007; Liu et al., 2010). Increasing *PLS* expression leads to decreased ethylene signaling, while increasing ethylene concentration increases ethylene responses. Therefore, application of exogenous IAA has antagonistic effects on ethylene signaling that regulates PIN levels. In addition, the increase of auxin concentration due to exogenous IAA application increases PIN levels. The overall effects of exogenous application of IAA lead to an increase in PIN levels. When ACC is exogenously applied, both endogenous ethylene and auxin concentrations increase, and *PLS* expression levels decrease (Chilley et al., 2006; Liu et al., 2010). Increase in ethylene concentration and decrease in PLS expression synergistically enhance ethylene responses. Therefore, when ACC is exogenously applied, auxin, ethylene and PLS all synergistically enhance PIN levels. Therefore, exogenous application of ACC leads to an increase in PIN levels.

The relationship between cytokinin and PIN levels implies an additional regulatory role of cytokinin in addition to its regulation to auxin, ethylene and PLS levels. This regulatory relationship is derived as follows. When cytokinin is exogenously applied, both endogenous cytokinin and ethylene concentrations increase, but *PLS* expression decreases (Liu et al., 2010). However, there are two opposite experimental observations for cytokinin effects:

endogenous auxin either decreases (Eklof et al., 1997; Nordstrom et al., 2004) or increases (Jones et al., 2010).

Both decreased PLS protein and increased ethylene concentration synergistically enhance ethylene signaling (Casson et al., 2002; Liu et al., 2010). Following our analysis above, this increases PIN levels. When cytokinin positively regulates auxin biosynthesis (Jones et al., 2010), exogenous application of cytokinin increases endogenous auxin concentration and this positively regulates PIN levels. Therefore, when cytokinin is exogenously applied, changes in auxin, ethylene, and *PLS* expression all lead to the increase in PIN levels. However, it has been shown experimentally that exogenous application of cytokinin results in a reduction of PIN levels (Ruzicka et al., 2009). This implies that cytokinin has an additional role in regulating PIN levels, in addition to its regulation of auxin, ethylene, and PLS levels.

When cytokinin negatively regulates auxin biosynthesis, exogenous application of cytokinin decreases endogenous auxin concentration (Eklof et al., 1997; Nordstrom et al., 2004). The decrease in auxin concentration reduces PIN levels. However, the decrease in *PLS* expression and the increase in ethylene simultaneously enhance PIN levels. In the *pls* mutant, auxin concentration is low and ethylene concentration remains approximately unchanged (Liu et al., 2010). As analyzed above, due to the strong interaction between PLS protein and ETR1, PIN levels increase (**Figures 1A,B**) even though the auxin concentration has been reduced to a large extent (Petrásek et al., 2006; Figure 4C in Chilley et al., 2006). Based on experimental data (Chilley et al., 2006), we estimate that in *pls* roots, auxin concentration is 0.14 μM, compared with 0.23 μM in wild-type (Liu et al., 2010). Following exogenous application of cytokinin, an additional factor, i.e., an increase in ethylene concentration, also enhances ethylene signaling responses. Therefore, PIN levels should increase. However, experimental work shows that exogenous application of cytokinin results in the reduction of PIN levels (Ruzicka et al., 2009). Therefore, an explanation of the experimental results requires an additional regulatory role for cytokinin in controling PIN levels, and this is included in **Figure 3**.

In addition, PIN endocytic internalization is inhibited by auxin (Paciorek et al., 2005). Therefore, we have included in the network the inhibition by auxin of the cycling between PINpm (PIN at plasma membrane) to PINpi (PIN in cytosol). Therefore, by integrating our experimental data (**Figure 1**) with the experimental data in the literature, a hormonal crosstalk network of auxin, cytokinin and ethylene is revealed (**Figure 3**).

HORMONAL CROSSTALK NETWORK AND ROOT GROWTH

As described in **Figures 2** and **3**, the concentrations of all three hormones (auxin, ethylene, and cytokinin) in root growth are mutually regulated by a hormonal crosstalk network. Therefore, they cannot change independently. Any genes that affect either the transport or biosynthesis of one of the three hormones have roles in the concentrations of all three hormones, as we demonstrated for the interaction of PIN and PLS. Auxin distribution is a versatile mechanism mediating a broad range of developmental responses (Petrásek and Friml, 2009). Both ethylene and cytokinin have roles in cell division (Ortega-Martínez et al., 2007; Dello Ioio et al., 2007). Therefore, an improved understanding of the roles of hormones and genes in root growth requires the analysis of hormonal crosstalk in space and time. For example, in *pls*, root elongation rate is slower than in wild-type (Casson et al., 2002). Due to the action of the hormonal crosstalk network (**Figure 3**) and as evidenced by experimental measurements, auxin concentration decreases, cytokinin concentration increases and ethylene concentration remains approximately unchanged (Casson et al., 2002; Chilley et al., 2006; Liu et al., 2010). As auxin concentration regulates elongation (Liu et al., 2010) and cytokinin concentration regulates the rates of cell division (Dello Ioio et al., 2007), the reduction of root elongation rate in *pls* is due to the changes in both auxin and cytokinin concentrations, as ethylene concentration remains approximately unchanged in *pls*.

DISCUSSION

Transport-mediated, differential auxin distribution is a versatile mechanism mediating a broad range of developmental responses (Petrásek and Friml, 2009). The PIN-based auxin transport network can integrate various endogenous and environmental signals that modulate polarity or subcellular trafficking of PIN proteins, which are considered to be major regulatory mechanisms for PIN activity (Kleine-Vehn and Friml, 2008; Grunewald and Friml, 2010).

Nonetheless, experimental analyses have shown also that PIN levels in *Arabidopsis* vary in response to a range of hormones. Auxin positively regulates levels of several PIN proteins in different developmental contexts (Blilou et al., 2005; Laskowski et al., 2006; Chapman and Estelle, 2009; Vanneste and Friml, 2009) by a signaling pathway regulating transcription (Woodward and Bartel, 2005). Ethylene also upregulates PINs (e.g., PIN2) to remove auxin from the more distal region of the root tip (Ruzicka et al., 2007). Moreover, cytokinin negatively regulates PIN levels (Ruzicka et al., 2009). It is also evident that ethylene activates the biosynthesis of auxin locally in the root tip (Stepanova et al., 2007; Swarup et al., 2007), and that both auxin and cytokinin can synergistically activate the biosynthesis of ethylene (Chilley et al., 2006; Stepanova et al., 2007).

However, ethylene can also be synthesized without exogenous auxin and cytokinin application, such as in its role in root hair production (Tanimoto et al., 1995). When PIN levels change following a change in the concentration/response of a given hormone, it does not necessarily mean that the given hormone predominantly regulates PIN levels. This is because changing the concentration/response of a given hormone may also change the concentrations/responses of other hormones. As shown in this work, PIN levels are simultaneously regulated by auxin, ethylene, and cytokinin via the action of PLS. Therefore, PINs and hormones form an entangled network, and any perturbation in the network will cause changes in other components. As a result, auxin concentration is regulated by these and diverse interacting hormones via a hormonal crosstalk network, as demonstrated in the **Figure 3**.

This work demonstrates that integration of experimental measurements with existing knowledge in the literature is able to reveal how PIN1, PIN2, and three hormones (auxin, ethylene, and cytokinin) form an entangled network via the action of PLS. Our methodology involves two major steps. First, the PIN levels are measured (**Figure 1A**) and quantified (**Figure 1B**). Quantification

of images shows the trends of the PIN levels (**Figure 1B**). Second, integrating experimental trends into existing knowledge reveals the crosstalk of PIN1, PIN2, auxin, ethylene, and cytokinin via the action of PLS. As all components in **Figure 3** form an entangled network, changing one component leads to changes in the others. Therefore, we propose that, in order to reveal the key regulatory points in the network, novel modeling methodology should be developed to dissect the regulation of the hormonal crosstalk network in the future.

The *Arabidopsis* genome contains eight *PIN* genes (Grunewald and Friml, 2010; Peer et al., 2011). Different PINs may have different locations and they may play different roles in auxin biology (Grunewald and Friml, 2010; Peer et al., 2011). For example, PIN1 and PIN2 exhibit primarily polar localizations on the plasma membrane while PIN3, PIN4, and PIN7 exhibit both polar and apolar plasma membrane localizations (Peer et al., 2011). In addition, hormones may regulate PIN levels differentially. For example, cytokinin can negatively regulate levels of PIN1, PIN2 and PIN3, but it positively regulates PIN7 levels (Ruzicka et al., 2007). In the current paper we construct the interaction network of PIN1, PIN2, auxin, ethylene, and cytokinin via the action of PLS. Following the methodology developed in this work, the interaction networks between other PINs, hormones and other genes could be constructed by measuring data similar to those described in **Figures 1A,B**. Moreover, as described in **Figure 2**, populating the hormonal crosstalk network in a spatial setting should be able to further model how auxin gradients are dependent on hormonal crosstalk in root development.

In addition, other phytohormones such as gibberellin and brassinosteroids are also important signals in the regulation of root development (Depuydt and Hardtke, 2011; Garay-Arroyo et al., 2012; Vanstraelen and Benkov, 2012). Although the effects of gibberellin and brassinosteroids on root development have been subjected to mathematical modeling studies (Middleton et al., 2012; van Esse et al., 2012), the networks describing their crosstalk with other hormones have not been constructed. The principle developed in this work can be used to further integrate the hormonal crosstalk for other phytohormones and genes in the future.

MATERIALS AND METHODS

PLANT MATERIALS

Wild-type (Col-0, C24) ecotypes and the *pls* and *pls etr1* mutants of *Arabidopsis thaliana* have been described previously (Topping and Lindsey, 1997; Casson et al., 2002; Chilley et al., 2006). *pls* DR5::GFP seedlings were generated by crossing (Liu et al., 2010). For *in vitro* growth studies, seeds were stratified, surface-sterilized and plated on growth medium (half-strength Murashige and Skoog medium (Sigma, Poole, UK), 1% sucrose, and 2.5% phytagel (Sigma) at 22 ± 2°C as described (Casson et al., 2009). For silver application experiments, seeds were germinated aseptically on growth medium or growth medium containing 10 μM silver nitrate.

MICROSCOPY AND IMAGE ANALYSIS

Confocal images (for GFP imaging) were taken with a Bio-Rad Radiance 2000 microscope (Bio-Rad, Hemel Hempstead, UK) after counterstaining tissues with 10 mg/ml propidium iodide as described (Casson et al., 2009).

For comparisons of PIN protein signal intensities, at least three independent experiments were carried out. For each experiment at least 10 roots were evaluated with five random regions selected for signal intensity quantification for each. All fluorescence signals were evaluated on a Zeiss LSM 5 Exciter or Leica TCS SP2 confocal laser scanning microscopes. The same microscope settings were used for each independent experiment, and pixel intensities were taken into account when the images between controls and samples were compared. The average fluorescence intensity was measured with ImageJ (National Institutes of Health, http://rsb.info.nih.gov/ij). Statistics were evaluated with Excel (Microsoft). Results were visualized as average intensities with error bars representing standard deviation of the mean.

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The identification and differential expression of *Eucalyptus grandis* pathogenesis-related genes in response to salicylic acid and methyl jasmonate

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Two important role players in plant defence response are the phytohormones salicylic acid (SA) and jasmonic acid (JA); both of which have been well described in model species such as *Arabidopsis thaliana*. Several pathogenesis related (PR) genes have previously been used as indicators of the onset of SA and JA signaling in *Arabidopsis*. This information is lacking in tree genera such as *Eucalyptus*. The aim of this study was to characterize the transcriptional response of PR genes (*EgrPR2*, *EgrPR3*, *EgrPR4*, *EgrPR5*, *and EgrLOX*) identified in *Eucalyptus grandis* to SA and methyl jasmonate (MeJA) treatment as well as to qualify them as diagnostic for the two signaling pathways. Using the genome sequence of *E. grandis*, we identified candidate *Eucalyptus* orthologs *EgrPR2*, *EgrPR3*, *EgrPR4*, *EgrPR5*, *and EgrLOX* based on a co-phylogenetic approach. The expression of these genes was investigated after various doses of SA and MeJA (a derivative of JA) treatment as well as at various time points. The transcript levels of *EgrPR2* were decreased in response to high concentrations of MeJA whereas the expression of *EgrPR3* and *EgrLOX* declined as the concentrations of SA treatment increased, suggesting an antagonistic relationship between SA and MeJA. Our results support *EgrPR2* as potentially diagnostic for SA and *EgrPR3*, *EgrPR4*, *and EgrLOX* as indicators of MeJA signaling. To further validate the diagnostic potential of the PR genes we challenged *E. grandis* clones with the fungal necrotrophic pathogen *Chrysoporthe austroafricana*. The tolerant clone showed high induction of *EgrPR2* and decreased transcript abundance of *EgrPR4*. Pre-treatment of the susceptible genotype with 5 mM SA resulted in lesion lengths comparable to the tolerant genotype after artificial inoculation with *C. austroafricana*. Thus expression profiling of *EgrPR2* and *EgrPR4* genes could serve as a useful diagnostic approach to determine which of the two signaling pathways are activated against various pathogens in *Eucalyptus*.

Keywords: *Eucalyptus*, salicylic acid, methyl jasmonate, PR genes, defence, *PR2, PR4*

INTRODUCTION

The defence mechanisms that are employed by plants to deter pathogens have been well-studied in various model organisms such as *Arabidopsis thaliana*. These model systems have created a foundation for understanding general host responses to pathogens. Following the plants perception of an invading pathogen, a plethora of defences responses are activated. Among these responses is the activation of various phytohormone signaling molecules including salicylic acid (SA), jasmonic acid (JA), ethylene (ET), abscisic acid (ABA), auxin, cytokinins (CK), gibberelins (GA), and brassinosteriods (BR). In particular, the phytohormones SA and JA have been extensively investigated in various pathosystems. These studies have shown that biotrophic pathogens are impeded by the activation of the SA pathway whereas necrotrophic pathogens are targeted by induction of JA and ET signaling pathways (Glazebrook, 2005). Each of these signaling cascades has been shown to involve the activation of certain signature defence genes, e.g., Pathogenesis Related (PR) genes, which can be representative of the induction of a pathway (Reymond and Farmer, 1998).

Stimulation of the SA pathway can be represented by an increase in the expression levels of *PR1*, *PR2*, and *PR5* defence genes (Kunkel and Brooks, 2002; Delaure et al., 2008). *Arabidopsis* SA signaling mutants *npr1*, *nim1*, and *sai1* as well as plants expressing the bacterial salicylate hydroxylase (*nahG*) are all impaired in their ability to induce expression of the *PR1*, *PR2*, and *PR5* thereby indicating that these PR candidates can be used as a measure of SA signaling induction (Cao et al., 1994; Delaney et al., 1995; Shah et al., 1997). In the case of *eds* and *pad* mutants, there is a lack of SA signaling thereby allowing for increase in JA signaling due to the lack of antagonism by SA (Zhou et al., 1998; Gupta et al., 2000; Nawrath et al., 2002;

Glazebrook et al., 2003). Transgenic plants over-expressing these SA signature defence genes have also been shown to result in increased resistance against pathogens such as *Phytophthora parasitica* and *Alternaria alternata* (Alexander et al., 1993; Jach et al., 1995). Induction of a derivative of JA, MeJA, can be represented in *Arabidopsis* by an increase in the expression levels of *PR3*, *PR4*, Vegetative Storage Protein (*VSP*), and Lipoxygenase (*LOX*). Over-expression of these proteins has also been shown to confer resistance to *Phytophthora nicotianae* and *Rhizoctonia solani* (Boter et al., 2004; Mishina and Zeier, 2007; Kusajima et al., 2010). Mutants of the JA signaling pathway in *Arabidopsis*, e.g., *fad3/7/8*, *coi1*, *and jar1* have been shown to inhibit the expression of *PR3*, *PR4*, *VSP*, and *LOX* and thus increase the susceptibility of the mutant lines to numerous pathogens (Staswick et al., 1998; Vijayan et al., 1998; Norman-Setterblad et al., 2000). Additional JA mutants, *mpk4* and *ssi2*, display increased levels of *PR1*, *PR2*, and *PR5* whilst impaired in JA defence gene expression, thereby indicating that these mutants are involved in JA and SA antagonism (Petersen et al., 2000; Kachroo et al., 2001; Shah et al., 2001). Consequently *PR3*, *PR4*, and *LOX* defence genes can be used as indicators for the onset of JA signaling. One can thus refer to *PR2* and *PR5* as signature defence response genes for SA and *PR3*, *PR4*, and *LOX* as signature defence response genes for JA. Although there have been significant advances in the understanding of plant defences in model systems, signature defence genes associated with SA and JA in woody plants such as *Eucalyptus* has not been extensively explored.

Eucalyptus species and hybrid clones are commercially planted because of their valuable wood and fiber properties which have been exploited by the pulp and paper industry. Due to the importance and value associated with this genus of hardwood trees, the initiative to sequence the genome of *Eucalyptus grandis* was undertaken by the US Department of Energy (DOE—Joint Genome Institute) in 2008. Currently, the first annotated version of the genome, released in January 2011, is available through Phytozome v7.0 and consists of 4952 scaffolds including 11 linkage groups/chromosomal assemblies (Phytozome, 2010). This resource provides a useful platform for elucidating various physiological aspects of *Eucalyptus*, such as their responses to biotic and abiotic factors. Although *Eucalyptus* trees are generally disease tolerant, they can and do succumb to diseases caused by a wide range of pathogens (Wingfield et al., 2008). A stepping stone for improving our understanding of *Eucalyptus* responses would be to identify genes associated with the SA and JA signaling pathways in these trees. The first aim of this study was to identify *Eucalyptus* orthologs of signature defence genes specific for the SA (*PR2* and *PR5*) and JA (*PR3*, *PR4*, and *LOX*) signaling pathways using sequence information from other plant species and the *E. grandis* genome sequence. Secondly we aimed to characterize the expression profiles of the putative orthologs using reverse transcriptase quantitative PCR (RT-qPCR). Transcript profiling that was conducted under mock induction of the signaling pathways revealed dose-dependent induction of the orthologous signature defence genes, as well as key time points for their expression. Furthermore, the orthologous genes were found to corroborate the antagonistic relationship observed between SA and JA in *Arabidopsis*. The ability of these putative signature defence genes to respond to fungal infection by *Chrysoporthe austroafricana* was examined in tolerant (TAG5) and susceptible (ZG14) *E. grandis* genotypes (Van Heerden et al., 2005). Expression profiling of these signature genes revealed the possible involvement of SA in defence against *C. austroafricana*.

MATERIALS AND METHODS

PLANT MATERIAL

Disease free *E. grandis* (Clone A, Mondi Tree Improvement Research) plantlets were propagated *in vitro* and following rooting the plantlets were transferred to Jiffy pots and grown at 25–28°C under long day (16 h) conditions under light intensity of 300–500 lum/sqf. Potted cuttings of *E. grandis* clonal genotypes, ZG14 and TAG5 (Mondi) with a stem diameter of 1 cm, were subsequently used for the infection trial with *C. austroafricana* and kept under the same conditions as stated above.

PHYLOGENETIC IDENTIFICATION OF PUTATIVE ORTHOLOGS FOR SIGNATURE DEFENCE GENES ASSOCIATED WITH SA AND MeJA

The *Arabidopsis thaliana* amino acid sequences of the genes of interest were obtained from The *Arabidopsis* Information Resource (TAIR, version 10) (https://www.arabidopsis.org). A BLASTP similarity search was conducted against the predicted *E. grandis* proteome (first *ab initio* and homology-based annotation) using the amino acid sequence as a query. This analysis was performed in Phytozome v7.0 (www.phytozome.net) and predicted *E. grandis* transcripts with *e*-values $<10^{-50}$ were downloaded. Putative *Populus trichocarpa* orthologs of the gene of interest were retrieved from NCBI and added to the analysis using the same BLAST parameters. Aligned sequences were imported into MEGA v5.01 (Tamura et al., 2011) for the construction of a neighbor joining (NJ) tree. Confidence in the clades was substantiated by a bootstrap value calculated after 10,000 permutations. For the maximum likelihood (ML) analysis, the aligned sequences were assessed using Prottest 3.0 (Abascal et al., 2005) and PhyML 3.0 (Guindon and Gascuel, 2003) was used to perform the ML analysis using the parameters of the best model obtained from the Prottest results. Confidence in the clades was substantiated by a bootstrap value calculated after 1000 permutations. Furthermore the expression pattern of the selected gene model across different tissues was assessed on the *Eucalyptus* Genome Integrative Explorer (EucGenIE, http://eucgenie.bi.up.ac.za, Mizrachi et al., 2010). Following the identification of putative orthologs in *E. grandis* based on the expression data and NJ and ML trees, primers were designed and verified in Phytozome v7.0 using a BLASTN similarity search against the *E. grandis* genome (**Table 1**). *Eucalyptus* orthologs for *PR1a* (AT2G14610), *VSP1* (AT5G24780), and *PDF1.2* (AT5G44420) could not be identified based on the phylogenetic approach and were thus not assessed further.

DOSE RESPONSE OF PUTATIVE ORTHOLOGOUS SIGNATURE DEFENCE GENES FOR SA AND MeJA SIGNATURE DEFENCE GENES

SA and MeJA phytohormones were administered to *E. grandis* (clone A) plantlets by spraying the aerial portions with varying concentrations of the inducers until run-off. The following inducer concentrations were assessed: 25 μM, 50 μM,

Table 1 | Primer sequence of *Eucalyptus* target signature defence genes and reference genes assessed using RT-qPCR.

Primer name	Forward primer (5′–3′)	Reverse primer (5′–3′)	Amplicon size (bp)
SA SIGNATURE DEFENCE GENES			
**EgrPR2*	GCTCTACAACCAGCGCAATATC	GCCAACTGCTATGTACCTGAAC	214
EgrPR5	CCTGTTGGACGTCAACGCC	GTCGTCGTACTCGAAGATT	167
JA SIGNATURE DEFENCE GENES			
EgrPR3	CGGCCGCGAAGTCGTTCCC	AACTATAACTACGGGCAAT	277
EgrPR4	ATGCCGTGAGCGCATACTG	GCGTGTTGGTCCTGGTGTT	156
EgrLOX2	ATGAACACTTGCTTCCATT	TCCTACCATACGTGAACAA	165
REFERENCE GENES			
EgrARF	TGCGTACCGAGTTGTTGAGG	GTTGCACAGGTGCTCTGGAT	195
EgrFBA	TGAAGACATGGCAAGGAAGG	GTACCGAAGTTGCTCCGAAT	190
EgrIDH	TGGAACTGTTGAGTCTGG	TTAGGACCATGAATGAGGAG	59

**Egr, E. grandis.*

100 μM, 250 μM, 500 μM, 1 mM, and 5 mM. Sodium salicylate (Riedel-de Haen, Seelze, Germany) was used to prepare the SA solutions (adjusted to pH 7.0 with NaOH solution) with the addition of 0.1% Tween® 20 (Sigma-Aldrich, Missouri, USA). MeJA (methyl jasmonate 95%, Sigma-Aldrich) was prepared with the addition of 0.1% ethanol (100%) as well as 0.1% Tween® 20 (Sigma-Aldrich). Control plants for SA treatment were sprayed with distilled water containing 0.1% Tween® 20. The control plants for the MeJA treatment were sprayed with distilled water containing 0.1% Tween® 20 and 0.1% ethanol. Aerial parts of the plantlets were harvested 24 h post-treatment (hpt). Three biological replicates of consisting of five plants each was harvested for the control and treated samples.

INVESTIGATION OF THE EXPRESSION PROFILES OF PUTATIVE *E. grandis* ORTHOLOGS OVER A TIME COURSE

Phytohormones, SA and MeJA, were administered to *E. grandis* (clone A) plantlets as described in the previous section. A single concentration selected from the dose response experiment for SA and MeJA was assessed at the following time points: 6, 12, 24, and 48 hpt. Controls were harvested at each individual time point as well as at time zero which refers to the time prior to the application of inducers. Three biological replicates consisting of five plants each was harvested for the control and treated samples at the different time points.

INFECTION TRIAL WITH *CHRYSOPORTHE AUSTROAFRICANA*

Ramets of two *E. grandis* clones, TAG5 and ZG14 trees, with an approximate stem diameter of 1 cm were inoculated with the fungus *C. austroafricana* CMW2113 as previously described (Roux et al., 2003). Lesion lengths were recorded and plant material (stem tissue, 1 cm above and below the lesion) was harvested at 48 h post-inoculation, the earliest time point at which confirmation of infection was observed, as well as 2 and 6 weeks post-inoculation (wpi). Three biological replicates consisting of three trees each was harvested for the control and inoculated samples. Re-isolation of the fungus was performed by excising a piece from the periphery of the lesion after 6 weeks and placing the block on 2% Malt Extract Agar (Merck, Gauteng, South Africa). Confirmation of infection by *C. austroafricana* was done by observing the culture morphology after 5 days.

RNA EXTRACTION AND FIRST STRAND cDNA SYNTHESIS

Total RNA was extracted from the plant powder using a modified cetyl-trimethyl-ammonium-bromide (CTAB) extraction protocol (Zeng and Yang, 2002). Extracted samples were treated with RNase-free DNaseI enzyme (Qiagen Inc, Valencia, CA) and subsequently column purified using the RNeasy® MinElute Kit (Qiagen Inc) as per the manufacturer's instructions. Purified RNA (1 μg) was used as the template for reverse transcription using Improm II reverse transcriptase enzyme (Promega, Wisconsin, USA).

REVERSE TRANSCRIPTASE QUANTITATIVE PCR (RT-qPCR) ANALYSIS

Reverse transcriptase quantitative PCR was performed according to the Minimum Information for Publication of Quantitative Real-Time PCR Experiments guidelines (MIQE) (Bustin et al., 2009). For each target, three biological replicates and three technical replicates per biological replicate was performed. The LightCycler® 480 SYBR Green I Master Mix (2× concentration) kit (Roche, Mannheim, Germany) was used to perform the RT-qPCR experiments on the LightCycler® 480 Real-Time PCR system (Roche Diagnostics, GmBH, Basa, Switzerland) according to the manufactures instructions. Reactions were set up in 11 μl volumes containing: 1 μl (1:10 diluted cDNA template), 5 μl LightCycler® 480 SYBR Green I Master Mix, 0.5 μM of each primer, and water to make up the total volume. For each primer pair, a negative no template control was included. Samples were normalized to a combination of the following reference genes: *ADP ribosylation factor (EgrARF)*, *Fructose bisphosphate aldolase (EgrFBA)*, and *NADP-isocitrate dehydrogenase (EgrIDH*, Boava et al., 2010). Relative quantification and normalization was performed using *qBASE*plus v1.0 (Hellemans et al., 2007). The datasets were tested for normality using the Shapiro–Wilk's test with the statistical software package Analyse-it® (Analyse-it Software, Ltd., Leeds, UK). The pairwise comparison Kruskal–Wallis test ($p < 0.05$) was applied to investigate significant differential expression unless otherwise stated.

RESULTS

PHYLOGENETIC IDENTIFICATION OF PUTATIVE ORTHOLOGS FOR PR GENES ASSOCIATED WITH THE SALICYLIC ACID AND JASMONIC ACID SIGNALING PATHWAYS IN *Eucalyptus grandis*

Putative orthologs of defence genes that are known to be responsive to the SA and JA signaling pathways from *Arabidopsis* were identified in *E. grandis* using BLAST algorithms and phylogenetic analyses (**Table 2**). All of the genes, except for *EgrPR2*, had predicted transcripts that were congruent with the annotated sequence of *E. grandis* located on Phytozome v7.0. Further investigation into *EgrPR2* revealed a region on scaffold 1:33791675_33792649 that had the highest similarity to the *Arabidopsis* candidate. Therefore an *ab initio* prediction of this region was performed using GeneMark (designated GM_*Egrandis*_V1_Scaffold1) and the result of this was included in the phylogenetic tree. The *Arabidopsis PR2* gene formed a clade with GM_*Egrandis*_V1_Scaffold1 that was accompanied by a strong bootstrap statistical support in the ML phylogenetic tree (Results not shown) and the GeneMark predicted gene model therefore was selected as the putative ortholog (**Table 2**).

EXPRESSION PROFILING OF THE PUTATIVE ORTHOLOGOUS *EgrPR* GENES AT VARIOUS CONCENTRATIONS OF SA AND JA REVEALS DOSE-SPECIFIC INDUCTION

Following the identification of putative orthologs of signature defence genes of the SA and JA signaling pathways, we investigated the expression profile of the candidates under various doses of phytohormone application. Putative orthologous defence signature genes for the SA pathway, *EgrPR2* and *EgrPR5* both displayed increased transcript abundance at 25 μM SA (**Figure 1A**). Although both targets had increased transcript abundance at 25 μM, *EgrPR2* had a much higher increase (16-fold compared to the control) at 5 mM and therefore this concentration was used for further experiments. Putative orthologs for the following candidates, *EgrPR3*, *EgrPR4*, and *EgrLOX2* were profiled as signature defence genes of the JA pathway. *EgrPR3* and *EgrPR4* exhibited increased transcript abundance at a common concentration of 100 μM. *EgrPR3* was also significantly increased at 25 μM and 5 mM but the fold change was lower than at 100 μM for *EgrPR4* (**Figure 1B**). Although the expression of *EgrLOX2* was significantly induced at 1 mM, it was decided to proceed with 100 μM

Table 2 | Predicted gene models and corresponding genomic scaffold regions selected as putative orthologs for the SA and MeJA defence signature genes in *E. grandis*.

Gene	TAIR ID	Predicted gene model	Genomic scaffold region
EgrPR2	AT3G57260	*GM_*Egrandis*_V1_Scaffold1	Scaffold_1: 33791675–33792649
EgrPR3	AT3G12500	*Eucgr.I01495*	Scaffold_9: 25149898–25151718
EgrPR4	AT3G04720	*Eucgr.B02124*	Scaffold_2: 42319519–42320281
EgrPR5	AT1G75040	*Eucgr.A00487*	Scaffold_1: 7623283–7624480
EgrLOX2	AT3G45140	*Eucgr.J00825*	Scaffold_10: 8809509–8814780

**No predicted transcript on Phytozome v7.0 for the selected scaffold region.*

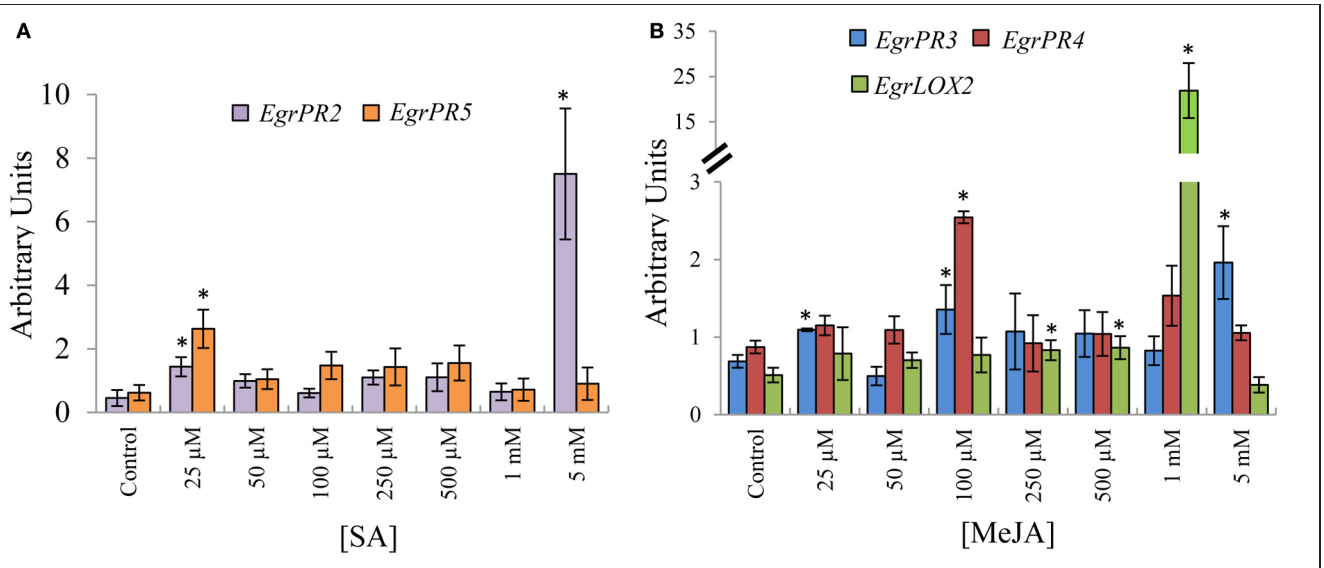

FIGURE 1 | Relative transcript abundance of *EgrPR* signature defence genes under hormone treatment. (A) Putative SA signature defence genes following normalization with *EgrARF* and *EgrFBA*. **(B)** Putative MeJA signature defence genes following normalization with *EgrARF* and *EgrIDH*. The y-axis represents the relative transcript abundance ratios expressed in arbitrary units. The x-axis shows the respective concentration range that was applied to the aerial parts of *E. grandis* plants. Error bars show the standard error of the mean of the biological replicates ($n = 3$) sampled after 24 hpt. Significance, indicated by *, is relative to the control for each target and was calculated by the Kruskal–Wallis test ($p < 0.05$).

for further experiments as both *EgrPR3* and *EgrPR4* exhibited significant differential expression at this concentration.

EXPRESSION OF *EgrPR* DEFENCE GENES VALIDATE SA–JA ANTAGONISM IN *E. grandis*

To investigate the hypothesis that SA and JA display an antagonistic relationship, the candidates were assessed by profiling the SA defence signature genes in material induced with MeJA and *vice versa*. The antagonistic relationship between SA and JA was clearly validated to occur in *E. grandis* in tissue treated with the phytohormone at selected concentrations. *EgrPR2* was suppressed at higher concentrations of MeJA relative to the control (**Figure 2A**). *EgrPR3* expression was reduced at 100 µM, 250 µM, 1 mM, and 5 mM whereas *EgrLOX2* was significantly lower at 100 µM, 1 mM, and 5 mM SA. *EgrPR4* had higher abundance at 25 µM SA and was not repressed at any of the other concentrations (**Figure 2B**).

TIME-DEPENDENT EXPRESSION OF PUTATIVE *EgrPR* GENES IDENTIFIES KEY POINTS OF INDUCTION

To investigate the expression profile of the suite of signature defence genes over a time course, 100 µM MeJA and 5 mM SA was applied to aerial portions of the *E. grandis* (clone A) tissue culture plants and the harvested material was profiled over various time points. The relative expression values for each time point was compared to the $T = 0$ control as well as the time specific control using the Kruskal–Wallis test (Note that the significance indicated on the graphs is only in relation to the time specific control). The $T = 0$ control was included in the experiment to indicate the basal level of gene expression prior to any treatment. Transcript abundance of the SA signature defence gene candidate, *EgrPR2* was significantly increased at 12, 24, and 48 hpt with a drastic peak at 24 hpt followed by a decline at 48 hpt (**Figure 3A**). *EgrPR5* displayed a gradual increase in expression from 6 to 48 hpt, with the expression of the target showing statistical significance all the time points except 12 hpt (**Figure 3B**). Signature defence genes for JA, *EgrPR3*, *EgrPR4*, and *EgrLOX2* all displayed altered levels of expression at the various time points (**Figures 3C–E**). *EgrPR4* transcript levels increased progressively from 6 to 48 hpt, with all the time points being statistically significant (**Figure 3D**). Notably the level at which *EgrPR2* and *EgrPR4* are expressed at 24 hpt was approximately the same level as was observed in the dose response experiment, thereby indicating reproducibility of the results.

EXPRESSION PROFILING OF THE PUTATIVE ORTHOLOGOUS DEFENCE GENES DURING INFECTION BY A PATHOGEN QUALIFIES THE POTENTIAL OF THE CANDIDATES TO BE DIAGNOSTIC OF SA AND MeJA AND IMPLICATES SA IN DEFENCE AGAINST *C. austroafricana*

The potential of these defence signature genes to be used as diagnostic markers was investigated under pathogen stress by employing the *E. grandis*—*C. austroafricana* pathosystem. Using the Kruskal–Wallis statistic test a significant difference ($p = 0.0295$) was observed between the lesion lengths of TAG5 (4.8 ± 2.1 cm) and ZG14 (8.2 ± 3 cm) at 6 wpi whereas no significance was observed at 48 h and 2 wpi. In TAG5, the SA signature gene *EgrPR2* showed significant differential expression at 2 and 6 wkpi (**Figure 4A**). In TAG5, the JA signature genes, *EgrPR4* significantly decreased at 2 wpi and increased once again at 6 wpi. Despite significant up-regulation of *EgrPR4* at 6 wpi in TAG5 compared to its control, the level to which it was induced was lower than *EgrPR2* levels (**Figure 4B**). In ZG14, the level of expression of *EgrPR2* was only significantly up-regulated at 6 wpi (**Figure 4A**) whereas the expression of *EgrPR4* transcripts was found to be significantly up-regulated at 2 and 6 wpi (**Figure 4B**). The pre-treatment of the susceptible genotype of *Eucalyptus* with 5 mM SA, prior to manual inoculation with *C. austroafricana*, resulted in a smaller lesion lengths (5 ± 0.5 cm) compared to the untreated plants (7 ± 0.6 cm) at 5 wpi (One-Way ANOVA, $p < 0.05$). These lesion lengths were comparable to lesions found on the tolerant genotype (4.8 ± 0.4 cm).

DISCUSSION

PR genes have been shown to be indicators of the SA and MeJA signaling pathways and can be termed signatures of these pathways. This study aimed to identify orthologs of signature

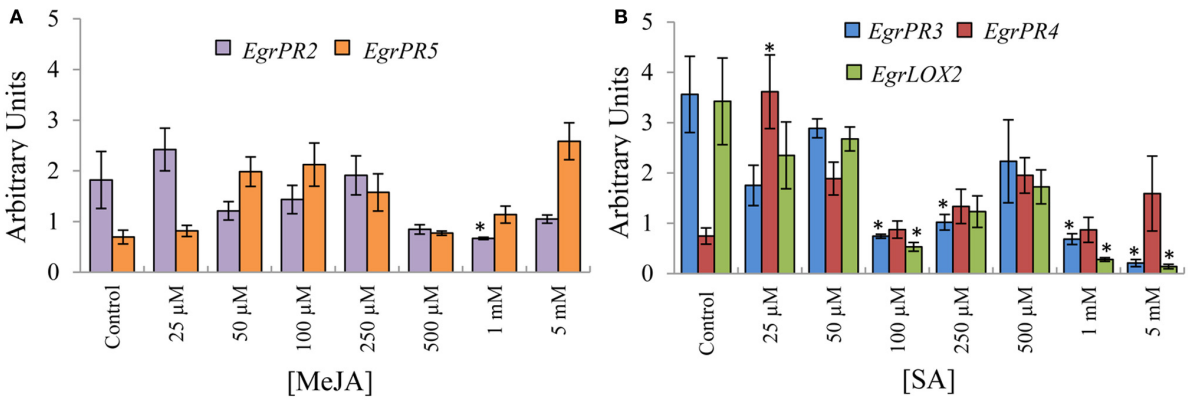

FIGURE 2 | Relative transcript abundance of the putative orthologs for the *EgrPR* defence genes in tissue treated with the opposite phytohormone. The y-axis represents the relative expression ratios expressed in arbitrary units. Putative SA signature defence genes **(A)** were normalized with *EguIDH* and *EgrARF* whereas the putative MeJA signature defence genes **(B)** were normalized with *EgrARF* and *EgrFBA*. The x-axis represents the concentration range for the applied inducer. Error bars show the standard error of the mean of the biological replicates ($n = 3$). Significance, indicated by *, is relative to the control in each graph and was calculated by the Kruskal–Wallis test ($p < 0.05$).

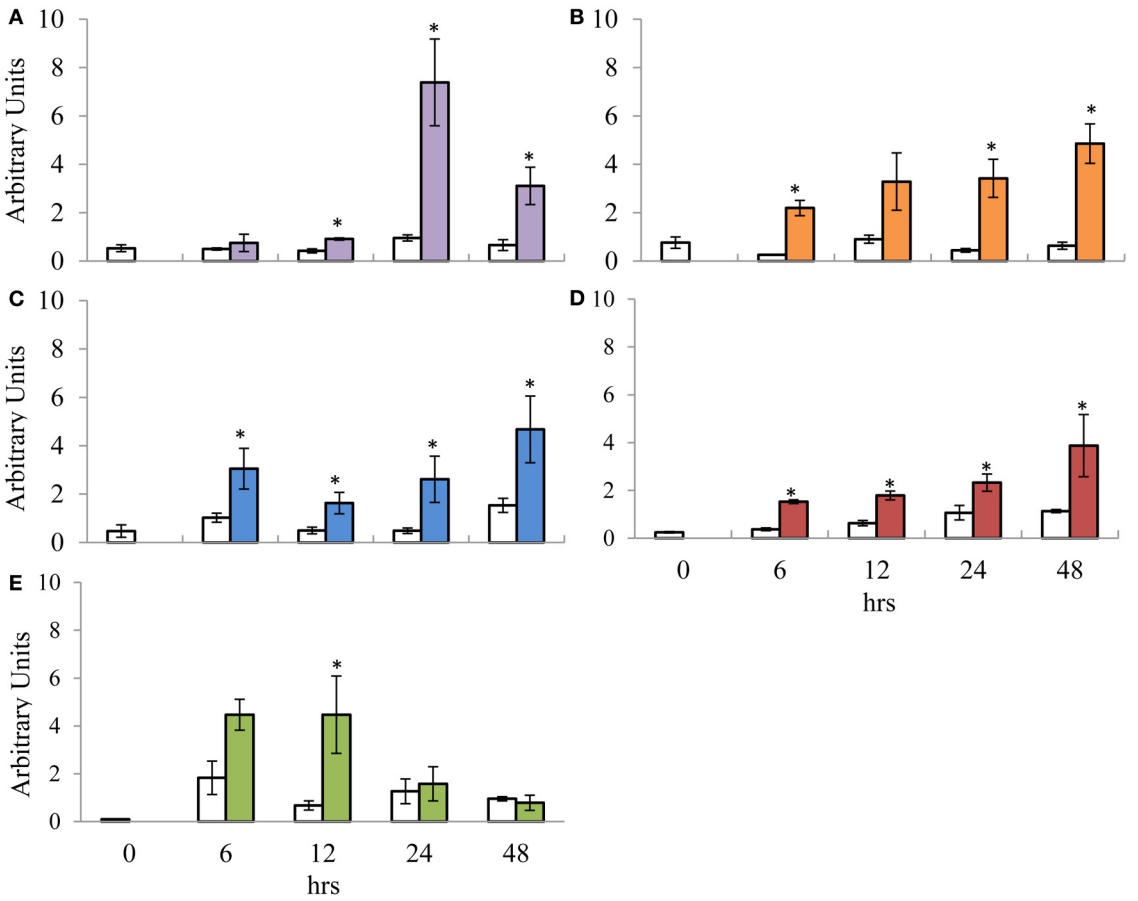

FIGURE 3 | Relative transcript abundance of putative orthologs for SA and MeJA signature defence genes assessed during the time course trial. (A) *EgrPR2*; **(B)** *EgrPR5*; **(C)** *EgrPR3*; **(D)** *EgrPR4*; **(E)** *EgrLOX2*. The y-axis represents the relative expression ratios expressed in arbitrary units. The x-axis represents the time course (h) post-treatment with 5 mM SA **(A** and **B)** and 100 μM MeJA **(C–E)**. Samples were normalized with *EgrARF* and *EgrFBA*. Error bars are show the standard error of the mean of the biological replicates ($n = 3$). White boxes represent the control samples whereas the colored boxes represent the treated samples. Significance between the control and treated samples is indicated by * at a specific time point and was calculated by the Kruskal–Wallis test ($p < 0.05$).

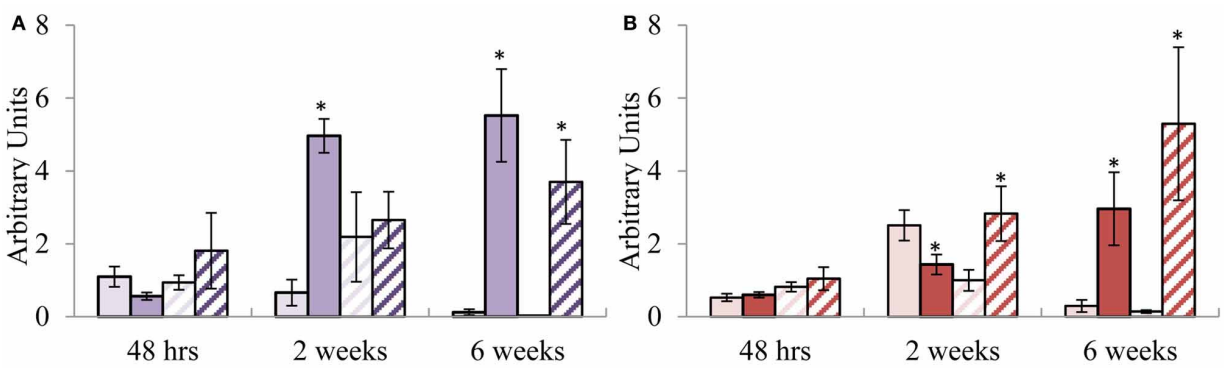

FIGURE 4 | Relative transcript abundance of putative orthologs for *EgrPR* signature defence genes during infection with *C. austroafricana*. (A) *EgrPR2*; **(B)** *EgrPR4*. The y-axis represents the relative expression ratios expressed in arbitrary units. The x-axis represents the time points post-inoculation at which the samples were analyzed. Error bars show the standard error of the mean of the biological replicates ($n = 3$). Samples were normalized to *EgrARF* and *EgrFBA*. Light and dark solid boxes represent the TAG5 control and inoculated samples respectively whereas the light and dark striped boxes represent the ZG14 control and inoculated samples respectively. Significance, indicated by *, is relative to the control and was calculated by the Student's *t*-test ($p < 0.05$).

defence genes for the SA and MeJA signaling pathways from *A. thaliana* in *E. grandis* using sequence similarity and phylogenetic analysis. Phylogenetics provides a solid starting point for selecting candidates to investigate, however it does not provide definitive evidence that the selected gene is the true functional ortholog (Chen et al., 2007). *Eucalyptus*, *Populus*, and *Arabidopsis* share an ancient hexaploidization event and therefore on average there should be three genes in each species relative to the ancestor (Jaillon et al., 2007). These genes may have undergone various gene loss and/or duplication events which have changed this number for many genes and gene families thereby possibly creating multiple functional orthologs. The putative orthologous signature defence genes identified here provide suitable candidates for further investigation in complementation and functional studies to better understand the role of these genes in *E. grandis*.

ORTHOLOGS FOR *EgrPR* SIGNATURE DEFENCE GENES EXHIBIT DOSE-SPECIFIC INDUCTION AND PATHWAY SPECIFICITY FOR *EgrPR2*, *EgrPR4*, AND *EgrLOX*

Based on the premise that the candidates identified through phylogeny were defence signature genes for SA and JA, we subsequently investigated the expression of these targets under various doses of phytohormone treatment. The concentrations used in this study were based on experiments conducted in *A. thaliana* and on the level of the phytohormone following a pathogen challenge in other model organisms (Rasmussen et al., 1991; Jung et al., 2007). The transcript abundance levels of the putative SA signature defence genes, *EgrPR2* and *EgrPR5* were increased (**Figure 1A**) by application of the inducer which was consistent with literature in *Arabidopsis* (Reymond and Farmer, 1998; Kunkel and Brooks, 2002; Delaure et al., 2008). Furthermore, when these genes were evaluated for their specificity to the SA pathway, it was clearly demonstrated that expression levels of *EgrPR2* was suppressed by higher concentrations of MeJA (**Figure 2A**). These results suggest that in *E. grandis EgrPR2* could serve as a diagnostic signature gene for the SA pathway. Expression of the MeJA defence signature genes, *EgrPR3* and *EgrLOX2* were significantly differentially regulated at varying concentrations of this phytohormone. These signature genes were additionally repressed at high concentrations of SA treatment confirming the suppressive effect of SA on MeJA responses. Transcripts of *EgrPR4* were found to be up-regulated by the application of MeJA, but showed no differential expression under SA treatment other than at 25 μM. Nonetheless, *EgrPR4* can be utilized as a defence signature for MeJA as expression levels of this gene were significantly altered upon application of that phytohormone. The data suggests that the known antagonistic relationship between MeJA and SA in *Arabidopsis* may also occur in *Eucalyptus*. All of the MeJA responsive defence signature genes profiled in this study were found to be diagnostic to the MeJA pathway in *E. grandis* and could serve as suitable markers for the pathway. Although SA and JA predominantly have an antagonistic relationship (Pieterse et al., 2009), there have been situations whereby these pathways act synergistically (Mur et al., 2006; Lazniewska et al., 2010). The outcome of the interaction between SA and JA seems to be largely dependent of the timing of activation and the concentration of the phytohormones (Mur et al., 2006).

TIME DEPENDENT EXPRESSION PROFILES SUGGEST THAT MeJA AND SA SIGNATURE DEFENCE GENES IN *E. grandis* ARE DIFFERENTIALLY REGULATED AS EARLY AS 6 hpt

To further elucidate the expression profiles of the *Eucalyptus* signature defence genes, we investigated the response of the candidates over time. The time at which a host's defences are activated has a crucial role in determining the outcome of a pathogen interaction. Susceptibility may not only be due to the lack of required artillery (e.g., defence genes), but also to the delayed activation of the genes required to curb the pathogen (Loon, 2009). Elucidating the time dependent expression profiles of the putative orthologous signature genes under mock induction of the signaling pathways would provide a glimpse into how the genes would respond under pathogen conditions. SA hormone levels in tobacco plants infected with tobacco mosaic virus (Malamy et al., 1990, 1996) parallels the expression profile observed for *EgrPR2* in this study (**Figure 3A**) under application of SA in *E. grandis*. In contrast to *EgrPR2*, *EgrPR5* was shown to gradually increase over the time points with a maximum expression level detected at 48 hpt (**Figure 3B**). This suggests that the signature defence genes identified in this study respond to mock induction of the signaling pathway in a similar manner as they would under pathogen incursion. Tobacco plants that have been treated with exogenous MeJA displayed time course patterns similar to that found in *E. grandis* for *LOX* and *PR3* (Bell and Mullet, 1993). In *E. grandis*, *EgrLOX2* transcript levels were significantly up-regulated as early as 12 hpt followed by a decline at 24 hpt (**Figure 3E**). This could indicate a possible role for *EgrLOX2* in the early stages of defence activation in a host as this gene is involved in jasmonate biosynthesis. *EgrPR3* displayed a similar profile with the level of transcripts increasing from 6 to 48 hpt in *E. grandis* (**Figure 3C**) compared to increasing levels from 8 to 24 h post-MeJA treatment in tobacco (Rickauer et al., 1997). A microarray time course study in which *Arabidopsis* plants were treated with MeJA revealed that *EgrPR4* transcripts began to increase as early as 1 h then slowly declined by 24 h (Jung et al., 2007). Conversely in *E. grandis*, *EgrPR4* increased from 6 hpt with the maximum expression level detected at 48 hpt (**Figure 3D**). Although the time points differ between the two organisms, the general trend of expression remains the same. The observed increase in the transcript levels of *EgrPR3* and *EgrPR4* over time could also be due to the role of these proteins in the host during defence. Both of these genes encode for products that target and alter the cell wall composition of a fungal pathogen and during infection an increase in expression would be beneficial in preventing the spread of the pathogen (Selitrennikoff, 2001).

PATHOGENICITY EXPERIMENTS CONDUCTED WITH *C. austroafricana* ESTABLISHES THE DIAGNOSTIC POTENTIAL OF THE *EgrPR* SIGNATURE DEFENCE GENES AND ELUCIDATES THE IMPORTANCE OF SA IN DEFENCE AGAINST THIS PATHOGEN

In *Arabidopsis*, the involvement of a specific signaling pathway during an interaction with a pathogen can be elucidated by the diagnostic ability of the assigned signature genes. This study examined the diagnostic potential of the putative orthologous

signature genes for SA and MeJA found in *Eucalyptus* upon infection with *C. austroafricana*. It was found that at 2 wpi there was no substantial difference in lesion length between TAG5 and ZG14 whilst at 6 wpi there was a significant lesion difference, suggesting that during the initial 2 weeks following infection the tolerant host was able to initiate a certain response to curb the spread of the disease. Interestingly, the signature defence gene expression profiles that were observed in the two hosts suggest a probable role of SA in the tolerance mechanism of TAG5. In the incompatible interaction (TAG5 and *C. austroafricana*), at 2 and 6 wpi, *EgrPR2* transcripts were considerably up-regulated compared to the control, whereas up-regulation only occurred at 6 wpi in the compatible interaction (ZG14 and *C. austroafricana*) and to a lower lever (**Figure 4A**). In addition, the level of MeJA signaling at 2 wpi was lower in the incompatible interaction compared to the compatible interaction as indicated by the expression levels of *EgrPR4* (**Figure 4B**). The antagonistic relationship between SA and MeJA evidently occurs within these hosts at this time point and could possibly have a key role in determining the outcome of the interaction with *C. austroafricana*. From other plant species, *PR2* is known to encode for the β-1, 3-glucanase enzyme which facilitates the enzymatic degradation of the glucan component of fungal cell walls (Theis and Stah, 2004). In TAG5, the elevated level of *EgrPR2* could contribute to confining the spread of *C. austroafricana* by hydrolyzing the β-1, 3-glucan component of the cell wall. In the review by Selitrennikoff (2001), it's hypothesized that this particular glucan component maybe abundant in the hypal apex of a growing fungus and degradation of the β-1, 3-glucan may lead to a loss in rigidity of the cell wall thereby resulting in cell lysis and eventual cell death. *EgrPR2* was significantly up-regulated at the later time points (2 and 6 wpi) but not at the early time point of 48 hpi (**Figure 4A**) suggesting that the lack of an early response could be a partial reason as to why TAG5 is tolerant but not fully resistant against *C. austroafricana*. Based on the premise that SA may facilitate tolerance, ZG14 plants were sprayed with 5 mM SA to determine if this hormone would increase the tolerance of this host. A significant reduction in the lesion lengths of ZG14 treated with SA was observed and the lesions were of similar length to that seen in the tolerant TAG5 plants. Induction of systemic resistance in *E. urophylla* upon application of 5 mM SA has been previously documented (Ran et al., 2005). *EgrPR4* encodes a hevein-like protein which acts like a chitin binding protein by targeting the β-chitin component of the cell wall. These proteins migrate to the cell walls of an invading fungus and disrupt the formation of the septa and hyphal tips (Selitrennikoff, 2001; Theis and Stah, 2004). In ZG14, *EgrPR4* was elevated at 2 wpi however the host was still susceptible to *C. austroafricana*. A possible explanation for this is that the level to which this gene is expressed was not high enough to curb the pathogen. Timing of defence gene expression is crucial in a pathogen interaction and the lack of significant *EgrPR4* expression at 48 h in TAG5 or in ZG14, may contribute to the ability of *C. austroafricana* to proliferate within these hosts during the initial 2 weeks of infection.

Our results suggest that *EgrPR2* and *EgrPR4* were diagnostic of SA and MeJA signaling pathways respectively against *C. austroafricana* as SA was recognized as playing a role in enhancing tolerance against the pathogen in *Eucalyptus*. It is possible that other signaling pathways may have a role in contributing to resistance in this interaction. The involvement of SA in facilitating a defence response to a necrotrophic pathogen is in contrast to the published literature from *Arabidopsis* which implicates the involvement of the MeJA pathway (Glazebrook, 2005). In spite of this, there have been studies that have shown that SA could also assist in impeding necrotrophic pathogens (Ferrari et al., 2003; Azaiez et al., 2009). It may also be possible that in tree species the roles of SA and MeJA in pathogen defence could differ from what is known in *Arabidopsis*.

This study provides a first step toward understanding hormone mediated defence responses of *Eucalyptus* trees. It is envisaged that expression profiling of the diagnostic markers, *EgrPR2* and *EgrPR4*, can be adopted as a tool to determine which of the two major defence pathways are active against different pathogens in *Eucalyptus* in future.

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Tomato transcriptome and mutant analyses suggest a role for plant stress hormones in the interaction between fruit and *Botrytis cinerea*

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Fruit–pathogen interactions are a valuable biological system to study the role of plant development in the transition from resistance to susceptibility. In general, unripe fruit are resistant to pathogen infection but become increasingly more susceptible as they ripen. During ripening, fruit undergo significant physiological and biochemical changes that are coordinated by complex regulatory and hormonal signaling networks. The interplay between multiple plant stress hormones in the interaction between plant vegetative tissues and microbial pathogens has been documented extensively, but the relevance of these hormones during infections of fruit is unclear. In this work, we analyzed a transcriptome study of tomato fruit infected with *Botrytis cinerea* in order to profile the expression of genes for the biosynthesis, modification and signal transduction of ethylene (ET), salicylic acid (SA), jasmonic acid (JA), and abscisic acid (ABA), hormones that may be not only involved in ripening, but also in fruit interactions with pathogens. The changes in relative expression of key genes during infection and assays of susceptibility of fruit with impaired synthesis or perception of these hormones were used to formulate hypotheses regarding the involvement of these regulators in the outcome of the tomato fruit–*B. cinerea* interaction.

Keywords: plant-pathogen, ripening, resistance, susceptibility, ethylene, salicylic acid, jasmonic acid, microarray

INTRODUCTION

Disease resistance or susceptibility of a plant depends not only on the specific plant–pathogen combination, but also on the developmental stage of the host tissues. The ripening process of fleshy fruit is an example of a developmental transition that coincides with increased susceptibility to pathogens. Ripening involves a complex network of regulatory and hormone-mediated pathways leading to significant changes in the physiological and biochemical properties of the fruit (Giovannoni, 2004). Among the ripening events, modifications in cell wall structure and composition, conversion of starch into simple sugars, changes in apoplastic pH and redox state, and decline in the concentration of antimicrobial metabolites contribute to susceptibility of fruit to pathogens (Prusky and Lichter, 2007; Cantu et al., 2008a,b). The enhanced susceptibility of ripe fruit to pathogens could be a default outcome of ripening or, alternatively, could be promoted by some, but not all, ripening processes (Cantu et al., 2009).

Fruit pathogens exhibit necrotrophic, biotrophic, or hemibiotrophic lifestyles (Prusky and Lichter, 2007; Cantu et al., 2008b), categories that reflect different infection strategies (Glazebrook, 2005). Necrotrophs, such as the ascomycete, *Botrytis cinerea*, cause necrosis by deploying hydrolytic enzymes (Van Kan, 2006), secreting toxins (Govrin et al., 2006; Dalmais et al., 2011) and/or hijacking the plant's enzymatic machinery (Cantu et al., 2009). Biotrophs depend on the integrity of plant host tissues and have developed strategies to deceive the host to obtain nutrients without inducing plant defenses or cell death (Perfect et al., 1999; Glazebrook, 2005). Hemibiotrophs are those pathogens that switch lifestyles at different developmental phases and/or in certain environmental conditions (Glazebrook, 2005; Kleemann et al., 2012). Therefore, the infection strategies of different pathogens challenge the competency of the plant host to respond and deploy effective defense mechanisms.

Tomato (*Solanum lycopersicum*) has served as a model organism to study fruit ripening (Giovannoni, 2004) and has emerged as an informative experimental system to characterize the molecular regulation of the ripening-related susceptibility to pathogens, in particular to necrotrophic fungi, such as *B. cinerea* (Powell et al., 2000; Flors et al., 2007; Cantu et al., 2008a, 2009). *B. cinerea* fails to develop in unripe (mature green, MG) tomato fruit, but as fruit start their ripening program and become ripe (red ripe, RR), concurrently they become more susceptible to infections, which lead to rapid breakdown of host tissues and extensive microbial colonization (Cantu et al., 2009).

The roles of the plant stress hormones, ethylene (ET), salicylic acid (SA), jasmonic acid (JA), and abscisic acid (ABA), in the control of plant developmental processes and the initiation of defense mechanisms against necrotrophic, biotrophic, or hemibiotrophic pathogens have been documented mostly for vegetative tissues (Doares et al., 1995b; Díaz et al., 2002; Wasternack,

2007; AbuQamar et al., 2008; Asselbergh et al., 2008; Bari and Jones, 2009; Pieterse et al., 2009; Cutler et al., 2010; López-Gresa et al., 2010; El Oirdi et al., 2011; Rivas-San Vicente and Plasencia, 2011; Nambeesan et al., 2012; Pieterse et al., 2012; Vandenbussche and Van Der Straeten, 2012). However, our understanding of how these hormones influence plant–pathogen interactions in fruit is still limited.

The gaseous hormone, ET, is involved in the control of terminal developmental programs, such as organ abscission, leaf and flower senescence, and fleshy fruit ripening (Patterson and Bleecker, 2004; Barry and Giovannoni, 2007; Klee and Giovannoni, 2011; Graham et al., 2012; Pech et al., 2012; Wang et al., 2013). ET also modulates plant resistance and susceptibility to pathogens. Thus, from one point of view, ET controls a variety of immune responses in conjunction with other signaling networks; but from another perspective, it promotes senescence or ripening, processes which facilitate infection by pathogens (Van Loon et al., 2006; Cantu et al., 2009; Van Der Ent and Pieterse, 2012).

JA influences flower development and may be involved in some ripening processes, depending on the plant species (Peña-Cortés et al., 2004). The best-known function of JA is to regulate plant immune responses against insects and pathogens, particularly necrotrophs (Glazebrook, 2005; Browse, 2009). JA may also play a role in resistance against abiotic stresses, including mechanical stress, salinity, and UV irradiation (Ballaré, 2011).

SA is a phenolic compound with hormonal features that is crucial for the establishment of basal defenses, effector-triggered immunity, and both local and systemic acquired resistance (Durrant and Dong, 2004; Vlot et al., 2009). SA is typically involved in the activation of plant defenses against biotrophs and hemibiotrophs, but it also appears to enhance susceptibility to necrotrophs by antagonizing the JA signaling pathway through the regulatory protein NPR1 and by inhibition of auxin signaling (Glazebrook, 2005; Beckers and Spoel, 2006; Koornneef et al., 2008; Spoel and Dong, 2008).

ABA regulates many aspects of plant development, including seed dormancy and germination, and plays a significant role in tolerance to abiotic stress (Fujita et al., 2006; Wasilewska et al., 2008). ABA also can influence the outcome of plant–microbe interactions. Negative and positive roles have been described for this hormone depending on the pathosystem, developmental stage of the host, and/or the environmental conditions in which the plant–pathogen interaction occurs (Mauch-Mani and Mauch, 2005; Ton et al., 2009; Robert-Seilaniantz et al., 2011). In general, ABA suppresses plant resistance mechanisms by antagonizing SA- and JA/ET-dependent immune responses (Anderson et al., 2004; Mohr and Cahill, 2007; Sánchez-Vallet et al., 2012), thereby promoting susceptibility (Spoel and Dong, 2008). In addition, negative regulation involving systemic acquired resistance activation and ABA synthesis has been documented (Yasuda et al., 2008).

Genome-wide transcriptional profiling studies have been valuable in the study of hormonal signaling during plant–pathogen interactions (Glazebrook, 2005) because they enable researchers to monitor the activation or suppression of multiple pathways simultaneously. We used hybridization-based microarray data obtained from tomato fruit infected with *B. cinerea* to characterize the patterns of expression of genes involved in hormone biosynthesis and signaling to infer the potential role of stress hormones in fruit–pathogen interactions. The expression profiles of important genes were validated and extended by qRT-PCR using independent biological material at different stages of infection. We integrated the gene expression results with susceptibility phenotypes of fruit compromised in hormone synthesis and perception, in order to provide a model describing how ET, SA, JA and ABA influence the susceptibility of tomato fruit to *B. cinerea*.

MATERIALS AND METHODS

TRANSCRIPTOME ANALYSIS HORMONE-RELATED GENES

Genes that have been previously described as involved in the synthesis, modification, signaling, and response of ET, SA, JA, and ABA were selected based on their functional annotation from the Arabidopsis Hormone Database (AHD) 2.0 (http://ahd.cbi.pku.edu.cn) (Jiang et al., 2011). The amino acid sequences of the 414 selected genes were retrieved from the Arabidopsis TAIR10 collection (http://arabidopsis.org) and used as queries in a BLASTP search (*e*-value $\leq 1e^{-3}$, low complexity filter "on") against all of the predicted proteins in the tomato (*Solanum lycopersicum*) genome sequence (ITA2.3 release; http://solgenomics.net). A total of 326 sequences with identity greater than 60% and with alignment coverage more than 70% of the query length were considered putative tomato homologs of the Arabidopsis hormone-related proteins. In addition, the sequences of 19 known tomato protein gene sequences related to ET synthesis and signaling pathways were added to the dataset. Corresponding unigene sequences and Affymetrix array chip probes were then obtained, respectively, from GenBank (http://ncbi.nlm.nih.gov/genbank/) and Affymetrix (http://www.affymetrix.com/analysis/) to extract the normalized hybridization values from the microarray analysis of *Botrytis cinerea*-infected tomato fruit (Cantu et al., 2009; http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE14637) at the MG and RR stages and at 1 day post-inoculation (dpi). The resulting dataset (141 tomato genes) was used to identify significant ($P \leq 0.05$) fold changes in ET, SA, JA, and ABA-related genes that are in common or uniquely regulated by infection of MG and RR fruit by *B. cinerea* and by ripening of healthy fruit.

PLANT MATERIAL

The *NahG* tomato line (cv. Moneymaker) expressing the *Pseudomonas putida* SA hydroxylase gene (*NahG*) under regulation of the constitutive promoter 35S were developed by Brading et al. (2000) and kindly provided by Dr. J. Jones (John Innes Centre, Norwich, UK). The *sitiens* tomato mutant and its wild-type background cv. Moneymaker were contributed by the Tomato Genetics Research Center (TGRC; UC Davis, CA). Tomato (*Solanum lycopersicum*) cv. Ailsa Craig (AC), the *NahG* transgenic line, the *sitiens* mutant line, and their wild-type non-transgenic control line (cv. Moneymaker) were grown in greenhouse and field conditions during 2008, 2009, and 2012 in Davis, California. Fruit were tagged at 3 days post-anthesis (dpa) and harvested at 31 dpa for MG fruit and at 42 dpa for RR fruit. Ripening stages of the fruit were confirmed by the color, size, and texture.

FUNGAL CULTURE AND FRUIT INOCULATION

B. cinerea (B05.10) was provided by Dr. J. A. L. van Kan (Department of Phytopathology, Wageningen University). Conidia, collected from sporulating cultures grown on 1% potato dextrose agar (Difco), were counted and diluted to 500 conidia μL^{-1} for inoculations. Fruit were disinfected and inoculated as in Cantu et al. (2008a). Briefly, on the day of harvest fruit were surface sterilized by submersion in a solution of 10% (v/v) bleach followed by three deionized water rinses. At the time of inoculation fruit were wounded at seven sites to a depth of 2 mm and a diameter of 1 mm. Six out of the seven sites were inoculated with 10 μL of a water suspension containing 5000 conidia of *B. cinerea* and the seventh site was mock-inoculated with 10 μL of sterile water (wounded control). Healthy fruit were not wounded or inoculated. All fruit samples were incubated at 20°C in high humidity. Susceptibility was determined daily for 3 dpi as disease incidence (percentage inoculation sites showing symptoms of tissue maceration or soft rot). The evaluation of susceptibility was repeated with three separate harvests of fruit using 10–15 fruits per experiment. The significance of the susceptibility data was analyzed by ANOVA followed by Tukey's *post-hoc* test using R (R Foundation for Statistical Computing). For percentage values, statistical analysis was carried out after angular transformation.

ETHYLENE AND 1-MCP TREATMENTS

Fruit were placed in air-tight chambers containing either 10 μL/L ET, low (12 nL/L), or high (450 nL/L) levels of 1-methylcyclopropene (1-MCP; SmartFresh©, kindly contributed by AgroFresh Inc.) for 18 h at 20°C. As controls, fruit at the same stage were placed in an identical closed chamber without ET or 1-MCP. Immediately after treatment, fruit were divided into three replication groups and inoculated with *B. cinerea* and assessed for disease incidence as described above.

RNA ISOLATION

To confirm the gene expression changes identified in the re-analysis of the microarray hybridization data, additional MG and RR fruit (cv. AC) were inoculated as above with *B. cinerea* or kept uninoculated (i.e., healthy). Fruit pericarp and epidermal tissues were collected after 1 and 3 days post-inoculation (dpi) and high-quality RNA was isolated. Five biological replicates were produced per sample and each replicate consisted of independent pools of 3–5 fruits. Two grams of tissue per sample were ground in liquid nitrogen and 10 mL of the RNA extraction buffer (CTAB 2% v/v, PVP 2% v/v, 100 mM Tris pH 8, 2 M NaCl, 25 mM EDTA, 0.5 g/L spermidine, 10 mM β-mercaptoethanol) were added. The samples were immediately incubated for 5 min at 65°C. Two extractions with one equal volume of chloroform:isoamyl alchohol (24:1, v/v) followed by centrifugation at 4000 rpm for 45 min at 4°C were performed. The supernatant was recovered and 1/10 volume of 1M KOAc was added followed by centrifugation at 4000 rpm for 20 min at 4°C. The supernatant was collected and 1/4 volume of 10 M LiCl was added. Samples were incubated overnight at −20°C and then centrifuged at 4000 rpm for 45 min at 4°C. The supernatant was discarded and the RNA pellet was further purified using the RNeasy Plant Mini Kit (Qiagen®). DNAse treatment (RNase-Free DNase Set, Qiagen®) was done in column during the purification step. The RNA was resuspended in 35 μL of nuclease-free water. The RNA concentration and purity were measured using NanoDrop 2000c Spectrophotometer (Thermo Scientific, Inc.). The RNA integrity was checked by agarose gel electrophoresis.

QUANTITATIVE RT-PCR

cDNA was synthesized from the prepared RNA using M-MLV Reverse Transcriptase (Promega). qRT-PCR was performed on a StepOnePlus PCR System using Fast SYBR Green Master Mix (Applied Biosystems). All qRT-PCR reactions were performed with the following cycling conditions: 95°C for 10 min, followed by 40 cycles of 95°C for 3 s and 60°C for 30 s. Tomato actin (Solyc03g078400) was used as reference gene and process in parallel with the genes of interest. Primer efficiencies were calculated using 4-fold cDNA dilutions (1:1, 1:4, 1:16, 1:64, and 1:256) in duplicate as well as checking for amplification in a negative control without DNA. The efficiencies of the primer sets used in this study were all above 90% (**Table S3**). Specificity of the primers was checked by analyzing dissociation curves ranging from 60 to 95°C. The $2^{-\Delta\Delta}CT$ method (Livak and Schmittgen, 2001) was used to normalize and calibrate transcript values relative to the endogenous constitutive gene (actin, Solyc03g078400) control. Within analyses, the same calibrator was used for all genes so the scales of their linearized values are comparable. Data presented is from 3 to 5 biological replicates per treatment and per stage.

RESULTS AND DISCUSSION

TRANSCRIPTOMIC ANALYSIS AND VALIDATION OF HORMONAL-RELATED GENES DURING FRUIT INFECTION BY *B. cinerea*

Although the complete sequence of the tomato genome is available (The Tomato Genome Consortium, 2012), an integration of genome annotations with functional information is required to assign biological importance to gene sequences and generate a framework for the study of developmental processes and signaling networks. The study of stress hormonal pathways in tomato fruit has focused mainly on the characterization of ET-related genes involved in the initiation of ripening (Barry and Giovannoni, 2007; Klee and Giovannoni, 2011; Pech et al., 2012). The roles of the stress hormones, SA, JA, and ABA, for the outcomes of fruit infections have not been extensively investigated.

We previously used microarray hybridization technology to characterize the expression changes of ripening-related genes in relation to the increased susceptibility to *B. cinerea* of ripe fruit. Using RNA from tomato fruit at two ripening stages, MG and RR at 1 dpi with *B. cinerea*, we profiled the expression of several cell wall modifying genes (e.g., polygalacturonase, expansin, and glucanases) and few hormone-related genes (e.g., *ACS2*, *ACO5*, *AOS*) (Cantu et al., 2009). The shortage of functional annotations for genes represented on the microarray has limited the identification of genes involved in hormonal pathways related to stress and pathogen responses.

Here we report (1) the identification of a set of 345 hormone-related tomato genes, which includes 19 known ET-related genes and 326 tomato genes that show significant homology to Arabidopsis genes involved in ET, SA, JA, and ABA pathways;

(2) the re-annotation of the hormone-related genes on the Affymetrix Tomato Chip, and (3) the transcriptional changes of these hormonal-related genes in response to *B. cinerea* using published microarray results (Cantu et al., 2009).

Hormone-related Arabidopsis gene sequences were retrieved from the AHD 2.0 (Jiang et al., 2011) and BLASTP searches were used to identify their homologous copies in the tomato genome (minimum *e*-value $< 1e^{-3}$; alignment coverage >70% of the query length; identity >60%). We selected the AHD 2.0 because it is currently the most comprehensive and up-to-date database of hormone-related genes; it includes 1318 gene accessions for eight different plant hormones, which had been extracted from 906 scientific papers published before August 2010. From this database, we identified 128 genes related to ET, 72 genes related to SA, 55 genes related to JA, and 159 genes related to ABA pathways (Jiang et al., 2011).

Among the homologous tomato genes identified, 141 genes (**Table S1**) were found to be expressed in tomato fruit based on the microarray data. Of these 141 genes, we focused on those with significant changes in expression ($P \leq 0.05$) that (1) were in common during infection of tomato fruit by *B. cinerea* regardless of the ripening stage, (2) that were responses to *B. cinerea* but are specific to the ripening stage and phenotype of the fruit (i.e., MG: resistant and RR: susceptible), and (3) that were common in response to infection and as a consequence of ripening. As result, we identified 65 stress hormone-related genes that showed differential expression in response to *B. cinerea* (**Figure 1**).

Relative expression changes of 20 hormone-related genes (8 ET genes, 3 SA genes, 2 JA genes, 6 ABA genes, and 1 gene related to multiple hormones) were measured by qRT-PCR using independent preparations of RNA from *B. cinerea*-infected (1 dpi) and equivalent healthy tomato fruit at MG and RR stages, in order to validate the results from the microarray analysis (**Figure 3**; **Table S2**). Additionally, gene expression was measured at 3dpi to determine whether the up- or down-regulation of the expression of these genes is maintained or modified as infection progresses (**Figure 3**; **Table S2**).

For the 20 genes analyzed, 88% of all expression comparisons, i.e., infection of MG fruit (MG infected vs. healthy), infection of RR fruit (RR infected vs. healthy), and ripening (RR healthy vs. MG healthy) were observed in both the microarray and in the qRT-PCR data. However, by qRT-PCR only 59% of the gene expression changes were significant ($P \leq 0.05$), mostly because of inter-sample variability (**Table S2**); in fact, the qRT-PCR coefficient of variation (CV; 20.88%) was almost three times higher than the microarray CV (7.06%). Even with the high CV of the qRT-PCR experiments, there was a strong correlation between the microarray and the qRT-PCR data (Pearson coefficient $R = 0.76$, $P = 2.04e^{-7}$) (**Figure 2**).

In the following sections, the expression profiles of genes involved in ET, JA, SA, and ABA biosynthesis and signaling are presented and discussed in light of the susceptibility to *B. cinerea* of fruit that are either hormone-insensitive or hormone-deficient.

ETHYLENE (ET)

The expression of 50% of the ET biosynthetic genes identified in fruit was altered as consequence of infection with

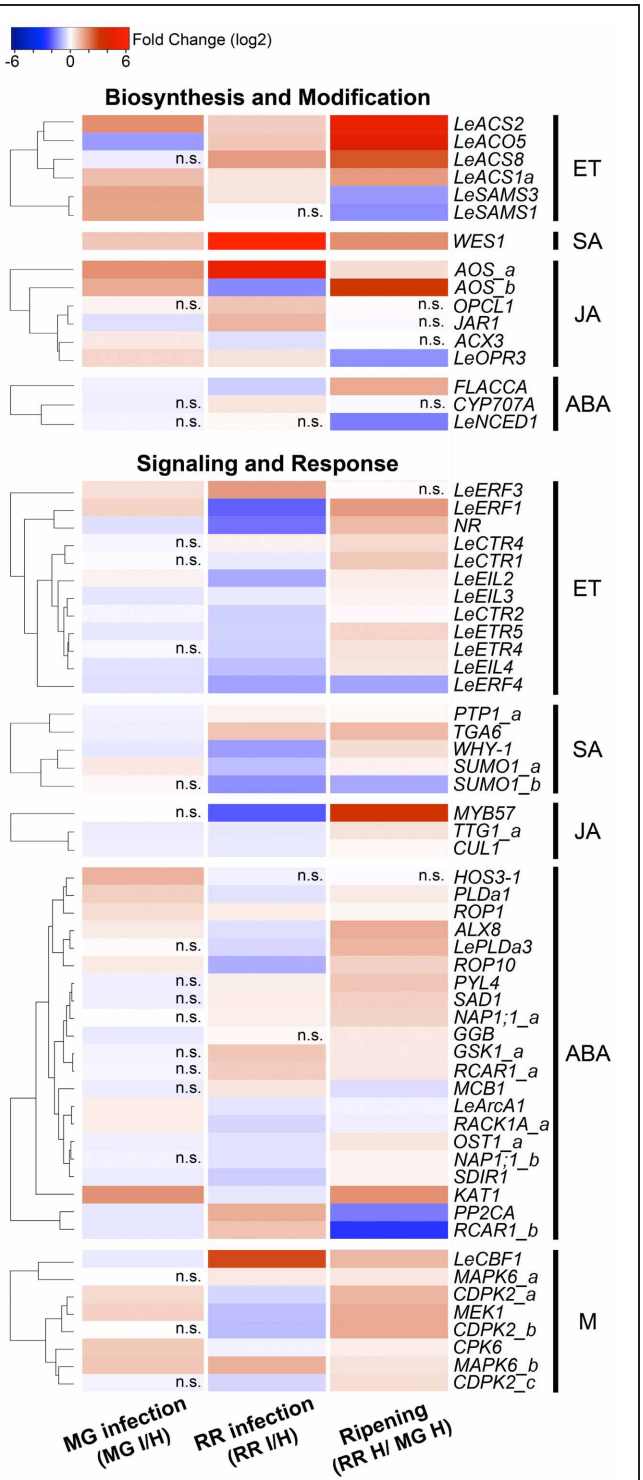

FIGURE 1 | Stress hormone-related genes identified in the microarray analysis that show expression changes as consequence of fruit infection or ripening. Genes involved in in ethylene (ET), salicylic acid (SA), jasmonic acid (JA), abscisic acid (ABA), and multiple (M) hormonal pathways are clustered according to similarities in their expression pattern calculated by Euclidean distance. The colors in the heatmap represent the intensity of the log2-fold expression changes. Non-significant comparisons ($P > 0.05$) are marked in the figure as n.s.

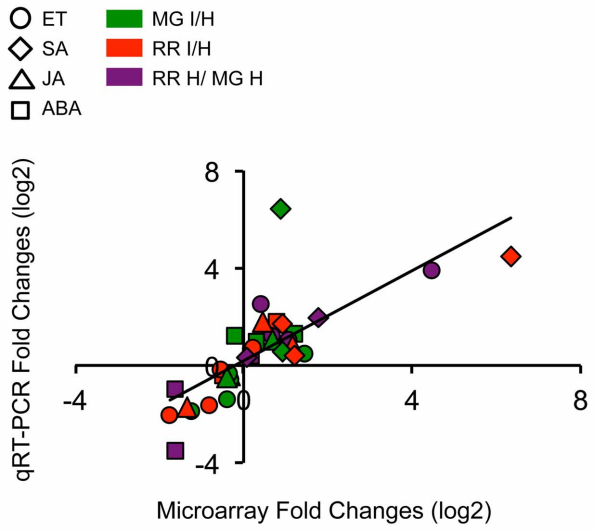

FIGURE 2 | Scatter plot shows expression changes (log2-fold) measured by microarray hybridizations and by qRT-PCR analysis of selected hormone-related genes. Results are plotted for genes that show significant ($P \leq 0.05$) up- or down-regulation in tomato fruit after *B. cinerea* infection and ripening. A linear trendline is shown.

B. cinerea (**Figure 1**; **Table S1**). Three patterns of transcriptional reprogramming were identified in the microarray analysis: (1) increased expression of S-adenosyl-L-methionine (SAM) synthetase genes, *LeSAMS1*, and *LeSAMS3*, which decline during ripening of healthy fruit (Van De Poel et al., 2012a); (2) up-regulation of two members of the 1-aminocyclopropane-1-carboxylic acid (ACC) synthase (ACS) gene family; and (3) down-regulation of an ACC oxidase (ACO) gene in *B. cinerea* infected MG fruit.

Increases in *LeSAMS1* and *LeSAMS3* expression have been detected in tomato vegetative tissues under high salinity conditions and following ABA treatment, suggesting a link between SAM and stress tolerance (Espartero et al., 1994). Besides being a substrate for ET synthesis, SAM is also utilized for the production of polyamines (PAs) and is the primary methyl-donor for modification of essential macromolecules (Van De Poel et al., 2012b). Both ET and PAs, and possibly the relative concentrations of each, mediate biotic and abiotic stress responses in fruit and vegetative tissues (Bitrián et al., 2012; Nambeesan et al., 2012). PAs have been shown to reduce the rate of fruit ripening while ET accelerates it (Mehta et al., 2002; Nambeesan et al., 2010). Therefore, enhanced SAM production and changes in the relative synthesis or abundance of ET/PA may be associated with resistance to pathogen infection, particularly in MG fruit for which the up-regulation of *LeSAMS3* after *B. cinerea* inoculation was validated by qRT-PCR; expression increased further at a later time during the infection process (i.e., 3 dpi) (**Figure 3**).

Tomato ACS and ACO isoforms are differentially expressed depending on the developmental process; some are specifically associated with ripening (e.g., *LeACS1a*, *LeACS2*, *LeACS4*, *LeACO1*, *LeACO3*, and *LeACO4*) while others act preferentially in vegetative tissues and immature fruit (Cara and Giovannoni, 2008; Yokotani et al., 2009; Klee and Giovannoni, 2011; Pech et al., 2012). These expression patterns relate to different systems of ET production, described later. From the microarray analysis, premature increased expression of two ACS genes involved in the tomato ripening process, *LeACS1a* and *LeACS2*, occurs in *B. cinerea*-infected MG fruit, which might suggest that pathogen infections activate the synthesis of ET, thereby accelerating the onset of the ripening process and subsequently inducing susceptibility as proposed by Cantu et al. (2009). On the other hand, down-regulation of the ET biosynthetic gene *LeACO5* only in MG fruit as consequence of infection (**Figures 1**, **3**; **Tables S1, S2**) can be interpreted as a counteracting effort by the plant to control the pathogen-induced increase in ET production.

Infection of fruit affects the expression of 40% of the ET signaling components that are transcribed in fruit (**Figure 1**; **Table S1**). Expression of the ET receptors *LeETR4*, *LeETR5* and *NR* decrease after pathogen inoculation at both fruit ripening stages (**Figure 1**), and the down-regulation was validated in RR fruit at 1 and 3 days after *B. cinerea* infection for both *LeETR5* and *NR* genes (**Figure 3**; **Table S2**). ET receptors are negative regulators of the signaling pathway (Hua and Meyerowitz, 1998), and both their de-phosphorylation and degradation are induced upon ET binding, thereby activating responses to the hormone (Kevany et al., 2007; Kamiyoshihara et al., 2012). However, during fruit ripening, increases in the transcript levels of these receptors do not correlate with protein accumulation or receptor activity (Kevany et al., 2007). Therefore, the impact on ET perception caused by the down-regulation of the expression of the ET receptors observed during infection of fruit should be evaluated further by examining receptor protein levels and phosphorylation state. For example, the reduction in ET sensitivity caused by mutation in the *NR* receptor (i.e., constitutive receptor activation) was shown to enhance resistance of tomato leaves to several pathogens (Lund et al., 1998) and to reduce susceptibility of tomato fruit to *B. cinerea* infection (Cantu et al., 2009).

The expression of the primary ET response factors *LeEIL3* and *LeEIL4* is suppressed as a consequence of exposure of tomato fruit to *B. cinerea* and up-regulated during fruit ripening (**Figure 1**; **Table S1**). The down-regulation after fruit infection was validated for *LeEIL4* (**Figure 3**), while for *LeEIL3* only the suppression in infected MG fruit was statistically significant (**Table S2**). The *LeEIL1-4* genes encode redundant transcription factors that bind to secondary response elements in order to activate downstream ET responses (Tieman et al., 2001). In Arabidopsis leaves infected with the bacterial pathogen *Pseudomonas syringae*, the ET response factors EIN3 and EIL1 appear to negatively regulate plant immune responses by disrupting the pathogen-induced accumulation of SA (Chen et al., 2009). Thus, the decrease in *LeEIL4* and *LeEIL43* expression during fruit infection may represent a plant strategy to modulate the intensity of the ET response to *B. cinerea*, and/or to avoid the repression of SA biosynthesis.

The expression of other ET signaling component genes (with the exception of *LeERF4*) also is enhanced during ripening, but specific expression changes after infection depend on the ripening stage of the fruit (**Figure 1**; **Tables S1, S2**). For example, the protein kinase *LeCTR4* is up-regulated in infected RR fruit, and *LeERF1* expression increased in infected MG fruit but is

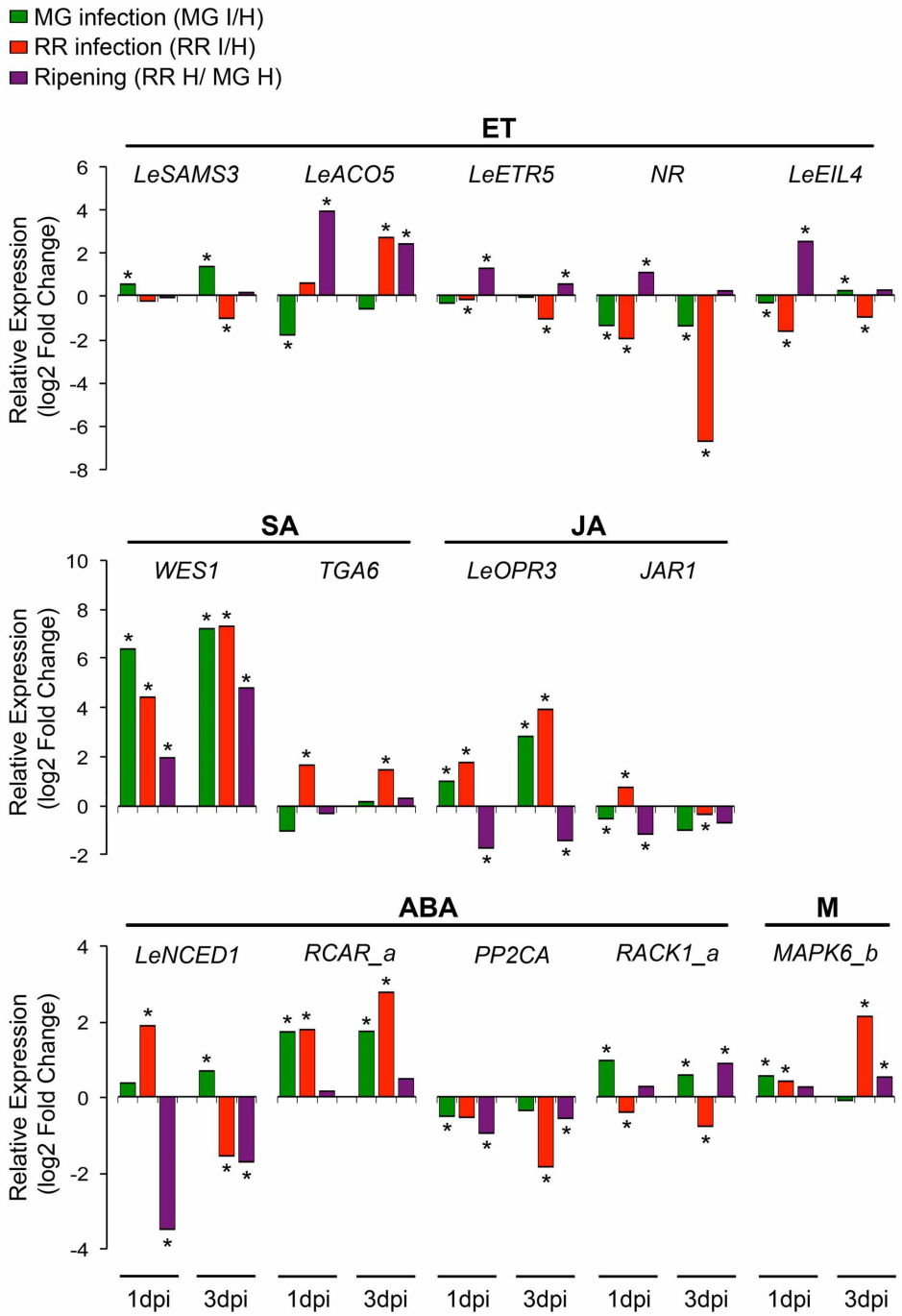

FIGURE 3 | Changes in the relative expression of representative hormone-related genes after infection of fruit by *Botrytis cinerea* and during ripening. Changes (log2-fold) in expression of genes in ethylene (ET), salicylic acid (SA), jasmonic acid (JA), abscisic acid (ABA), and multiple (M) hormonal pathways caused by *Botrytis* infection in fruit at two ripening stages (MG I/H and RR I/H) or by ripening of healthy fruit (RR H/ MG H) were determined by qRT-PCR at two time points (1 and 3 days post-infection, dpi). Asterisks indicate significant fold changes ($^*P \leq 0.05$).

reduced in infected RR fruit. Even though LeERF1 has been reported to induce fruit ripening and softening (Li et al., 2007), its over-expression also is associated with resistance of RR tomato fruit to the necrotroph, *Rhizopus nigricans* (Pan et al., 2013). In addition, ERF1 serves as an intersection point between ET and JA response pathways triggering plant defenses, particularly against necrotrophs (Lorenzo and Solano, 2005; Pieterse et al., 2012). By qRT-PCR no change in expression of *LeERF1* was detected in infected RR fruit; therefore, further analyses using additional biological material, including infections of fruit with

other pathogens, are necessary to reliably assess the regulation of *ERF1* expression in responses to infections.

Experimental observations have suggested that low concentrations of ET are required to induce defense responses in fruit prior to pathogen infection (Ku et al., 1999; Akagi et al., 2011), while high and/or persistent ET levels have been related to increased pathogen susceptibility (Marcos et al., 2005). ET production in fruit is considered to be under the control of two systems, designated Systems 1 and 2. The role of each system is specific to the plant species (climacteric vs. non-climacteric) and developmental stage (Pech et al., 2012). System 1 is characterized by low levels of ET synthesis due to auto-inhibition and is present throughout early fruit development and during ripening of non-climacteric fruit (e.g., strawberry, grape, citrus, and pepper). System 2 refers to the autocatalytic synthesis of ET that is active at the onset of ripening in climacteric fruit (e.g., tomato, apple, peach, and avocado) and that leads to high levels of accumulated hormone (Yokotani et al., 2009; Klee and Giovannoni, 2011; Pech et al., 2012). It is possible that ET is generated in unripe fruit after pathogen recognition under System 1 and that this pathogen-induced concentration of ET specifically activates the expression of defense genes and/or other resistance pathways, but once the ET levels surpass a threshold, induction of System 2 and the associated climacteric ripening, or the activation of senescence/ripening pathways in non-climacteric fruit, may lead to enhanced susceptibility regardless of the defense mechanisms activated. Therefore, ET can act as a promoter of susceptibility or resistance depending on its levels in the tissue and on the developmental stage of the host; in the case of fruit, this corresponds to the point at which the tissue is competent to respond to different ET concentrations.

The hypothesis that ET responses during tomato fruit infection depend on the concentration and perception of this hormone is supported by the results shown in **Figure 4**. In this experiment, tomato fruit at MG and RR stages were pre-treated with either high levels of ET (10 μL/L), or low (12 nL/L) or high (450 nL/L) levels of the ET inhibitor, 1-MCP, prior to inoculation with *B. cinerea*. 1-MCP, which disrupts ET responses by essentially irreversibly binding to the plant cell ET receptors and maintaining their phosphorylation state (Kamiyoshihara et al., 2012), has been widely used to study ripening and disease development in fruit (Blankenship and Dole, 2003; Watkins, 2006; Cantu et al., 2009; Zhang et al., 2009b). Pre-treatment of fruit with ET had no effect on infections of MG fruit by *B. cinerea*; these fruit were about to enter the climacteric phase of ripening and were capable of perceiving the hormone. Pre-treatment with ET also did not affect infections of RR fruit, which had already established ET-induced ripening processes. Pre-treatment with low levels of 1-MCP initially reduced infections in both MG and RR fruit; however, resistance was maintained only in MG fruit in which the climacteric increase of ET was delayed. Pre-treatment with high levels of 1-MCP prematurely induced susceptibility in MG fruit but did not influence RR fruit infections. These observations suggest that low concentrations of 1-MCP may block some but not all ET receptors probably because of limited amounts of the inhibitor and continuing *de novo* generation of receptors. Thus, ET might be perceived in an appropriate concentration to promote resistance in the presence of low 1-MCP levels. In contrast, high 1-MCP levels may block ET perception longer and, thereby, hamper resistance response mechanisms that rely on ET perception. Previous studies also confirmed that application of high concentrations of 1-MCP (>450 nL/L) prior to inoculation with other pathogens (e.g., *Colletotrichum* spp., *Dothiorella* spp., *Penicillium* spp.) often induces rapid decomposition of climacteric and non-climacteric fruit, while application of low concentrations (5–100 nL/L) tends to reduce or stop infections (Ku et al.,

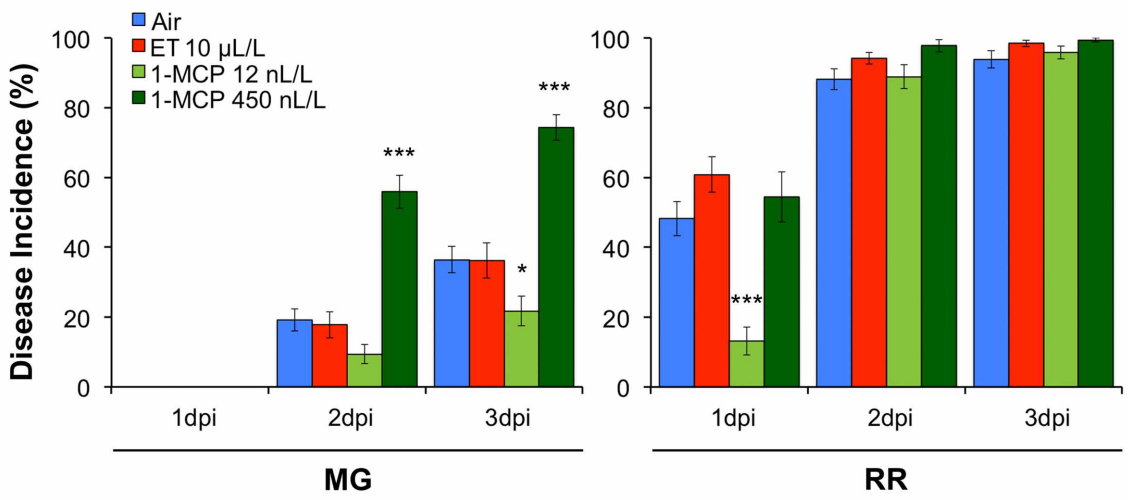

FIGURE 4 | Effect of ethylene (ET) and the ET-perception inhibitor 1-MCP on tomato fruit susceptibility to *Botrytis cinerea*. Disease incidence (% of inoculation sites with soft rot symptoms at 1, 2, and 3 days post-inoculation, dpi) for infections of MG (31 days post-anthesis, dpa) and RR (42 dpa) wild-type tomato fruit (cv. Ailsa Craig). Immediately prior to inoculation and within 2 h of harvest, fruit were treated for 18 h with air, 10 μL/L ET and 12 nL/L 1-MCP or 450 nL/L 1-MCP. Asterisks indicate significant differences within treatments at a given time point and developmental stage (* $P \leq 0.05$, *** $P \leq 0.001$).

1999; Porat et al., 1999; Hofman et al., 2001; Bower et al., 2003; Janisiewicz et al., 2003; Adkins et al., 2005; Marcos et al., 2005).

ET-mediated defenses are generally effective for controlling biotrophs, but are frequently inadequate against necrotrophs (Van Loon et al., 2006; Cantu et al., 2009; Van Der Ent and Pieterse, 2012). Certain necrotrophic pathogens, such as *Penicillium digitatum* and *B. cinerea*, are capable of producing ET, possibly as a virulence factor (Achilea et al., 1985; Cristescu et al., 2002; Zhu et al., 2012) and/or to induce ET synthesis in the host, thus promoting premature senescence or ripening (Marcos et al., 2005; Swartzberg et al., 2008; Cantu et al., 2009). However, it is not possible to distinguish experimentally in infected tissues between the ET synthesized by the pathogen or by the host. While it is known that ET is synthesized by *B. cinerea* using the 2-keto-4-methylthiobutyric acid pathway (Cristescu et al., 2002) rather than the ACC pathway used in plants, the genes responsible for ET biosynthesis by *B. cinerea* have not been identified so inferences about total ET abundance based on biosynthetic gene expression of both organisms cannot be made yet. The dissimilar roles of ET in necrotrophic and biotrophic infections may relate to the model of ET concentration-dependent responses of plant tissues. Low levels of ET may effectively control both biotrophs and necrotrophs, but higher ET levels may favor only necrotrophic infections. Whether a pathogen is capable of perceiving ET and responding to the hormone during its development or when interacting with the host is also relevant in infections and should be explored further.

SALICYLIC ACID (SA)

Two routes of SA biosynthesis had been described in plants, the isochorismate (IC) pathway and the phenylalanine ammonia-lyase (PAL) pathway, but neither pathway has been completely resolved (Dempsey et al., 2011). SA synthesis in response to pathogen infection and abiotic stress is apparently preferentially by the IC pathway (Wildermuth et al., 2001; Garcion et al., 2008; Tsuda et al., 2008), while the PAL pathway may have a minor contribution in local resistance (Ferrari et al., 2003). No significant changes in gene expression in either SA biosynthesis pathway were detected in the microarray analysis. Only the expression of *WES1*, a SA-modification enzyme, increased as consequence of ripening and infection, as shown in the microarray and validation studies (**Figure 1**; **Table S1**). Further up-regulation of *WES1* was also observed later in infection (3 dpi) in both MG and RR fruit (**Figure 3**). WES1 catalyzes SA–Asp conjugation (Zhang et al., 2007). The SA–Asp conjugate is considered to be an inactive form of SA and a target for catabolism (Dempsey et al., 2011). Thus, this result may suggest that SA inactivation occurs during fruit ripening and is a generalized response of tomato fruit to pathogen challenge regardless of the ripening stage. Moreover, SA can influence the levels of other hormones, including ET (Ding and Yi Wang, 2003), and in fruit it could interfere with the regulation of ripening. Further characterization of the SA synthesis pathways and studies of the hormone's production/modification during fruit development are needed to understand fully its impacts on fruit–pathogen interactions.

SA signaling occurs via NPR1-dependent and -independent pathways (Vlot et al., 2009). NPR1 is a transcriptional co-regulator of SA responses and has been recently identified as a receptor of SA in plants (Wu et al., 2012). In the NPR1-dependent pathway, NPR1 monomers interact with members of the TGA family of bZIP transcription factors to regulate expression of SA-responsive genes (Kesarwani et al., 2007; Vlot et al., 2009). TGA factors can be activators or repressors depending on the presence of SA and their ability to form specific protein complexes (Pontier et al., 2001; Zhang et al., 2003). From the microarray and qRT-PCR results, the down-regulation of a tomato homolog of *TGA6* in MG fruit (1 dpi) and its up-regulation in RR fruit (at 1 and 3 dpi) suggest that this gene may serve as a control point to modulate SA signaling during fruit–pathogen interactions (**Figures 1**, **3**; **Tables S1, S2**). Tomato TGAs have been previously implicated in resistance against biotrophs (Ekengren et al., 2003) and can be recruited by necrotrophic pathogens to induce susceptibility (Rahman et al., 2012).

Independently from NPR1, the protein kinases MAPK3 and MAPK6 have been shown to be important in systemic acquired resistance and priming for resistance (Menke et al., 2004; Beckers et al., 2009; Galletti et al., 2011). Pre-treatment with low concentration of SA prior to pathogen encounters induces the accumulation of inactive MAPK3 and MAPK6 in vegetative tissues and once an infection occurs, these kinases are rapidly activated to enhance the expression of defense genes (Beckers et al., 2009). The phosphatases, PTP1 and MKP1, inactivate both MAPK3 and MAPK6 and therefore suppress the downstream SA signaling pathway (Bartels et al., 2009). In infected fruit, a significant decrease in expression of a *PTP1* homolog is observed only in resistant (i.e., MG) fruit, which may lead to the activation of the MAPKs. In particular, a tomato homolog of *MAPK6* (i.e., *MAPK6_b*) appears to be significantly up-regulated in MG fruit after *B. cinerea* inoculation (1 dpi) (**Figures 1**, **3**; **Tables S1, S2**). These results indicate that SA responses via the MAPK pathway may be distinct from those mediated by NPR1 and that these responses may be necessary for both basal and induced defenses in MG fruit.

The susceptibility of the *NahG* tomato line, which does not accumulate SA (Brading et al., 2000), provides additional support for the hypothesis that some SA responses can contribute to resistance in fruit (**Figure 5A**). When we inoculated *NahG* fruit with *B. cinerea* conidia, the fruit at the MG stage were significantly more susceptible to *B. cinerea* infection than their wild-type counterparts and did not generate the localized necrotic response surrounding the inoculation site that is common in resistant unripe fruit [i.e., a lignified and suberized layer of necrotized cells; **Figure 5A**; (Cantu et al., 2009)]. The localized necrotic response in MG fruit is associated with an oxidative burst that is visible within 18 h after pathogen inoculation (Cantu et al., 2009), which could be potentiated by SA as part of a positive feedback loop between this hormone and reactive oxygen species (Overmyer et al., 2003; Vlot et al., 2009). On the other hand, RR fruit from *NahG* and wild-type plants were equally susceptible to *B. cinerea* and no necrotic response was evident with either genotype (data not shown). These results suggest that unripe MG fruit are capable of promoting SA-mediated responses, possibly independently from those influenced by NPR1 (e.g., MAPK-related),

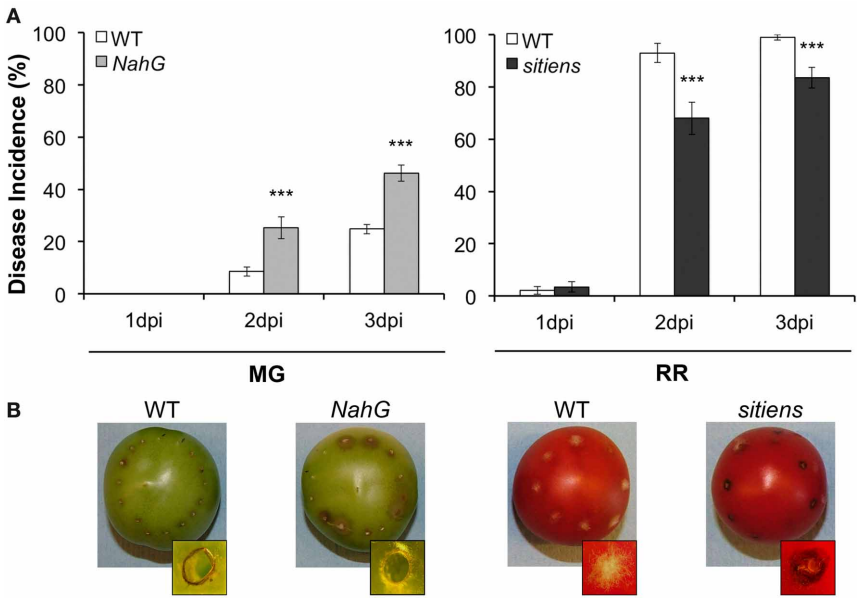

FIGURE 5 | Susceptibility of *NahG* and *sitiens* tomato fruit to *Botrytis cinerea*. (A) Disease incidence (% of inoculation sites with soft rot symptoms at 1, 2, and 3 days post-inoculation, dpi) of *NahG* MG stage fruit (31 days post-anthesis, dpa) and *sitiens* RR stage fruit (42 dpa) compared to the isogenic wild-type (WT) cultivar Moneymaker. Asterisks indicate significant differences between genotypes at a given time point and developmental stage (*** $P \leq 0.001$). **(B)** Representative-inoculated fruit (3 dpi) for each genotype. Insets in all frames show a magnification of an inoculation site, viewed from above the fruit surface (3 dpi). WT fruit at MG stage and *sitiens* fruit at RR stage present a dark necrotic ring that limits the disease symptoms, whereas MG *NahG* fruit or RR WT fruit do not display this inoculation site-localized necrotic zone.

and thereby, may prime fruit for resistance without favoring susceptibility.

JASMONIC ACID (JA)

The increase in expression of JA biosynthetic and the subsequent accumulation of JA occurs locally as a consequence of pathogen, insect or physical damage to plant tissues (Cheong et al., 2002; Wasternack, 2007; Browse, 2009). Up-regulation of three tomato homologs encoding JA biosynthetic enzymes, allene oxide synthase (AOS), 12-oxo-cis-10,15-phytodienoic acid (OPDA) reductase 3 (OPR3), and 3-oxo-2-(cis-2′-pentenyl)-cyclopentane-1-octanoic acid (OPC)-8:CoA ligase (OPCL1) was observed during infections of MG and RR fruit (**Figure 1**; **Table S1**). The expression of the *OPR3* homolog was confirmed in *B. cinerea*-infected fruit after 1 and 3 dpi (**Figure 3**; **Table S2**). In addition, up-regulation of a *JAR1* homolog is detected in RR fruit at 1 dpi (**Figures 1**, **3**; **Tables S1, S2**), but at 3 dpi its expression is down-regulated in both MG and RR tissues (**Figure 3**; **Table S2**). JAR1 is a GH3 acyl-adenylase that conjugates isoleucine to JA, activating the hormone (Staswick and Tiryaki, 2004; Thines et al., 2007) and it is required to activate JA-related responses of Arabidopsis leaves against necrotrophic infection (Staswick et al., 1998).

In the microarray data, transcriptional changes in response to *B. cinerea* are only evident for homologs of two downstream JA-responsive factors (*MYB57* and *TTG1_a*) and a member of the SCFCOI1 complex (*CUL1*). Transcriptional reprogramming of important JA-signaling components (e.g., *COI1*, *MYC2*) was not evident during tomato fruit infection or during ripening (**Figure 1**; **Table S1**), which may indicate that activation of JA-related defenses in fruit occurs *via* other signaling pathways. In contrast, when *B. cinerea* infects petunia flowers it was been reported that expression of *COI1* is activated in the absence of ET signaling (Wang et al., 2013), which indicates that JA signaling pathways could be differentially activated as consequence of fungal infection depending on the plant tissue and the presence/absence of endogenous ET levels. Both JA and ET synergistically activate the expression of a large set of defense genes (Thomma et al., 2001; Glazebrook, 2005; Lorenzo and Solano, 2005) through the transcription factors, ERF1 and ORA59 (Lorenzo and Solano, 2005; Pré et al., 2008). These shared JA- and ET-regulated responses are preferentially triggered when ET is present, while responses unique to JA are induced mostly in the absence of ET (Lorenzo et al., 2004; Pieterse et al., 2009).

SA and JA signaling pathways are generally considered antagonistic (Beckers and Spoel, 2006; Koornneef et al., 2008; Spoel and Dong, 2008; Pieterse et al., 2009, 2012). The antagonism is dependent on NPR1 and influenced by the hormone concentration and the timing of the SA/JA signal initiation (Mur et al., 2006; Koornneef et al., 2008; Leon-Reyes et al., 2010). This interplay between SA and JA might reduce fitness costs from the unnecessary deployment of defenses and could serve as a regulatory mechanism allowing plants to adjust their defense strategies in response to the pathogen's lifestyle (Pieterse et al., 2009; Van Der Ent and Pieterse, 2012). However, some pathogens can exploit the SA/JA antagonism for their own benefit (Alkan et al., 2011; El Oirdi et al., 2011); for example, *B. cinerea* produces an elicitor of

SA responses through the NPR1-dependent pathway, which leads to the inactivation of two JA-response genes, *Proteinase I* and *II*, that are required for resistance against necrotrophs (El Oirdi et al., 2011).

ET can counteract the negative effects of NPR1 on JA responses, but it also enhances the NPR1-dependent expression of SA defense genes (De Vos et al., 2006; Spoel et al., 2007; Leon-Reyes et al., 2009). Leon-Reyes et al. (2010) proposed that the concurrent activation of ET and JA pathways promotes plant insensitivity to subsequent SA-mediated suppression of JA-dependent defenses, which then favors effective resistance against pathogens of different lifestyles. Hence, localized synthesis and perception of JA, ET, and SA at the appropriate relative concentration and timing appear to be required for plant resistance. During infections of fruit, ET, SA, and JA networks might interact to stimulate defenses. Nonetheless, accumulation of susceptibility factors as a consequence of ET-triggered senescence/ripening and the antagonism between SA and JA responses may represent opposing influences in the fruit–pathogen interaction and, thus, lead to susceptibility.

ABSCISIC ACID (ABA)

Increased expression of the tomato 9-cis-epoxycarotenoid dioxygenase 1 (*LeNCED1*), a key ABA biosynthetic gene, occurs during early infection (1 dpi) of susceptible (RR) fruit (**Figures 1**, **3**; **Tables S1, S2**), which suggests a link between ABA synthesis and fruit susceptibility. Several plant pathogens, including *B. cinerea*, generate ABA during infection or use effectors to induce its production by the host, facilitating senescence/ripening and subsequent colonization of the ripened tissue (Siewers et al., 2004, 2006; De Torres-Zabala et al., 2007, 2009).

ABA has been involved in fruit ripening of climacteric and non-climacteric fruit (Zhang et al., 2009a; Koyama et al., 2010; Jia et al., 2011; Soto et al., 2013). Exogenous treatments of ABA induce the expression of the ripening-associated ET biosynthetic genes *LeACS2*, *LeACS4*, and *LeACO1*, thereby, triggering ET production and ripening (Zhang et al., 2009a). In tomato fruit, expression of the 9-cis-epoxycarotenoid dioxygenase 1 (*LeNCED1*) increases at the onset of ripening prior to the ET climacteric rise (Zhang et al., 2009a). A slight induction of *LeNCED1* was detected in infected MG fruit (1 and 3 dpi), which could have been prematurely induced to initiate climacteric ripening; however, a significant decrease in expression occurs at the late stage of ripening (**Figure 3**; **Table S2**). The development and analysis of a genetic knock-out mutant line in *LeNCED1* will be instrumental to understand the impact of ABA synthesis during the increase in ripe fruit susceptibility.

The expression of *FLACCA*, a tomato molybdenum cofactor synthase that is involved in ABA biosynthesis, increases as consequence of ripening, but it is reduced in response to the *B. cinerea* infection (**Figure 1**; **Table S1**). These observations indicate that the plant may reduce the expression of *FLACCA* in an effort to contain the rise in ABA production caused by the pathogen colonization; however, experimental evidence is needed to test this hypothesis.

The interaction between tomato fruit and *B. cinerea* results in significant changes in the expression of 37% genes involved in the ABA signaling pathway (**Figure 1**; **Table S1**). Alterations in regulators of ABA signaling/responses (e.g., receptors and transcription factors) are detected as well as changes in membrane protein channels (e.g., *KAT1*).

In general, increased expression of the *PYL/PYR/RCAR* receptors was observed in RR fruit (**Figure 1**; **Table S1**). The PYL/PYR/RCAR receptors are positive regulators of ABA response by blocking the PP2Cs inhibitors (Raghavendra et al., 2010; Cutler et al., 2010). In Arabidopsis, suppression of three PP2C clade A phosphatases results in constitutive activation of ABA signaling and increased susceptibility to fungal infection (Sánchez-Vallet et al., 2012). In agreement with these results, significant up-regulation of a *RCAR1* homolog (*RCAR_a*) and down-regulation of a PP2C homolog in infected RR fruit at 1 and 3 dpi provides further support for a positive relationship between ABA responses and susceptibility (**Figure 3**; **Table S2**).

Enhanced expression of suppressor genes (e.g., tomato homologs of *HOS3a* and *RACK1*) throughout the ABA hormone-signaling network is detected after inoculation with *B. cinerea* of resistant MG fruit (**Figures 1**, **3**; **Tables S1, S2**). In contrast to the increased expression in MG fruit, the homolog *RACK1_a* is significantly down-regulated in RR fruit at 1 and 3 dpi (**Figure 3**; **Table S2**). Previous studies have demonstrated a role for RACK1 in the activation of defense mechanisms in response to pathogens in rice. The rice *RACK1* homolog (i.e., *RACK1A*) triggers ROS production, defense gene expression, and disease resistance by interacting with OsRac1, a Rac/Rop small GTPase involved in basal immune responses (Nakashima et al., 2008). It is plausible that tomato homolog of *RACK1* has a similar role in fruit by controlling infections in MG fruit.

The contribution of ABA to the enhanced susceptibility of ripe fruit is supported by the disease development assays with the tomato *sitiens* mutant which fails to synthesize ABA (Harrison et al., 2011). Inoculation of RR *sitiens* fruit with *B. cinerea* resulted in a significant decrease in disease incidence when compared to the infected wild-type RR fruit (**Figure 5B**). Interestingly, about 40% of the inoculated sites in RR *sitiens* fruit displayed the typical localized necrotic response of wild-type MG green fruit (**Figure 5B**). MG *sitiens* fruit are as resistant as MG wild-type fruit (data not shown). The molecular mechanisms that mediate the reduction of susceptibility in RR *sitiens* fruit are not known; however, analysis of necrotrophic infections in leaves of *sitiens* plants suggest that a strong induction of defense-related genes (e.g., PR-1), the oxidative burst, and an increase in cuticle permeability might be crucial for the resistant phenotype of this mutant (Asselbergh et al., 2007; Curvers et al., 2010).

CONCLUDING REMARKS

Plants modulate the ET, SA, JA, and ABA hormone networks to induce immune responses against the attacks by various classes of pathogens (Pieterse et al., 2012). Recent studies indicate that other hormones such as auxin, gibberellins, cytokinins, cell wall oligogalacturonides, and brassinosteroids might also be implicated in responses to pathogens either directly or by interacting with other hormones (Doares et al., 1995a; Bari and Jones, 2009). The interactions among hormones provide the plant with a powerful regulatory potential, but also give opportunities for pathogens to

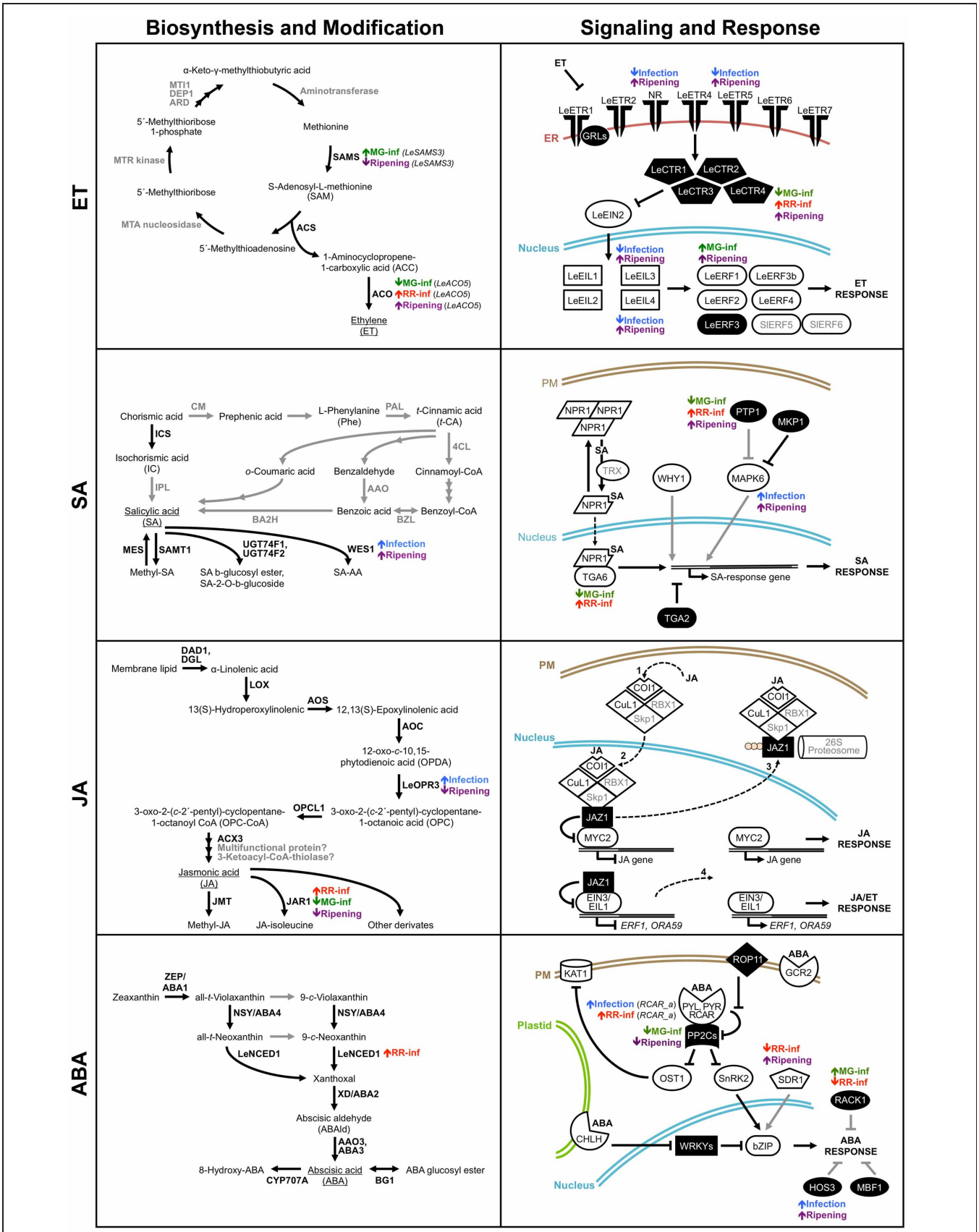

FIGURE 6 | Continued

FIGURE 6 | Overview of key expression changes of genes involved in genes in ethylene (ET), salicylic acid (SA), jasmonic acid (JA), and abscisic acid (ABA) pathways during the tomato fruit–*Botrytis cinerea* interaction. Schematic depictions of the ET, SA, JA, and ABA biosynthesis/modification and signaling/response pathways summarize the microarray analysis and qRT-PCR results and highlight changes in transcript abundance affected by fungal infection or by ripening *per se* (Cantu et al., 2009). Proteins identified in the microarray analysis with significant homology to Arabidopsis genes or known ethylene-related genes are in black bold font; whereas proteins that were not detected in our study or are hypothetical are indicated in gray bold font. Black solid lines indicate well-characterized steps or interactions, while gray solid lines refer to steps/interactions that have not been experimentally confirmed. Dashed lines refer to protein translocation between cellular compartments. In the signaling pathways, solid white figures correspond to positive regulators of hormonal responses while solid black figures indicate negative regulators. Gene expression changes caused by *B. cinerea* infections of tomato fruit at two ripening stages (MG-inf and RR-inf), that are common to infection of fruit at both stages (Infection), or that occur during ripening of healthy fruit (Ripening) are identified next to the appropriate proteins in the pathways. Up-regulation of gene expression is depicted by a short up arrow and down-regulation by a short down arrow. The detailed microarray and qRT-PCR results are presented in **Tables S1, S2** in the supplementary material and the references used to build this figure are listed in **Table S4**.

manipulate the plant defense-signaling networks to their advantage (Van Der Ent and Pieterse, 2012). Plants in their natural environments infrequently interact with a single pathogen species, rather they are impacted by microbial communities, herbivores, and other plants, all of which could individually, collectively or cooperatively influence responses to contact with pathogens. This complexity should be taken into account when studying plant–pathogen associations.

In fruit, high levels of ET and ABA, which stimulate senescence/ripening processes, may facilitate colonization by necrotrophs. The balance between SA and JA responses seems to be crucial for resistance in unripe fruit, while ABA production correlates with ripe fruit susceptibility. ET, at appropriate concentrations, also contributes to the resistance of fruit by activating JA and/or ET responses and possibly by blocking the antagonistic effect of SA on JA signaling. Hence, the role of plant hormones in promoting fruit resistance or susceptibility depends on the interaction of several factors, including: (1) the concentration of the hormones, (2) the timing of the synthesis and perception of the hormones, (3) the competence of the host tissue to respond to active forms of the hormones, (4) the localization of the plant's response to the hormones, and (5) the pathogen's infection strategy, including its own production of hormones.

The interaction between tomato fruit and *B. cinerea* causes transcriptional reprograming of multiple plant hormone networks simultaneously, and, depending on the developmental stage of the fruit contributes to either resistance or susceptibility outcomes. In **Figure 6**, we provide an overview of key expression changes of genes involved in biosynthesis, modification, signaling, and response pathways of the hormones (i.e., ET, SA, JA, and ABA) that, based on our transcriptome profiling analysis and validation, we propose to be part of the regulation of the resistance-to-susceptibility transition associated with ripening and healthy fruit ripening. Analytical methods that allow the simultaneous profiling of multiple signaling molecules that are produced during fruit infections (Müller and Munné-Bosch, 2011), will shed further light on the signaling networks that control fruit susceptibility in the context of ripening, but the challenge of identifying whether the hormones are synthesized by the host or by the pathogens will still be a limitation.

New strategies to study complex gene networks involved in hormone signaling in fruit–pathogen interactions, including the analysis of natural or induced mutants (i.e.: TILLING populations) in both plants and pathogens, the use of laser micro-dissection and cell-specific transcriptomics, and metabolomics can contribute novel important information to our understanding of the biological and ecological importance of plant development in modulating resistance and susceptibility. From an applied perspective, evaluating the specific hormonal events that promote fruit susceptibility may facilitate the development of commodities that ripen successfully and yet are less susceptible to pathogen infection.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://www.frontiersin.org/Plant_Cell_Biology/10.3389/fpls.2013.00142/abstract

Table S1 | Microarray expression data for the 141 putative hormone-related genes expressed in fruit. The table includes the Arabidopsis (TAIR, http://arabidopsis.org) and tomato accessions (Sol Genomics Network, http://solgenomics.net), the Affymetrix probes and annotations, the gene names, and the log2-fold changes of the comparisons between inoculated and control fruit (i.e., MG I/H and RR H/I) or during ripening (i.e., RR H/MG H). Different putative tomato homologs for the same Arabidopsis gene are distinguished by a letter after the gene name, for example *MAPK6_a* and *MAPK6_b*.

Table S2 | Candidate stress hormone-related genes used for qRT-PCR analysis. Changes in relative expression (log2) between infected and control fruit (i.e., MG I/H and RR H/I) at 1 dpi or during ripening (i.e., RR H/MG H) of the 20 genes used in the validation of the microarray results, and their correspondent changes at 3 dpi. Non-significant changes ($P \leq 0.05$) are indicated in gray font.

Table S3 | Primer sequences used for qRT-PCR.

Table S4 | References utilized to build the diagrams in Figure 6.

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ABA crosstalk with ethylene and nitric oxide in seed dormancy and germination

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Dormancy is an adaptive trait that enables seed germination to coincide with favorable environmental conditions. It has been clearly demonstrated that dormancy is induced by abscisic acid (ABA) during seed development on the mother plant. After seed dispersal, germination is preceded by a decline in ABA in imbibed seeds, which results from ABA catabolism through 8′-hydroxylation. The hormonal balance between ABA and gibberellins (GAs) has been shown to act as an integrator of environmental cues to maintain dormancy or activate germination. The interplay of ABA with other endogenous signals is however less documented. In numerous species, ethylene counteracts ABA signaling pathways and induces germination. In Brassicaceae seeds, ethylene prevents the inhibitory effects of ABA on endosperm cap weakening, thereby facilitating endosperm rupture and radicle emergence. Moreover, enhanced seed dormancy in *Arabidopsis* ethylene-insensitive mutants results from greater ABA sensitivity. Conversely, ABA limits ethylene action by down-regulating its biosynthesis. Nitric oxide (NO) has been proposed as a common actor in the ABA and ethylene crosstalk in seed. Indeed, convergent evidence indicates that NO is produced rapidly after seed imbibition and promotes germination by inducing the expression of the ABA 8′-hydroxylase gene, *CYP707A2*, and stimulating ethylene production. The role of NO and other nitrogen-containing compounds, such as nitrate, in seed dormancy breakage and germination stimulation has been reported in several species. This review will describe our current knowledge of ABA crosstalk with ethylene and NO, both volatile compounds that have been shown to counteract ABA action in seeds and to improve dormancy release and germination.

Keywords: abscisic acid, dormancy, ethylene, germination, hormone, nitric oxide, seed

INTRODUCTION

Survival of plant species mainly relies on the sexual reproduction to give birth to new individuals. In flowering plants, the seed is the main unit of dispersal and allows colonization of new geographic areas. As a consequence of the double fertilization process, a mature angiosperm seed contains a diploid embryo and protective layers comprising the triploid endosperm, a nourishing tissue for the embryo, and the seed coat of maternal origin. During development on the mother plant, after embryogenesis completion, reserve accumulation takes place and is followed, in so-called orthodox seeds, by an intense dehydration leading to low seed water content upon dispersal. In many species, a dormant state is also induced during the maturation phase, preventing pre-harvest germination and allowing seed survival until environmental conditions become suitable for germination and seedling establishment (Bentsink and Koornneef, 2008; Finkelstein et al., 2008; North et al., 2010).

Dormancy has been defined as a developmental state in which a viable seed fails to germinate under favorable environmental conditions (Bewley, 1997), but different definitions and classifications have been proposed. Finch-Savage and Leubner-Metzger (2006) summarized a classification proposed by Baskin and Baskin (2004), based on the fact that dormancy results from physiological and developmental (or morphological) properties of the seed. Dormancy is therefore divided in five classes: (1) physiological dormancy (PD) can be released by different stratification (moist chilling) treatments depending on its depth, (2) morphological dormancy (MD) is due to a delay of embryo development, (3) morphophysiological dormancy (MPD) is combining both PD and MD, (4) physical dormancy (PY) is correlated with seed coat impermeability to water and needs disruption of the seed coat (scarification) to be released, and finally (5) combinational dormancy combining PY and PD. Most species display a non-deep PD corresponding to a dormancy that can be released, depending on the species, by gibberellin (GA) treatment, stratification, scarification, or a period of dry storage (after-ripening). In this case, seeds generally combine a coat-imposed dormancy due to the covering layers of the seed (seed coat and endosperm) that prevent the radicle protrusion, and an embryo dormancy due to its incapacity to induce radicle growth.

When dormancy is released, seeds can germinate under favorable conditions, specific to each species. The germination process, that begins with seed imbibition and finishes with a developed plantlet, is divided in three distinct phases of water uptake. Phase

I starts with a fast water uptake and the activation of respiratory metabolism and transcriptional and translational activities. During phase II water uptake ceases, seed reserve mobilization begins and testa rupture occurs. Later, in the third phase water uptake resumes and endosperm rupture allows radicle protrusion; then starts the post-germination phase with high water uptake, mobilization of the major part of reserves and first cell divisions, until the complete seedling development (Bewley, 1997; Nonogaki et al., 2010; Weitbrecht et al., 2011). Germination *sensu stricto* ends with radicle protrusion. It is often described has the resulting consequence of the growth potential of the embryo and the resistance of the surrounding layers. Endosperm weakening is an essential part of the modification of seed envelopes for the progress of germination and involves the activation of cell-wall modifying enzymes (Finch-Savage and Leubner-Metzger, 2006; Endo et al., 2012; Linkies and Leubner-Metzger, 2012). After dormancy release, storage/imbibition of non-dormant seeds in unfavorable conditions for germination can trigger a secondary dormancy. This is a way to protect seeds against germination too late in the year and induce a seasonal cycling of dormancy level in seeds (Cadman et al., 2006; Footitt et al., 2011).

The regulation of seed dormancy and germination by the hormonal balance between abscisic acid (ABA) and GA, in response to environmental signals, is well documented in a number of recent reviews (Finkelstein et al., 2008; Seo et al., 2009; Nambara et al., 2010; Nonogaki et al., 2010; Weitbrecht et al., 2011; Graeber et al., 2012; Rajjou et al., 2012). The present review will describe recent knowledge about key players in the ABA metabolism and signaling pathways that control dormancy induction and maintenance and convergent evidences supporting the role of two other signaling compounds, nitric oxide (NO) and ethylene, in dormancy breakage and germination, and their interactions with ABA metabolism and signaling pathways.

ABA HOMEOSTASIS AND SIGNALING IN DORMANCY CONTROL

ABA SYNTHESIS

Abscisic acid is formed by cleavage of C_{40} oxygenated carotenoids, also called xanthophylls, which are produced in plastids from C_5 precursors (Ruiz-Sola and Rodríguez-Concepción, 2012). Key genes encoding enzymes of the ABA biosynthesis pathway have been identified through mutant selection for altered germination phenotypes, giving further evidence of the major role of ABA in the regulation of seed dormancy and germination (**Figure 1**). For instance, the first ABA-deficient mutant, identified in *Arabidopsis thaliana*, was isolated in a GA biosynthesis mutant *ga1* suppressor screen, on its ability to germinate in the absence of GA. It was shown to be defective in zeaxanthin epoxidase (ZEP) activity, like a *Nicotiana plumbaginifolia* mutant selected later on its early germination phenotype (Koornneef et al., 1982; Marin et al., 1996). ZEP catalyzes the epoxidation of zeaxanthin into violaxanthin and is encoded, in *Arabidopsis*, by the *ABA1* gene (Audran et al., 2001; Xiong et al., 2002). Violaxanthin is then converted into neoxanthin, by neoxanthin synthase (NSY), likely encoded by the *Arabidopsis ABA4* gene (Dall'Osto et al., 2007; North et al., 2007). Despite impairment in *ABA4* function completely prevents neoxanthin synthesis, the *aba4* mutant exhibits no obvious dormancy

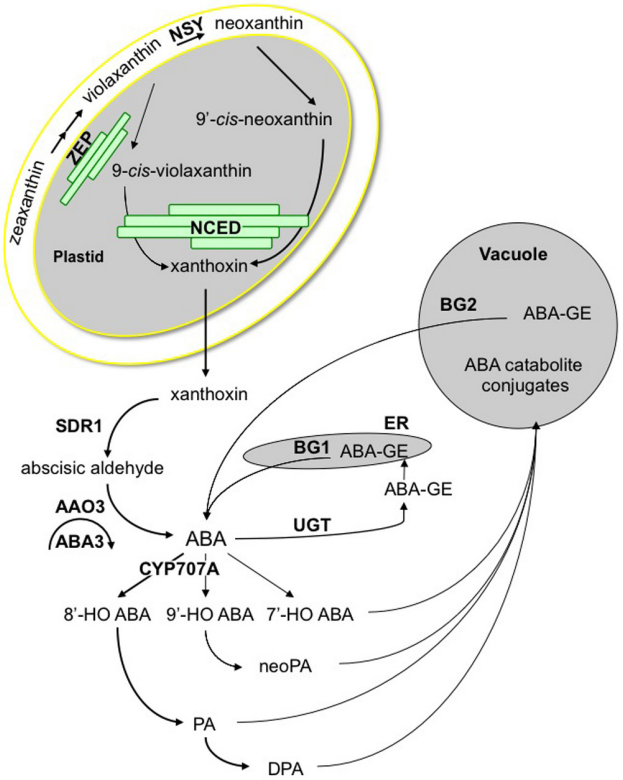

FIGURE 1 | ABA metabolism pathway. Zeaxanthin conversion into violaxanthin is catalyzed by zeaxanthin epoxidase (ZEP). ABA4 is involved in the synthesis of neoxanthin, which is then *cis*-isomerized, together with violaxanthin, by an unknown isomerase. Carotenoid cleavage is catalyzed by a family of 9-*cis*-epoxycarotenoid dioxygenases (NCED) to form xanthoxin. Xanthoxin moves to the cytosol by an unknown mechanism and is converted into abscisic aldehyde by a short-chain dehydrogenase reductase (SDR1), which is then oxidized into ABA by an abscisic aldehyde oxidase (AAO3). Sulfuration of AAO3 molybdenum co-factor by ABA3 is necessary for enzyme activity. The 8′-hydroxylation by CYP707A enzymes is thought to be the predominant pathway for ABA catabolism. Hydroxy-groups of ABA and its catabolites, phaseic acid (PA), neoPA, and dihydrophaseic acid (DPA) are targets for conjugation. ABA-glucose ester is formed by ABA glucosyltransferases (UGT) and hydrolyzed by glucosidases, including BG1 and BG2.

phenotype, due the formation of *cis*-violaxanthin by an alternate pathway (North et al., 2007). Both *cis*-violaxanthin and *cis*-neoxanthin cleavage gives rise to xanthoxin, the C_{15} aldehyde precursor of ABA. Since *cis*-isomerization of violaxanthin and neoxanthin is required prior to cleavage, an unknown isomerase might be involved. The *VIVIPAROUS14* (*VP14*) gene in maize (*Zea mays*) has been shown to encode a 9-*cis*-epoxycarotenoid dioxygenase (NCED), which catalyzes the oxidative cleavage of either 9′-*cis*-neoxanthin or 9-*cis*-violaxanthin (Schwartz et al., 1997; Tan et al., 1997). *NCED* genes have been then identified in a number of other plant species (Nambara and Marion-Poll, 2005). In *Arabidopsis*, *VP14*-related gene family is composed of nine members, five of which (*NCED2*, *NCED3*, *NCED5*, *NCED6*, and *NCED9*) encode xanthoxin-producing enzymes (Iuchi et al., 2001; Toh et al., 2008).

In *Arabidopsis* plastids, ZEP is associated mainly to envelope and slightly to thylakoid membranes (**Figure 1**). In contrast

NSY/ABA4 is presumably tightly bound to the envelope since this protein is predicted to contain four transmembrane domains and is exclusively found in the envelope fraction (Joyard et al., 2009). In contrast, NCED proteins have been detected either in stroma or thylakoid membrane-bound compartments, or both (Tan et al., 2003). In addition, recent VP14 structural analysis suggested that this enzyme might penetrate the surface of thylakoid membrane to access and transfer carotenoid substrates to its catalytic center (Messing et al., 2010). The scattered location of ZEP, NSY, and NCED suggests that the production of xanthoxin inside plastids may require transport mechanisms of lipid-soluble carotenoid molecules, which are not currently understood. Since the following enzymatic reactions take place in the cytosol, xanthoxin is also presumed to migrate from plastid to cytosol by a still unknown mechanism.

Abscisic aldehyde is synthesized from xanthoxin, by an enzyme belonging to short-chain dehydrogenase/reductase family, which is named SDR1 and is encoded by the *ABA2* gene in *Arabidopsis* (Rook et al., 2001; Cheng et al., 2002; Gonzalez-Guzman et al., 2002). The oxidation of the ABA-aldehyde is the final step of ABA biosynthesis, and is catalyzed by an abscisic aldehyde oxidase. In *Arabidopsis*, four homologous aldehyde oxidase (*AAO*) genes have been characterized, but only one of them, *AAO3*, encodes a protein that has proven activity on abscisic aldehyde (Seo et al., 2000). Activity of this molybdoenzyme requires the activation of its molybdenum co-factor (Moco) by addition of a sulfur atom to the Mo center, which is catalyzed by a Moco sulfurase, which has been named ABA3 in *Arabidopsis* (Bittner et al., 2001; Xiong et al., 2001).

ABA CATABOLISM

Abscisic acid inactivation is a crucial mechanism to fine-tune ABA levels, which occurs by either oxidation or conjugation (**Figure 1**). The major catabolic route is the 8′-hydroxylation of ABA by the CYP707A subfamily of P450 monooxygenases (Kushiro et al., 2004; Saito et al., 2004). Spontaneous 8′-hydroxy-ABA isomerization gives rise to phaseic acid (PA), which is then converted to dihydrophaseic acid (DPA) by a still unknown reductase. ABA can also be hydroxylated at the C-7′ and C-9′ positions. As 8′-hydroxylation, 9′-hydroxylation is catalyzed by CYP707A as a side reaction, and neoPA is then formed by spontaneous isomerization (Zhou et al., 2004; Okamoto et al., 2011). The conjugation of ABA with glucose to form the ABA-glucose ester (ABA-GE) is catalyzed by an ABA glucosyltransferase, and in *Arabidopsis* only UGT71B6 exhibits a selective glucosylation activity toward the natural enantiomer (+)-ABA (Lim et al., 2005; Priest et al., 2006). Subsequent hydrolysis of conjugates constitutes an alternative pathway for ABA synthesis in response to dehydration stress. Two glucosidases BG1 and BG2, localizing respectively in the endoplasmic reticulum or the vacuole, have been identified (Lee et al., 2006; Xu et al., 2012).

Deficiency in either ABA synthesis or ABA inactivation by 8′-hydroxylation leads to strong dormancy phenotypes, respectively dormancy loss or strengthening (Nambara and Marion-Poll, 2005; Seo et al., 2009; Nambara et al., 2010). In contrast, reports on functional analysis of mutant or overexpressing lines in ABA conjugation or ABA-GE hydrolysis did not yet describe the implication of these processes in dormancy control. Nevertheless, ABA conjugation may contribute to ABA breakdown upon germination, as shown in lettuce (*Lactuca sativa*; Chiwocha et al., 2003).

ABA SIGNALING PATHWAY

Genetic analyses suggest that PYR/PYL/RCAR (pyrabactin resistance1/PYR1-like/regulatory components of ABA receptor) ABA receptors, clade A type 2C protein phosphatases (PP2C) and group III sucrose non-fermenting1-related protein kinase2 (SnRK2) subfamily are essential core components of the upstream signal transduction network that regulates ABA-responsive processes, including dormancy and germination (reviewed in Cutler et al., 2010). PYR/PYL/RCAR proteins constitute a 14-member family, belonging to the START-domain superfamily, also called Bet v I-fold (Ma et al., 2009; Park et al., 2009). ABA binding induces receptor conformation changes allowing the formation of a protein complex with PP2C and the inhibition of phosphatase activity (**Figure 2**). The clade A PP2C, including ABA INSENSITIVE1 (ABI1) and ABI2, also interact with three SnRK2 (SnRK2.2, SnRK2.3, and SnRK2.6) and, in the absence of ABA, dephosphorylate a serine residue whose phosphorylation is required for kinase activity (Soon et al., 2012). When ABA is present, PP2C binding to the receptor releases inhibition of SnRK2 activity, which can phosphorylate downstream targets.

Other types of receptors and a large number of genes, whose mutations alter ABA germination sensitivity, have been reported to participate in ABA signaling. In particular, regulatory mechanisms such as RNA processing, RNA/protein stability or chromatin remodeling have an important role. However, they will not be detailed here, since their role in ABA crosstalk with ethylene and NO in seeds still requires further investigation. In *Arabidopsis* seeds, extensive evidence including mutant phenotypes strongly supports a central role of the PYR/PYL, PP2C, SnRK2 complex in ABA signaling (reviewed in Cutler et al., 2010; Nambara et al., 2010). Germination of a *pyr/pyl* sextuple mutant is highly insensitive to ABA, as also observed for the *snrk2.2 snrk2.3 snrk2.6* triple mutant (Fujii and Zhu, 2009; Gonzalez-Guzman et al., 2012). Moreover the *snrk2* triple mutant exhibits loss of dormancy and even seed vivipary under high humidity conditions (Nakashima et al., 2009). Conversely, in accordance with PP2C being negative regulators of ABA signaling, germination in triple *pp2c* mutants was slower than in wild type and was inhibited by very low ABA concentrations (Rubio et al., 2009). In contrast, the gain-of-function mutations *abi1-1* and *abi2-1*, which prevent PP2C binding to PYL/PYR/RCAR, lead to ABA insensitivity and reduced dormancy (Ma et al., 2009; Park et al., 2009).

Basic leucine zipper transcription (bZIP) factors of the ABA-RESPONSIVE ELEMENTS (ABRE) BINDING FACTOR/ABA RESPONSE ELEMENT BINDING FACTOR/ABA INSENSITIVE5 (ABF/AREB/ABI5) clade have been shown in different species to constitute SnRK2 downstream targets and regulate ABRE containing genes (Johnson et al., 2002; Kobayashi et al., 2005; Umezawa et al., 2009). Several family members are expressed at different seed stages and exhibit partially redundant or antagonistic functions, and ABI5 appears to have a predominant role in the regulation of a subset of late embryogenesis abundant (LEA) proteins during late seed development (Bensmihen et al., 2002;

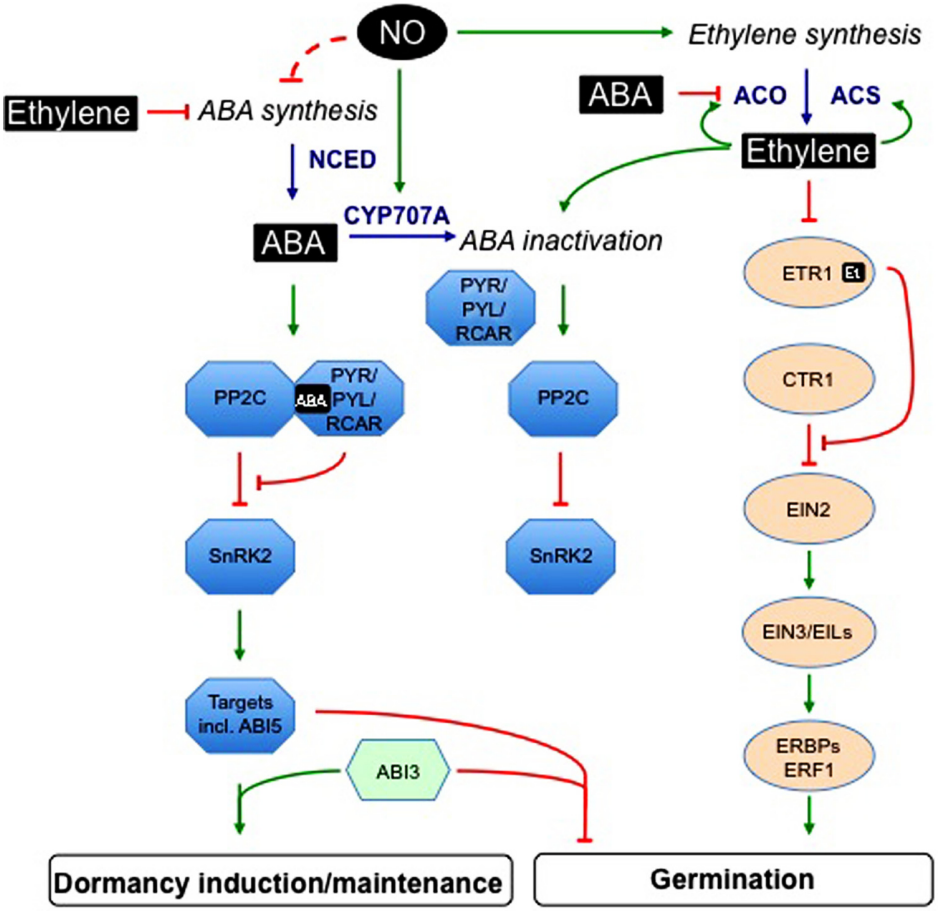

FIGURE 2 | Interactions between ethylene, abscisic acid, and nitric oxide signaling pathways in the regulation of seed germination and dormancy. This scheme is based on genetic analyses, microarray data, and physiological studies on seed responsiveness to ABA, ethylene, or NO. ABA binding to PYR/PYL/RCAR receptor induces the formation of a protein complex with PP2C and the inhibition of phosphatase activity. In the absence of ABA, PP2C dephosphorylate SnRK2. When ABA is present, PP2C binding to the receptor releases inhibition of SnRK2 activity, which can phosphorylate downstream targets, including ABI5-related transcription factors. Interactions between ABI3 and ABI5 mediate transcriptional regulation of ABA-responsive genes. Ethylene positively regulates its own biosynthesis, by acting on ACC synthesis catalyzed by ACS and subsequent conversion to ethylene by ACO. This last step is also subject to ABA inhibition. Ethylene is perceived by receptors (among which ETR1) located in the endoplasmic reticulum; its binding leads to the deactivation of the receptors that become enable to recruit CTR1. Release of CTR1 inhibition allows EIN2 to act as a positive regulator of ethylene signaling pathway. EIN2 acts upstream of nuclear transcription factors, such as EIN3, EILs, and ERBPs/ERFs. Ethylene down-regulates ABA accumulation by both inhibiting its synthesis and promoting its inactivation, and also negatively regulates ABA signaling. In germinating seeds, NO enhances ABA catabolism and may also negatively regulate ABA synthesis and perception. Moreover, NO promotes both ethylene synthesis and signaling pathway. ABA, abscisic acid; ABI3, ABA insensitive3; ABI5, ABA insensitive5; ACC, 1-aminocyclopropane 1-carboxylic acid; ACO, ACC oxidase; ACS, ACC synthase; CTR1, constitutive triple response 1; CYP707A, ABA-8′-hydroxylase; EIL, EIN3-like; EIN, ethylene-insensitive; EREBP, ethylene-responsive element binding protein; ERF, ethylene response factor; Et, ethylene; ETR1, ethylene receptor1; NCED, 9-cis-epoxycarotenoid dioxygenase; NO, nitric oxide; PP2C, clade A type 2C protein phosphatases; PYR/PYL/RCAR, pyrabactin resistance1/PYR1-like /regulatory components of ABA receptor; SnRK2, group III sucrose non-fermenting-1-related protein kinase 2; a dashed line is used when regulatory targets are not precisely identified.

Finkelstein et al., 2005). *abi5* mutation confers ABA-insensitive germination, but it does not impair seed dormancy, suggesting that other factors might be involved in dormancy induction (Finkelstein, 1994). Nevertheless, ABI5 has been clearly proven to act as a major inhibitor of germination processes in imbibed seeds, notably through its up-regulation by stress-induced ABA accumulation (Lopez-Molina et al., 2001; Piskurewicz et al., 2008). ABI3/VIVIPAROUS1 (VP1) interacts with ABI5 for the regulation of a number of ABA-responsive genes during seed maturation and germination (Lopez-Molina et al., 2002; Piskurewicz et al., 2008, 2009). However, in contrast to *abi5*, *abi3* mutants do not only exhibit ABA-resistant germination, but also other phenotypes including desiccation intolerance and precocious germination. They share these maturation defects with *fusca3* (*fus3*) and *leafy cotyledon2* (*lec2*) mutants, which, like *abi3*, carry mutations in B3 transcription factor family genes. These factors form a complex network regulating the expression of reserve storage and LEA genes by their binding to RY motif, and it has been suggested that the lack of dormancy induction in mutants might indirectly result from early seed developmental defects (Gutierrez et al., 2007;

Finkelstein et al., 2008; Santos-Mendoza et al., 2008; Graeber et al., 2012). Nevertheless *fus3* mutation has been shown to affect ABA levels in developing seeds (Gazzarrini et al., 2004). In addition, ABA-specific phenotypes of *abi3/vp1* mutants strongly suggest an involvement in ABA-regulated dormancy induction, but downstream dormancy genes still remain elusive. Nevertheless, one of these might be the recently identified *seed dormancy4* (*Sdr4*) gene in rice, which encodes a nuclear protein of unknown function (Sugimoto et al., 2010).

The *Arabidopsis DELAY OF GERMINATION1* (*DOG1*) gene, whose precise function is still unknown, has been identified as a major regulator of seed dormancy (Bentsink et al., 2006). In accordance, protein accumulation in dry seeds well correlates with dormancy depth, and both transcript and protein levels are increased upon cool conditions of seed maturation, which increase seed dormancy (Kendall et al., 2011; Nakabayashi et al., 2012). Despite *dog1* dormancy phenotypes are similar to ABA synthesis and signaling mutants, current evidence suggests that DOG1 and ABA act in independent pathways. Nevertheless regulation of dormancy depth by DOG1 requires a functional ABA signaling pathway (Nakabayashi et al., 2012), and DOG1 has been reported to be implicated in the ABA-mediated sugar signaling pathway, together with ABI4, an APETALA2 transcription factor involved in reserve mobilization at germination (Penfield et al., 2006; Teng et al., 2008). Another mutation, named *despierto* (*dep*), also causes dormancy loss (Barrero et al., 2010). *DEP* gene encodes a C3HC4 RING (Really Interesting New Gene)-finger protein, whose targets are unknown. In addition to similarity in mutant phenotypes, expression of both *DEP* and *DOG1* genes is maximal during late seed development and decreases during imbibition. Moreover *dep* mutation reduces *DOG1* transcript levels in developing seeds and vice versa. It also down-regulates the expression of several ABA biosynthesis and signaling genes, including *NCED6*, *NCED9*, and *ABI3*, suggesting its action in dormancy induction may involve the ABA signaling pathway (Barrero et al., 2010).

SPATIOTEMPORAL REGULATION OF ABA LEVEL AND SIGNALING IN DORMANCY AND GERMINATION

Abscisic acid is produced in all seed tissues (testa, endosperm, embryo), as suggested by the spatiotemporal expression of ABA biosynthesis genes (Lefebvre et al., 2006; Frey et al., 2012). However, ABA accumulated in seeds also originates from synthesis in vegetative tissues and transport to the seed (Frey et al., 2004; Kanno et al., 2010). Several ABA transporters have been recently identified, which belong to either the ATP-binding cassette (ABC) or nitrate transporter 1 (NRT1)/peptide transporter (PTR) families (Kang et al., 2010; Kuromori et al., 2010; Kanno et al., 2012). ABC transporter G family member 25 (ABCG25) functions as a plasma membrane ABA exporter, whereas both ABCG40 and AIT1 (ABA IMPORTER1) are plasma membrane uptake transporters. Despite mutations in these three genes induce alterations in germination sensitivity to ABA, suggesting a possible function in seeds, the precise contribution of any of them to either ABA supply from mother plant to seeds or its translocation between maternal and/or embryonic seed tissues needs further investigation. Another ABC transporter gene, ABCG22, has been reported to be involved in ABA-regulated water stress tolerance, but its function in ABA transport remains uncertain (Kuromori et al., 2011). ABA levels are maximal during mid-seed development, with a large fraction produced in maternal tissues (Karssen et al., 1983; Kanno et al., 2010). Maternal ABA has a major contribution to the regulation of many aspects of seed development, but only ABA produced by zygotic tissues at late maturation stages imposes dormancy (Karssen et al., 1983; Frey et al., 2004).

Carotenoid cleavage by NCED and ABA inactivation by CYP707A 8′-hydroxylase have been proven to constitute key regulatory steps for the control of ABA levels, which affect seed dormancy and germination in response to environmental cues (Nambara and Marion-Poll, 2005; Seo et al., 2009; Nambara et al., 2010). Among the five *Arabidopsis NCED* genes, *NCED6* and *NCED9* exhibit the highest expression levels in developing seeds and show distinctive expression patterns. *NCED6* is specifically expressed in endosperm, whereas *NCED9* expression is detected in testa and embryo. Furthermore mutant analysis indicated that ABA production in both embryo and endosperm contributes to dormancy induction (Lefebvre et al., 2006; Frey et al., 2012). In barley (*Hordeum vulgare*), the two *HvNCED* genes also exhibit differential spatiotemporal patterns of expression. In contrast to *HvNCED2*, *HvNCED1* transcript levels vary depending on environmental conditions during grain development and modulate ABA accumulation at late maturation stages (Chono et al., 2006). ABA inactivation by CYP707A during seed maturation also regulates dry seed ABA levels and dormancy depth, as deduced from *cyp707a* mutant analysis (Okamoto et al., 2006). Moreover, the seed dormancy increase under cold-maturation conditions is not only correlated with *DOG1* up-regulation, as mentioned above, but also with *CYP707A2* down-regulation (Kendall et al., 2011).

Upon imbibition, dormancy maintenance and germination are also regulated by both ABA catabolism and neo-synthesis. A decrease in ABA levels at imbibition has been observed in both dormant and non-dormant seeds in several species; nevertheless dormant seeds maintain higher ABA levels and in accordance exhibit lower *CYP707A* transcript levels, as shown in *Arabidopsis* and barley (Millar et al., 2006). Barley *HvABA8'OH1* transcripts were detected in coleorhiza cells near the root apex and *Arabidopsis CYP707A2* in endodermis and micropylar endosperm next to the radicle (Millar et al., 2006; Okamoto et al., 2006). Moreover, it is well documented in several species that unfavorable light or temperature conditions prevent germination by coordinated regulation of *NCED* and *CYP707A* gene expression (Seo et al., 2006; Gubler et al., 2008; Toh et al., 2008; Leymarie et al., 2009; Argyris et al., 2011). Furthermore, dormancy cycling by seasonal variation of soil temperature has been recently linked to the regulation of ABA metabolism and signaling genes. Deep dormancy in winter is correlated with increased ABA levels and *NCED6* expression, together with that of *DOG1* and *MOTHER OF FLOWERING LOCUS T* (*MFT*). *MFT* encodes a phosphatidylethanolamine-binding protein, which is regulated by *ABI3* and *ABI5*, and feedback regulates ABA signaling by repressing *ABI5* (Xi et al., 2010). In contrast, shallow dormancy in summer is correlated with a reduction in ABA levels and an up-regulation of *CYP707A2* and *ABI2*, which negatively regulates ABA signaling (Footitt et al., 2011).

In *Arabidopsis*, despite endosperm consists in a single cell layer in mature seeds, convergent evidence demonstrated its major role in ABA control of seed dormancy and germination. Firstly, whereas the removal of whole seed coat (endosperm and testa) releases mechanical constraints and allows development of embryos dissected from dormant seeds, the preservation of the endosperm after testa removal maintains dormancy (Bethke et al., 2007a). Secondly, using a "seed coat bedding assay," Lee et al. (2010) showed that diffusion of endospermic ABA from dormant seed envelopes could prevent growth of non-dormant embryos, including those of ABA-deficient *aba2* mutants. In isolated embryos, translocated ABA was able to induce ABI5 protein accumulation, whose level was correlated with dormancy maintenance. In addition, in a previous study, *ABI5* transcript was detected in the embryo and the micropylar endosperm of imbibed seeds, suggesting a role in the inhibition of both embryo growth and endosperm rupture by ABA (Penfield et al., 2006). The tissue-specificity of ABA sensitivity is also likely regulated by the spatiotemporal expression of upstream ABA signaling components, as suggested by the differential expression of PYR/PYL genes in embryo and/or endosperm of imbibed seeds (Gonzalez-Guzman et al., 2012).

ETHYLENE BIOSYNTHESIS, SIGNALING, AND ABA CROSSTALK IN SEED GERMINATION

ETHYLENE BIOSYNTHESIS AND SIGNALING

Ethylene biosynthesis pathway in germinating seeds is the same as that described in other plant organs (**Figure 3**), in which *S*-adenosyl-methionine (*S*-AdoMet) and 1-aminocyclopropane-1-carboxylic acid (ACC) are the main intermediates (Yang and Hoffman, 1984; Wang et al., 2002; Rzewuski and Sauter, 2008). The first step of ethylene biosynthesis is the conversion of *S*-AdoMet to ACC catalyzed by ACC synthase (*S*-adenosyl-L-methionine methylthioadenosine-lyase, ACS), the by-product being 5′-methylthioadenosine (MTA), which is recycled back to methionine through the Yang cycle (Yang and Hoffman, 1984; Kende, 1993). The second step corresponds to the oxidation of ACC by ACC oxidase (ACO) to form ethylene, CO_2, and hydrogen cyanide (HCN). Cyanide produced during this final step of ethylene synthesis is detoxified to β-cyanoalanine by β-cyanoalanine synthase (β-CAS). Both ACS and ACO are encoded by a multigene family. In *Arabidopsis*, nine active *ACS* genes have been characterized (Yamagami et al., 2003; Wang et al., 2005; Dong et al., 2011). Most of them can be induced by cycloheximide (*ACS2*, *ACS4*, *ACS6*), wounding (*ACS2*, *ACS4*), and ethylene treatment (*ACS2*, *ACS6*; reviewed in Wang et al., 2002). In addition, *ACS6* can also be induced by cyanide (Smith and Arteca, 2000) or ozone treatment (Vahala et al., 1998). ACO activity controls *in vivo* ethylene production and has fundamental contribution during seed germination (Matilla and Matilla-Vazquez, 2008; Linkies and Leubner-Metzger, 2012).

In *Arabidopsis*, five membrane-localized receptors have been identified: ethylene resistant 1 (ETR1), ETR2, ethylene response sensor 1 (ERS1), ERS2, and ethylene insensitive 4 (EIN4; **Figure 2**). Among them, ETR1 and ERS1 contain three transmembrane domains in the N-terminus and a histidine kinase domain in the C-terminus. In contrast, ETR2, EIN4, and ERS2 have four transmembrane regions and a serine–threonine kinase domain in the C-terminus (Kendrick and Chang, 2008). Binding of C_2H_4 to the receptors occurs in the hydrophobic N-terminal part of the receptor dimer and requires a copper co-factor (Hall et al., 2007). The signaling pathway of C_2H_4 is controlled by CTR1 (constitutive triple response 1), a serine–threonine protein kinase that acts as a negative regulator, downstream of the receptor and upstream of EIN2. C_2H_4 binding results in the inactivation of the receptor–CTR1 complex, and in turn allows activation of a kinase cascade controlling EIN2 and its transcription factors in the nucleus such as EIN3, EIL1, ethylene-responsive element binding proteins (EREBPs)/ethylene-responsive factors (ERFs), which activate the transcription of ethylene-responsive genes (Wang et al., 2002; Liu et al., 2004; Rzewuski and Sauter, 2008; Yoo et al., 2008; Stepanova and Alonso, 2009). EIN2 works downstream of CTR1 and upstream of EIN3 (Alonso et al., 1999). Recently, Qiao et al. (2009) demonstrated that EIN2 protein level is regulated through its degradation by the proteasome in the presence of the hormone via 2 F-Box proteins ETP1 and ETP2; in the presence of C_2H_4, ETP1 and ETP2 levels are low, thus increasing EIN2 protein level.

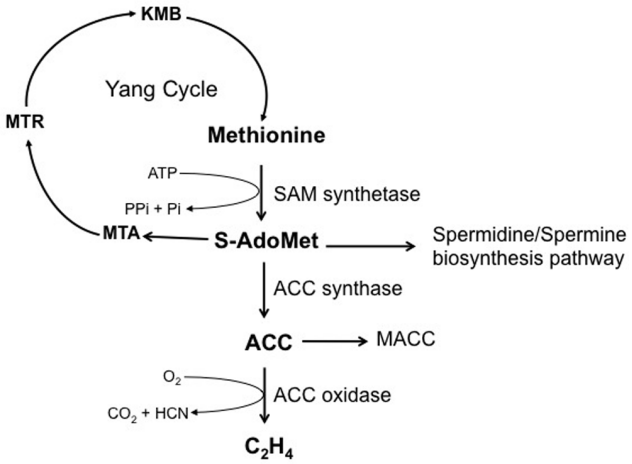

FIGURE 3 | Ethylene biosynthesis pathway. *S*-adenosyl-methionine (*S*-AdoMet) is synthesized from the methionine by the *S*-adenosyl-methionine synthetase (SAM synthetase) with one ATP molecule expensed per *S*-AdoMet synthesized. *S*-AdoMet is then converted to 1-aminocyclopropane-1-carboxylic acid (ACC) by ACC synthase, 5′-methylthioadenosine (MTA) being a by-product. MTA is recycled to methionine by successive enzymatic reactions involving various intermediates (MTR, 5-methylthioribose; KMB, 2-keto-4-methylthiobutyrate), which constitute the methionine (Yang) cycle. *S*-AdoMet is also the precursor of the spermidine/spermine biosynthesis pathway. Ethylene production is catalyzed by the ACC oxidase using ACC as substrate, and generates carbon dioxide and hydrogen cyanide. Malonylation of ACC to malonyl-ACC (MACC) reduces ACC content and consequently ethylene production.

SEED RESPONSIVENESS TO EXOGENOUS ETHYLENE

The influence of ethylene on seed germination is well documented (Corbineau and Côme, 1995; Kepczynski and Kepczynska, 1997; Matilla, 2000; Matilla and Matilla-Vazquez, 2008). Ethylene, ethephon (an ethylene-releasing compound), or ACC (the precursor of ethylene) stimulate seed germination in numerous

species, among which several parasitic plants such as *Orobanche ramosa* (Chun et al., 1979) and some *Striga* species (Egley and Dale, 1970; Bebawi and Eplee, 1986). Application of ethylene promotes germination of either primary dormant or secondary dormant seeds (**Table 1**). It breaks seed coat-imposed dormancy in cocklebur (*Xanthium pennsylvanicum*; Katoh and Esashi, 1975; Esashi et al., 1978), subterranean clover (*Trifolium subterraneum*; Esashi and Leopold, 1969), *Rumex crispus* (Taylorson, 1979) and *Arabidopsis* (Siriwitayawan et al., 2003), and embryo dormancy in apple (*Malus domestica*; Kepczynski et al., 1977; Sinska and Gladon, 1984), sunflower (*Helianthus annuus*; Corbineau et al., 1990), and beechnut (*Fagus sylvatica*; Calvo et al., 2004a). It can also overcome thermodormancy in lettuce (Abeles, 1986) or secondary dormancy in sunflower (Corbineau et al., 1988), *Amaranthus caudatus* (Kepczynski et al., 1996a), and *Amaranthus paniculatus* (Kepczynski and Kepczynska, 1993). Likewise, it stimulates germination of non-dormant seeds placed in non-optimal conditions (Kepczynski and Kepczynska, 1997; Matilla, 2000). For example, it can overcome the inhibition of germination imposed by high temperatures (Abeles, 1986; Gallardo et al., 1991) or osmotic agents (Negm and Smith, 1978; Kepczynski and Karssen, 1985), and alleviates the salinity effect in numerous halophytes (Khan et al., 2009).

The stimulatory effect of exogenous ethylene increases with hormone concentration, and the efficient concentrations range from 0.1 to 200 $\mu L\ L^{-1}$, depending on species and depth of their dormancy. Ethylene at 1.25 $\mu L\ L^{-1}$ allows 100% germination of dormant *Arabidopsis* seeds incubated at 25°C in darkness, when dormant sunflower seeds required 12.5 $\mu L\ L^{-1}$ to fully germinate at 15°C. Breaking of dormancy during chilling of apple seeds, or during dry storage of sunflower achenes, results in an increasing sensitivity to ethylene (Sinska, 1989; Corbineau and Côme, 2003). In *Stylosanthes humilis*, non-dormant seeds are at least 50-fold more sensitive to ethylene than freshly harvested dormant ones (Ribeiro and Barros, 2006). Improvement of dormant seed germination does not require a continuous application of ethylene; a short treatment in the presence of this compound is sufficient to improve germination of dormant seeds in various species (Schönbeck and Egley, 1981; Corbineau and Côme, 2003; Kepczynski et al., 2003). Seed responsiveness to ethylene decreases during prolonged pre-incubation under conditions favoring the maintenance of dormancy, probably due to an induction of a secondary dormancy (Speer et al., 1974; Esashi et al., 1978; Jones and Hall, 1984; Corbineau and Côme, 2003).

INVOLVEMENT OF ETHYLENE BIOSYNTHESIS AND SIGNALING IN SEED GERMINATION

Ethylene production begins as the imbibition phase starts and increases with the germination progression. Its development differs among species (reviewed in Kepczynski and Kepczynska, 1997; Matilla, 2000; Matilla and Matilla-Vazquez, 2008), however, the radicle protrusion through the seed coat is always associated with a peak of ethylene release. A close relationship between the ability to produce ethylene and seed vigor has been reported in numerous species (Samimy and Taylor, 1983; Gorecki et al., 1991; Khan, 1994; Chonowski et al., 1997), and ACC-dependent C_2H_4 production was proposed as a marker of seed quality (Corbineau, 2012).

Table 1 | Species whose seed dormancy is broken by ethylene or ethephon, an ethylene-releasing compound, or 1-aminocyclopropane-1-carboxylic acid (ACC).

Species	Type of dormancy	Reference
Amaranthus caudatus	Primary and secondary dormancies	Kepczynski and Karssen (1985); Kepczynski et al. (1996a, 2003)
Amaranthus paniculatus	Secondary dormancy	Kepczynski and Kepczynska (1993)
Amaranthus retroflexus	Primary dormancy	Kepczynski et al. (1996b)
Arabidopsis thaliana	Primary dormancy	Siriwitayawan et al. (2003)
Arachis hypogaea	Primary dormancy	Ketring and Morgan (1969)
Chenopodium album	Primary dormancy	Machabée and Saini (1991)
Fagus sylvatica	Embryo primary dormancy	Calvo et al. (2004a)
Helianthus annuus	Embryo primary dormancy	Corbineau et al. (1990)
	Secondary dormancy	Corbineau et al. (1988)
Lactuca sativa	Thermodormancy	Speer et al. (1974)
	Secondary dormancy	Abeles (1986)
Pyrus malus	Embryo primary dormancy	Kepczynski et al. (1977); Sinska and Gladon (1984)
Rumex crispus	Primary and secondary dormancies	Taylorson (1979); Samimy and Khan (1983)
Stylosanthes humilis	Primary dormancy	Ribeiro and Barros (2006)
Trifolium subterraneum	Primary dormancy	Esashi and Leopold (1969)
Xanthium pennsylvanicum	Primary and secondary dormancies	Katoh and Esashi (1975); Esashi et al. (1978)

Ethylene production depends on both ACS activity that modulates ACC content, and the activity of ACO, the key enzyme that converts ACC into ethylene. Evolution of ethylene production during germination is associated with an increase in ACO activity, as well as a progressive accumulation of *ACS* and *ACO* transcripts, with generally a sharp increase during endosperm rupture or/and radicle protrusion (Gomez-Jimenez et al., 1998; Matilla and Matilla-Vazquez, 2008; Linkies et al., 2009; Iglesias-Fernandez and Matilla, 2010; Linkies and Leubner-Metzger, 2012). In *Sisymbrium officinale*, *SoACS7* level is very low during seed imbibition, a more notable expression being detected when endosperm rupture reached 50–100%, whereas *SoACO2* expression is detected at early stages during seed imbibition, and then rises during the germination process (Iglesias-Fernandez and Matilla, 2010). Similarly, expression of *PsACO1* in pea (*Pisum sativum*; Petruzzelli et al., 2003) and *BrACO1* in turnip (*Brassica rapa*; Rodriguez-Gacio et al., 2004) is maximal at radicle emergence. In two Brassicaceae species, *Arabidopsis* and *Lepidium sativum*, *ACO1* and *ACO2* have been demonstrated to be the major *ACOs* involved in ethylene synthesis in seeds (Linkies et al., 2009; Linkies and Leubner-Metzger, 2012). In *Lepidium sativum*, the correlation between *ACO1* and *ACO2* transcript accumulation with *in vivo* ACO enzyme activity suggests that *ACO* is regulated at the transcriptional level during germination.

Ethylene has been shown to regulate its own synthesis by inducing *ACO* transcription (Lin et al., 2009). It is required for the stimulation of *ACO* gene expression in pea (Petruzzelli et al., 2000, 2003), beechnut (Calvo et al., 2004b), and turnip (Puga-Hermida et al., 2003). In contrast, expression of *SoACS7* in *Sisymbrium officinale* and *PsACS1* in pea is not affected (Petruzzelli et al., 2000, 2003; Iglesias-Fernandez and Matilla, 2010).

Induction of thermodormancy is often associated with a reduced ethylene production, which may result in chickpea (*Cicer arietinum*) from a greater ACC-malonyltransferase activity and an *S*-AdoMet channeling toward the polyamine pathway, thus reducing ethylene precursor availability (Martinez-Reina et al., 1996), or also from ACO activity inhibition, as observed in chickpea and sunflower (Corbineau et al., 1988; Gallardo et al., 1991). Incubation at high temperature (35°C) of lettuce seeds induces a reduction in ethylene production (Prusinski and Khan, 1990), associated with a complete repression of *LsACS1* and a reduced expression of *ACO-A* (homologous to *AtACO4*; Argyris et al., 2008).

In contrast, treatments (chilling, GA, HCN…) that break seed dormancy often lead to an increase in ethylene production (reviewed in Kepczynski and Kepczynska, 1997; Matilla and Matilla-Vazquez, 2008). Cyanide treatment, which breaks embryo dormancy in apple and sunflower, stimulates ethylene production (Oracz et al., 2008; Gniazdowska et al., 2010). In apple 5-day-old seedlings, it increases ACS and ACO activities (Bogatek et al., 2004), whereas in sunflower it reduces *in vivo* ACC-dependent ethylene production (i.e., *in vivo* ACO activity) and *HaACS* and *HaACO* expression (Oracz et al., 2008). However, in *Arabidopsis*, cold stratification down-regulates the expression of *ACOs*, but results in transient expression of ACS (Narsai et al., 2011; Linkies and Leubner-Metzger, 2012).

Studies using inhibitors of ACS activity (AVG: aminoethoxyvinylglycine; AOA: amino-oxyacetic acid), ACO activity ($CoCl_2$; α-AIB: α-aminoisobutyric acid), or ethylene action (2,5 NBD: 2,5-norbornadiene; STS: silver thiosulfate) demonstrated that ethylene evolved by seeds plays a promotive role in germination and dormancy breakage (Kepczynski et al., 1977, 2003; Sinska and Gladon, 1989; Corbineau et al., 1990; Esashi, 1991; Longan and Stewart, 1992; Gallardo et al., 1994; Hermann et al., 2007). Conversely, application of exogenous ACC stimulates germination of various ethylene-sensitive seeds such as lettuce (Fu and Yang, 1983), sunflower (Corbineau et al., 1990), cocklebur (Satoh et al., 1984), *Amaranthus caudatus* (Kepczynski, 1986) and *Amaranthus retroflexus* (Kepczynski et al., 1996b), chickpea (Gallardo et al., 1994), sugar beet (*Beta vulgaris*; Hermann et al., 2007). Thermodormancy in lettuce, *Amaranthus caudatus* and chickpea is also reversed by exogenous ACC (Gallardo et al., 1996; Kepczynski et al., 2003). This stimulatory effect of ACC suggests that dormancy might be related to low C_2H_4 production due to insufficient levels of endogenous ACC, i.e., low ACS activity.

Analysis of mutant lines altered in ethylene biosynthesis and signaling pathway demonstrated the involvement of ethylene in regulating seed germination. Mutations in *ETHYLENE RESISTANT1* (*ETR1*) and *ETHYLENE INSENSITIVE2* (*EIN2*) genes result in poor germination and deeper dormancy compared to wild type, in contrast *constitutive triple response1* (*ctr1*) seeds germinate slightly faster (Bleecker et al., 1988; Leubner-Metzger et al., 1998; Beaudoin et al., 2000; Subbiah and Reddy, 2010). *ERFs* genes might also play a key (pivotal) role in ethylene responsiveness and germination regulation (Leubner-Metzger et al., 1998; Pirrello et al., 2006). In beechnut, Jimenez et al. (2005) demonstrated that the expression of *FsERF1*, a transcription factor involved in C_2H_4 signaling and sharing high homology with *Arabidopsis ERFs*, increases during dormancy release in the presence of ethephon or after chilling. In sunflower, *ERF1* expression is fivefold higher in non-dormant than in dormant embryos, and also markedly stimulated by gaseous HCN, which breaks dormancy (Oracz et al., 2008). Beechnut *FsERF1* is almost undetectable in dormant seeds incubated under high temperature conditions that maintain dormancy, or in the presence of germination inhibitors, either ABA or AOA, an inhibitor of ethylene biosynthesis, but increases during moist chilling that progressively breaks dormancy (Mortensen et al., 2004; Jimenez et al., 2005). In tomato (*Solanum lycopersicon*), *SlERF2* transcript accumulation is higher in germinating seeds than in non-germinating ones, and its overexpression in transgenic lines results in premature seed germination (Pirrello et al., 2006). Interestingly, in lettuce seeds, expression of genes involved in ethylene signaling (*CTR1*, *EIN2*, and *ETR1*) is less affected by high temperature than that of biosynthesis genes (*ACS* and *ACO*; Argyris et al., 2008).

CROSSTALK BETWEEN ETHYLENE AND ABA

Effect of ABA on ethylene metabolism

The antagonistic interaction between ABA and C_2H_4 during germination was demonstrated in numerous species (Leubner-Metzger et al., 1998; Beaudoin et al., 2000; Ghassemian et al., 2000; Kucera et al., 2005; Matilla and Matilla-Vazquez,

2008). In *Arabidopsis* and *Lepidium sativum*, ethylene counteracts the inhibitory effects of ABA on endosperm cap weakening and endosperm rupture (Linkies et al., 2009). ABA also increases the ethylene requirement to release primary and secondary dormancies (Kepczynski and Kepczynska, 1997; Corbineau and Côme, 2003; Kepczynski et al., 2003). Inhibition of germination by ABA is associated with a reduction in ethylene production (Kepczynski and Kepczynska, 1997; Matilla, 2000). ABA clearly inhibits *in vivo* ACO activity, and this inhibition correlates with a decreased accumulation of *ACO* transcripts (Bailly et al., 1992; Petruzzelli et al., 2000, 2003; Linkies et al., 2009). In *Arabidopsis*, the accumulation of *ACO1* transcripts in both the embryo and endosperm during germination is inhibited by ABA, and the high levels of *ACO1* transcripts in ABA-insensitive mutants suggests the regulation of *ACO* expression by ABA (Penfield et al., 2006; Carrera et al., 2008; Linkies et al., 2009). In the embryo, *ACO2* transcript accumulation is also inhibited by ABA (Penfield et al., 2006). In *Lepidium sativum*, inhibition of both *ACO1* and *ACO2* by ABA is restricted to the endosperm cap (Linkies et al., 2009). In accordance, microarray analysis in *Arabidopsis aba2* mutant detected an up-regulation of *ACO* transcript accumulation (Cheng et al., 2009). Moreover, inhibition of shoot growth in tomato ABA-deficient mutants, *flacca* and *notabilis*, and in *Arabidopsis aba2* results from increased ethylene production (Sharp et al., 2000; LeNoble et al., 2004). In contrast to pea, chickpea, *Lepidium sativum*, and *Arabidopsis*, there is an ABA-mediated up-regulation of ACC accumulation and *ACO* expression in sugar beet seeds (Hermann et al., 2007).

Effect of ethylene on ABA metabolism and signaling

Treatment with exogenous ethylene or ACC does not affect ABA content nor expression of genes involved in ABA biosynthesis in *Lepidium sativum* (Linkies et al., 2009) and sugar beet (Hermann et al., 2007). Nevertheless, seeds of *Arabidopsis* ethylene-insensitive mutants, *etr1* and *ein2*, exhibit higher ABA content than wild type and consistently germinate more slowly (Kende et al., 1998; Beaudoin et al., 2000; Ghassemian et al., 2000; Chiwocha et al., 2005; Wang et al., 2007). ABA-GE levels are reduced in *etr1–2* seeds; increased ABA accumulation might therefore be attributed to a decrease in ABA conjugation (Chiwocha et al., 2005). However, ethylene may also regulate other enzymatic steps, since a microarray analysis reported *NCED3* up-regulation in *ein2* and *CYP707A2* down-regulation in *etr1-1* (Cheng et al., 2009). High ABA levels in *ein2* were also associated with an up-regulation of *ABA1* (Wang et al., 2007), which was, however, not detected on microarrays (Cheng et al., 2009).

Several reports suggest that, during germination, ethylene not only acts on ABA metabolism to reduce ABA levels, but also negatively regulates ABA signaling (Gazzarrini and McCourt, 2001; Kucera et al., 2005). Indeed, mutations that reduce ethylene sensitivity (e.g., *etr1*, *ein2*, and *ein6*) result in an increase in ABA sensitivity, while increased ethylene sensitivity in *ctr1* and *eto1* reduces ABA sensitivity (Beaudoin et al., 2000; Ghassemian et al., 2000; Brady and McCourt, 2003; Chiwocha et al., 2005; Kucera et al., 2005; Linkies et al., 2009; Subbiah and Reddy, 2010). Mutations in *CTR1*, for example, enhance the ABA insensitivity of *abi1-1* seeds, when C_2H_4-insensitive mutants like *ein2* reduce it (Beaudoin et al., 2000). However, no significant difference in ABA sensitivity is observed in *ein3*, *ein4*, *ein5*, and *ein7* (Subbiah and Reddy, 2010).

In addition, overexpression in *Arabidopsis* seeds of a beechnut tyrosine phosphatase, *FsPTP1*, reduces dormancy, through both ABA signaling down-regulation and *EIN2* up-regulation, suggesting that the negative role of *FsPTP1* in ABA signaling might result from modulation of C_2H_4 signaling (Alonso-Ramirez et al., 2011). This central role of EIN2 in mediating cross-links between hormonal response pathways has also been reported in plant response to abiotic and biotic stresses (Wang et al., 2007).

Despite the existence of interactions between the ABA and ethylene signaling pathways, genetic evidence indicates that they may mainly act in parallel, since double mutants obtained by crossing ethylene mutants (*ctr1*, *ein1*, *ein3*, and *ein6*) with the *aba2* mutant exhibit phenotypes resulting from both ABA deficiency and altered ethylene sensitivity (Cheng et al., 2009).

NITRIC OXIDE HOMEOSTASIS, SIGNALING AND CROSSTALK WITH ABA AND ETHYLENE

NITRIC OXIDE: CHEMICAL NATURE AND REACTIVITY

Nitric oxide is an inorganic, uncharged, gaseous free radical that can readily diffuse through cell membranes. Upon production, released NO can adjust to the cellular redox environment leading to the formation of diverse biologically active compounds referred to as reactive nitrogen species (RNS; Stamler et al., 1992). Thus, its biological half-life is assumed to be in the order of seconds depending on the redox environment and the initial amount (Saran et al., 1990). While NO production can be beneficial at relatively low levels, uncontrolled accumulation, referred to as nitrosative stress, can result in detrimental consequences in plant cells. A strict control of NO levels is therefore required for cell survival. The regulation of NO biosynthesis, localization, and duration along with the control of NO removal (or storage) is therefore of paramount importance in determining the biological consequences of NO accumulation and thus for its role as secondary messenger (Besson-Bard et al., 2008; Moreau et al., 2010; Baudouin, 2011). The chemical reactivity of NO makes it an unusual signal molecule that can readily act on a wide range of targets, especially proteins (Besson-Bard et al., 2008). The signal it mediates can also be modulated along the signal transduction pathways depending on the biological environment, thus adding to the complexity of NO signaling.

THE DISTINCT PATHWAYS FOR NITRIC OXIDE BIOSYNTHESIS IN PLANTS AND THEIR RELATIVE CONTRIBUTION IN SEEDS

Due to their importance as basis for NO-mediated signaling, the biosynthesis pathways of NO in plants have been the subjects of intense investigations during the last decade (Besson-Bard et al., 2008; Corpas et al., 2009; Moreau et al., 2010; Gupta et al., 2011). The existence of several sources of NO associated with enzymatic or non-enzymatic reactions has been reported but only a few have been completely elucidated so far. Here we will mainly focus on the reactions proven or suggested to be relevant in the context of seed physiology (**Figure 4**), as NO synthesis was previously reviewed in Simontacchi et al. (2007) and Sirova et al. (2011).

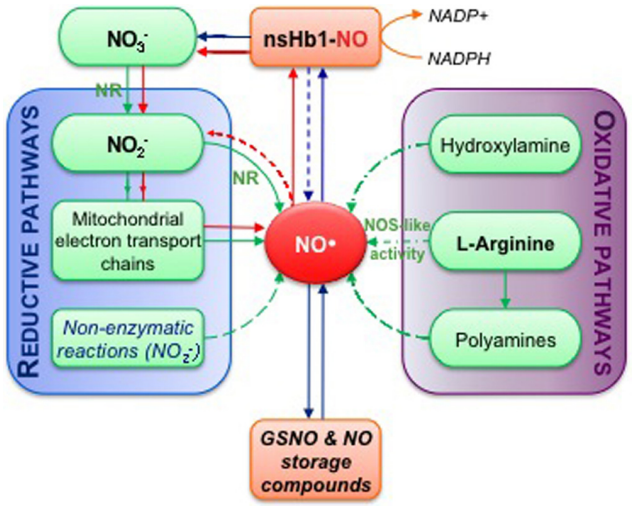

FIGURE 4 | Simplified overview of NO biosynthesis and homeostasis in plant cells. This scheme is inspired from Moreau et al. (2010). Nitrate (NO_3^-) assimilation produces nitrite (NO_2^-) in a reaction catalyzed by nitrate reductase (NR). The subsequent reduction of nitrite into NO can occur enzymatically, either through NR activity or mitochondrial electron transport chains, and *via* non-enzymatic reactions (reductive pathways). Alternatively, NO synthesis can result from oxidative reactions from hydroxylamine, polyamines or L-arginine (L-Arg; oxidative pathways). NO synthesis from L-Arg could account for the nitric oxide synthase-like (NOS-like) activity detected in plants. The pool of NO is then influenced by non-symbiotic hemoglobin 1 (nsHb1) dioxygenase activity, which converts NO into NO_3^-. NO can also react with reduced glutathione or thiol groups leading to the reversible formation of *S*-nitrosothiols (e.g., GSNO, *S*-nitrosoglutathione; *S*-nitrosylated proteins). Red arrows highlight the so-called nitrate-NO cycle that may take place under hypoxia. Green arrows correspond to biosynthesis reactions while blue arrows indicate reactions involved in NO homeostasis.

Nitric oxide synthase-like activity

In animals, NO biosynthesis is mainly catalyzed by three isoforms of NO synthase (NOS; Alderton et al., 2001). These enzymes metabolize L-arginine (L-Arg) into L-citrulline and NO *via* the following reaction:

$$\text{L-Arg} + \text{NAD(P)H,H}^+ + \text{O}_2 \Rightarrow$$
$$\text{L-citrulline} + \text{NAD(P)}^+ + \text{H}_2\text{O} + \text{NO}$$

To date, despite the identification of a green alga NOS (Foresi et al., 2010), the search for a NOS homolog enzyme in higher plants only encountered failure, although biochemical assay highlighted the existence of a NOS-like activity in several plant tissues and organelles (Fröhlich and Durner, 2011). Moreover, exogenous application of NOS inhibitors (structural analogs of L-Arg such as L-NAME, *N*-nitro-L-arginine methyl ester) significantly reduced NO release under diverse conditions in several plant species (Crawford, 2006). Using these approaches, a NOS-like activity was detected in sorghum (*Sorghum bicolor*) and soybean (*Glycine max*) imbibed seeds (Simontacchi et al., 2007). Nonetheless, the liability of such proofs is now debated in light of the discovery of other L-Arg-dependent NO synthesis pathways (Tun et al., 2006). Moreover, the recent finding that L-NAME can affect NO production by interfering with nitrate reductase (NR) activity discredits its use as a NOS inhibitor in plants (Rasul et al., 2012). Thus, after more than a decade of intense research in the area and despite the proven occurrence of L-Arg-dependent NO biosynthesis, the mere existence of NOS is now questioned in plants (Fröhlich and Durner, 2011).

Nitrate reductase

Apart from its well-known role in nitrate reduction and assimilation, the cytosolic NR has been shown to catalyze the reduction of nitrite to NO, using NAD(P)H as electron donor, both *in vitro* and *in vivo*, *via* the following reaction (Yamasaki et al., 1999; Rockel et al., 2002):

$$\text{NAD(P)H} + 3\text{H}_3\text{O}^+ + 2\text{NO}_2^- \Rightarrow \text{NAD(P)}^+ + 2\text{NO} + 5\text{H}_2\text{O}$$

In vivo, NR would be responsible at least in part for the basal level of NO production with a low reduction efficiency (in the order of 1% of the total NR activity). However, the nitrite reductase activity of NR (NR-NiR) can drastically increase under certain conditions such as oxygen deprivation (Rockel et al., 2002). Overall, conditions leading to NR-mediated nitrite production exceeding the rate of nitrite removal can lead to a substantial increase in NO production by NR. Both the nitrate and nitrite reductase activities of NR are tightly controlled by post-translational modifications (PTM; Lillo et al., 2004; Park et al., 2011; Wang et al., 2011). In *Arabidopsis*, NR and NR-NiR activities are stimulated by sumoylation mediated by the E3 SUMO ligase AtSIZ1 (Park et al., 2011). Furthermore, the H_2O_2-induction of NO biosynthesis in *Arabidopsis* roots was recently proposed to depend on mitogen-activated protein kinase 6 (MPK6)-mediated phosphorylation of one of the NR isoforms (Ser 627 in *Arabidopsis* NIA2; Wang et al., 2010, 2011). Moreover, NO was reported to inhibit NR activity in wheat leaves (Rosales et al., 2011). In *Arabidopsis* seedlings, GA may also negatively regulate light-induced NR activity at post-translational level (Zhang et al., 2011).

Distinct studies reported an implication of NR in NO-mediated signal transduction pathways (Bright et al., 2006; Neill et al., 2008; Gupta et al., 2011). In seeds, the NO-mediated positive effect of NO_2^- and NO_3^- on dormancy release supports an involvement of nitrite-dependent reductive pathways in NO biosynthesis, possibly via NR-NiR activity or at least depending on NR activity in the case of exogenous NO_3^- (Bethke et al., 2006b). Accordingly, NR activity was detected concomitantly with a NOS-like activity in soybean and sorghum embryonic axes, both enzymatic activities appeared to parallel the accumulation of NO upon seed imbibition (Simontacchi et al., 2007).

In *Arabidopsis*, NR is encoded by two homologous genes, *NIA1* and *NIA2* (Wilkinson and Crawford, 1991). The relative contribution of these two isoforms to NO production was suggested to differ with a possible predominant involvement of NIA1 in NO production (Baudouin, 2011). Despite NO has been demonstrated to break seed dormancy (Bethke et al., 2006b; Liu et al., 2009), NR involvement in *Arabidopsis* seed germination remains unclear. Two distinct research groups assessed the germination characteristics of the *nia1nia2* double mutant (also named G′4-3), obtained by Wilkinson and Crawford (1993). In the first study, G′4-3 seeds

were found to be less dormant than wild type seeds (Alboresi et al., 2005), but more dormant in the second (Lozano-Juste and Leon, 2010). Differences in culture environments of mother plants, germination conditions or duration of seed storage may explain these contrasted results (Clerkx et al., 2004; Matakiadis et al., 2009).

Polyamines and hydroxylamines

Upon exogenous application of the polyamines, spermine (spm) and spermidine (spd), a rapid NO production from *Arabidopsis* seedlings has been observed under aerobic conditions (Tun et al., 2006). In plants, the tri-amine Spd and tetra-amine Spm are formed by successive additions of aminopropyl groups [resulting from *S*-AdoMet decarboxylation] to the diamine putrescine (Put; reviewed in Wimalasekera et al., 2011a). Put can be synthesized either from L-Arg (by L-Arg decarboxylase) or from L-ornithine (by ornithine decarboxylase). However, as *Arabidopsis* lacks ornithine decarboxylase activity, polyamines are exclusively produced from L-Arg (Hanfrey et al., 2001). Thus, NO biosynthesis from polyamines can be considered as a L-Arg-dependent pathway in *Arabidopsis*.

Plant cells are also able to produce NO through hydroxylamine oxidation and this reaction is promoted by reactive oxygen species (ROS) accumulation (Rümer et al., 2009). Thus, NO might be responsible for the positive effect of exogenous hydroxylamines on seed germination (Hendricks and Taylorson, 1974). However, the relevance of such pathway to NO synthesis remains unclear.

Nitric oxide production in the apoplast

The existence of a root specific plasma membrane nitrite-NO reductase (Ni-NOR) was reported in tobacco (*Nicotiana tabacum*; Stöhr et al., 2001). This enzyme would catalyze the reduction of nitrite into NO in the apoplast and could act in tandem with a plasma membrane-bound NR (PM-NR; Eick and Stöhr, 2012). Its implication has been proposed in several physiological processes in roots (Stöhr and Stremlau, 2006), but has not been so far investigated in seeds.

The non-enzymatic reduction of nitrite to NO can also occur under acidic pH and could be promoted by the presence of reductants (Mallick et al., 2000):

$$2HNO_2 \Rightarrow NO + NO_2 + H_2O \Rightarrow 2NO + 2O_2 + H_2O$$

This non-enzymatic reaction may be of paramount importance in seeds as an intense NO production was observed during early *Arabidopsis* seed imbibition next to the aleurone layer (Liu et al., 2009). Sodium nitroprusside (SNP) releases dormancy by generating both NO and cyanide. In C24 dormant seeds, the cell impermeable NO scavenger, cPTIO (2-(4-carboxyphenyl)phenyl-4,4,5,5-tetramethylimidazoline-1-oxyl 3-oxide), was demonstrated to efficiently impede SNP dormancy release, suggesting that the apoplast might be either an important pathway for NO movement or a site for NO production (Bethke et al., 2006b).

Mitochondrial respiration

Depending on the oxygen availability, several hemeproteins can either act as NO scavengers or NO producers. In hypoxic mitochondria, deoxyhemeproteins can catalyze a NR-independent nitrite reduction into NO using electrons from the electron transport chain (Planchet et al., 2005). The re-oxidation of NO into nitrite can then occur either non-enzymatically inside the mitochondria, or in the cytosol, through the nicotinamide adenine dinucleotide phosphate (NADPH)-dependent dioxygenase activity of class-1 non-symbiotic hemoglobin (nsHb1) that metabolizes NO into nitrate, which is subsequently reduced into nitrite by NR (Igamberdiev and Hill, 2004; Perazzolli et al., 2004). These reactions constitute the so-called hemoglobin-NO cycle (displayed in red in **Figure 4**; Igamberdiev et al., 2010). nsHb1 proteins participate in NO scavenging, thereby playing an essential role in NO homeostasis. Accordingly, modulation of nsHb1 expression in plants was shown to directly impact NO levels at distinct developmental stages including in seeds (Hebelstrup and Jensen, 2008; Thiel et al., 2011) and in diverse environmental conditions (Dordas, 2009; Cantrel et al., 2011).

The very active mitochondrial respiration upon seed imbibition may result in an oxygen consumption exceeding the atmospheric diffusion, thus leading to localized hypoxia in germinating seeds (Benamar et al., 2008). In such conditions, nitrite-dependent NO production may occur in mitochondria and modulate respiration through reversible NO-mediated inhibition of cytochrome *c* oxidase (COX), thereby regulating oxygen consumption to avoid anoxia (Benamar et al., 2008). Therefore, this nitrite-dependent NO biosynthesis in mitochondria may be of significant importance in germinating seeds. However, its possible role in NO-mediated dormancy release has not yet been established.

Overall, current evidence supports the co-existence of several distinct NO biosynthesis pathways in seeds. Their relative contribution is probably highly dependent on both oxygen and ROS levels that may change along the time-course of imbibition. Further investigations will be required to elucidate the regulation of NO accumulation during seed imbibition.

S-nitrosoglutathione: a reversible "storage" pool of nitric oxide?

As for plant hormones, any mechanism directly influencing NO levels besides biosynthesis pathways may have a pivotal role in the regulation of NO signaling. In particular, since NO can react with reduced glutathione (GSH) to form *S*-nitrosoglutathione (GSNO), GSNO has been proposed to constitute a storage and transport form for NO in plants and seeds (Sakamoto et al., 2002). Such modulation of NO storage pool would have a significant impact on NO levels. GSNO can further be metabolized by the GSNO reductase (GSNOR). Accordingly, *gsnor* mutants have multiple phenotypes suggesting GSNOR involvement in several growth and developmental processes including seed germination (Lee et al., 2008; Holzmeister et al., 2011; Kwon et al., 2012).

MOLECULAR TARGETS OF NITRIC OXIDE IN SEEDS

Due to its chemical nature, NO is highly reactive and can interact with diverse molecules in plant cells. A number of NO-regulated genes have been identified in plants (Besson-Bard et al., 2009). These genes encode proteins involved in a wide range of functions from signal transduction to stress responses. However, the main challenge remains to pinpoint the direct molecular targets of NO, which are still poorly documented in plants. However, it is generally assumed that proteins constitute direct relevant NO targets.

Besides its capacity to bind to transition metals of metalloproteins, NO can cause protein PTM, such as cysteine *S*-nitrosylation or tyrosine nitration (Moreau et al., 2010). These modifications remain poorly characterized in plants and particularly in seeds. However, as discussed below, there is strong experimental evidence indicating that NO signaling in seeds could principally rely on PTM of specific proteins (Delledonne, 2005).

Many *S*-nitrosylated proteins identified in plants are implicated in various metabolic processes (Lindermayr et al., 2005; Abat et al., 2008; Romero-Puertas et al., 2008; Tanou et al., 2009; Palmieri et al., 2010). In dry *Arabidopsis* seeds, a β-subunit of the mitochondrial ATP synthase complex was found to be *S*-nitrosylated, suggesting that NO could participate in the regulation of the seed energy status (Arc et al., 2011). In wheat seeds, a parallel increase in NO and protein *S*-nitrosylation was reported during *sensu stricto* germination (Sen, 2010). At least 13 modified proteins were detected, but not identified. In recalcitrant *Antiaris toxicaria* seeds, desiccation impedes subsequent germination by enhancing H_2O_2 accumulation (Bai et al., 2011). This stress is associated with an increased carbonylation and a reduced *S*-nitrosylation of the antioxidant enzymes of the ascorbate-GSH pathway. Conversely, NO pre-treatments promote germination of desiccated seeds through PTM pattern reversion that enhances antioxidant enzyme activities (Bai et al., 2011). The balance between carbonylation and *S*-nitrosylation of these proteins was proposed to act as molecular switch tuning their activity according to the redox environment (Lounifi et al., 2013).

CROSSTALK BETWEEN NO, ETHYLENE, AND ABA

In stomatal guard cells, ABA-induced stomatal closure is mediated by the successive accumulation of ROS and NO, acting as secondary messengers in ABA signaling (Neill et al., 2008). Even though similar actors are present in seeds, the picture is quite different, as both ROS and NO counteract ABA-inhibition of seed dormancy release and germination (Bethke et al., 2006b; Liu et al., 2010). This obvious discrepancy of NO action between seeds and stomata highlights the specificity of the seed signaling pathways (**Figure 2**).

In imbibed seeds, the application of ABA biosynthesis inhibitors, fluridone or norflurazon, reduces ABA neo-synthesis and promotes dormancy release and germination. In tomato seeds, the NO scavenger, cPTIO, was shown to prevent germination stimulation by fluridone (Piterkova et al., 2012). Conversely, in dormant *Arabidopsis* C24 seeds, SNP enhances the positive effect of norflurazon on germination and also decreases seed sensitivity to exogenous ABA (Bethke et al., 2006a). Taken together, these results suggest that NO reduces both ABA accumulation and sensitivity. In agreement, pharmacological experiments demonstrated that NO enhances *CYP707A2* gene expression in *Arabidopsis* seeds (Liu et al., 2009). Indeed, during the first stage of seed imbibition, a rapid accumulation of NO, possibly at the endosperm layer, was suggested as required for rapid ABA catabolism and dormancy breaking. A similar NO accumulation during imbibition was also observed in germinating seeds from other species (Simontacchi et al., 2007). Recently, in *Arabidopsis*, NO was suggested to act upstream of GA in a signaling pathway leading to vacuolation of protein storage vacuoles in aleurone cells, a process inhibited by ABA (Bethke et al., 2007a). Since the growth of isolated embryos was unaffected by NO donors or scavengers, the endosperm layer might be the primary site of NO synthesis and action in seeds, and in accordance was shown to perceive and respond to NO (Bethke et al., 2007a). Besides its effect on the hormonal balance, it has been speculated that NO may accelerate flux through the pentose phosphate pathway by indirectly increasing the oxidation of NADPH (Hendricks and Taylorson, 1974; Bethke et al., 2007b). An increase in glucose catabolism via this pathway may in turn promote dormancy release (Roberts and Smith, 1977).

Several lines of evidence suggest that NO crosstalk with ABA and ethylene may involve protein modifications. Among the proteins recently identified as candidates for a regulation by tyrosine nitration in *Arabidopsis* seedlings (Lozano-Juste et al., 2011), at least two may be involved in the interplay between ABA and NO in seeds. The first one is the Moco sulfurase ABA3 that catalyzes the conversion from the de-sulfo to the sulfo form of the Moco (Wollers et al., 2008). The de-sulfo form of Moco (also call the "oxo" form) is the co-factor of NR, involved in nitrite and NO generation in plants while the sulfo form is the co-factor of the aldehyde oxydase required for the last step of ABA synthesis (Mendel, 2007). If proven, modulation of ABA3 activity by nitration could affect the equilibrium between ABA and NO production in plants. The second protein is the E3 SUMO ligase AtSIZ1 recently demonstrated to stimulate NR and NR-NiR activities, and negatively regulate ABA signaling by ABI5 sumoylation (Miura et al., 2009; Park et al., 2011). Thus, such modifications could have an important impact in seeds. Similarly, PTM contribution in the NO regulation of ethylene action has been also reported. In *Arabidopsis*, the up-accumulation of NO under hypoxia stimulates ethylene biosynthesis, possibly through PTM of key enzymes such as ACS and ACO by *S*-nitrosylation (Hebelstrup et al., 2012). In contrast, ethylene biosynthesis can be reversibly inhibited by NO through *S*-nitrosylation of methionine adenosyltransferase (MAT), leading to the reduction of the *S*-AdoMet pool (Lindermayr et al., 2006).

S-AdoMet is the precursor of ethylene and polyamines, thus a negative feedback regulation may exist between ethylene and the polyamine-dependent NO biosynthesis. Consistently, NO and ethylene accumulation are negatively correlated in ripe fruits (Manjunatha et al., 2012). In addition, exogenous Spm was shown to reduce ethylene production in apple seeds (Sinska and Lewandowska, 1991). Accordingly, an antagonism may exist between a positive polyamine effect mediated by NO and a negative effect due to a competition with ethylene biosynthesis for *S*-AdoMet. Furthermore, a copper amine oxidase (CuAO1) involved in polyamine catabolism has also been shown to regulate NO biosynthesis and participate to ABA signaling (Wimalasekera et al., 2011b). Indeed, seedlings of *Arabidopsis cuao1* mutant are impaired in both polyamine and ABA-induced NO synthesis, and mutant seeds also display a reduced sensitivity to exogenous ABA during germination (Wimalasekera et al., 2011b).

As mentioned above, in Brassicaceae species, ethylene positively regulates seed germination by stimulating the weakening and rupture of seed testa and endosperm by counteracting the inhibitory action of ABA on radicle protrusion (Linkies et al., 2009). In apple embryos, inhibition of ethylene biosynthesis prevents the

promotion of dormancy release and germination by NO donors (Gniazdowska et al., 2007). Dormancy breaking of apple seeds by NO induces a transient production of ROS, stimulating ethylene accumulation thanks to an increase in both ACS and ACO activity (Gniazdowska et al., 2010). NO may also act on ethylene signaling since EREBPs were described as a class of transcription factors induced by NO (Parani et al., 2004). Moreover during tobacco seed germination, EREBP-3 that is transiently induced just before endosperm rupture is stimulated by ethylene and inhibited by ABA (Leubner-Metzger et al., 1998). Therefore, a synergic link seems to exist, at different levels, between NO and ethylene during seed germination, that counteracts ABA action.

CONCLUSION

Significant advances have been recently obtained in the understanding of the ABA and ethylene metabolism and signaling pathways. In contrast, current knowledge on NO biosynthesis, signaling and action is far too incomplete, especially in seeds, and would require further investigation. Future research efforts should also lead to the identification of downstream target genes of signaling components, in order to fully understand how ABA is able to induce and maintain dormancy, or ethylene to stimulate germination. Moreover unraveling the role of post-translational mechanisms will be particularly crucial to developing a deeper understanding of hormonal pathways and deciphering NO regulatory network.

Nitric oxide and ethylene crosstalk with ABA involves interactions at multiple levels in metabolism and signaling pathways. It would be important to discriminate the hierarchy among these signaling pathways, identify major regulatory nodes and determine whether the environmental factors, which regulate germination and dormancy, modulate this hierarchy.

Moreover, control of seed dormancy and germination involves distinct physiological processes, in tissues of different origin, to achieve a coordinated regulation of embryo arrest or growth and surrounding structure maintenance or rupture. Although hormonal signaling networks in seeds and whole plants share common components, sets of specific regulatory factors, among which only few are known, are likely working in restricted seed territories. Current research combining genetic tools and recent technologies including microdissection, transcriptome profiling, high-throughput proteomics, metabolomics, and system biology, should help to identify missing regulatory components and unravel complex interactions between signal transduction pathways.

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A dynamic interplay between phytohormones is required for fruit development, maturation, and ripening

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Plant species that bear fruit often utilize expansion of an ovary (carpel) or accessory tissue as a vehicle for seed dispersal. While the seed(s) develop, the tissue(s) of the fruit follow a common progression of cell division and cell expansion, promoting growth of the fruit. Once the seed is fully developed, the fruit matures and the surrounding tissue either dries or ripens promoting the dissemination of the seed. As with many developmental processes in plants, plant hormones play an important role in the synchronization of signals between the developing seed and its surrounding fruit tissue(s), regulating each phase of fruit development. Following pollination, fruit set is achieved through a de-repression of growth and an activation of cell division *via* the action of auxin and/or cytokinin and/or gibberellin. Following fruit set, growth of the fruit is facilitated through a relatively poorly studied period of cell expansion and endoreduplication that is likely regulated by similar hormones as in fruit set. Once the seeds reach maturity, fruit become ready to undergo ripening and during this period there is a major switch in relative hormone levels of the fruit, involving an overall decrease in auxin, gibberellin, and cytokinin and a simultaneous increase in abscisic acid and ethylene. While the role of hormones in fruit set and ripening is well documented, the knowledge of the roles of other hormones during growth, maturation, and some individual ripening components is sketchy.

Keywords: fruit development, ripening, hormonal regulation

BACKGROUND

The fruiting body of flowering (angiosperm) plants has evolved to best aid seed protection and dispersal. A diverse range of fruit types within angiosperm species exists and these variations are exemplified between fleshy fruits, that have evolved with an enlargement of the tissue surrounding the seed to create attractive flesh for seed dispersing animals, and "dry" fruit, that split open (dehisce) to release the seed *via* abiotic dispersal mechanisms. Evolutionary studies have revealed that plant species producing fleshy fruit have evolved from ancestral dry fruit producing species, suggesting common mechanisms between dry and fleshy fruit (Knapp, 2002). Pulling upon the literature from across different species, we have revealed common trends in the hormonal regulation of the different stages of fruit development (**Figure 1**). In all cases, dry or fleshy fruit undergo a progression of specific steps including: fruit set, fruit growth, maturation, and ripening/senescence. The crosstalk between hormones that occurs during most of these steps is scarce, nevertheless with the advent of genomic and high throughput technologies there has been significant progress in characterizing hormones and the expression of associated downstream genes in both model and non-model organisms. In this review, we aim to give an overview of the way plant hormones interact to control these different developmental steps and the switch(es) between them as well as highlight areas that require further research to understand these complex processes.

FRUIT SET

Fruit set is the first step in fruit development; it is established during and soon after fertilization. Seed bearing plants have a unique double fertilization event with two pollen nuclei fertilizing the embryo and the endosperm (Dumas et al., 1998; Raghavan, 2003; Hamamura et al., 2012). The role of hormones during embryo development and seed maturation has been well reviewed (for example: Gutierrez et al., 2007; Sun et al., 2010). The fertilization event leads to the development of the seed that de-represses cell division and fruit growth in a synchronized manner (review: Fuentes and Vivian-Smith, 2009). Fruit set has traditionally been attributed to the action of three hormones, auxin, and/or gibberellin, and/or cytokinin (Mariotti et al., 2011). Application of these hormones alone can trigger fruit development to a certain extent and, in many plant species, application in combination will induce normal fruit growth even in the absence of fertilization (parthenocarpy; Nitsch, 1952; Crane, 1964; Gillaspy et al., 1993; Vivian-Smith and Koltunow, 1999), indicating that an interplay between these hormones is necessary for fruit set and fruit growth. In many species, auxin and cytokinin levels in the seed increase during seed development until maturity (Nitsch, 1950; Blumenfeld and Gazit, 1970; Varga and Bruinsma, 1976; Yang et al., 2002; Devoghalaere et al., 2012) and in pea, removal of the seed leads to reduced gibberellin biosynthesis in the pericarp (García-Martínez and Carbonell, 1980; Ozga et al., 1992). These observations led

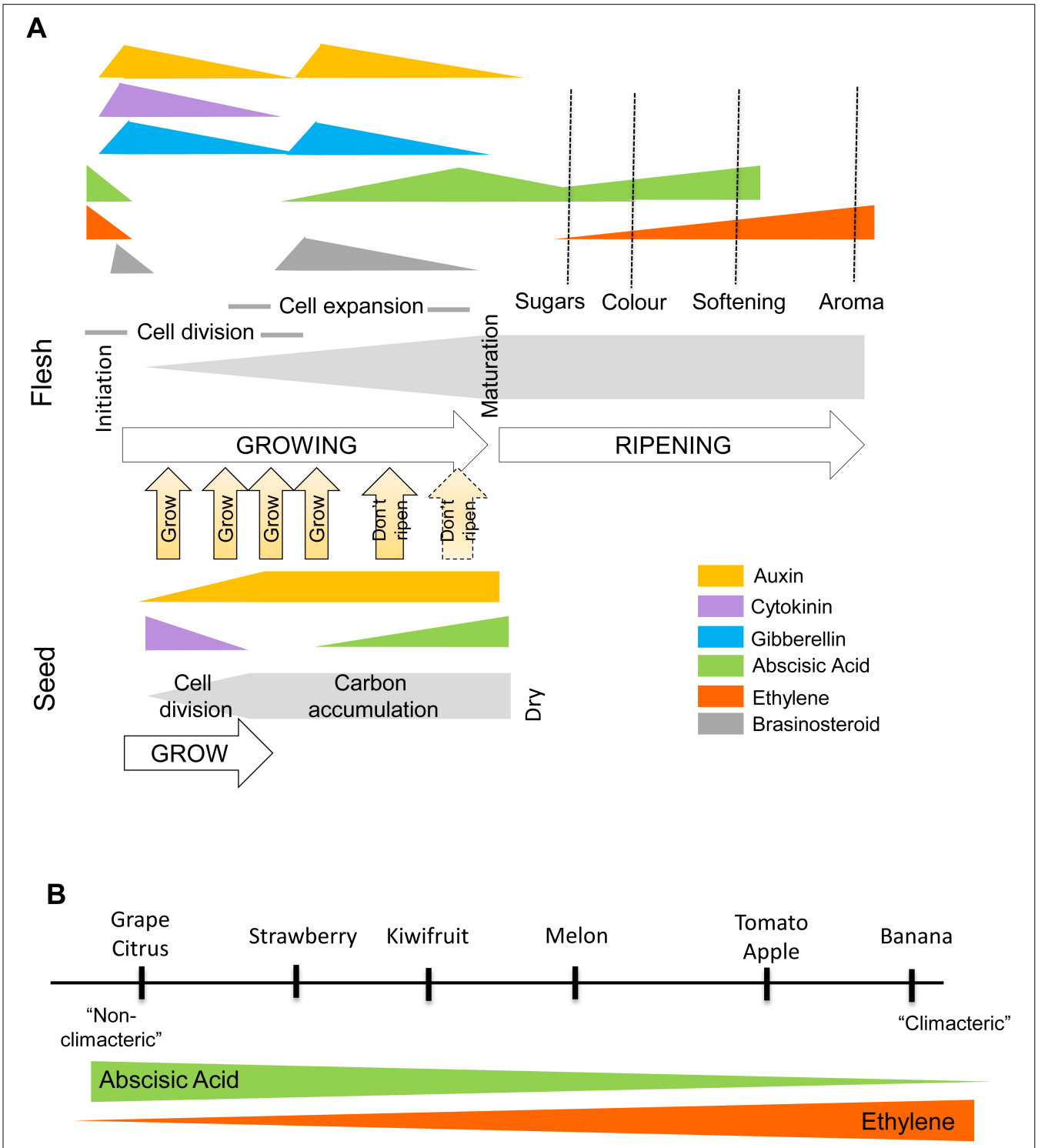

FIGURE 1 | (A) Hormonal changes that occur in a generic fruit during development and ripening. Differential hormone concentrations occur in the seed and the surrounding tissue with the developing seed influencing its environment. Multiple studies have shown that increases in auxin, cytokinin, gibberellin, and brassinosteroid at fruit set, and an involvement of auxin, gibberellin, and brassinosteroid at fruit growth. For fruit maturation there is an inhibition of auxin transport from the seed and increase in ABA. This triggers the ripening/senescence program which leads to an increase in ABA and/or ethylene biosynthesis and response in the surrounding tissue. **(B)** The spectrum of ripening dependencies to ABA and ethylene. All fruit appear to respond to ABA and ethylene. In historically considered "climacteric fruit," ABA indirectly regulates ripening through ethylene. In "non-climacteric" fruit, the ABA has a more dominant role but the fruit still have ethylene-dependant ripening characters.

to the "seed control" hypothesis where the seeds communicate through hormones to the surrounding tissue(s) to promote fruit growth through firstly cell division and later on cell expansion (Ozga et al., 2002).

At the molecular level, the main advances have been on how gibberellin and auxin pathways interact to promote fruit set in both dry fruit, such as *Arabidopsis thaliana* (*Arabidopsis*), and fleshy fruit, such as tomato (de Jong et al., 2009a; Carrera et al., 2012; Ruan et al., 2012). Early studies showed that elevated levels of gibberellins and auxin are present in fruits from plants that exhibit parthenocarpy (Talon et al., 1990) and auxin levels increase during seed development while gibberellin levels increase in the ovaries following fertilization (Olimpieri et al., 2007; Hu et al., 2008). In *Arabidopsis*, fruit development induced by auxin occurs solely through activation of gibberellin signaling and the current, simplified model, of auxin and gibberellin action is the following: auxin, synthesized in the ovules on fertilization is transported to the pericarp where it induces gibberellin biosynthesis (Zhao, 2010). In turn, the newly synthesized gibberellin will lead to the release of growth repression (Fuentes et al., 2012). There are additional layers of regulation, for example, it has been shown that a threshold level of gibberellins in the gynoecium is required to initiate auxin biosynthesis, providing a feedback loop (Vivian-Smith and Koltunow, 1999). Tomato fruit set can be achieved by application of auxin or gibberellin. Auxin appears to act partly through gibberellin, as it can induce gibberellin biosynthesis early during fruit development (Serrani et al., 2008), but each hormone seems to also play a specific role on its own. Auxin-induced fruit contain many more cells compared to gibberellin-induced fruits, which contain fewer larger cells (Bungerkibler and Bangerth, 1983). One of the key players in gibberellin–auxin crosstalk is an auxin response factor (ARF), *SlARF7*, which when mutated causes parthenocarpic fruit development. The mutated fruit display a thick pericarp with large cells having a similar appearance to gibberellin-induced fruit. Molecular analysis has showed that *SlARF7* was partly controlling both auxin and gibberellin signaling (de Jong et al., 2009b, 2011). This pathway was further characterized through the analysis of the tomato *procera* parthenocarpic mutant, with a constitutive gibberellin response, and indicate that activation of the gibberellin signaling pathway after fertilization also controls *SlARF7* expression (Carrera et al., 2012).

Cytokinin levels also increase after pollination (Matsuo et al., 2012). Although cytokinins are generally considered to play a critical role in the stimulation of cell division during fruit development (Wismer et al., 1995; Srivastava and Handa, 2005), very few experimental data support the involvement of this hormone in the initial cell division phase of fruit growth (Mariotti et al., 2011). It is well known that cytokinin promotes cell proliferation at shoot apical meristems and interact closely with auxin (Murray et al., 2012) and are likely to function in a similar manner in the developing gynoecia (Lindsay et al., 2006; Bartrina et al., 2011). A recent study in *Arabidopsis* showed that cytokinin plays at least two roles during fruit development: an early proliferation-inducing role at the medial region of the developing gynoecia and a later role during formation of fruit valve margins (Marsch-Martinez et al., 2012). Finally brassinosteroids might also have a role in fruit set (Fu et al., 2008), however, the interaction with other hormones has not been investigated.

While auxin, gibberellins, and cytokinin levels are increasing at fruit set, abscisic acid (ABA) levels decrease (Hein et al., 1984; Kojima et al., 1993). Consistent with these observations, a transcriptomic analysis showed that mRNA levels of several ABA biosynthesis genes decrease after pollination, while expression of ABA degradation genes increases (Vriezen et al., 2008). ABA has also been shown to counteract the effect of gibberellin on fruit set in pea (García-Martínez and Carbonell, 1980). Expression of ethylene biosynthesis and signaling genes also decrease after pollination while in unpollinated tomato ovaries ethylene biosynthesis and signaling genes are highly expressed.

Overall, these data demonstrate that fruit set relies on a fine balance between plant hormones; the concerted action of auxin and/or gibberellin and/or cytokinin (dependency toward a specific hormone will likely depend on the plant species) will ultimately lead to activation of core cell cycle genes. We can also speculate that ABA and ethylene could have an antagonistic effect on fruit set but this will require further investigation (**Figure 1A**).

FRUIT GROWTH

The developing seed continually sends signals to the surrounding tissue to expand and there is usually a positive correlation between seed number and fruit size (Nitsch, 1970). The developing fruit must also signals back to the rest of the plant so that it is provided with enough nutrients and does not abort. The extent of growth of the fruit from anthesis to maturity is extremely variable; in some species the fruit enlarge relatively little while in others they may increase in volume many thousand times. Unique to fleshy fruit, concomitant with cell expansion, there is an accumulation of storage products and an increase in sugar accumulation (Coombe, 1976). While fruit expansion is a key event, there is little literature covering the role of hormones in the transition for the division to the expansion phases and to the sustained growth of the fruit. Drawing on literature outside the fruit environment it is clear that cell expansion is regulated by auxin, gibberellin, and brassinosteroid (Davies, 2010; Pattison and Catala, 2012).

Cell enlargement depends on both cell wall loosening and increases in turgor pressure (Cosgrove, 2005). While auxin mostly controls cell division during fruit set, it is thought to play an important role during the growth phase by influencing cell enlargement together with gibberellins (Csukasi et al., 2011). In tomato, the maintenance of auxin gradients, through the precise localization of auxin transporters, such as the PIN transporters, will be essential for fruit growth (Pattison and Catala, 2012). A transcriptomic approach focusing on the cell expansion phase revealed that in the growing exocarp and locular tissues, a range of cell wall-related proteins are up-regulated during the expansion stage of the fruit, as well as sugar transport proteins and various glycolytic enzymes. Some genes belonging to the expansins, endo-xyloglucan transferase and pectate lyases families have been shown to be regulated by either auxin, gibberellin, or both in tomato (de Jong et al., 2011; Carrera et al., 2012). A genome-wide approach in apple, focusing on the role of auxin during cell expansion, showed that auxin action potentially involves an *ARF* gene, which is linked to

quantitative trait loci (QTLs) for fruit size (Devoghalaere et al., 2012). ABA has also been associated with the expansion phase in tomato (Gillaspy et al., 1993) and ABA-deficient mutants have a reduced fruit size (Nitsch et al., 2012). The source of these hormones originates mostly from the seed and has to be transported to the surrounding tissue and/or is synthesized directly in the expanding tissue but, expect for auxin, our current knowledge is, however, limited in this area.

FRUIT MATURATION

Fruit maturity is a developmental point where the fruit has reached the competence to ripen, but has yet to start the ripening process. Auxin and maybe cytokinin appear to be key regulators of fruit maturation. Genetic studies have shown that the tomato *ripening inhibitor* (*rin*) mutant that displays a non-ripening phenotype, have higher levels of auxin and cytokinin at breaker stage compared to wild-type fruit (Davey and Van Staden, 1978; Rolle and Chism, 1989). The suppression of a *rin*-like *MADS*-box gene in apple (Ireland et al., 2013), resulted in a maintenance of high auxin concentration during fruit maturation and fruit that did not ripen (Ireland et al., 2013; Schaffer et al., 2013). In *Arabidopsis* and *Brassica napus*, a low auxin is required for seed dehiscence (pod shatter) to occur (Chauvaux et al., 1997; Sorefan et al., 2009). A mutation in *INDEHISCENT* (*IND*) results in high levels of auxin within the valve margins of the dehiscence zone compared to wild-type controls and it has been postulated that this high intracellular auxin at least partially inhibits dehiscence (Sorefan et al., 2009). In tomato, reduction of auxin by the over-expression of a *Capsicum chinense* auxin-conjugating enzyme (*GH3*) leads to decreased auxin and an increased sensitivity to ethylene at an earlier stage of development (Liu et al., 2005). In strawberry, when achene's are removed from immature fruit, precocious ripening of the receptacle occurs (Given et al., 1988), this ripening can be stopped by the application of exogenous auxin. During fruit growth, auxin levels in the seed are higher than in the surrounding fruit tissue (Devoghalaere et al., 2012) and this suggests as the seeds become dormant, auxin biosynthesis or transport to the rest of the fruit is inhibited, allowing the mature fruit to ripen. This appears to be supported across fruit species as addition of auxin to mature fruit invariably delays ripening (Vendrell, 1985; Manning, 1994; Davies et al., 1997; Aharoni et al., 2002). It should also be noted that although seeds have a strong influence on maturity, parthenocarpic fruit still ripen suggesting a developmental regulation may also be involved.

The role of cytokinin during fruit maturation is less well documented but cytokinin-deficient *Arabidopsis* fruit show non-synchronous ripening with fewer viable seeds compared to controls suggesting cytokinin also has a role in the regulation of silique maturation and ripening (Werner et al., 2003). Finally decreases in free cytokinin and auxin levels are also observed before ripening in orange and grape (Minana et al., 1989; Bottcher et al., 2011).

One of the challenges in future work will be to better understand the molecular mechanisms underlying fruit maturation and interaction between these hormones.

FRUIT RIPENING/SENESCENCE

The progression of fruit ripening or senescence is a complex process involving changes to the metabolic and physiological traits of a fruit. In all fruit, in the tissue surrounding the seed, there is a color change and a change in cell wall composition causing either a dehiscence or a softening (Klee and Giovannoni, 2011). Unique to fleshy fruit there is often a breakdown of stored carbohydrates to sugars and a decrease in acidity along with an increase in flavor and aroma volatiles (Klee and Giovannoni, 2011). The control of ripening appears to be achieved predominantly through the ripening hormones ABA and ethylene (reviews: Fedoroff, 2002; Giovannoni, 2004; Setha, 2012), ethylene being the most studied. Fruit types that have a strong requirement for ethylene to ripen such as tomatoes, peaches, bananas, apples, and melon have previously been labeled climacteric and the role of ethylene in both these fruit types has been extensively reviewed (for example, Bapat et al., 2010; Paul et al., 2012). In peaches and tomato, indole-3-acetic acid (IAA) has also been reported to have some crosstalk with ethylene during ripening as (i) production of ethylene can be concomitant with an increase of IAA and (ii) auxin-signaling components can be up-regulated by ethylene and vice versa (Jones et al., 2002; Trainotti et al., 2007). In fruit that have a lower requirement of ethylene to ripen (referred as non-climacteric fruit such as grape and citrus), ABA appears to have a stronger role (Setha, 2012). It has been shown that in the climacteric fruits tomato and banana, there is an increase in ABA preceding an increase in ethylene. Exogenous application of ABA induces ethylene through the biosynthesis genes (Jiang et al., 2000; Zhang et al., 2009), while a suppression of ABA leads to a delay in fruit ripening (**Figure 1B**; Sun et al., 2012a). In the dry dehiscent fruit *Arabidopsis*, again ABA increases with silique maturation (Kanno et al., 2010) and has been linked with the promotion of dehiscence, an ethylene mediated event (Child et al., 1998; Kou et al., 2012).

While there is a considerable amount of literature on fruit ripening, researchers have often only focused on a small number of physiological changes to document the ripening process. For example color change and/or fruit firmness are often used as a surrogate for ripening, with other ripening characters completely overlooked. It is becoming clear that some ripening traits are independently controlled from each other (Johnston et al., 2009; Ireland et al., 2013). The use of single physiological marker(s) may hence lead to a misrepresentation of this complex process. Here we have summarized the literature based on how different traits respond to hormones rather than considering ripening as one single process.

SUGAR ACCUMULATION

There is little literature on the hormonal control of starch hydrolysis and the resulting sugar accumulation. There have been a number of studies that have documented the metabolic changes that occur during maturation and ripening (Fait et al., 2008; Osorio et al., 2011, 2012), though the link between hormonal control and metabolite accumulation is limited; however, Johnston et al. (2009) observed in apple that, while this could progress independently of ethylene, it was highly sensitive to ethylene. In melon, the application of exogenous ABA was shown to promote starch hydrolysis (Sun et al., 2012b), different from growth section, however, this was confounded by the fact that the ABA also increased the ethylene levels.

COLOR CHANGE

Much of the literature documents the control of color change during fruit ripening. This is achieved by a combination chlorophyll loss (degreening) and production of secondary color metabolites such as carotenoids and anthocyanins. Color change in many fruit species is associated with an increase of ABA and/or ethylene. In apple, the degreening occurs independently of ethylene but ethylene can accelerate the process (Johnston et al., 2009). Citrus and melon also both require ethylene for the degreening of the skin. The production of secondary color metabolites is strongly ethylene regulated in tomato, though some intermediates can be produced in the absence of ethylene. Application of ABA to tomato fruit results in an enhanced onset of breaker stage compared to controls, further implicating ABA as being positive regulator of ripening in tomato (Buta and Spaulding, 1994). In grape and strawberry, the color change is strongly regulated by ABA (Deytieux et al., 2005; Jia et al., 2011), though application of 1-methylcyclopropene (1-MCP; an inhibitor of ethylene response) can delay this process, suggesting that ethylene may play a role (Chervin et al., 2004). There are also reports of color change being inhibited by brassinosteroids in grape and strawberry (Symons et al., 2006; Chai et al., 2013).

CELL WALL HYDROLYSIS

There is a considerable set of literature covering ripening related changes in the cell wall (review: Brummell, 2006). Depending on the fruit type these can manifest as a formation of a dehiscence zone, or through the softening of the flesh tissue. In each case there is a suite of cell wall-related genes that are up-regulated, and in many instances each is differentially regulated. In the case of fruit softening, loss of a single gene can be compensated by other gene action (Powell et al., 2003). In apple and melon, there are both ethylene-independent and ethylene-dependent softening which can be observed in the differential regulation of cell wall-related genes. In banana, it has been shown that ABA can act synergistically with ethylene to promote softening (Lohani et al., 2004) and in grape ABA has been shown to cause fruit softening (Cantin et al., 2007).

Studies of *Arabidopsis* silique dehiscence indicate that ethylene, jasmonic acid, and ABA work in conjunction with each other to promote normal floral organ abscission *via* the up-regulation of genes like *POLYGALACTURONASE* (*ADPG1*; Ogawa et al., 2009). In *Arabidopsis*, a delayed dehiscent phenotype is associated with reduction in the ability of *Arabidopsis* fruit to produce ethylene and that a wild-type time to dehiscence can be restored with treatment of exogenous ethylene (Child et al., 1998; Patterson, 2001). Finally salicylic acid has been shown to delay softening in banana (Srivastava and Dwivedi, 2000).

FLAVOR AND AROMA PRODUCTION

In apple, aroma volatiles are the least ethylene sensitive, and most ethylene-dependant of the ripening traits. Consistent with this, there are a significant number of publications linking the production of aroma with ethylene (Flores et al., 2002; Botondi et al., 2003; Defilippi et al., 2005; Schaffer et al., 2007). There is, however, remarkably little literature examining if other hormones contribute to the regulation of volatile production in fruit.

SUMMARY

It is clear that there is still considerable work needed to better understand the way that hormones interact during fruit development. While there are areas that have been quite extensively covered such as fruit set and the role of ethylene in fruit ripening, there are considerable gaps in our understanding of the hormonal control and crosstalk of other areas, such as fruit expansion, endoreduplication, starch hydrolysis, and flavor development. While much of the physiology is now documented there are considerable opportunities to further our molecular understanding of these complex processes.

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Disease resistance or growth: the role of plant hormones in balancing immune responses and fitness costs

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Plant growth and response to environmental cues are largely governed by phytohormones. The plant hormones ethylene, jasmonic acid, and salicylic acid (SA) play a central role in the regulation of plant immune responses. In addition, other plant hormones, such as auxins, abscisic acid (ABA), cytokinins, gibberellins, and brassinosteroids, that have been thoroughly described to regulate plant development and growth, have recently emerged as key regulators of plant immunity. Plant hormones interact in complex networks to balance the response to developmental and environmental cues and thus limiting defense-associated fitness costs. The molecular mechanisms that govern these hormonal networks are largely unknown. Moreover, hormone signaling pathways are targeted by pathogens to disturb and evade plant defense responses. In this review, we address novel insights on the regulatory roles of the ABA, SA, and auxin in plant resistance to pathogens and we describe the complex interactions among their signal transduction pathways. The strategies developed by pathogens to evade hormone-mediated defensive responses are also described. Based on these data we discuss how hormone signaling could be manipulated to improve the resistance of crops to pathogens.

Keywords: abscisic acid, auxin, hormone crosstalk, pathogens, salicylic acid, trade-off, virulence factor

INTRODUCTION

In their natural environments, plants are under continuous biotic stress caused by different attackers (e.g., bacteria, fungi, viruses, oomycetes, and insects) that compromise plant survival and offspring. Given that green plants are the ultimate source of energy for most organisms, it is not surprising that plants have evolved a variety of resistance mechanisms that can be constitutively expressed or induced after pathogen or pest attack (Glazebrook, 2005; Panstruga et al., 2009). Plants have developed molecular mechanisms to detect pathogens and pests and to activate defense responses. The plant innate immune system relies in the specific detection by plant protein recognition receptors (PRRs) of relatively conserved molecules of the pathogen called pathogen-associated molecular patterns (PAMPs). This resistance response is known as PAMP-triggered immunity (PTI). Successful pathogens secrete effector proteins that deregulate PTI. To counteract this, plant resistance (R) proteins recognize effectors and activate effector-triggered immunity (ETI; reviewed in Dodds and Rathjen, 2010).

A fine-tune regulation of these immune responses is necessary because the use of metabolites in plant resistance may be detrimental to other physiological processes impacting negatively in other plant traits, such as biomass and seed production (Walters and Heil, 2007; Kempel et al., 2011). These physiological constrains, together with other factors such as the co-existence of plants with natural attackers, have contributed to drive the evolution of a dynamic and complex network system. Defense layers from separate cellular components and from diverse physiological processes are interconnected to reduce the inherent fitness cost of being well-defended (Chisholm et al., 2006; Panstruga et al., 2009; Schulze-Lefert and Panstruga, 2011). The resistance response is regulated by phytohormones, that are small molecules which synergistically and/or antagonistically work in a complex network to regulate many aspects of plant growth, development, reproduction, and response to environmental cues (Pieterse et al., 2009; Santner et al., 2009; Jaillais and Chory, 2010). Recent progresses have been made in understanding the complex hormone network that governs plant immunity, giving rise to a database containing information of the hormone-regulated genes (e.g., in *Arabidopsis thaliana*) and the phenotypic description of hormone-related mutants (Peng et al., 2009). In parallel, it has been found that pathogens have developed sophisticated molecular mechanisms to deregulate the biosynthesis of hormones and/or to interfere with hormonal signaling pathways, thus, facilitating the overcoming of plant defense mechanisms (Jones and Dangl, 2006; Dodds and Rathjen, 2010). The essential roles of salicylic acid (SA) and ethylene (ET)/jasmonic acid (JA)-mediated signaling pathways in resistance to pathogens are well described (Robert-Seilaniantz et al., 2011a). SA signaling positively regulates plant defense against biotrophic pathogens, that need alive tissue to complete their life cycle, whereas ET/JA pathways are commonly required for resistance to necrotrophic pathogens, that degrade

plant tissue during infection, and to herbivorous pests (Glazebrook, 2005; Bari and Jones, 2009). Several exceptions for this general rule have been described, and thus SA pathway is also required for plant resistance to particular necrotrophic pathogens, whereas ET/JA pathways were found to be essential for resistance to some biotrophic pathogens (Berrocal-Lobo et al., 2002; Robert-Seilaniantz et al., 2011a). Additionally, other hormones such as auxins and abscisic acid (ABA), originally described for their function in the regulation of plant growth processes and the response to abiotic stresses, have recently emerged as crucial players in plant–pathogen interactions (Mauch-Mani and Mauch, 2005; Kazan and Manners, 2009; Ton et al., 2009; Fu and Wang, 2011). All the phytohormone pathways are linked to each other in a huge, complex and still obscure network. For example, ET, ABA, auxin, gibberellins, and cytokinins pathways are considered as hormone modulators of the SA–JA signaling backbone (Pieterse et al., 2012).

To develop hormone-based breeding strategies aiming to improve crop resistance to pathogens, we need to understand the intricate regulation of hormone homeostasis during plant–pathogen interactions, and how pathogens interfere with this hormone regulation. Indeed, manipulation of a plant hormone pathway can result in enhanced resistance to a particular pathogen, but it could also have a strong negative effect on plant growth and resistance to a distinct type of pathogen with a different life style (Holeski et al., 2012). In this review, we will discuss novel insights on the complex role of phytohormones in balancing plant innate immunity and development, with a special focus on the regulatory crosstalk of auxins, SA, and ABA. We will also learn about decoy strategies employed by the attackers to disturb hormone-mediated defense responses in plants, and we will describe how misregulation of these hormone pathways leads to strong effects on developmental features and on disease resistance to pathogens. Finally, we will discuss the potential of manipulating hormone homeostasis/signaling to improve crop resistance to pathogen.

HORMONE REGULATORY NETWORKS IN DISEASE RESISTANCE

AUXINS

Auxins are a group of molecules including IAA (indole-3-acetic acid) that regulate many aspects of plant development, such as apical dominance, root gravitropism, root hair, lateral root, leaf, and flower formation, and plant vasculature development (Kieffer et al., 2010; Swarup and Péret, 2012). Both direct and indirect effects of auxins on the regulation of pathogen resistance responses in plants have been described (Kazan and Manners, 2009). Indirect effects may be caused by auxins regulation of development-associated processes, such as cell wall architecture, root morphology, and stomata pattern. For example, treatment of rice with IAA impaired the resistance to *Xanthomonas oryzae* pv. *oryzae* probably as a consequence of the activation of the biosynthesis of cell wall-associated expansins that lead to cell wall loosening, which facilitates pathogen growth (Ding et al., 2008).

Auxins can negatively impact plant defense by interfering with other hormone signaling pathways or with PTI (Robert-Seilaniantz et al., 2011a). The bacterial PAMP flg22, a peptide from flagellin protein (Boller and Felix, 2009; Pel and Pieterse, 2012), induces an *Arabidopsis* microRNA (miR393), which negatively regulates the mRNA levels of auxins receptors TIR1 (transport inhibitor response 1), AFB2 (auxin signaling F-box 2), and AFB3. Thus, the flg22-triggered suppression of auxin signaling leads to increased resistance to the bacterium *Pseudomonas syringae* pv. *tomato* DC3000 (*Pst*DC3000) and also to the oomycete *Hyaloperonospora arabidopsidis* (Navarro et al., 2006; Robert-Seilaniantz et al., 2011b). The flg22-induced resistance to these biotrophic pathogens was explained by the observed induction of the SA signaling pathway. Supporting this hypothesis, it was found independently that treatment of *Arabidopsis* leaves with flg22 induces SA accumulation (Tsuda et al., 2008).

In *Arabidopsis*, SA treatment stabilizes the Aux/IAA proteins, leading to down-regulation of the expression of auxin-related genes. Moreover, the enhanced susceptibility to *P. syringae* pv. *maculicola* 4326 (*Psm*4326) of plants expressing the *NahG* gene (encoding a bacterial salicylate hydroxylase that degrades SA) is partially reverted by the *axr2-1* mutation, that disrupts auxin signaling, further indicating that auxin signaling is part of the SA-induced resistance signaling pathway (Wang et al., 2007). Interaction between SA and auxins was further clarified by the characterization of the regulatory pattern of *GH3.5* gene, which is involved in auxin homeostasis in *Arabidopsis* plants. Lines overexpressing *GH3.5* have lower levels of Aux/IAA proteins, overexpression of SA signaling pathway and enhanced resistance to *P. syringae* (Park et al., 2007). Moreover, these transgenic lines also displayed enhanced resistance to abiotic stress and induction of the ABA regulatory pathway (Park et al., 2007).

The conjugated auxin–aspartic acid (IAA–Asp) has been recently reported to play a key role in regulating resistance to the necrotrophic fungus *Botrytis cinerea* and *Pst*DC3000. In *Arabidopsis*, tomato, and *Nicotiana benthamiana* infected with these pathogens there is an enhanced expression of *GH3.2* and *GH3.4* genes, which encode two enzymes required for conjugation of auxins with Asp. Thus, upon pathogen infection, accumulation of IAA–Asp takes place, promoting the development of disease symptoms in infected plants (Gonzalez-Lamothe et al., 2012). The negative effects of auxins on the activation of plant resistance is further supported by the observed enhanced susceptibility of auxin-treated rice to *X. oryzae* (Ding et al., 2008) and of auxin-treated *Arabidopsis* to *Pst*DC3000 (Navarro et al., 2006) and *Fusarium culmorum* (Petti et al., 2012). Disruption of auxin signaling in *Arabidopsis* mutants, such as *axr1*, *axr2*, and *axr3*, leads to enhanced resistance to *F. oxysporum* (Kidd et al., 2011). Nevertheless, auxins have also been shown to positively regulate *Arabidopsis* immunity as *axr2-1* and *axr1-1* mutants were more susceptible than wild-type plants to the necrotrophic fungi *B. cinerea* and *Plectosphaerella cucumerina* (Llorente et al., 2008).

One of the biosynthetic pathways of auxins is partially shared with those required for the biosynthesis of tryptophan-derived antimicrobials, such as indole glucosinolates and camalexin. This might lead to competition for the biosynthetic precursor of auxin and antimicrobials (Barlier et al., 2000; Grubb and Abel, 2006). The recently characterized *Arabidopsis wat1* (*walls are thin1*) mutant exhibits specific enhanced resistance to vascular pathogens such as *Ralstonia solanacearum*. This response was associated to a misregulation of tryptophan derivatives (i.e., lower

levels of auxin and indole glucosinolates) specifically in roots, resulting in enhanced levels of SA which is, like tryptophan, a chorismate-derivative (Denancé et al., 2013). Collectively, these data demonstrate that auxins play a central role in balancing plant resistance responses.

ABSCISIC ACID

Abscisic acid is an isoprenoid compound that regulates developmental processes, such as seed development, desiccation, and dormancy (Wasilewska et al., 2008). In addition, the function of ABA as a regulator of abiotic stress has been thoroughly described (Shinozaki and Yamaguchi-Shinozaki, 2007). ABA has also emerged as a complex modulator of plant defense responses (Asselbergh et al., 2008; Feng et al., 2012; Sánchez-Vallet et al., 2012). ABA can function as a positive or a negative regulator of plant defense depending on the plant–pathogen interaction analyzed (Mauch-Mani and Mauch, 2005; Asselbergh et al., 2008; Ton et al., 2009). ABA-impaired (biosynthesis or signaling) mutants in tomato (*sitiens*) and *Arabidopsis* (*abi1-1*, *abi2-1*, *aba1-6*, *aba2-12*, *aao3-2*, and *pyr1pyl1pyl2pyl4*) were shown to overexpress defensive-signaling pathways, leading to enhanced resistance to different pathogens such as *B. cinerea*, *P. syringae*, *F. oxysporum*, *Plectosphaerella cucumerina*, and *Hyaloperonospora parasitica* (Audenaert et al., 2002; Mohr and Cahill, 2003; de Torres-Zabala et al., 2007; de Torres Zabala et al., 2009; Garcia-Andrade et al., 2011; Sánchez-Vallet et al., 2012). Negative interactions of ABA with the major hormones involved in plant defense response (SA, JA, and ET) have been described by means of exogenous hormone treatments (Yasuda et al., 2008; de Torres Zabala et al., 2009; Sánchez-Vallet et al., 2012). For instance, almost 65% of the up-regulated genes and 30% of the down-regulated genes in *aba1-6* mutant were found to be up- or down-regulated by either ET, JA, or SA treatment (Sánchez-Vallet et al., 2012). Remarkably, these genes constitutively up-/down-regulated in *aba1-6* mutant were differentially expressed in *Arabidopsis* wild-type plants inoculated with *Plectosphaerella cucumerina*, indicating that they form part of the defensive responses activated upon pathogen infection (Sánchez-Vallet et al., 2012). In addition, ABA plays a direct role in regulating R (resistance) protein activity. ABA and exposition of plants to high temperature both reduce the nuclear accumulation of SNC1 (suppressor of *npr1-1*, constitutive1) and RPS4 (resistant to *Pseudomonas syringae* 4) compromising disease resistance to *P. syringae* (Mang et al., 2012).

Abscisic acid can also positively regulate the resistance to some pathogens, such as *Alternaria brassicicola*, *R. solanacearum*, and *Pythium irregulare*, as ABA-deficient and-insensitive mutants (*abi1-1*, *abi2-1*, *abi4-1*, *aba1-6*, *aba2-12*, *aao3-2*, and *npq2-1*) were found to be more susceptible than wild-type plants to these pathogens (Adie et al., 2007; Hernandez-Blanco et al., 2007; Flors et al., 2008; Garcia-Andrade et al., 2011). In *Arabidopsis*, ABA has been shown to be required for JA biosynthesis that is essential for resistance to *Pythium irregulare* (Adie et al., 2007). This contrasts with the negative interaction of ABA- and JA-signaling in the modulation of *Arabidopsis* resistance to the necrotrophic fungus *Plectosphaerella cucumerina* (Sánchez-Vallet et al., 2012). Similarly, although ABA and SA have been shown to function antagonistically in the control of the resistance to some pathogens, they trigger stomata closure to avoid penetration of the bacteria *P. syringae* in *Arabidopsis* (Melotto et al., 2006). Plant treatment with flg22 is known to interfere with ABA signaling to induce stomata closure. The ABA- or flg22-induced stomata closure are impaired in lines overexpressing HSC70-1 (heat shock cognate70-1) and mutants in HSP90 (heat shock protein90; Clément et al., 2011), resulting in an increased susceptibility to both virulent and avirulent strains of *P. syringae* (Hubert et al., 2003; Takahashi et al., 2003; Noël et al., 2007). ABA is a key hormone in *Arabidopsis* response to *R. solanacearum* infection, as 40% of the genes up-regulated during the development of wilting symptoms were related to ABA, including those encoding proteins for ABA biosynthesis [i.e., 9-*cis*-epoxycarotenoid dioxygenase3 (NCED3)] or signaling [i.e., ABA-insensitive1 (ABI1) and ABI5; Hu et al., 2008]. More recently, it has been shown that pre-inoculation of *Arabidopsis* with an avirulent strain of *R. solanacearum* activates plant resistance to virulent isolates of this bacterium, and this resistance was correlated with the enhanced expression of ABA-related genes that resulted in a hostile environment for the infection development. These results suggest that ABA may be used in biological control of bacterial wilt caused by *R. solanacearum* (Feng et al., 2012).

SALICYLIC ACID

The function of SA in activating resistance against pathogens has been thoroughly described. In *Arabidopsis*, SA is synthesized from chorismate (a precursor of tryptophan and, consequently, of auxins) *via* two pathways, either through phenylalanine or through isochorismate (reviewed in Vlot et al., 2009). This second pathway, in which SID2/ICS1 (salicylic acid induction deficient 2/isochorismate synthase 1) is involved, is activated upon pathogen infection, such as *Erysiphe* or *P. syringae*, and after plant recognition of pathogen effectors or PAMPs (Tsuda et al., 2008; Vlot et al., 2009). Deficiency of SA biosynthesis in *sid2-1* mutant leads to reduced resistance response in *Arabidopsis* plants (Nawrath and Métraux, 1999). SA is a regulator of plant resistance to biotrophic and hemibiotrophic pathogens, such as *Hyaloperonospora arabidopsidis* and *P. syringae*, and it also regulates systemic acquired resistance (SAR), a well-studied type of induced resistance (Glazebrook, 2005). In addition, SA is a central regulator of immunity. It interacts with other signaling pathways (e.g., ET and JA pathways), as a strategy to induce the proper resistance responses and to reduce the associated fitness costs (Vlot et al., 2009; Thaler et al., 2012).

NPR1 (non-expressor of PR genes 1), a well-known central player in SA signaling (Cao et al., 1997), and NPR3 and NPR4 proteins have been recently described as SA receptors (Fu et al., 2012; Wu et al., 2012). NPR1 localizes at the cytosol as an oligomer, and in the presence of SA, redox changes occurs in NPR1 that lead to the dissociation of NPR1 complex and to the translocation of the corresponding monomers to the nucleus. There, NPR1 protein activates the transcription of defensive genes, such as *PR* (*pathogenesis-related protein*), by interacting with TGA (TGACG sequence-specific binding protein) transcription factors (Dong, 2004; Tada et al., 2008; Robert-Seilaniantz et al., 2011a). In *Arabidopsis*, EDS1 (enhanced disease susceptibility 1) is a major node required both for SA-dependent basal resistance against

virulent pathogens and for the activation of the ETI mediated by the TIR–NB–LRR (Toll-interleukin receptor domain–nucleotide binding domain–leucine rich repeat) resistance proteins (Parker et al., 1996; Falk et al., 1999). EDS1 protein is present in distinct pools at nuclei and cytoplasm, and these two EDS1 locations are required for a complete immune response (Garcia et al., 2010). Several EDS1 interactors have been identified, including PAD4 (phytoalexin deficient4), RPS4, RPS6, SAG101 (senescence-associated gene101), SRFR1 (suppressor of RPS4-RLD1), and SNC1 (Feys et al., 2001, 2005; Bhattacharjee et al., 2011; Heidrich et al., 2011; Rietz et al., 2011). The EDS1–PAD4 complex is necessary for basal resistance and activation of SA-defense response (Rietz et al., 2011). Indeed mutations in EDS1 and PAD4 lead to reduce resistance to pathogens such as *Hyaloperonospora parasitica* and deficiency of the SA signaling pathway (Parker et al., 1996; Falk et al., 1999; Jirage et al., 1999). Transcriptional regulation of SA-defensive genes is also mediated by HDA19 (histone deacetylase19) that repressed SA-mediated basal defense to *Pst*DC3000 (Choi et al., 2012). Up-regulation of SA marker genes (*PR1*, *PR2*, *ICS1*, *EDS1*, *PAD4*) and over-accumulation of SA take place in *hda19* mutant, which correlates with its enhanced resistance phenotype to *Pst*DC3000 pathogenic bacteria. Indeed, HDA19 targets *PR1* and *PR2* promoters to regulate gene expression. The mutation *hda19* causes hyper-acetylation of histones in the promoters of *PR* genes and priming of SA-associated plant defense (Choi et al., 2012).

Negative crosstalk between SA and JA signaling pathways has been thoroughly described (Gimenez-Ibanez and Solano, 2013). For example, *WRKY33*, a positive regulator of JA-related genes, is a repressor of the SA pathway. In the *wrky33* mutant there is an enhanced expression of several SA-regulated genes (*SID2*/*ICS1*, *EDS5*/*SID1*, *PAD4*, *EDS1*, *NIMIN1*, *PR1*, *PR2*, *PR3*) and increased accumulation of SA levels. In turn, SA induction contributes to down-regulate JA-signaling, and to increase the susceptibility of *wrky33* plants to necrotrophic fungi (Birkenbihl et al., 2012; Sánchez-Vallet et al., 2012). NPR1 is a regulator of SA-mediated suppression of the JA/ET signaling pathway, as revealed using *npr1* mutant (Spoel et al., 2003). The *Arabidopsis* mediator subunit 16 (MED16) was recently described to be a positive regulator of SA-induced defense response and a negative regulator of JA/ET signaling pathway (Zhang et al., 2012). The negative crosstalk between SA and JA is exploited by *P. syringae* strains producing the phytotoxin coronatine (COR), an structural mimic of the active JA-Ile, to suppress SA signaling (Uppalapati et al., 2005; Zheng et al., 2012). *P. syringae* strains impaired in production of COR have reduced virulence on *Arabidopsis* wild-type plants but not on SA-deficient lines (e.g., *sid2* and *NahG*; Brooks et al., 2005). In a search for *Arabidopsis* mutants in which the virulence of COR-deficient *Pst*DC3000 mutant was recovered, several *scord* (*susceptible to coronatine-deficient Pst*DC3000) mutants were found to be defective in SA signaling (Zeng et al., 2011). For instance, *scord3* mutant plants are impaired in EDS5/SID1, a key protein required for SA biosynthesis, and consequently it has reduced SA levels compared with wild-type plants (Zeng et al., 2011), further corroborating the role of SA in resistance to pathogens.

DECOY STRATEGIES OF PATHOGENS: MANIPULATION OF THE HOST HORMONE MACHINERY

PATHOGENS PRODUCE AND DEGRADE HORMONES

Auxins

Many pathogenic microbes and plant growth promoting rhizobacteria have evolved complete pathways for auxin biosynthesis with tryptophan as the main precursor (Spaepen et al., 2007). Auxin-producing phytopathogenic bacteria are mostly, but not exclusively, gall-inducing microbes. They include, for instance, *Agrobacterium tumefaciens* (Liu and Nester, 2006), *Agrobacterium rhizogenes* (Gaudin and Jouanin, 1995), *Erwinia chrysanthemi* (Yang et al., 2007), *Erwinia herbicola* (Brandl and Lindow, 1998), *Pseudomonas fluorescens* (Suzuki et al., 2003), *P. putida* (Leveau and Lindow, 2005), *Pseudomonas savastanoi* (Glickmann et al., 1998), *P. syringae* (Glickmann et al., 1998), *R. solanacearum* (Sequeira and Williams, 1964; Valls et al., 2006), and *Rhodococcus fascians* (Vandeputte et al., 2005). In *R. solanacearum*, auxin biosynthesis is governed by HrpG, a major regulator of bacterial virulence and response to metabolic signals (Valls et al., 2006). In *Agrobacterium tumefaciens*, two genes required for conversion of tryptophan to auxin are localized on the T-DNA region of the Ti plasmid injected into plant cells. Auxin biosynthesis is necessary for tumor gall formation and for pathogenicity of *Agrobacterium* (Lee et al., 2009): auxins negatively regulate the expression of genes necessary for the transfer of *Agrobacterium* T-DNA in plants and also inhibit the growth of several bacterial species *in vitro* (Liu and Nester, 2006).

Auxin biosynthesis in fungal pathogens seems to be limited to a few species. In *Ustilago maydis*, *U. esculenta*, and *U. scitaminea* auxin is produced (Chung and Tzeng, 2004; Reineke et al., 2008). In this case, auxin does not seem to be required for *U. maydis*-induced tumor formation or for pathogenicity, as a mutant defective in four genes encoding key auxin biosynthetic enzymes was compromised in auxin levels but not in tumor formation (Reineke et al., 2008). Additionally, other fungi have enzymatic tools to produce auxins, such as *Colletotrichum gloeosporioides* f. sp. *aeschynomene*, *Colletotrichum acutatum*, and *F. proliferatum* (Robinson et al., 1998; Chung et al., 2003; Maor et al., 2004; Tsavkelova et al., 2012). Nevertheless, the production of auxins by fungal pathogens has not been clearly demonstrated to be a virulent factor that favors plant colonization.

Abscisic acid

Several fungal species produce ABA, including *B. cinerea*, *Rhizoctonia solani*, *Ceratocystis fimbriata*, and *Rhizopus nigricans* (Dörffling et al., 1984; Inomata et al., 2004a). ABA biosynthesis by *B. cinerea* requires a cluster of four genes, *BcABA1* to *BcABA4* (Hirai et al., 2000; Inomata et al., 2004b; Siewers et al., 2004, 2006). Unlike plants, fungi, such as *B. cinerea* and *Cercospora* sp., use the mevalonate pathway to produce ABA (Hirai et al., 2000; Inomata et al., 2004a). The role of ABA as a *B. cinerea* virulence factor has not been fully demonstrated, but several published data support this hypothesis: (i) ABA biosynthesis in the fungus is stimulated by the host plant (Kettner and Därffling, 1995); (ii) exogenous treatment with ABA increased disease symptoms caused by the fungus on roses (Shaul et al., 1996); and (iii) ABA contributes to susceptibility to *B. cinerea* and other pathogens by suppressing

defense responses in plants (Audenaert et al., 2002; Sánchez-Vallet et al., 2012).

Salicylic acid

Although SA biosynthesis has not been described in plant pathogens, it is known that some plant-associated bacteria can degrade salicylate. Indeed, the enzyme salicylate hydroxylase (NahG), that catalyzes the formation of catechol from salicylate, has been identified in various bacteria, such as *P. putida* and *P. fluorescens* (You et al., 1991; Chung et al., 2001).

PATHOGEN EFFECTORS INTERFERE WITH HORMONE SIGNALING IN PLANTS

Effectors are proteins secreted by pathogens during infection to deregulate host immune responses. One common strategy implemented by effectors is the manipulation of the homeostasis of plant phytohormones, resulting in deactivation of the appropriate defense response (Robert-Seilaniantz et al., 2007; Bari and Jones, 2009; **Figures 1** and **2**).

Bacteria and phytoplasma

In addition to the common example of the phytotoxin COR produced by *P. syringae* strains to manipulate the plant hormonal balance (Zheng et al., 2012), many phytopathogenic bacteria have developed large repertoires of type III effectors (T3E) which are necessarily injected through the syringe-like type III secretion system inside plant cells to deregulate plant immunity (**Figure 1**; Jones and Dangl, 2006; Boller and He, 2009; Büttner and He, 2009). The roles of bacterial effectors in plant immunity have been extensively reviewed elsewhere (Cui et al., 2009; Rivas and Genin, 2011; Deslandes and Rivas, 2012; Dou and Zhou, 2012; Howden and Huitema, 2012). *Xanthomonas* sp. bacteria synthesized TAL (transcription activator-like) effectors, such as AvrBs3 from *X. axonopodis* pv. *vesicatoria* (formerly *X. campestris* pv. *vesicatoria*), that are imported to the plant nuclei where they activate the expression of host target genes (Boch et al., 2009; Moscou and Bogdanove, 2009; Bogdanove et al., 2011). Five targets, designed as *up-regulated by AvrBs3 1 to 5* (*UPA1–5*), are auxin-induced genes members of the SAUR (small auxin up RNA) family (Marois

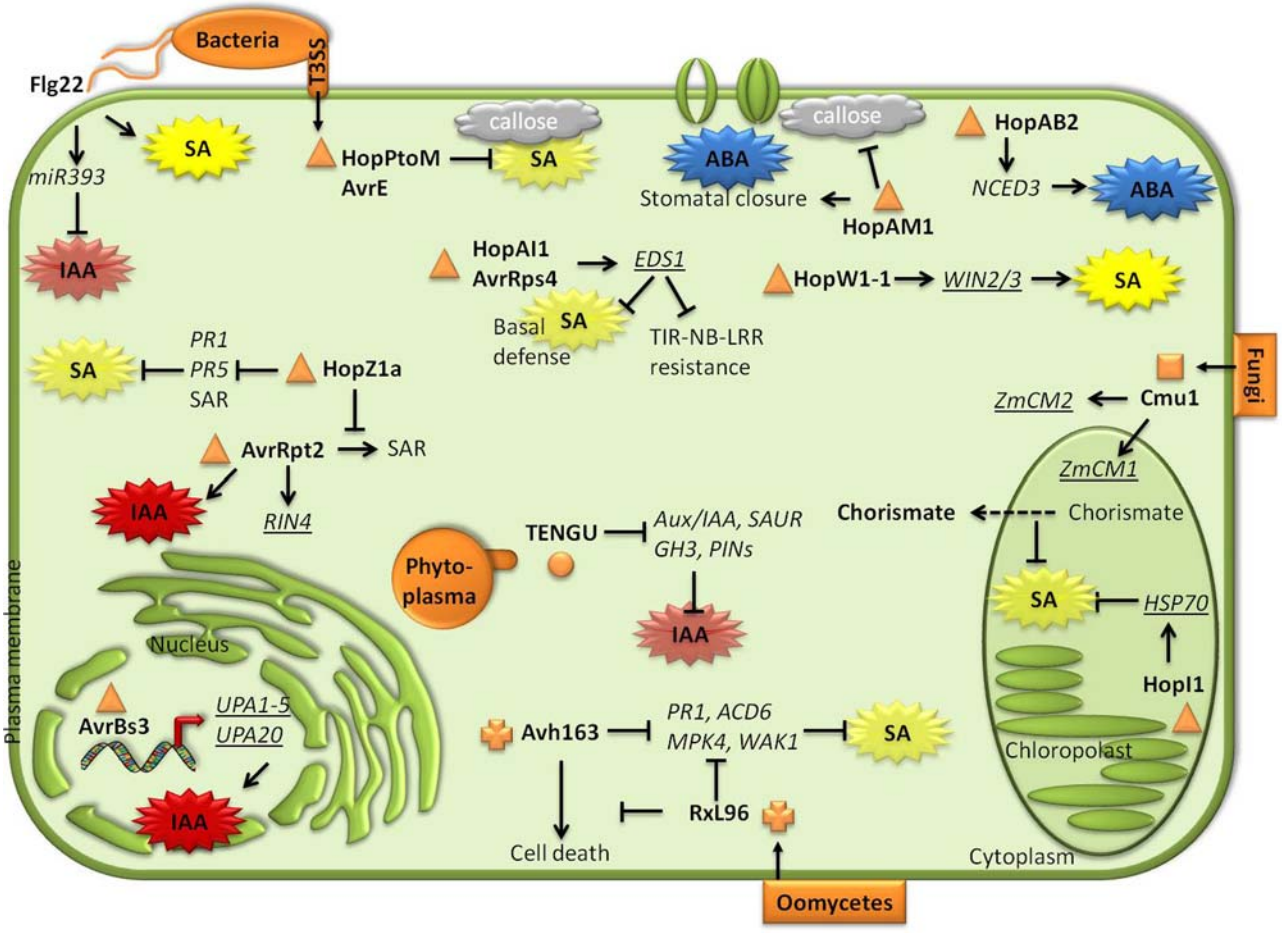

FIGURE 1 | Decoy strategies elaborated by pathogens and pests to interfere with plant hormone biosynthesis/signaling pathways. Phytopathogenic bacteria, phytoplasmas, fungi, and oomycetes secrete various effectors inside plant cells during infectious process. Once in the host cells, some effectors specifically bind to (underlined), induce and/or decrease (arrows/crossed lines) target gene expression or protein activity. Consequently, ABA-, SA-, or Auxin-mediated defense mechanisms are activated/repressed.

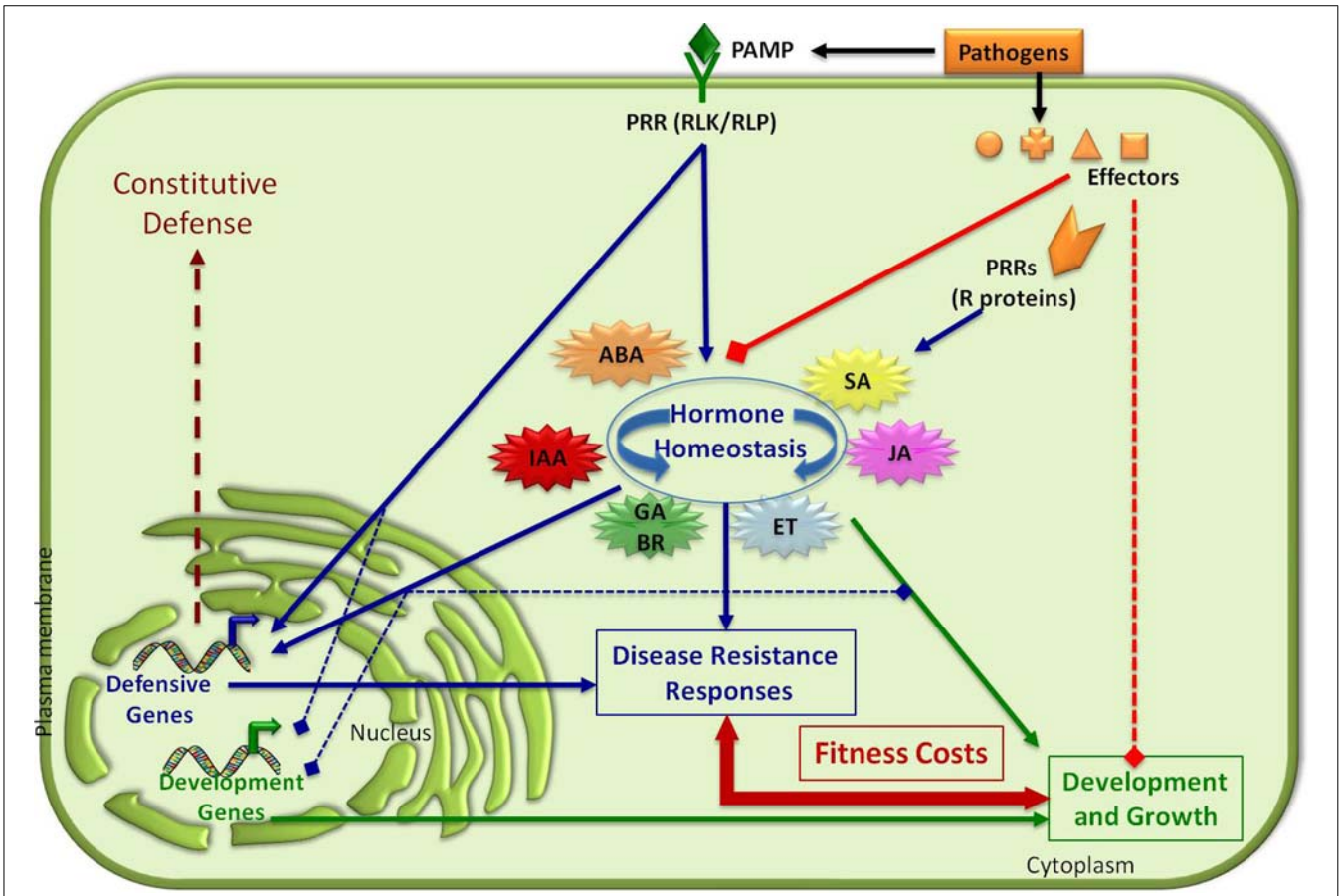

FIGURE 2 | Balancing plant immune responses and fitness costs. Plant disease resistance responses are induced upon recognition of PAMPs/effectors from pathogens and pests by plant PRR proteins. This recognition modulates plant hormonal homeostasis and transcriptional reprograming of defensive genes. The activation of these inducible resistance responses (PTI and/or ETI) negatively regulates the expression of developmental-associated genes impacting on plant fitness costs. Effectors from pathogens interfere with hormonal balance and the activation of PTI and ETI. Pathogens can also negatively impact plant growth and developmental-associated processes (transcriptional expression of genes, negative regulation of signaling pathways, etc.; see text for details). Positive and negative interactions are indicated by arrows and squares, respectively. GA, gibberellic acid; BR, brassinosteroids.

et al., 2002). Additionally, induction of the TAL target *UPA20* provokes cell hypertrophy, a feature which is characteristic of auxin accumulation (Kay et al., 2007). Auxin is a susceptibility factor in *Arabidopsis* plants infected with *Pst*DC3000, and consequently, auxin was hypothesized to be a potential target for bacterial effectors. Thus, the cysteine protease bacterial effector AvrRpt2 triggers auxin signaling pathway to enhance bacterial virulence in *Arabidopsis* lines lacking the resistance gene that normally recognizes this T3E. Transgenic plants expressing AvrRpt2 accumulated higher auxin levels and showed a constitutive activation of the auxin signaling pathway. Additionally, auxin levels in *Arabidopsis* leaves inoculated with *Pst*DC3000*avrRpt2* were higher than those in plants infected with *Pst*DC3000 (Chen et al., 2007), indicating that AvrRpt2 modulates auxin pathway to enhance bacterial virulence, but this effect was found to be independent of SA (Chen et al., 2004). Auxin signaling seems to be a preferential target of phytoplasmas, some bacteria-like, obligate plant pathogens belonging to the class of Mollicutes that require sap-feeding insect herbivores as vectors for transmission to plants (Sugio et al., 2011). Indeed, TENGU (tengu-su inducer) is an effector of *Candidatus* phytoplasma asteris that, when expressed in *Arabidopsis* transgenic lines, causes dwarfism and abnormal reproductive organogenesis and flower sterility. These phenotypes, which are similar to the disease symptoms provoked by the phytoplasma, have been associated to alterations in hormone balance. Microarray analysis of transgenic *Arabidopsis* plants expressing TENGU demonstrated that many auxin-related genes were down-regulated, including genes of the *Aux/IAA*, *SAUR*, *GH3*, and *PIN* families (Hoshi et al., 2009). Thus, TENGU effector could interfere with auxin signaling in plants.

Several *P. syringae* effectors target SA. HopPtoM and AvrE are repressors of SA-dependent callose deposition but do not affect SA-responsive genes in *Arabidopsis* infected leaves (DebRoy et al., 2004). The effector HopI1 (previously named HopPmaI), that is essential for the virulence of *P. syringae* pv. *maculicola* (*Pma*) in *Arabidopsis*, *N. benthamiana*, and *N. tabacum*, has been found to be a modulator of SA-mediated defense responses. Indeed, the expression of HopI1 in *Arabidopsis acd6-1* (*accelerated cell*

death6-1) mutant reduces the enhanced SA levels and the constitutive induction of defense responses characteristic of this mutant (Jelenska et al., 2007). Another effector, HopZ1a, a cysteine protease from *P. syringae* that interferes with SA signaling, is able to suppress *Pst*DC3000-induced expression of *PR1* and *PR5* and the SAR induced either by *Pst*DC3000 (virulent) or *Pst*DC3000*avrRpt2* (avirulent) pathogens (Macho et al., 2010). Thus, HopZ1a contributes to *Pst* virulence by suppressing SA-mediated defenses that takes place during ETI induced by other effectors such as AvrRpt2. EDS1, a key regulatory node of basal and induced resistance, is also targeted by bacterial pathogen effectors. AvrRps4 and HopA1, two *Pst*DC3000 effectors, bind to EDS1 interfering with the interaction between EDS1 and TIR–NB–LRR resistance proteins, and consequently preventing the activation of the immune response (Bhattacharjee et al., 2011; Heidrich et al., 2011). In contrast to other effectors, HopW1-1, that forms part of the T3E repertoire of *Pma*, but not of that of *Pst*DC3000 (Guttman et al., 2002), induces resistance in the Ws accession of *Arabidopsis* to *Pma* (Lee et al., 2008). This effect of HopW1-1 on Ws was corroborated by the fact that *Pst*DC3000 strain expressing HopW1-1 has reduced growth and caused weak disease symptoms in the Ws plants. In a yeast two-hybrid screen, three *Arabidopsis* HopW1-1-interacting proteins (WIN2, WIN3) were found to bind to the effector (Lee et al., 2008). The enhanced resistance triggered by HopW1-1 was not caused by activation of a hypersensitive response, but it was dependent on an enhanced accumulation of SA. Indeed, *pad4* mutants were almost completely compromised in their resistance response to HopW1-1.

HopAM1 contributes to *P. syringae* virulence by manipulating ABA-mediated responses in plants: it enhances stomata closure, suppresses infection-triggered callose deposition, and inhibits seed germination. Remarkably, HopAM1 increased *P. syringae* virulence on *Arabidopsis* plants grown under water-stressed conditions (Goel et al., 2008). *Arabidopsis* lines expressing HopAM1 showed enhanced colonization by the avirulent *Pst*DC3000 *hrcC*$^{-}$ mutant, impaired in T3SS, and did not develop callose-rich papillae that are normally induced by *hrcC*$^{-}$ strain in wild-type plants (Goel et al., 2008). An effector of *P. syringae* pv. *phaseolicola*, HopAB2, promotes virulence on *Arabidopsis* and bean plants, and suppresses basal resistance to *Pst*DC3000 *hrpA*$^{-}$, a mutant compromised in T3SS (de Torres et al., 2006). Expression of HopAB2 in *Arabidopsis* plants induces the expression of *NCED3*, resulting in enhanced biosynthesis of ABA, which interferes with the accumulation of SA levels and the activation of SA-mediated resistance (de Torres-Zabala et al., 2007). Thus, HopAM1 and HopAB2 are suppressors of defense mechanisms by enhancing ABA responses and promoting disease susceptibility in plants.

Filamentous pathogens: oomycetes and fungi

Oomycete genomes contain a class of cytoplasmic proteins known as RXLRs that contain a conserved RXLR amino acid motif (arginine, any amino acid, leucine, arginine; Rehmany et al., 2005; Morgan and Kamoun, 2007). Two effectors from this class, HaRxL96 from *Hyaloperonospora arabidopsidis*, the causal agent of downy mildew on *Arabidopsis*, and its ortholog PsAvh163 from *Phytophthora sojae*, which causes soybean rot disease, interfere with plant immunity (Anderson et al., 2012). Remarkably, *Arabidopsis* plants expressing HaRxL96 or PsAvh163 became more susceptible to virulent and avirulent pathogens, indicating that these effectors repress basal resistance and ETI. In fact, the induction of SA-defensive genes, but not SA biosynthesis, that take places upon infection with avirulent strains of *Hyaloperonospora arabidopsidis*, was suppressed in the transgenic lines expressing HaRxL96 or PsAvh163, indicating that these effectors interfere with SA signaling to trigger plant susceptibility to oomycetes (Anderson et al., 2012).

Filamentous extracellular or obligate fungal pathogens secrete effectors via hyphae or haustoria (Stergiopoulos and de Wit, 2009; de Jonge et al., 2011). *U. maydis* is a basidiomycete fungus that causes smut disease on maize and its relative teosinte (Brefort et al., 2009; Djamei and Kahmann, 2012). Maize infection by *U. maydis* results in the repression of SA-associated *PR1* defense gene expression during the early biotrophic phase of the interaction, while auxin production in the host is induced later during tumor formation (Doehlemann et al., 2008). One of the most highly expressed genes of *U. maydis* during plant colonization is the Cmu1 effector, a chorismate mutase protein (Skibbe et al., 2010). Cmu1 is required for full virulence since the induction of tumors is significantly reduced in a *U. maydis cmu1* mutant (Djamei et al., 2011). Once inside plant cells, Cmu1 is localized in the cytoplasm, the nucleus and guard cells and it is spread to neighbor cells through plasmodesmata. A yeast two-hybrid analysis showed that Cmu1 interacts with two maize chorismate mutases, ZmCm1 and ZmCm2, which are found in plastids and cytoplasm in plants, respectively. Interestingly, SA levels were higher in maize inoculated with a *cmu1* mutant than with a wild-type strain, resulting in an increased resistance of the mutant to *U. maydis*. It was hypothesized that Cmu1 could act together with ZmCm2 in the plant cytoplasm to enhance the flow of the SA-precursor chorismate from the plastid (where SA biosynthesis takes place) to the cytosol. Consequently, in plastids, less chorismate would be available for SA biosynthesis (Djamei et al., 2011). These results indicate that SA biosynthesis pathway of maize is hijacked by *U. maydis* as a mechanism of virulence. Interestingly, such a mechanism was also described for the soybean cyst nematode *Heterodera glycines* and the root-knot nematode *Meloidogyne javanica* (Bekal et al., 2003; Doyle and Lambert, 2003). The virulence factor of *Cladosporium fulvum* Avr2 targets the tomato papain-like cysteine protease (PLCP) RCR3 and *Phytophthora*-inhibited protease 1 (PIP1) in order to deregulate basal immunity. RCR3 and PIP1 are specifically induced by treatment of tomato plants with the SA analog benzothiadiazole (BTH). Therefore, Avr2 seems to interfere with tomato SA signaling pathway (Shabab et al., 2008).

FITNESS COSTS OF DEFENSE RESPONSES REGULATED BY PHYTOHORMONES

The involvement of many plant growth regulatory phytohormones in the control of plant resistance responses to both biotic and abiotic stresses indicates the existence of a tight interconnection between two physiological processes: development and adaptation to environmental cues. The regulatory potential of the hormone network allows plants to quickly respond to environmental

changes and, thus, to use the limited nutrient resources in a cost-efficient manner. This hypothesis is based on the idea that being well-defended (i.e., having strong, pre-existing defensive mechanisms) may not always be the best defensive strategy, most likely because allocation of metabolites and proteins to resistance may constrain other plant physiological processes (Walters and Heil, 2007; Manzaneda et al., 2010; Kempel et al., 2011). In line with this hypothesis, it is generally believed that hormone-induced resistance evolved to save energy under enemy-free conditions, as they will only incur energy costs when these defensive mechanisms are activated upon pathogen infection or insect attack (Walters and Heil, 2007). However, pathogens and pests evolve to get adapted to the continuous exposure to defensive genetic traits (i.e., antibiotic or antideterrent proteins and/or metabolites). Therefore, it is also possible that hormone-induced resistance evolved to slow down the potential adaptation of putative attackers to these biochemical barriers (Walters and Heil, 2007). All these physiological constrains, together with the co-existence of plants with natural attackers, have evolutionary driven the selection of plant innate immune system.

In different plant species there have been characterized mutants or transgenic lines showing constitutive activation of defensive mechanisms and enhanced resistance to particular pathogens. These resistance phenotypes are generally associated with the misregulation of particular hormone signaling pathways (Robert-Seilaniantz et al., 2011a). The characterization of these mutants and transgenic plants has contributed to the identification of the molecular components involved in hormone biosynthesis and signaling pathways, and to the discovery of cross-regulatory nodes among these signaling pathways. Thus, *Arabidopsis* mutants constitutively overexpressing a specific hormone-dependent pathway (SA, ET, JA, ET + JA, etc.) show enhanced resistance to particular type of pathogens (reviewed by Robert-Seilaniantz et al., 2011a; Holeski et al., 2012). However, this enhanced, constitutive resistance negatively impact plant fitness as these mutants have phenotypic alterations such as dwarfism, spontaneous lesions in different organs, accelerated senescence, delayed flowering, sterility, or reduced seed production (for a review, see Robert-Seilaniantz et al., 2011a; Holeski et al., 2012; Thaler et al., 2012). These data indicate that plants have genetic determinants to fine-tune fitness/resistance balance. An example of this fine-tune regulation is represented by the SA receptor NPR3, that is a negative regulator of defensive response during *Arabidopsis* early flower development through its interaction with NPR1 and TGA2. Remarkably, the *nrp3* plants exhibit increased resistance to *P. syringae* infection of immature flowers, but showed reduced fitness in comparison to that of wild-type plants (Shi et al., 2012).

Alteration of a particular hormone signaling pathway generally results in the miss-regulation of other signaling pathways due to the described complex regulatory network that exist among hormones. Thus, the negative cross-regulations among hormone pathways, such as auxin, ABA, and SA described in this review, lead to alterations in the pattern of resistance to natural attackers. That is, enhanced resistance to a particular pathogen (i.e., necrotroph) can be achieved in some of these mutants, but they generally undergo increased susceptibility to a different one (i.e., biotroph; Spoel et al., 2007; Robert-Seilaniantz et al., 2011a). In some particular cases, such as in the ABA-deficient mutant *aba1*, broad spectrum resistance to both necrotrophic and biotrophic pathogen is observed, but this phenotype is also linked to a reduced adaptation of the mutant to abiotic stresses such as drought (Audenaert et al., 2002; de Torres-Zabala et al., 2007; de Torres Zabala et al., 2009; Garcia-Andrade et al., 2011; Sánchez-Vallet et al., 2012). As in nature, plants are exposed to many different biotic agents, but also to abiotic stress, hormone homeostasis is critical in the establishment of appropriate and effective defensive responses of plant against natural attackers and/or abiotic stresses in an ecological context (**Figure 2**). In line with the hypothesis of the critical role of hormones in balancing growth and response to environmental cues, it has been recently demonstrated that brassinosteroids, that control several developmental-associated processes, also modulate the efficiency of PTI in *Arabidopsis* (Belkhadir et al., 2012). The interaction between these two types of environmental stresses (biotic and abiotic) requires a complex adaptive molecular response involving many factors that we are just starting to understand (reviewed in Atkinson and Urwin, 2012).

Expressing constitutive resistance by the modification of hormone homeostasis/signaling encounters the risk of allocating resources to defense in the absence of natural pathogens and of impairing defensive mechanisms against particular natural attackers. One alternative to the constitutive, long-lasting activation of induced resistance is to fine-tune plant resistance mechanism by modulating the "immunological memory" of plants, as it has been described in animals (Conrath, 2011). An interesting phenomenon in this context is the so-called "priming" that is a condition whereby plants that have been subjected to prior attack will respond more quickly or more strongly to a subsequent attack. Given that resources are not committed until the threat returns, priming is thought to be a relatively low-cost mechanism of advancing plant defense (Conrath, 2011). Remarkably, the resistance response in primed plants treated with a low, non-effective concentration of a defensive hormone is also faster and stronger than that in non-primed plants (Conrath, 2011). It has been recently demonstrated the existence of an epigenetic regulation of priming, which explain the lack of significant transcriptional changes in primed plants unless they are exposed to the priming agent/hormone (Luna et al., 2012; Slaughter et al., 2012). The genetic control of priming shows similarities to the genetic mechanisms that regulate transgenerational defense induction in plants, such as the SA-dependent SAR and the inherited JA-dependent defense (Holeski et al., 2012; Thaler et al., 2012; Zheng et al., 2012). Similarly, transgenerational priming has been also described (Luna et al., 2012; Slaughter et al., 2012). All these epigenetically inherited changes in defense can strongly alter plant responses to jasmonate and salicylate in offspring and therefore might negatively impact plant resistance to particular type of pathogens (Latzel et al., 2012; Luna et al., 2012).

Though all the published data clearly indicate a fitness cost associated to the constitutive activation of hormone-mediated resistance mechanisms, it must be considered that these experiments were generally performed under laboratory conditions, without nutrient limitations and ecological constraints (i.e., plants were infected with just one pathogen). Long-term experiments

with model and crop plants under field conditions should be done to determine the potential use of hormone-mediated resistance in crop protection, as these experiments will provide information on the hormone-mediated effectiveness of disease control, but also on plant trade-offs and changes in the population structure of pathogens and pests. Also, a better understanding of the molecular and genetic mechanisms regulating hormone-mediated resistance would be required to successfully manipulate hormone homeostasis/signaling and improve crop resistance to pathogens.

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Open or close the gate – stomata action under the control of phytohormones in drought stress conditions

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Two highly specialized cells, the guard cells that surround the stomatal pore, are able to integrate environmental and endogenous signals in order to control the stomatal aperture and thereby the gas exchange. The uptake of CO_2 is associated with a loss of water by leaves. Control of the size of the stomatal aperture optimizes the efficiency of water use through dynamic changes in the turgor of the guard cells. The opening and closing of stomata is regulated by the integration of environmental signals and endogenous hormonal stimuli. The various different factors to which the guard cells respond translates into the complexity of the network of signaling pathways that control stomatal movements. The perception of an abiotic stress triggers the activation of signal transduction cascades that interact with or are activated by phytohormones. Among these, abscisic acid (ABA), is the best-known stress hormone that closes the stomata, although other phytohormones, such as jasmonic acid, brassinosteroids, cytokinins, or ethylene are also involved in the stomatal response to stresses. As a part of the drought response, ABA may interact with jasmonic acid and nitric oxide in order to stimulate stomatal closure. In addition, the regulation of gene expression in response to ABA involves genes that are related to ethylene, cytokinins, and auxin signaling. In this paper, recent findings on phytohormone crosstalk, changes in signaling pathways including the expression of specific genes and their impact on modulating stress response through the closing or opening of stomata, together with the highlights of gaps that need to be elucidated in the signaling network of stomatal regulation, are reviewed.

Keywords: stomata, guard cells, phytohormones, abiotic stress, ABA, jasmonic acid, crosstalk

INTRODUCTION

Stomata are specialized epidermal structures that are essential for plant survival and productivity. These structures consist of two guard cells around a pore. Every stoma is a molecular valve that acts in gas exchange, mainly CO_2 and O_2, which is necessary for optimal photosynthesis and which restricts water loss by modulating the transpiration level. The genes that are involved in the process of stomata development were crucial for the movement of plants from water to land during evolution since stomata facilitated gas exchange while limiting desiccation. The stomatal morphogenesis pathway has been identified in detail in *Arabidopsis thaliana* through investigations of many mutants with an impaired stomatal pattern or with other morphological defects in their epidermal cells. Cell distribution and differentiation require a balance between proliferation and cell specification in time and space. The differentiation of stomata is preceded by at least one asymmetric as well as a few symmetric cell divisions. It requires three different types of precursor cells: the meristemoid mother cell (MMC), meristemoids and the guard mother cell (GMC). The last step of stomatal development is the differentiation of the stoma itself within the structure of the guard cells. The number and pattern of stomata varies in different organs in *A. thaliana*. A common feature of patterning is that stomata are separated from each other by at least one epidermal cell. This pattern ensures the presence of neighbor cells for ion exchange, which is necessary for the regulation of the aperture width. For this reason, neighbor cells are part of a stomatal complex (Nadeau and Sack, 2002; Nadeau, 2009; Lau and Bergmann, 2012; Pillitteri and Torii, 2012; Vatén and Bergmann, 2012). Recent research has shown that the mode of action of stomata depends on the integration of environmental and intracellular signals. Many environmental factors such as CO_2 concentration, biotic and abiotic stresses, and additionally different plant hormones, can modulate stomatal reaction. For plants that encounter dehydration stress, the most essential factor is the ability of stomata to close and thus prevent excess water loss. Opening and closing is achieved by the swelling and shrinking of the guard cells, which is driven by ion exchange; cytoskeleton reorganization and metabolite production; the modulation of gene expression and the posttranslational modification of proteins (reviewed in Kim et al., 2010). Swelling of the guard cells results in stomata opening since the content of ions and osmolites within them makes them bigger and thus able to move away from each other making the stomatal aperture larger. In contrast, closing is an opposite mechanism and results in the shrinking of the guard cells when the efflux of ions occurs.

Stomatal closure is the earliest plant response to water deficit (Schroeder et al., 2001b). This rapid reaction is regulated by a complex network of signaling pathways, in which the major and the best-known player, abscisic acid (ABA), acts in concert with jasmonates (JA), ethylene, auxins, and cytokinins (Nemhauser et al., 2006; Huang et al., 2008). The complexity of the response is mainly dependent on the initial threshold of stress and individual plant's

stress history. Generally, ABA and JA are positive regulators of stomatal closure, while auxin and cytokinins are positive regulators of stomatal opening. The mode of action of ethylene is ambiguous because it can act as a positive or negative regulator, depending on the tissue and conditions (Nemhauser et al., 2006; Huang et al., 2008).

This paper presents a comprehensive review of the genetic and molecular basis of stomata action under the control of phytohormones, particularly when response to drought stress is considered.

OPEN OR CLOSE THE GATE – THE ROLE OF ABA, ION CHANNELS, AND DIURNAL CYCLE IN STOMATAL MOVEMENTS REGULATION

THE REGULATORY ROLE OF ION CHANNELS LOCALIZED IN THE GUARD CELL MEMBRANE IN THE OPENING AND CLOSING STOMATA

The guard cell turgor is dynamically adjusted to environmental conditions and hormonal signals in order to facilitate the proper gas exchange and prevent excessive water loss. Mature guard cells do not have plasmodesmata and for this reason most influx and efflux of solutes occurs via ion channels, transporters, and pumps that are localized in the plasma membrane (PM). The action of ion channels, transporters, and pumps that are essential for stomatal function is well documented and supported by molecular studies involving mutants in the genes encoding these protein. During the opening of the stomata, the H^+-ATPase pump mediates the efflux of H^+ from the guard cells. In plants, H^+-ATPases belong to the multi-gene family of the P-type ATPases, with 11 genes in *Arabidopsis*, which are all expressed in the guard cells (Ueno et al., 2005). In the guard cells, the action of H^+-ATPase activity is positively regulated by blue light and auxins, whereas Ca^{2+} and ABA act as negative regulators. The efflux of H^+ hyperpolarizes the PM and leads to K^+ uptake via activation of inward K^+ rectifying channels, such as KAT1 (potassium channel in *Arabidopsis thaliana* 1), KAT2 (potassium channel in *Arabidopsis thaliana* 2), and AKT1 (*Arabidopsis thaliana* K^+ transporter 1) (Schachtman et al., 1992; Pilot et al., 2001; Szyroki et al., 2001). Another signal that activates the influx of K^+ via K^+ channels is the acidification of the apoplast as a result of H^+ extrusion from the guard cells. K^+ uptake is balanced by counter-ions, mainly Cl^- obtained from the apoplast, malate^{2-} that is derived from starch breakdown or NO_3^-. The last one is transported from the apoplast by a nitrate transporter AtNRT1.1 (CHL1) [nitrate transporter 1 (chlorina1)]. The importance of NO_3^- uptake was confirmed by an analysis of an *Arabidopsis clh1* mutant. The stomatal apertures of the *chl1* mutant were smaller than those of the wild-type when nitrate was supplied. Furthermore, the *chl1* mutant was drought tolerant (Guo et al., 2003). Ions supplied into the guard cells together with water transported via aquaporins generate the turgor that are necessary to keep stomata open (**Figure 1A**).

During stomatal closure, the inhibition of H^+-ATPase and the activation of anion channels together result in membrane depolarization. Anion channels such as rapid channels (R-type) and slow channels (S-type) facilitate the efflux of malate^{2-}, Cl^-, and NO_3^- (Roelfsema et al., 2004; Roelfsema and Hedrich, 2005). The decreased level of malate^{2-} in guard cells is also linked with the gluconeogenic conversion of malate^{2-} into starch (Willmer and Fricker, 1996). Membrane depolarization creates a driving force for the efflux of K^+ via K^+ outwardly rectifying channels such as GORK (guard cell outwardly rectifying K^+ channel) (Jeanguenin et al., 2008). An *Arabidopsis gork* mutant displayed impaired stomatal closure, thus confirming the important role of GORK in elimination K^+ ions and in the facilitation of stomatal closure (Hosy et al., 2003). Another event that accompanies stomatal closure is an elevation of the cytoplasmic Ca^{2+} concentration as a result of Ca^{2+}-release via channels situated in both the PM and in the tonoplast (MacRobbie, 2006). Ca^{2+} channels are encoded by genes from three gene-families: TPC1 (two-pore channel 1) (Peiter et al., 2005), CNGC (cyclic nucleotide gated channel) (Finn et al., 1996), and GLR (glutamate receptor) (Lacombe et al., 2001). Taken together, the efflux of solutes from the guard cells leads to a reduced turgor and stomatal closure (**Figure 1B**).

ABSCISIC ACID – HOW THE PROPER LEVEL OF THE MAIN REGULATOR OF STOMATAL MOVEMENTS IS ACHIEVED IN PLANTS

Abscisic acid has been postulated as a main regulator of stomatal movements but its proper functioning depends on the appropriate level of biologically active ABA within the plant cells. This is achieved by synchronized processes such as ABA biosynthesis, catabolism, conjugation/deconjugation, and transport. These processes, which are well recognized and studied in various species, have confirmed the function of many enzymes involved in the biosynthesis, catabolism, conjugation/deconjugation, and transport of ABA. The exception, not fully recognized yet, is ABA signal transduction pathway. Although ABA has been the focus of many research groups since the early 90s, there are still many questions in regards to the function of the proteins involved in ABA signaling, protein interactions or the impact of the components of signalosome on specific physiological responses. Therefore, with the progress in studies on ABA signaling, the state of knowledge and the already known interaction web should be updated and verified.

Abscisic acid is synthesized in the plastids and cytosol, mainly in the vascular parenchyma cells but also in the guard cells, through the cleavage of a C40 carotenoid precursor, followed by a two-step conversion of the intermediate xanthoxin into ABA via ABA-aldehyde (Taylor et al., 2000; Finkelstein and Rock, 2002; Schwartz et al., 2003; Endo et al., 2008; Melhorn et al., 2008). The pathway begins with isopentenyl pyrophosphate (IPP), which is the biological isoprene unit and the precursor of all terpenoids, as well as many plant hormones. The next step is the epoxidation of zeaxanthin and antheraxanthin into violaxanthin, which is then catalyzed by zeaxanthin epoxidase (ZEP) (Marin et al., 1996). After a series of violaxanthin modifications that are controlled by the enzyme ABA4, violaxanthin is converted into 9-cis-epoxycarotenoid (North et al., 2007). Oxidative cleavage of the major epoxycarotenoid 9-cis-neoxanthin by the 9-cis-epoxycarotenoid dioxygenase (NCED) yields a C15 intermediate – xanthoxin (Schwartz et al., 1997). This step is the last one that occurs in plastids. Xanthoxin is exported to the cytoplasm where a two-step reaction via ABA-aldehyde occurs. The first step is catalyzed by a short-chain alcohol dehydrogenase/reductase (SDR) that is encoded by the *AtABA2* (*ABA deficient 2*) gene (Rook et al., 2001; Cheng et al., 2002; Gonzalez-Guzman et al., 2002)

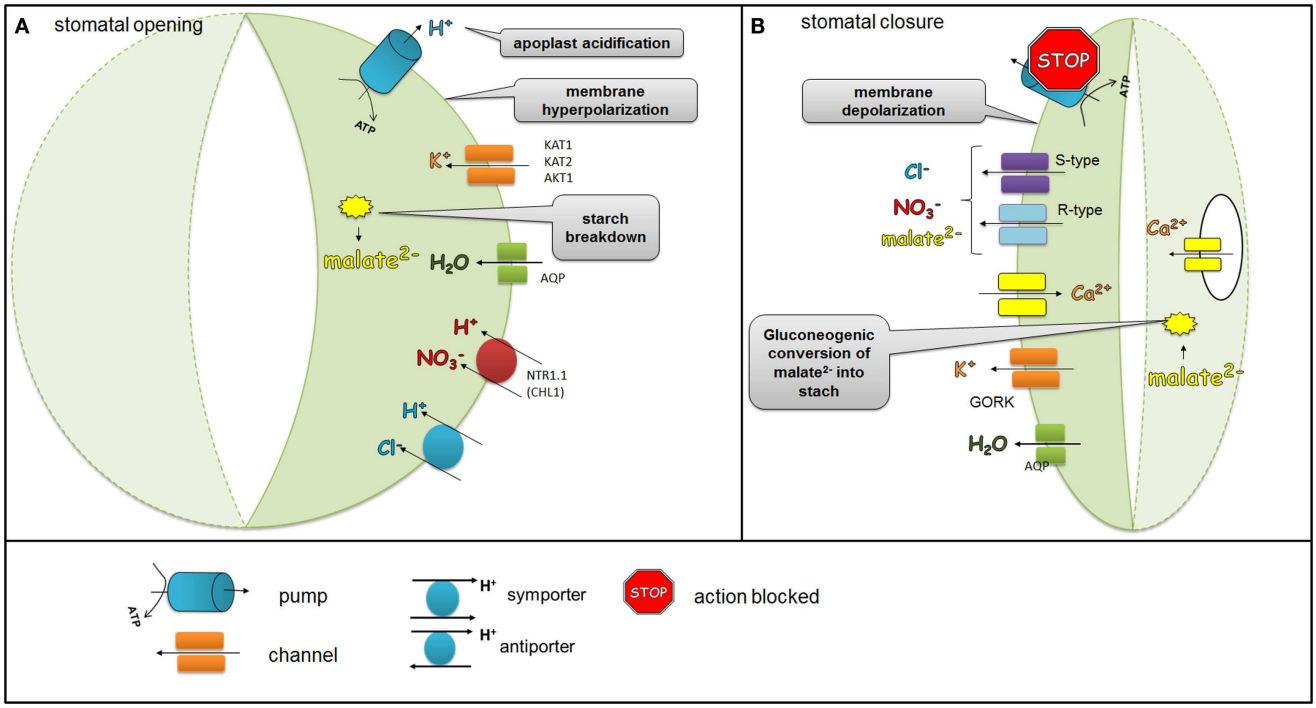

FIGURE 1 | Regulation of ion channels, pumps, and transporters localized in the plasma membrane of the guard cells during stomatal opening and closure. During stomatal opening **(A)** H^+-ATPase pumps H^+ from the guard cells and hyperpolarizes the membrane, which leads to the activation of K^+ inward rectifying channels (KAT1, KAT2, AKT1). Anionic species such as malate^{2-} from the breakdown of starch and transported NO_3^- and Cl^- ions contribute to the intracellular solute buildup that can mediate the import of sugars or can be used for the synthesis of sugars. Ions supplied into the guard cells together with water transported via aquaporins generate the turgor that is needed to keep stomata opened. During stomatal closure **(B)**, H^+-ATPase is inhibited and S-type and R-type anion channels are activated. As the plasma membrane is depolarized, S-type and R-type channels facilitate the efflux of malate^{2-}, Cl^-, and NO_3^-. At the same time, K^+ outwardly rectifying channels such as GORK are activated through the depolarization of the membrane, which leads to the efflux of K^+. The decreased level of malate^{2-} is also caused by the gluconeogenic conversion of malate into starch. The elevation of the Ca^{2+} concentration as a result of the release of Ca^{2+}- via channels situated in both the plasma membrane and in the tonoplast is another event that accompanies stomatal closure.

and that generates ABA-aldehyde. Then, the ABA-aldehyde oxidase (AAO) with the molybdenum cofactor (MoCo) catalyzes the last step in the biosynthesis pathway – the conversion of ABA-aldehyde into ABA (Seo et al., 2004) (**Figure 2A**). The appropriate level of active ABA is achieved not only through the biosynthesis and catabolism reactions performed by CYP707A1-4 (cytochrome P450, family 707, subfamily A, polypeptide 1, 2, 3, 4) (Kushiro et al., 2004; **Figure 2B**), but also by the inactivation of ABA through conjugation and deconjugation. ABA can be inactivated at the C-1 hydroxyl group by different chemical compounds that form various conjugates and that accumulate in vacuoles or in the apoplastic space (Dietz et al., 2000). The most widespread conjugate is ABA glucosyl ester (ABA-GE), which is catalyzed by ABA glucosyltransferase (Boyer and Zeevaart, 1982). Lee et al. (2006) identified the AtBG1 (beta-1,3-glucanase 1) protein that is responsible for the release of ABA from ABA-GE. Their findings showed that ABA deconjugation plays a significant role in providing an ABA pool that allows plants to adjust to changing physiological and environmental conditions (**Figure 2C**).

The ability of ABA to move long distances allows it to serve as a critical stress messenger. Kuromori et al. (2011) identified the ABA importer – ABCG22 (*Arabidopsis thaliana* ATP-binding cassette G22). The gene encoding this transporter is mainly expressed in the guard cells. In addition, the expulsion of ABA into the intercellular space is mediated by transporters such as ABCG25 (*Arabidopsis thaliana* ATP-binding cassette G25). ABCG25 is expressed primarily in vascular tissues where ABA is synthesized (Kuromori et al., 2010). ABA delivery to the guard cells promotes a cascade of reactions that lead to stomatal closure and that inhibit stomatal opening in order to prevent water loss (**Figure 2D**).

After ABA is received from ABC transporters by the guard cells, the PYR/PYL/RCAR (pyrabactin-resistance 1/pyrabactin-resistance like/regulatory component of ABA receptor) perceives ABA intracellularly and forms complexes that inhibit clade A of PP2Cs (protein phosphatase 2C), the negative regulators of ABA signaling, such as ABI1 (ABA insensitive 1), ABI2 (ABA insensitive 2), HAB1 (hypersensitive to ABA1) (Ma et al., 2009; Park et al., 2009; Santiago et al., 2009; Nishimura et al., 2010). The inactivation of PP2Cs allows downstream targets to be phosphorylated and activated – Sucrose Non-fermenting 1-Related subfamily 2 protein Kinases (SnRK2) (Fujii and Zhu, 2009; Fujita et al., 2009; Umezawa et al., 2009; Kim et al., 2010). ABA receptors, PP2Cs, and SnRKs form the core of the early ABA signaling cascade (**Figure 2E**).

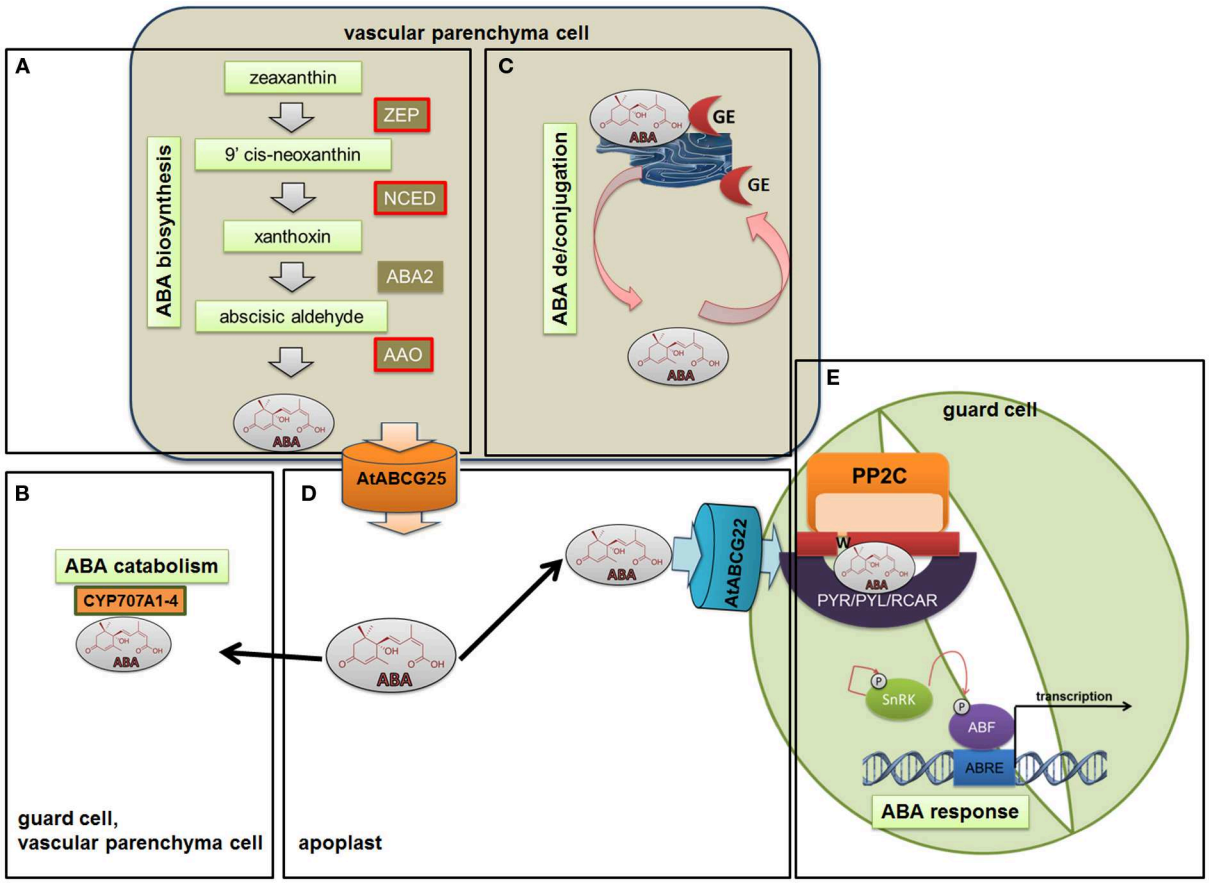

FIGURE 2 | Abscisic acid biosynthesis, catabolism, deconjugation, transport, and signaling. ABA biosynthesis **(A)** is mainly induced by upregulating *NCED3, ZEP*, and *AAO* genes. At the same time as the biosynthesis of ABA is induced, the catabolism **(B)** that is performed by CYP707A1-4 is inhibited. The balance between active and inactive ABA in the cell is achieved not only by the regulation of biosynthesis and catabolism but also by ABA conjugation and deconjugation. The most widespread conjugate is the ABA glucosyl ester (ABA-GE), which is catalyzed by ABA glucosyltransferase **(C)**. ABA delivery to the guard cells via ABCG transporters such as AGCG22 **(D)** promotes a cascade of reactions. The core of early ABA signaling involves ABA receptors – PYR/PYL/RCAR proteins, PP2Cs, and SnRKs **(E)**. After binding ABA to the receptor, the negative regulatory action of PP2Cs is inhibited and SnRKs are able to phosphorylate and activate downstream targets in order to transduce the ABA signal.

REGULATION OF STOMATAL MOVEMENTS DURING THE DIURNAL CYCLE – THE ROLE OF ABA

The ABA mode of action is linked to diurnal stomatal movements. It has been proposed that this link is based on both the molecular connections between ABA and circadian-clock pathways and on ABA biosynthesis and response to light (reviewed in Tallman, 2004). Although several studies have been carried out linking the diurnal cycle with ABA signaling, there is still a need for further research that would clarify this connection. It has been confirmed that the elevated ABA levels in the dark phase of the day are responsible for stomatal closure but, on the other hand, the molecular basis of the sensing CO_2 molecules by guard cells is still not well understood. This part of investigations still needs confirmation through the use of well-established methods.

In darkness, stomata are closed. This is probably caused by an intensive ABA accumulation through the biosynthesis of ABA in the guard cells and the simultaneous import of endogenous ABA from the apoplast to the guard cells using ABA transporters such as ABCG22 (Kuromori et al., 2011), while at the same time, ABA catabolism processes are disfavored. Elevated ABA levels cause stomata closure via the activation of an ABA signaling cascade, the efflux of Ca^{2+} from internal stores, the activation of S-type and R-type anion channels that lead to the efflux of Cl^-, malate^{2-}, and NO_3^- and the activation of the GORK channel that leads to the efflux of K^+. During the night, elevated levels of CO_2 in the leaves were observed due to respiration. It has been proved that CO_2 has a positive effect on the stomatal closure process. The guard cells probably do not sense CO_2 molecules but instead HCO_3^- is synthesized from CO_2 (Hu et al., 2010), which activates S-type channels and leads to the efflux of Cl^-, malate^{2-}, and NO_3^- (Xue et al., 2011) (**Figure 3A**).

At first light, a depletion of endogenous ABA is observed through xanthophyll cycling, the isomerization of ABA precursors and the activation of ABA catabolism enzymes, such as CYP450 (cytochrome P450). The degradation of ABA liberates the guard cells to extrude H^+ via H^+-ATPase (H^+-pump) and accumulate

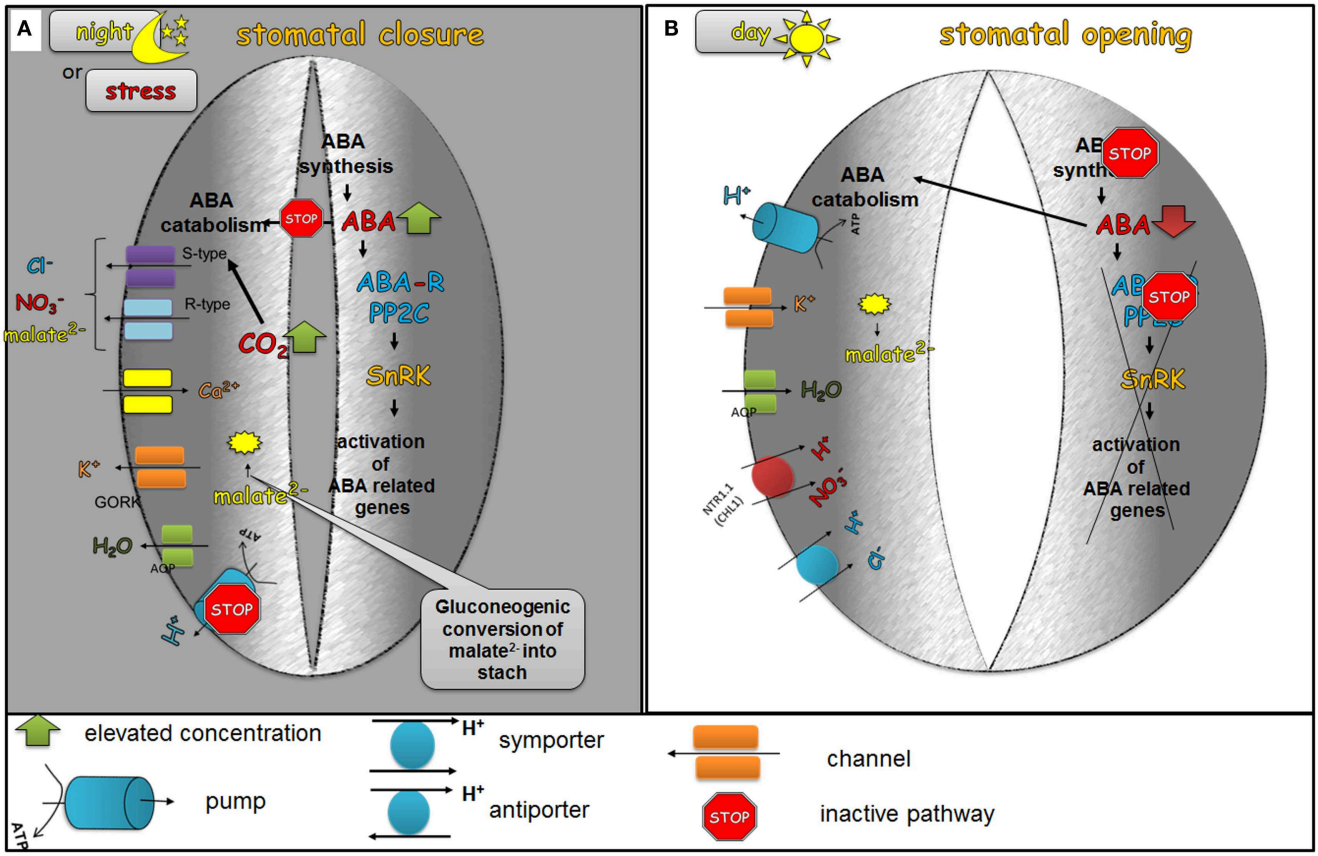

FIGURE 3 | The role of ABA in the diurnal regulation of stomatal movements. In the dark phase of the day **(A)**, ABA biosynthesis is favored and at the same time the catabolism of ABA is inhibited. As a result of these processes, elevated levels of ABA are present in the guard cells. ABA activates the efflux of Ca^{2+} from internal stores, the activation of S-type and R-type anion channels leading to the efflux of Cl^-, malate^{2-}, and NO_3^-, the activation of GORK channel, which leads to the efflux of K^+ and consequently to the closing of stomatal pores. The decreased level of malate^{2-} is also caused by the gluconeogenic conversion of malate into starch. In the dawn **(B)**, the first light promotes ABA catabolism processes and the level of ABA biosynthesis decreases, which leads to a decreased concentration of active ABA in the guard cells. Low endogenous ABA levels no longer inhibit H^+-ATPase (H^+-pump), which is then able to extrude H^+ from the guard cells. At the same time, the accumulation of water and ions, such as K^+, Cl^-, malate^{2-} occurs in order to generate the turgor that is needed to keep stomata open.

water and ions, such as K^+, Cl^-, malate^{2-} in order to generate the turgor needed to keep stomata open. K^+ uptake is mainly responsible for the rapid increase of the turgor and the opening of stomata during the dawn (Humble and Raschke, 1971; Talbott and Zeiger, 1996). The accumulation of sugars such as glucose, fructose and sucrose has been reported during the light phase of the day (Talbott and Zeiger, 1998). In the midday, ABA is delivered to the apoplast around the guard cells through the xylem transpiration stream and the guard cells are regulated by steady-state ABA concentrations (**Figure 3B**).

In the evening, ABA biosynthesis outweighs the ABA catabolism in the guard cells, which leads to stomatal closure (for review, see Tallman, 2004).

ABA ON THE WAY TO REACHING THE GUARD CELLS UNDER DROUGHT STRESS CONDITIONS

Under drought stress conditions, ABA would reach a concentration high enough to cause ion efflux and an inhibition of sugar uptake by the guard cells in the midday, thus reducing the apertures for the rest of the day. Analyses of ABA biosynthesis, catabolism, de/conjugation, and transport have been supported by various studies involving several species and different methods, such as mutant analysis, transcriptomics, proteomics, or immunohistochemical techniques. In order to define the role of ABA in stress response, the action of several components of the pathways mentioned were tested in response to stress. The engagement of such various techniques makes the state of knowledge in the field of ABA biosynthesis, catabolism, de/conjugation, and transport well supported and reliable.

It has been shown that ABA concentrations can increase up to 30-fold in response to drought stress (Outlaw, 2003). Water deficit promotes ABA biosynthesis via the upregulation of a key enzyme – NCED3. A significant increase in NCED transcript levels can be detected within 15–30 min after leaf detachment or dehydration treatment (Qin and Zeevaart, 1999; Thompson et al., 2000), which indicates that the activation of *NCED* genes can be fairly quick. Cheng et al. (2002) reported that the *AtNCED3*, *AtZEP* (*Zeaxanthin epoxidase*), and *AtAAO3* (*ABA-aldehyde oxidase*) genes could

be induced in *Arabidopsis* by ABA and studies in rice showed that *OsNCED3* expression was induced by dehydration (Ye et al., 2011). An immunohistochemical analysis, using antibodies raised against AtNCED3, revealed that protein is accumulated in the leaf vascular parenchyma cells in response to drought stress. This was not detected in non-stressed conditions. These data indicate that drought-induced ABA biosynthesis occurs primarily in the vascular parenchyma cells and that vascular-derived ABA might trigger stomatal closure via the transport to the guard cells (Endo et al., 2008). *AtNCED3* expression is upregulated by drought conditions across the species observed and decreases after rehydration.

Drought, like the dark part of a diurnal cycle, also promotes the deconjugation of the ABA-glucose ester (ABA-GE), which is stored in the vacuoles of leaf cells and also circulates in the plant (Xu et al., 2002; Seiler et al., 2011). Both processes, intensive ABA biosynthesis and ABA deconjugation, lead to the accumulation of high levels of biologically active ABA. ABA delivery to the guard cells via ABCG transporters, such as AGCG22 that was mentioned above, promotes a cascade of reactions that lead to stomatal closure and that inhibit stomatal opening in order to prevent water loss (**Figure 2**).

ABA TRIGGERS CHANGES IN ION HOMEOSTASIS IN THE GUARD CELLS, WHICH LEADS TO STOMATAL CLOSURE UNDER STRESS

The ABA signaling network that leads to stomatal closure under stress is activated by the perception ABA. This begins a cascade of reactions that leads to the reduced turgor of the guard cells through ABA modulation of ion channel activities, including the regulated efflux of anions and potassium ions and the inhibition of K^+ import. Recently, the core signalosome of ABA signaling including ABA receptors, phosphatases (PP2Cs), and kinases (SnRK2s) was established (Ma et al., 2009; Park et al., 2009; Santiago et al., 2009; Nishimura et al., 2010). Although its function is clear and confirmed by advanced molecular analysis, there is still a need to explain the impact of single components, such as kinases, on the regulation of the ion channels or the proton pump (e.g., AHA1), which is described below. On the other hand, the interaction between ABA regulated kinases SnRK2s and S-type anion channels, and the potassium inwardly rectifying channels, described below, has been well established and documented.

The inactivation of PP2Cs such as ABI1 and ABI2 by the complex ABA-receptor facilitates the phosphorylation and activation of a downstream target of phosphatases – SnRK2, such as SnRK2.2/D, SnRK2.3/E, and SnRK2.6/OST1/E, which are the key players in the regulation of ABA signaling and abiotic stress response (Fujii and Zhu, 2009; Fujita et al., 2009; Umezawa et al., 2009). Kinases are able to regulate the activity of ion channels and the proton pump. It was shown that ABA inhibits the action of a proton pump such as H^+-ATPase. The dominant *Arabidopsis* mutant *ost2* (*opened stomata 2*) in *AHA1* (H^+-ATPase 1 HA1) gene exhibited the constitutive activation of AHA1 H^+-ATPase, which in turn resulted in an inability to close stomata in response to ABA (Merlot et al., 2007). The molecular mechanism of the inhibition of AHA1 by ABA has not yet been fully elucidated. One of the most direct pieces of evidence of the regulation of H^+-ATPase by SnRK is the demonstration that specific calcium-stimulated kinase, PSK5 (a member of the SnRK3 kinase family), is able to phosphorylate the closest homolog of AHA1 – AHA2 (H^+-ATPase 1 HA2) in Ser392 localized in the C-terminus of the AHA2 protein. This reaction prevents the 14-3-3 protein, which is the main activator of AHA2 leading to the inhibition of H+-ATPase action, from binding (Fuglsang et al., 2007).

Sucrose Non-fermenting 1-Related subfamily 2 protein Kinases also regulate S-type anion channels and potassium inwardly rectifying channels such as SLAC1 (slow anion channel-associated 1) and KAT1 (K^+ channel in *Arabidopsis thaliana*), respectively. The first one is activated by SnRK2, whereas KAT1 is inhibited. *SLAC1* encodes the anion-conducting subunit of an S-type anion channel. In different species, S-type anion channels are activated in the guard cells by ABA, cytosolic Ca^{2+}, and phosphorylation events (Schmidt et al., 1995; Pei et al., 1997; Leonhardt et al., 1999; Raschke et al., 2003; Roelfsema et al., 2004; Mori et al., 2006). The *slac1* mutant displayed a strongly impaired response to a range of stomatal closing stimuli such as ABA and Ca^{2+} (Negi et al., 2008; Vahisalu et al., 2008). Increased SLAC1 activity causes an efflux of anions which results in depolarization of the membrane as a consequence of phosphorylation by SnRK. This in turn leads to the loss of K^+ cations from the cell through the K^+ efflux channel GORK (guard cell outward-rectifying K^+), which is activated by depolarization (Jeanguenin et al., 2008). KAT1 is an inward K^+ channel that allows an influx of K^+ inside the guard cell when the proton pump drives the PM to a negative potential. When plants encounter drought stress conditions and the ABA level rises, both the proton pumps (as mentioned above) and KAT1 are inactivated by SnRKs. It was shown that the activity of KAT1 is inhibited by an elevation of ABA and cytosolic Ca^{2+} (Schroeder and Hagiwara, 1989; Blatt and Armstrong, 1993; Grabov and Blatt, 1999) via phosphorylation by SnRK, which in turn results in a decreased influx of K^+ into the guard cells (Hubbard et al., 2010). The loss of K^+ and anions from the guard cells is accompanied by the efflux of water via aquaporins. Together, these events lead to a reduction of the turgor, which results in stomatal closure in response to ABA as a major signal of drought (**Figure 3A**).

Abscisic acid activates the Ca^{2+}-permeable channels in the PM of the guard cells and triggers an influx of Ca^{2+} into the cytoplasm of the guard cells through the release of the second messenger, inositol-1,4,5-triphosphate (IP_3), which in turn activates the Ca^{2+} channels that are located in the vacuole and endoplasmic reticulum (Schroeder and Hagiwara, 1990; Hamilton et al., 2000; Krinke et al., 2007; Kwak et al., 2008). Ca^{2+}-dependent protein kinases (CDPKs) are activated during drought stress and are able to control stomatal closure in an ABA-dependent manner. After ABA is perceived by a receptor, the action of PP2Cs such as ABI1 are inhibited. ABI1 was identified as a negative regulator of CPK21 (Ca^{2+} dependent protein kinase 21), which like SnRK phosphorylates SLAC1. SLAC1 phosphorylation, in turn, results in the activation of anion and the efflux of K^+ (Geiger et al., 2010). An increased cytosolic Ca^{2+} level activates the Ca^{2+}-dependent pathways that inhibit K^+ import and trigger the depolarization of the membrane. Mori et al. (2006) identified two calcium-dependent kinases – CPK3 (calcium-dependent protein kinase 3) and CPK6 (calcium-dependent protein kinase 6) as positive regulators of ABA signaling in the guard cells during water stress. Inactivation of both genes led to a reduction in the activation of S-type

channels by ABA and Ca^{2+}, the impairment of the ABA activation of Ca^{2+} permeable channels and a decreased sensitivity of stomata to ABA. Disruption of the regulatory subunit *RCN1* (*roots curl in NPA*) of the gene encoding PP2A (protein phosphatase 2A) led to a reduction of the ABA activation of anion channels and a decreased sensitivity of stomata to ABA (Kwak et al., 2002, **Figure 4**).

Another protein involved in ABA signaling in stomata is GPA1. GPA1 is a positive regulator in the ABA-mediated inhibition of stomatal opening. *Arabidopsis* plants lacking GPA1 (Gα subunit of G protein) showed a reduction in the inhibition of inward K^+ currents and a reduced guard cell ABA-insensitivity in stomatal opening (Wang et al., 2001). The mutants *era1* (*enhanced response to ABA1*) and *abh1* (*ABA hypersensitive 1*), which are deficient in a farnesyl transferase subunit and RNA cap-binding protein, respectively, are ABA hypersensitive and showed an enhanced ABA activation of S-type channels (Pei et al., 1998; Schroeder et al., 2001a; Hugouvieux et al., 2002; **Figure 4**). However, the exact molecular role of ERA1 or ABH1 in stomatal regulation should be clarified in future research.

During stomatal closure, slow vacuolar (SV) channels activated by cytosolic Ca^{2+} contribute to the release of Ca^{2+} from vacuoles. SV channels were shown to be calcium permeable and it was suggested that they facilitate a brief transient efflux of cations, including Ca^{2+}, from vacuoles (Ward and Schroeder, 1994).

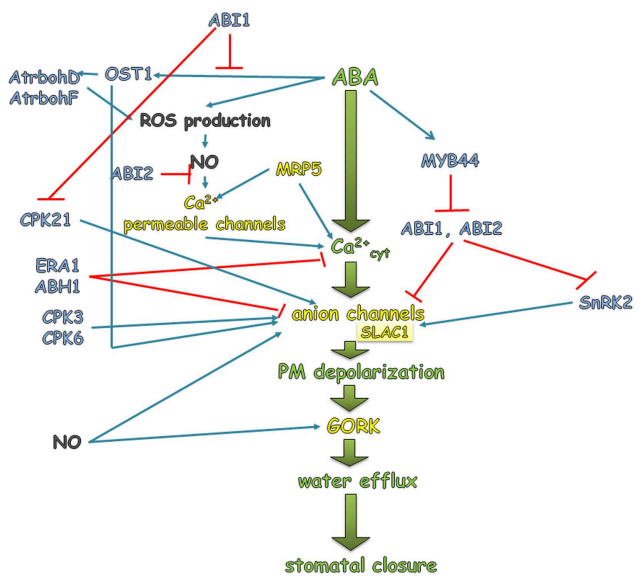

FIGURE 4 | ABA regulation of stomatal closure during drought stress. An increased level of endogenous ABA in response to drought activates a signal transduction pathway that involves a sequence of events such as the elevation of the cytosolic Ca^{2+} level, which consequently activates the anion channels (S-type and R-type), which leads to membrane depolarization. The latter activate GORK, which is responsible for extruding K^+ from the guard cells. Simultaneous with the efflux of K^+, an efflux of water is observed. Together, these events lead to a decrease in the turgor of the guard cells and to stomatal closure under drought conditions. The sequence of events, which is explained in detail in the main text and presented in green in the figure, is the core of the reactions that are induced or inhibited by different proteins that are activated by ABA. Blue arrows indicate activation, while red blunt ended lines indicate inhibition.

Several of the genes involved in the processes described above and more are presented in **Table 1** together with a description of mutant phenotypes.

NO AND ROS IN RESPONSE TO DROUGHT STRESS AND ABA

The guard cells generate reactive oxygen species (ROS) such as hydrogen peroxide (H_2O_2) and nitric oxide (NO) in response to ABA (Pei et al., 2000; Zhang et al., 2001). Exogenous H_2O_2 activates permeable Ca^{2+} channels in the PM of *Arabidopsis* guard cells and inhibits inward K^+ channels (Zhang et al., 2001; Kohler et al., 2003; Kwak et al., 2003). Taking into account the fact that ROS and NO signaling is not yet fully understood, there is a need for further analysis in order to elucidate their function, for example, the role of Ca^{2+} in ROS and NO action in guard cells should be clarified.

Reactive oxygen species production in *Arabidopsis* guard cells is mediated by two subunits of NADPH oxidase – AtrbohD (*Arabidopsis thaliana* respiratory burst oxidase homolog D) and

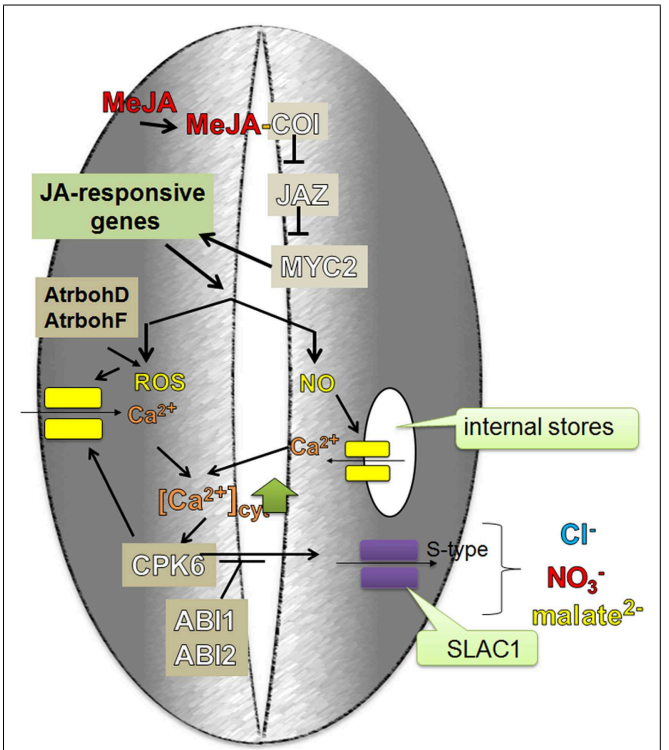

FIGURE 5 | Me-JA regulated stomatal closure during drought stress. MeJA, before it can be bound by a receptor in the plant cell, is converted into a biologically active form (+)-7-iso-Jasmonoyl-L-isoleucine (JA-Ile). JA-Ile is then bound by the receptor ^{SCF}COI complex that contains the coronatine insensitive1 (COI1) F-box protein. This interaction leads to the JAZ degradation which is negative regulator of MYC2. Inactive JAZ is not able to repress MYC2 function which in turn activates JA-responsive genes. MeJA induces the formation of ROS and NO, which activate the efflux of Ca^{2+} from internal stores and the influx from the apoplast by channels in plasma membrane. CPK6 acts downstream of NO and ROS signaling and therefore may be the target of an NO-stimulated influx of Ca^{2+} into the cytoplasm. As a feedback loop, MeJA-induced influx of Ca^{2+} into the cytoplasm activates CPK6, which in turn is able to activate the S-type anion channel – SLAC1, which then leads to the MeJA-stimulated stomatal closure.

Table 1 | Selected genes involved in the regulation of stomatal movement under stress.

Gene	Description	Mutant	Phenotype	Reference
ABH1	Encodes a nuclear cap-binding protein that forms a heterodimeric complex with CBP20 and is involved in ABA signaling	*abh1*	ABA hypersensitive, shows enhanced ABA activation of S-type channels	Schroeder et al. (2001a), Hugouvieux et al. (2002)
ABI1	Encodes the protein phosphatase 2C involved in abscisic acid (ABA) signal transduction. Negative regulator of stomatal closure promoted by ABA	*abi1*	Improper stomatal regulation leading to increased transpiration	Parcy and Giraudat (1997)
ABI2	Encodes the protein phosphatase 2C involved in abscisic acid (ABA) signal transduction. Negative regulator of stomatal closure promoted by ABA	*abi2*	Improper stomatal regulation leading to increased transpiration	Pei et al. (1997)
AHA1	Encodes a plasma membrane proton ATPase	*ost2*	Constitutively activated H+-ATPases, insensitivity to ABA persisted stomatal opening and a reduced ability to close stomata in response to drought	Merlot et al. (2007)
ALMT12	Encodes an anion transporter involved in stomatal closure	*almt12*	Impaired stomatal closure in response to ABA, darkness and CO_2	Meyer et al. (2010)
AtrbohD	Encodes the NADPH/respiratory burst oxidase protein D (RbohD).Interacts with AtrbohF	*atrbohd*	Impaired stomatal closure in response to ABA	Kwak et al. (2003)
AtrbohF	Encodes the NADPH/respiratory burst oxidase protein F (RbohF). Interacts with AtrbohD	*atrbohf*	Impaired stomatal closure in response to ABA	Kwak et al. (2003)
COI	Encodes a protein containing Leu-rich repeats and a degenerate F-box motif	*coi*	Disrupted activation of S-type anion channels	Munemasa et al. (2007, 2011)
CPK10	Encodes the calcium-dependent protein kinase whose gene expression is induced by dehydration and high salt	*cpk10*	Sensitive to drought, impaired stomatal closure	Zou et al. (2010)
CPK21	Encodes a member of the calcium-dependent protein kinase	*cpk21*	Tolerant to osmotic and drought stress	Franz et al. (2010)
CPK3	Encodes the calcium-dependent protein kinase 3 (CPK3), a member of the *Arabidopsis* CDPK gene family. CPK3 is expressed in both guard cells and mesophyll cells. Functions in guard cell ion channel regulation	*cpk3*	Reduction in ABA and Ca^{2+} activation of S-type channels, impaired ABA activation of Ca^{2+} permeable channels, decreased ABA sensitivity to stomatal closure	Mori et al. (2006)
CPK6	Encodes the calcium-dependent protein kinase 3 (CPK3), a member of the *Arabidopsis* CDPK gene family. CPK3 is expressed in both guard cells and mesophyll cells. Functions in guard cell ion channel regulation	*cpk6*	Reduction in ABA and Ca^{2+} activation of S-type channels, impaired ABA activation of Ca^{2+} permeable channels, decreased ABA sensitivity to stomatal closure	Mori et al. (2006), Munemasa et al. (2011)
ERA1	Encodes a beta subunit of farnesyl-trans-transferase, which is involved in meristem organization and the ABA-mediated signal transduction pathway. Mutant phenotypes were observed in meristem organization and response to abscisic acid and drought	*era1*	ABA hypersensitive and showed enhanced ABA activation of S-type channels	Pei et al. (1998)
ERF7	Encodes a member of the ERF (ethylene response factor) subfamily B-1 of the ERF/AP2 transcription factor family (ATERF-7). The protein contains one AP2 domain. Phosphorylated by PKS3 *in vitro*. Involved in ABA-mediated responses	*erf7*	Increased sensitivity of stomata to ABA compared to the wild-type, enhanced drought tolerance	Song et al. (2005)
GORK	Encodes a guard cell outward potassium channel. Belongs to the Shaker family K+ channel	*gork*	Impaired stomatal closure	Hosy et al. (2003)

(Continued)

Table 1 | Continued

Gene	Description	Mutant	Phenotype	Reference
GPA1	Encodes an alpha subunit of a heterotrimeric GTP-binding protein. GPA1 is a positive regulator in ABA-mediated inhibition of stomatal opening	*gpa1*	Reduction in the inhibition of inward K^+ currents, reduced guard cell ABA-insensitivity in stomatal opening	Wang et al. (2001)
KAT1	Encodes a potassium channel protein (KAT1)	*kat1*	No impairment of stomatal action, but potassium currents were altered	Szyroki et al. (2001)
MRP5	Encodes a high-affinity inositol hexakisphosphate transporter that plays a role in guard cell signaling and phytate storage. It is a member of the MRP subfamily/ABC transporter subfamily C	*mrp5*	Impaired ABA regulation of Ca^{2+} permeable channels, defects in S-type channel regulation	Suh et al. (2007)
MYB15	Encodes a member of the R2R3 factor gene family	*35S:myb15*	More sensitive to ABA-induced stomatal closure, improved drought tolerance	Ding et al. (2009)
MYB44	Encodes a member of the R2R3 factor MYB gene family involved in mediating plant responses to a variety of abiotic stimuli	*35S:myb44*	More drought tolerant	Jung et al. (2007)
MYB60	Encodes a putative transcription factor of the R2R3-MYB gene family. Transcript increases under conditions that promote stomatal opening (white and blue light) and decreases under conditions that trigger stomatal closure (ABA, desiccation, darkness) with the exception of elevated CO_2. Expressed exclusively in the guard cells of all tissues. It is required for light-induced opening of stomata	*myb60*	Reduced stomatal aperture which helps to limit water loss during a drought	Cominelli et al. (2005)
MYB61	Encodes the putative transcription factor. Expressed in guard cells, plays a role in the regulation of stomatal pore size	*myb61*	Larger stomatal pores than the wild-type	Liang et al. (2005)
NFYA5	Encodes a member of the CCAAT-binding transcription factor (CBF-B/NF-YA) family. Expression is upregulated in response to ABA and drought	*nfya5*	Hypersensitive to drought because their stomata are more open than the wild-type	Li et al. (2008)
NPX1	Encodes NPX1 (Nuclear Protein X1), a nuclear factor that regulates abscisic acid responses	*npx1*	Stomata were more closed than the wild-type in response to ABA and were more drought tolerant	Kim et al. (2009)
NRT1.1 (CHL1)	Encodes NRT1.1 (CHL1), a dual-affinity nitrate transporter. The protein is expressed in guard cells and functions in stomatal opening	*nrt1.1 (chl)*	Lower transpiration rate and tolerant to drought	Guo et al. (2003)
PUB18	Encodes a protein containing a UND, a U-box and an ARM domain	*pub18*	Hypersensitive to ABA-mediated stomatal closure	Seo et al. (2012)
PUB19	Encodes PUB19, a plant U-box armadillo repeat protein. Involved in the salt inhibition of germination together with PUB18	*pub19*	Hypersensitive to ABA-mediated stomatal closure	Liu et al. (2011)
SLAC1	Encodes a membrane protein with 10 predicted transmembrane helices. SLAC1 is a multispanning membrane protein that is expressed predominantly in the guard cells that play a role in regulating cellular ion homeostasis and S-type anion currents. SLAC1 is important for normal stomatal closure in response to a variety of signals including elevated CO_2, ozone, ABA, darkness and humidity. SLAC1:GFP localizes to the plasma membrane	*slac1*	Reduced stomatal closure response to ABA, CO_2, Ca^{2+} and ozone treatments	Vahisalu et al. (2008)

Pink indicates genes that encode the negative regulators of ABA signaling, blue indicates genes that encode ion channels, pump, and transporters localized in the plasma membrane of guard cells, green indicates genes that encode the Ca^{2+}-dependent protein kinases involved in the regulation of stomatal movements, brown indicates genes that encode the transcription factors involved in the regulation of stomatal movements.

AtrbohF (*Arabidopsis thaliana* respiratory burst oxidase homolog F). The significance of ROS involvement in stomatal closure was revealed by an analysis of the *atrbohD*/*atrbohF* double mutant, which showed impaired stomatal closure in response to ABA (Kwak et al., 2003). The protein, OST1 (open stomata1), displays dominant kinase activity during drought stress response and is able to activate NADPH oxidase (Sirichandra et al., 2009). Mutants in *OST1* showed a wilty phenotype in water deficit conditions because of the impairment of stomatal closure and ROS production (Mustilli et al., 2002; Yoshida et al., 2006; **Figure 4**).

Another crucial factor for stomatal closure is NO, which is generated in response to ABA (Neill et al., 2002, 2008). Exogenously applied NO donors triggered stomatal closure, whereas the application of an NO scavenger inhibited ABA-induced stomatal closure (Neill et al., 2002; **Figure 4**).

There is some evidence that both H_2O_2 and NO actions in the guard cells require calcium. In addition, H_2O_2 inhibits K^+ channel activity, induces cytosolic alkalization in the guard cells and promotes NO signaling in response to ABA (Zhang et al., 2001; Kohler et al., 2003; Wang and Song, 2008). Conversely, NO neither stimulates H_2O_2 synthesis nor does it require H_2O_2 for its action (Bright et al., 2006).

THE SECOND VIOLIN IN THE CONCERT OF STOMATAL CLOSURE – THE ROLE OF JASMONATES IN THE REGULATION OF STOMATAL MOVEMENT

Jasmonates are lipid-derived phytohormones that are involved in the regulation of vegetative and reproductive growth and the defense response against abiotic stress (Katsir et al., 2008). JA biosynthesis is induced by stress conditions (Wasternack, 2007) and many genes related to JA signaling are regulated by drought stress (Huang et al., 2008). The positive role of JA in the regulation of stomatal closure was observed in many studies (Gehring et al., 1997; Suhita et al., 2003, 2004; Munemasa et al., 2007). Similar to the ABA signaling pathway, JA signaling has been under intense investigation, particularly in relation to stress response. With the progress in research, many new components and their roles in JA-mediated stress response will be identified. Although the interaction between ABA and JA signaling pathways in stomata function has been established, there is still a need for further investigation and identification of the nodes linking these two signaling pathways, such as CPK6, which is described below.

When JA or methyl JA (MeJA) are applied exogenously to plants, they are converted into a biologically active form (+)-7-iso-Jasmonoyl-L-isoleucine (JA-Ile). JA-Ile is then bound by the receptor ^{SCF}COI complex that contains the coronatine insensitive1 (COI1) F-box protein (Fonseca et al., 2009; Sheard et al., 2010). This interaction leads to the degradation of the repressor protein, JAZ (Jasmonate ZIM-domain), by the 26S proteasome and as a result, to the activation of distinct JA response genes by MYC2 (MYC domain transcription factor 2) (Chini et al., 2007; Thines et al., 2007; Fernández-Calvo et al., 2011). In the absence of JA, JAZ inhibits MYC2, which is then unable to activate the transcription of JA-inducible genes (**Figure 5**).

Munemasa et al. (2011) identified CPK6 (Ca^{2+} dependent protein kinase 6), which had previously been studied by Mori et al. (2006) in regards to ABA signaling, as a positive regulator of MeJA signaling in the guard cells. CDPKs function as important cytosolic Ca^{2+} sensors in various plant physiological processes. Four kinases are involved in ABA signaling in *Arabidopsis* guard cells: CPK3, CPK6, CPK4, and CPK11; however, only mutations in the *CPK6* impaired MeJA-induced stomatal closure (Munemasa et al., 2011). Like ABA, MeJA activates S-type anion channels. In *coi1* (*coronatine insensitive 1*) and *cpk6* mutants, the activation of S-type anion channels was disrupted (Munemasa et al., 2007, 2011). Geiger et al. (2010) showed a direct interaction between CPK6 and the SLAC1 – S-type anion channel. The activation of SLAC1 by CPK6 was inhibited by the PP2Cs, ABI1, and ABI2, since *abi1* and *abi2* mutants exhibited insensitivity of stomata to MeJA, which leads to the inability of stomatal closure in response to MeJA (**Figure 6**).

The formation of ROS and NO in the guard cells is not only induced by ABA and ethylene but also by MeJA. It has been showed that both ROS and NO levels were decreased in MeJA-insensitive mutants (Munemasa et al., 2007). Suhita et al. (2004) showed that a disruption of both genes encoding NADPH oxidase, *AtrbohD* and *AtrbohF*, results in the impairment of MeJA-induced stomatal closure and ROS production. However, in the *cpk6 Arabidopsis* mutant, ABA- and MeJA-mediated the production of ROS and NO was not reduced. CPK6 acts downstream of NO and ROS signaling and therefore may be a target of the NO-stimulated influx of Ca^{2+} into the cytoplasm. As a feedback loop, MeJA-induced influx of Ca^{2+} into the cytoplasm activates CPK6, which in turn is able to activate the S-type anion channel – SLAC1 (**Figure 5**). This property of CPK6 makes it a node between the NO, ROS, ethylene and JA signaling pathways in ABA-induced stomatal closure (Munemasa et al., 2011; **Figure 6**).

Jasmonates interacts with the ABA pathway by increasing the influx of Ca^{2+}, which stimulates CDPK and the resulting cascade in order to close stomata. Munemasa et al. (2007) reported that ABA or MeJA treatment triggers a reduction in the stomatal aperture within 10 min. MeJA-induced Ca^{2+} levels were significantly lowered and stomatal closure was impaired when ABA biosynthesis inhibitors were added or when ABA-deficient mutants were studied. This suggests that jasmonate-induced changes in stomatal movements require endogenous ABA. In order to clarify this hypothesis, Hossain et al. (2011) examined the effect of 0.1 μM of ABA on MeJA-induced stomatal closure in *aba 2-2* (*ABA deficient 2-2*) mutants related to ABA biosynthesis. In the wild-type, 0.1 μM of ABA did not significantly induce either stomatal closure or Ca^{2+} oscillations. The authors did not observe stomatal closure in *aba2-2* when MeJA was applied without ABA, while in the presence of 0.1 μM ABA, MeJA induced stomatal closure.

WHEN ABA MEETS ETHYLENE

Ethylene is a gaseous phytohormone that is involved in the regulation of numerous plant processes such as seed germination, root-hair growth, leaf and flower senescence and abscission, fruit ripening, nodulation, and plant responses to stresses (Bleecker and Kende, 2000). It has been observed that ethylene can influence stomatal response via crosstalk with ABA; however, reports on its effect have been contradictory. Ethylene has been linked to the promotion of both stomatal closure (Pallas and Kays, 1982) and stomatal opening (Madhavan et al., 1983; Levitt et al., 1987;

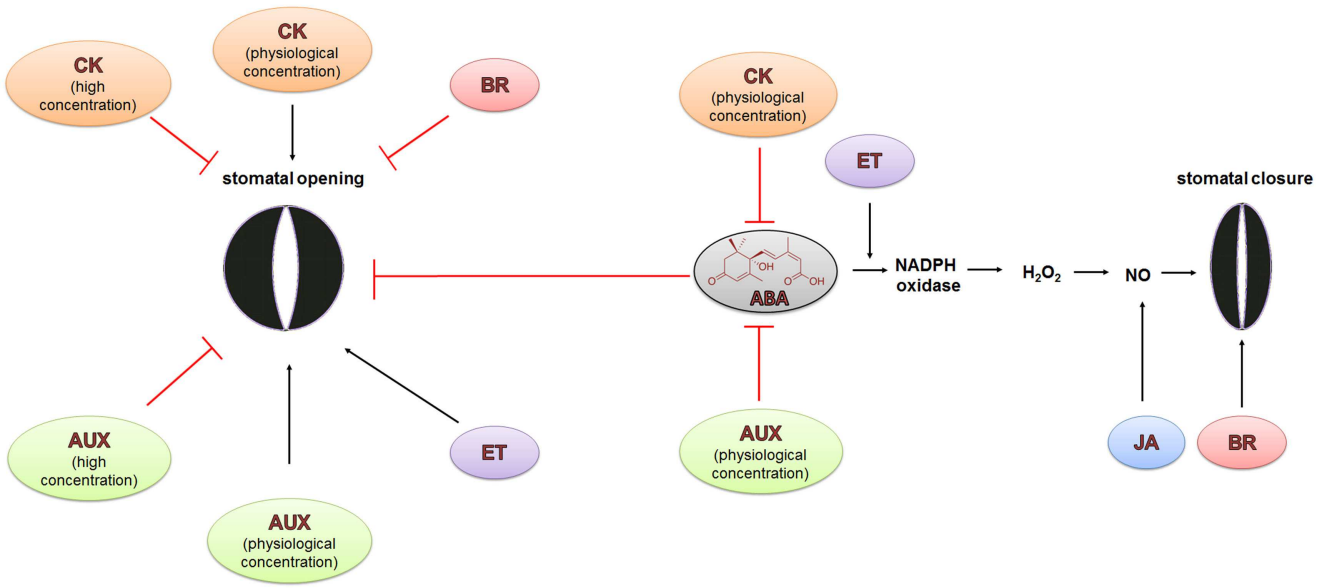

FIGURE 6 | Hormonal crosstalk in the regulation of stomatal closure and opening during water stress. The regulation of stomatal opening and closure is not only regulated by ABA, whose role is dominant, but also by other phytohormones. Jasmonates (JA) and brassinosteroids (BR) induce stomatal closure and inhibit stomatal opening under drought conditions, whereas the role of other hormones is ambiguous. Cytokinins (CK) and auxins (AUX) in low physiological concentrations promote stomatal opening while in high concentrations, they are able to inhibit this process. The role of ethylene (ET) is the most curious. It can stimulate the closing and opening of the stomata. The details are described in the text.

Merritt et al., 2001; **Figure 6**). These contradictory effects need to be verified. One possible reason could be related to the methods used for stomatal observation that use detached leaves. Experiments with detached leaves do not always reflect the real response to stress or other applied factors in plants.

Tanaka et al. (2005) showed that *Arabidopsis* plants exposed to gaseous ethylene first did not close their stomata after the application of ABA. This was clear evidence that ethylene repressed ABA action in stomatal closure. In a drought stressed *eto1* (*ethylene overproducer 1*) mutant, stomata closed more slowly and were less sensitive to ABA than in the drought-treated wild type (Tanaka et al., 2005). In order to elucidate the interaction between ethylene and ABA during stomatal response, epidermal peels from the wild-type and *eto1* were treated with ABA, ethylene, and both phytohormones. When ethylene was applied independently of ABA, it induced H_2O_2 synthesis within 30 min of the treatment. When ethylene was applied to the ABA-pretreated wild-type epidermal peels, an inhibition of stomatal closure was observed (Tanaka et al., 2005). Desikan et al. (2006) proved that ethylene-mediated stomatal closure is dependent on the H_2O_2 that is generated by NADPH oxidase. As was discussed above, H_2O_2 is one of the major molecules in ABA-induced stomatal closure.

There have been some studies that revealed both increased and decreased ethylene production in response to drought stress. However, most of them described experiments with detached leaves, which may not reflect the response of intact plants under drought conditions (Morgan et al., 1990; Abeles et al., 1992). Generally, elevated ABA concentrations limit the production of ethylene; and therefore a dramatic increase of ABA concentration during water stress probably causes a reduction in the production of ethylene (Sharp, 2002). The physiological mechanism of ethylene inhibition of the ABA-mediated stomatal closure may be related to the function of ethylene as a factor that ensures a minimum carbon dioxide supply for photosynthesis by keeping stomata half-opened under the stress conditions (Leung and Giraudat, 1998; Tanaka et al., 2005).

AUXINS AND CYTOKININS – AMBIGOUS PARTICIPATION IN STOMATAL MOVEMENTS

Auxins and cytokinins are major phytohormones that are involved in processes related to plant growth and development such as cell division, growth and organogenesis, vascular differentiation, lateral root initiation as well as gravi- and phototropism (Berleth and Sachs, 2001). Auxins typically play a positive role in stomatal opening but high concentrations of auxin can inhibit stomatal opening (Lohse and Hedrich, 1992; **Figure 6**). Auxins stimulate the PM H^+-ATPase in the guard cells. Proton efflux leads to the hyperpolarization of the membrane and results in an uptake of K^+. Low auxin concentrations activate inward K^+ channels leading to stomatal opening, whereas high auxin level promotes outward K^+ channels, while simultaneously inhibiting inward K^+ channels, which results in stomatal closure (Lohse and Hedrich, 1992; Blatt and Thiel, 1994).

The impact of cytokinins on stomatal movements is also ambiguous. It has been shown that an increased cytokinin concentration in xylem sap promotes stomatal opening and decreases sensitivity to ABA. Drought stress inhibits the synthesis of cytokinins in roots and its transport to shoots, which in turn results in stomatal closure (Pospísilova, 2003; Pustovoitova et al., 2003). However, stomatal response to exogenously applied cytokinins depends on

the concentration and cytokinin species (**Figure 6**). Generally, exogenous cytokinins and auxins can inhibit ABA-induced stomatal closure in diverse species (Stoll et al., 2000; Tanaka et al., 2006).

BRASSINOSTEROIDS PLAY IN THE SAME TEAM WITH ABA

Brassinosteroids (BR) are polyhydroxylated steroidal phytohormones that are involved in seed germination, stem elongation, vascular differentiation, and fruit ripening (Clouse and Sasse, 1998; Steber and McCourt, 2001; Symons et al., 2006). It has been shown that epibrassinolide (eBL) promotes stomatal closure and inhibits stomatal opening in epidermal peels of *Vicia faba* through the negative regulation of the inwardly rectifying K^+ channels that are responsible for the uptake of K^+ during stomatal opening (Haubrick et al., 2006; **Figure 6**). eBL is able to activate the transcription of drought-inducible genes in *Arabidopsis*, such as *RD29A* (*response to drought 29A*), *ERD10* (*early response to drought 10*), and *RD22* (*rehydration responsive 22*) (Kagale et al., 2007). Together, these results suggest that there is an interaction between BR and ABA in drought response that is related to stomatal closure.

THE-STATE-OF-ART AND WEAK POINTS IN OUR UNDERSTANDING OF STOMATAL MOVEMENTS

Stomata are epidermal pores on a plant's surface that are essential for the control of water balance in plants. Many factors that are responsible for the regulation of stomatal movements have been already identified, such as components of ABA and other phytohormone signaling pathways. The most important, and one that is supported by well-documented studies, is the interaction between ABA (when taking into account its biosynthesis, catabolism, de/conjugation, and core signalosome) and the pumps and ion channels in the guard cell PM, in the regulation of stomatal movements under the stress.

However, further analyses of the networks of protein interactions, the co-expression of genes, metabolic factors, etc. should provide new insights into the key regulators of drought response in relation to guard cell movements. Taking into account that phytohormone pathways are still under intensive investigations and there are still many gaps to be elucidated, many of the already established interactions may be changed as further progress in research is achieved.

There are ambiguous reports in regards to the role of some phytohormones, such as ethylene, auxins, or cytokinins, in the regulation of stomatal movement that need to be clarified. In addition, the interaction between the diurnal cycle and ABA pathway should be further investigated in order to achieve a full understanding of this process.

There are some points that should be highlighted as a possible cause of the ambiguous reports related to the action of the regulators of stomatal movements. The first of these is the technique that is used to observe the stomata. Most analyses of stomata under stress are based on stomatal aperture observations. Some studies rely on stomata replicas from plants treated with stress and control, and observed under the light microscopy. This method is simple and inexpensive but generates problems due to the type of material used for the replicas. The accuracy and precision in the determination of stomatal aperture width is limited by the resolution of the standard light microscope. In contrast, scanning microscopy (SEM) offers high resolution images of stomata but requires expensive equipment and is not suitable for collecting large numbers of probes (Lawson et al., 1998). Recently, a popular technique in stomatal observations is confocal microscopy (Cañamero et al., 2006). As long as a proper technique that is not controversial in regards to its influence on stomatal response is not applied, all aperture measurements will be under discussion.

Another crucial problem is that most reports describe experiments with detached leaves, which may not reflect the response of intact plants under drought conditions (Morgan et al., 1990; Abeles et al., 1992; Dodd, 2012). Franks and Farquhar (2007) addressed the problem of data integration in stomatal research. They pointed out the lack of the integration of mechanical and quantitative physical information about guard cells and adjacent cells in model of stomatal function. Such integration of data should allow gas-exchange regulation to be better described and predicted. As long as guard cells are considered as a model without their surroundings, the results obtained may not be relevant. Another problem noted by Franks and Farquhar (2007) is that research on the impact of various environmental factors on the stomatal regulation and stomatal density should be performed on and compared among several species, not only one. This would allow a full picture of a broad morphological and evolutionary spectrum of possibilities of stomata development, density, and movement regulation in response to stresses to be obtained.

Summarizing, there are still many questions about the techniques used for evaluating the stomatal response to stress. Further development of proper methods will bring us closer to a fuller and more relevant understanding of stomatal action. The great progress in molecular biology studies enable insights into the signaling pathways, identification of new components, and interactions between them to be gained.

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When stress and development go hand in hand: main hormonal controls of adventitious rooting in cuttings

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Adventitious rooting (AR) is a multifactorial response leading to new roots at the base of stem cuttings, and the establishment of a complete and autonomous plant. AR has two main phases: (a) induction, with a requirement for higher auxin concentration; (b) formation, inhibited by high auxin and in which anatomical changes take place. The first stages of this process in severed organs necessarily include wounding and water stress responses which may trigger hormonal changes that contribute to reprogram target cells that are competent to respond to rooting stimuli. At severance, the roles of jasmonate and abscisic acid are critical for wound response and perhaps sink strength establishment, although their negative roles on the cell cycle may inhibit root induction. Strigolactones may also inhibit AR. A reduced concentration of cytokinins in cuttings results from the separation of the root system, whose tips are a relevant source of these root induction inhibitors. The combined increased accumulation of basipetally transported auxins from the shoot apex at the cutting base is often sufficient for AR in easy-to-root species. The role of peroxidases and phenolic compounds in auxin catabolism may be critical at these early stages right after wounding. The events leading to AR strongly depend on mother plant nutritional status, both in terms of minerals and carbohydrates, as well as on sink establishment at cutting bases. Auxins play a central role in AR. Auxin transporters control auxin canalization to target cells. There, auxins act primarily through selective proteolysis and cell wall loosening, via their receptor proteins TIR1 (transport inhibitor response 1) and ABP1 (Auxin-Binding Protein 1). A complex microRNA circuitry is involved in the control of auxin response factors essential for gene expression in AR. After root establishment, new hormonal controls take place, with auxins being required at lower concentrations for root meristem maintenance and cytokinins needed for root tissue differentiation.

Keywords: adventitious rooting, auxin, receptors, jasmonic acid, cytokinin, nutrition, microRNAs, hormonal crosstalk

INTRODUCTION

If flowering is a key developmental process for sexual reproduction in plants, adventitious rooting (AR) occupies a central role in asexual propagation. Forestry, horticulture, and fruit crops depend to a large extent on the successful establishment of roots in cuttings and other propagules. Clonal propagation is of particular relevance to forestry, since genetic improvement in long lived species with large generation cycles is often limiting. Genetic gains from interspecific hybridization, mutations, and transgenic events can be captured and multiplied faster and more efficiently based on clonal propagation through AR of cuttings. Overall, the main application of AR is propagation by cuttings and its derived techniques adapted to clonal garden greenhouses and *in vitro* cultures, minicuttings and microcuttings, respectively (Assis et al., 2004). Therefore, rather than looking into the examples of developmentally programmed AR in intact plants, the focus of the present review is on AR of severed organs or in response to stressful conditions, such as flooding.

Most research on AR has been centered on the role of phytohormones, mainly auxins, and cutting physiological conditions. The role of stress responses associated with cutting severance and the relevance of mother plant status has often received less attention, although a shift in focus has been clearly taking place in the last two decades or so. Wound responses associated with cutting severance are integrated, and often necessary, in the steps leading to AR, and mother plant status is a key determinant of rooting propensity of cuttings derived from it. Therefore, the control of environmental variables of stock plants is rather relevant for the clonal propagation process. Clearly, a fundamental aspect governing AR responses to external and internal stimuli is cellular competence to respond. This developmental capacity to respond is responsible for many of the failures to obtain AR in mature cuttings, even upon careful manipulation of environmental variables and phytohormones that can modulate rooting.

The concept of adventitious root is based essentially on anatomical origin. Adventitious roots are formed in stems, leaves and

non-pericycle tissue in older roots, differing from primary roots, of embryonic origin, and lateral roots, which are derived from the pericycle layer (Li et al., 2009a). There are two main patterns of adventitious root development: direct and indirect. The tissues involved in the process of root development are most frequently the cambium and vascular tissues, which undergo the first mitotic divisions, leading directly to root primordia in the first pattern. In the indirect pattern of AR, albeit the same tissues often take part, the formation of a callus is observed prior to differentiation of root primordia. In both cases, before root primordia become distinguishable, clusters of usually isodiametric cells are formed (meristemoids; Altamura, 1996). In the indirect pattern of AR, a bottleneck is frequently observed, i.e., the establishment of an effective vascular connection between the newly formed root primordia and the stem. Poorly connected vasculature with the stem leads to non-functional roots, with negative consequences for cutting survival (Fleck et al., 2009).

Adventitious rooting is a complex process that can be affected by numerous variables, both internal and external. A large body of evidence has supported the existence of successive physiological phases in the process of adventitious root development, each with specific requirements that can even be antagonistic, but operate in complementary fashion. The most widely recognized AR phases are induction, initiation, and expression (Kevers et al., 1997).

The induction phase in cuttings or detached organs, such as leaves, is generally marked by the immediate consequences of the wounding response caused by severance. It encompasses the first hours after cutting removal, with a local increase in jasmonate, phenolic compounds and auxin at the cutting base, often associated with a transiently lower peroxidase (EC 1.11.1.7) activity, and the establishment of a sink for carbohydrates in the same zone (Schwambach et al., 2008; Ahkami et al., 2009). Peroxidases are heme-containing enzymes with catalytic action on diverse organic compounds, including indole-3-acetic acid (IAA), and their activity has been used as a biochemical marker of the rooting phases (Corrêa et al., 2012a). The induction phase is devoid of visible cell divisions and involves reprograming of target cells to the following establishment of meristemoids, which takes place in the initiation phase. Studying AR in apple, De Klerk et al. (1999) launched the concept of an early phase of dedifferentiation (0–24 h), taking place before the induction phase. In the concept of the present review, the dedifferentiation phase postulated by De Klerk et al. (1999) corresponds to the early steps of the induction phase. During initiation, besides cell divisions, meristemoids and development of root primordia, often a lower auxin and phenolic concentration and higher peroxidase activity are observed. The expression phase corresponds to the growth of root primordia through the stem tissues and the establishment of vascular connections between the newly formed root and the original stem cutting. For simplification purposes, it is not uncommon to join the initiation and expression phases under a single denomination of formation phase (Fett-Neto et al., 1992).

These overall changes in phytohormone balance, along with other less predominant but not unimportant changes to be discussed ahead, trigger a sequence of gene expression events that leads to proteomic changes, culminating with new root differentiation. Considering different systems and the fragmentary information available, these molecular events of gene expression and gene product accumulation can be putatively summarized in chronological sequence as follows: wounding and water balance stress-related, carbohydrate sink establishment, auxin transport systems, cell wall degradation and assembly, transcription factors involved in cell fate determination, replication machinery, transcription factors with roles in growth and differentiation (Brinker et al., 2004; Sorin et al., 2005; Ahkami et al., 2009).

At the molecular level, including the participation of various phytohormones, considerably more knowledge is available on lateral root development. Certainly, there is at least some overlap between the processes of lateral root development and AR. Most of the similarities include the requirement for an initial auxin increase, followed by a reduction, the participation of auxin transporters, cell wall dynamics, and the activity of specific transcription factors in these processes. Root growth responses to nutrient gradients, such as nitrate and phosphate (Desnos, 2008), seem to be another feature apparently shared between lateral and adventitious roots (Schwambach et al., 2005). Lateral root development depends at least partially on auxin activation of founder cells in the pericycle at the primary root differentiation zone, possibly mediated by an interaction of auxin with its receptor TIR1 (transport inhibitor response 1; Petricka et al., 2012). The role of root development inhibitors, such as cytokinins and strigolactones, which will be discussed ahead, also seems to be shared between lateral root development and AR. However, besides the usual histological origin, other very important differences exist between lateral and adventitious root development, most likely related to the often associated wound response and particular reorganization of auxin transport systems in the latter.

Although AR in intact plants may take place in certain conditions, such as flooding or programmed development, the typical AR features of stress signaling and major shifts in root–shoot correlative influences are usually present in excised plant parts, such as cuttings, hypocotyls and leaves. Rooting protocols based on pre-etiolated intact seedlings, commonly used to investigate AR in *Arabidopsis thaliana*, have roots formed mostly from the pericycle, which extends from primary roots into the hypocotyls of young seedlings, and do not face stresses capable of disrupting root–shoot correlative influences. A comparison of an intact seedling system with de-rooted older plants or with rooting of petioles of detached leaves showed significant differences, not only in root founding tissues, but also in auxin requirements, sensitivity, and rooting mutant phenotypes (Corrêa et al., 2012b). The fact that lateral and adventitious root developments have fundamental functional differences can be further highlighted by the opposite effects of ethylene on both processes, observed in studies with tomato (Negi et al., 2010). Perhaps the pathways leading to adventitious versus lateral root development could be viewed as different roads, which may intertwine in some portions, and end up leading to the same destination, i.e., new roots.

MOTHER PLANT STATUS – DEVELOPMENTAL COMPETENCE TO RESPOND AND THE RIGHT SUPPLIES FOR THE HURDLES AHEAD

In vegetative propagation, a mother plant provides cuttings with improved selected characteristics, and the formation of new

adventitious roots is essential for the restoration of the whole plant condition. Physiological and biochemical quality of mother plants, in addition to their genetic makeup, could limit rooting performance of cuttings derived therefrom (Osterc, 2009). The physiological condition of mother plants is directly affected by the environment in which they were raised or to which they were exposed, including light and temperature conditions, water and nutrient supplies (Moe and Andersen, 1988). Endogenous auxin, carbohydrate content, mineral nutrients, and other biochemical components, such as phenolics that could act as rooting co-factors or auxin transport modulators, may be affected by environmental factors and are transferred from the stock plants to the propagules when the cutting is severed. The content, metabolism, and interactions of these metabolites and components will influence early responses to wound and root induction of cuttings.

Auxin endogenous concentration varies over the course of rooting phases, and is needed at higher concentration during the induction phase for proper rooting (Kevers et al., 1997). In this context, high auxin content immediately after cutting severance originating from the mother plant, may result in improved rooting. As far as light treatments are concerned, shade conditions (low red:far-red ratios) induced auxin biosynthesis and increased IAA levels in *Arabidopsis* seedlings (Tao et al., 2008). Light availability and quality have been shown to affect auxin transport rate and its predominant anatomical path in the stem (Morelli and Ruberti, 2002). Sorin et al. (2005) described an interaction between light and auxin metabolism affecting *Arabidopsis* rooting. Mutants with low rooting capacity (*ago1*) had upregulated light responses and disturbed auxin homeostasis.

Mineral nutrition of stock plants is an important factor in determining AR capacity. The biosynthesis of one of the main auxin precursors, the amino acid tryptophan, requires zinc (Blazich, 1988; Marschner, 1995), which is also a structural component of the auxin receptor ABP1 (Auxin-Binding Protein 1; Tromas et al., 2010). Manganese and iron are co-factor and structural component of peroxidases, respectively. Therefore, these nutrients can affect this class of auxin catabolism enzymes (Campa, 1991; Fang and Kao, 2000). The appropriate management of light quality and fertilization schemes applied to mother plants, in a way to positive influence auxin biosynthesis, transport, and metabolism, could implicate in a better rooting response on subsequent cuttings produced by these stocks. The relevance of mineral nutrition for AR is highlighted by the fact that rooting phase-specific mineral nutrient compositions, optimized for cuttings themselves, have been shown to improve rooting and survival of *Eucalyptus globulus* plants (Schwambach et al., 2005). High nitrogen supply to stock plants and the resulting elevated N content in herbaceous cuttings have been shown to strongly promote AR (Druege et al., 2000, 2004; Zerche and Druege, 2009).

The initial content and composition of phenolic compounds are also transferred to cuttings from mother plants and the interaction of these metabolites with auxin and peroxidases may have effects on adventitious root formation (De Klerk et al., 1999). Flavonoids, a major class of phenolic compounds, can influence auxin transport (Peer and Murphy, 2007), mainly by interacting with efflux carrier PIN2 (PIN-FORMED 2) or affecting the distribution of other PIN proteins (Buer et al., 2010). Phenolics are also important in modulating peroxidase activity and could also act as antioxidants, preventing auxin degradation at cutting bases (De Klerk et al., 1999).

Several investigations have pointed out that the initial carbohydrate content of the cutting, should be enough to supply the energy reserves throughout the rooting period (Veierskov, 1988; Husen, 2008). On the other hand, there is evidence that carbohydrate allocation and distribution within the cutting could be more important than the content itself (Druege et al., 2000; Druege, 2009; Ruedell et al., 2013). Light and current photosynthesis of cuttings could play an important role in this scenario, influencing carbohydrate metabolism and reallocation (Hoad and Leakey, 1996; Rapaka et al., 2005). In the rooting recalcitrant *E. globulus*, donor plants grown in medium devoid of sugar and exposed to white irradiance promoted AR in cuttings, whereas presence of exogenous sugar in donor plant media favored rooting in the easy-to-root *E. saligna*, with no significant effects of irradiance (Corrêa et al., 2005). Appropriate light environment applied to mother plants may increase carbohydrate sink capacity at the root formation site in cuttings derived therefrom.

Maturation negatively affects the regenerative ability of plant material and, as a consequence, diminishes its AR potential. The content and profile of phenolic compounds, as well as the contents of carbohydrates and auxins, switch according to maturation state, correlating with rooting competence (Fernández-Lorenzo et al., 2005; Husen and Pal, 2007; Osterc et al., 2009). The use of juvenile-like material can help overcoming this limitation (Cameron et al., 2003; Kibbler et al., 2004). In vegetative propagation of trees, the use of minicutting technique, both in hydroponic or sand bed minihedges, affords a better environmental control of ministumps (mother plants), improving their physiological quality, and, consequently, the rooting propensity of the minicuttings obtained (Assis et al., 2004; Schwambach et al., 2008).

The molecular basis of rooting competence is an essential aspect of AR. In principle, even if all environmental variables are ideally manipulated so as to favor AR, unless developmental competence is present, responses to the root-promoting signals do not take place and rooting fails. Developmental responsiveness is likely dependent on presence and density of functional phytohormonal receptors and signaling pathways, particularly those for auxin. A detailed investigation on AR of hypocotyls (able to root proficuously upon exposure to auxin) and epicotyls (root poorly even in presence of auxin) of 50-day old seedlings of *Pinus taeda* showed that lack of rooting responsiveness in epicotyls was not related to auxin uptake, transport, distribution among cells, or metabolism. Localized fast cell division and root meristem organization were lacking in epicotyls (Diaz-Sala et al., 1996). Application of the auxin transport inhibitor *N*-(naphthyl)phthalamic acid (NPA) up to the first 3 days after cutting severance inhibited rooting without affecting auxin concentration or metabolic status at the rooting site, suggesting a role for auxin polarity in rooting capacity that would be different than simply moving auxin to the rooting zone.

Auxin capacity to trigger gene expression has been suggested as an early and critical point in AR competence of *Pinus taeda* stem cuttings, for example (Greenwood et al., 2001). In this system,

the inability to root in mature cuttings was apparently due to the lack of cells capacity to arrange themselves into root meristems in presence of auxin. Cell division and callus formation, however, occurred similarly, both in physiologically juvenile and mature cuttings, leading the authors to suggest the existence of an auxin transduction pathway specific to root meristem organization. Members of the expansin gene family are among the early auxin-induced genes during AR of pine cuttings, particularly in non-growing zones of the stem before cell divisions that result in root development (Hutchison et al., 1999). Some auxin-responsive transcription factors have been shown to play roles in the control of cell division leading to root primordia differentiation in cuttings of tree species (Sánchez et al., 2007; Solé et al., 2008; Vielba et al., 2011; Rigal et al., 2012) and are discussed in further detail in the Section "Cell Cycle and Division-New Meristems" below.

The loss of AR capacity at physiologically mature stages is often associated with the transition to flowering (phase shift from juvenile to adult stage). However, in specific organ parts or under specific culture conditions, loss of rooting capacity can take place and become easily noticeable at much earlier stages of development (e.g., seedling), providing interesting experimental systems to study this process in trees (Fett-Neto et al., 2001; Greenwood et al., 2001). Another useful model to study AR and the loss of rooting capacity is *Arabidopsis thaliana*. Using de-rooted hypocotyls of young (12 day old) and adult (26 day old) plants of the Landsberg ecotype, it was shown that AR was much slower in adult de-rooted plants and that endogenous polar auxin transport (evaluated with NPA application) was crucial for AR (Díaz-Sala et al., 2002). These authors also showed that rooting was not dependent on phase shift to reproductive phase, although a correlation was observed. The decline in rooting capacity was probably linked to age-related processes. A correlation between reduced AR capacity and flowering phase shift was also shown in detached leaves of *Arabidopsis* plants of the Columbia ecotype, but only in leaves harvested 2–3 weeks after bolting (Corrêa et al., 2012b). A possible link between flowering and AR of detached leaves was not observed by analyzing the AR kinetics in two early and two late flowering time mutants of each of two ecotypes, Antwerpen and Columbia (Corrêa et al., 2012b). Interestingly, Díaz-Sala et al. (2002) showed that AR in de-rooted hypocotyls of *Arabidopsis* adult plants depended on RGD (Arg-Gly-Asp) peptides (a family of peptides bearing this signature domain), although these were not sufficient for rooting to occur and had no effect on young plant hypocotyls. The RGD peptides may be important in causing changes to the plasma membrane of plant cells and their interaction with cell walls, perhaps affecting cytological events required for AR in adult plant hypocotyls. Taken together these data indicate that *Arabidopsis* and *in vitro* culture systems of tree species are useful tools to study developmental competence to AR.

FEATURES ASSOCIATED WITH EXOGENOUS AUXIN SUPPLY

In addition to the already mentioned effect of endogenous auxin in adventitious root formation, it is well-established that this phytohormone can also act when exogenously supplied, entering the stem via the cut surface of cuttings. In many rooting recalcitrant species, application of exogenous auxin is needed to achieve satisfactory rooting responses (Diaz-Sala et al., 1996; Fett-Neto et al., 2001). In these cases, endogenous auxin produced in the shoot apex and transported basipetally to the cut surface may be complemented by exogenously applied phytohormone aiming at improving the rooting response (Pop et al., 2011). The absence of a shoot apical meristem has not limited AR in *Eucalyptus* microcuttings exposed to exogenous auxin (Fogaça and Fett-Neto, 2005).

Uptake of exogenously provided auxin implicates in a new auxin transport route, which enters the cuttings mostly via the cut surface (Kenney et al., 1969; Guan and De Klerk, 2000) and may be taken up by cells both through a pH trapping mechanism (Rubery and Sheldrake, 1973) and through influx carriers (Delbarre et al., 1996). Most of the supplied auxin acts at the wound site, inducing cell dedifferentiation, leading to a new root meristem later on. A portion of the supplied auxin could also be redistributed along the cutting, mostly via the xylem transpiration route (Osterc and Spethmann, 2001). In this case, auxin influx and efflux carriers would not take significant part in the process, losing directionality of the polar auxin transport throughout the plant (auxin transport is discussed ahead in detail). In fact, auxin uptake may also occur through the phloem and a better rooting performance in *Prunus subhirtella* juvenile cuttings was related to this kind of absorption path (Osterc and Stampar, 2011). However, studies with auxin transport inhibitors provided evidence that rooting in *Pinus taeda* hypocotyls is improved when exogenous auxin is incorporated in the polar auxin transport system (Diaz-Sala et al., 1996). Much of the data from different reports on interactions of exogenous auxins with the polar auxin transport system is probably difficult to compare because of the use of different auxins in the various experiments, including synthetic forms, for which the transport systems are poorly known.

CARBOHYDRATE ALLOCATION

Carbohydrates contribute to the formation of adventitious roots by supplying energy and carbon necessary for cell divisions, establishment of the new root meristems and root formation itself. The efficient partitioning of carbohydrates between the new sink of developing roots at cutting base and the shoot meristem sink could be critical for AR (Druege, 2009). Ahkami et al. (2009) proposed that the early establishment of a carbohydrate sink at the rooting site is a key metabolic event in *Petunia hybrida* adventitious root formation. Pre-incubation of *Petunia* cuttings in the dark increased carbohydrate levels at their bases upon transfer to light, improving AR (Klopotek et al., 2010). Similarly, a higher content of soluble sugars and starch in the rooting zone were associated with higher rooting response in *Tectona grandis* cuttings (Husen and Pal, 2007). Higher accumulation of soluble carbohydrates and starch at the root formation zone in microcuttings was associated with improved rooting capacity of *E. globulus* without exogenous auxin. This condition was observed when cuttings were obtained from mother plants grown in medium devoid of sucrose and exposed for a few weeks to far-red irradiation-enriched environment (Ruedell et al., 2013). When mother plants were grown in sucrose containing medium, the positive effect of exposing stock plants to far-red enriched irradiance on microcutting rooting capacity was abolished. Inhibition of AR in carnation cuttings

by high carbohydrate content has also been proposed, although the importance of establishing an auxin-stimulated carbon sink was pointed out (Agulló-Antón et al., 2011).

Growth and differentiation of tissues can be modulated by carbohydrate signals through alterations in metabolic fluxes and carbohydrate concentrations during development, which may regulate gene expression (reviewed by Rolland et al., 2006). These carbohydrate signals are generated by photosynthesis and carbon metabolism in source and sink tissues and probably play a regulatory role in adventitious root induction (Druege, 2009). Interactions between phytohormones and carbohydrates are essential part of the sugar sensing and signaling network (Rolland et al., 2006): and a glucose and auxin signaling crosstalk was shown to be important for controlling root development and growth in *Arabidopsis thaliana* seedlings (Mishra et al., 2009). Auxin supply to *Dalbergia sissoo* cuttings enhanced the content of total soluble sugars and starch, promoting AR (Husen, 2008). Different carbon sources may affect the rooting capacity of eucalypt microcuttings in a rooting phase-dependent fashion, even in absence or with suboptimal supplied auxin concentrations, particularly in the difficult-to-root *E. globulus* (Corrêa et al., 2005). Taken together, available data suggest that low carbohydrate allocation to the root formation site may limit AR. Adequate supply of these compounds is a combined function of sink strength and the capacity of the source to meet sink demand (Druege, 2009). Carbohydrates play important roles, not only by providing energy and carbon chains for biosynthetic processes in new meristems and roots, but also by affecting gene expression, in co-action with auxin.

WOUND RESPONSE

Severance of a cutting from the donor plant has immediate consequences, including injury and the isolation from functional integrity of the whole plant condition, i.e., loss of root–shoot correlative influences (Druege, 2009). Excision of *Petunia* cuttings led to a fast and transient increase in the wound-phytohormone jasmonic acid (JA) and a continuous accumulation of soluble and insoluble carbohydrates during adventitious root formation (Ahkami et al., 2009). There is some evidence that AR is also influenced by ethylene production caused upon wounding during explant preparation, and a stimulatory role of endogenous ethylene would depend on achieving a relatively narrow concentration range (Mensuali-Sodi et al., 1995). In fact, for some *in vitro* studies, the use of anti-ethylene chemicals has resulted in improved rooting responses (De Klerk et al., 1999).

Adventitious rooting in cuttings may be compared to a stress-induced reprogramming of shoot cell fate. Acclimation to stress is often accompanied by metabolic re-adjustment. The alternative oxidase (AOX) plays a central role in determining reactive oxygen species equilibrium in plants and can be induced in response to diverse abiotic and biotic stress conditions (Santos-Macedo et al., 2012). Secondary metabolism during AR may be associated with AOX activity. Phenylpropanoid derivatives, especially phenolic acids and lignin, are known to be closely related to the regulation of cell division and differentiation. Enhanced accumulation of phenolic acids and some flavonoids was found to correlate with *in vitro* rooting (De Klerk et al., 1999). Moreover, a complex interaction between AOX and H_2O_2 signaling is apparent. Application of H_2O_2 could replace added auxin as a rooting agent in olive cuttings (Santos-Macedo et al., 2009) and the presence of an AOX inhibitor, salicylhydroxamic acid (SHAM), reduced rooting even in presence of exogenous auxin (Santos-Macedo et al., 2012).

Phenolic compounds are known to protect plants from oxidative stress (Jaleel et al., 2009) and allow the containment of excessive wound response that may inhibit subsequent regeneration processes (De Klerk et al., 2011). Phloroglucinol and ferulic acid displayed antioxidant action, protecting IAA from decarboxylation and the tissue from oxidative stress in *Malus* "Jork 9," thereby promoting AR. The decarboxylation was attributed to the wound response and did not occur to such an extent in non-wounded plant tissues. The action of the phenolic compounds suggests that, at least in part, rooting depends on the inhibition of IAA decarboxylation caused by wounding, so that more auxin is available to induce roots (De Klerk et al., 2011).

Hydrogen peroxide, a form of reactive oxygen, functions as a signaling molecule that mediates various physiological and biochemical processes, as well as controls responses to various stimuli in plants (Neil et al., 2002). Li et al. (2009b) showed that H_2O_2 might function as a signaling molecule involved in the formation and development of adventitious roots in mung bean seedlings. Production of H_2O_2 was markedly induced in indole-3-butyric acid (IBA)-treated seedlings suggesting that IBA induced overproduction of H_2O_2 and promoted AR via a pathway involving H_2O_2. In another study, Li et al. (2009c) suggested that the mechanism underlying the IBA and H_2O_2-mediated facilitation of adventitious root formation is the early decrease of peroxidase and ascorbate peroxidase activities in IBA and H_2O_2-treated seedlings. The decrease in activity of these enzymes would be relevant to generate the necessary high level of auxin and H_2O_2 required for adventitious root induction.

WATER RELATIONS

The availability of water is one of the most important factors favoring root development, as cuttings have to maintain a positive water balance while roots develop (Loach, 1988). Puri and Thompson (2003) carried out a study to examine the influence of three levels of initial water potential in stem cuttings of *Populus* (dried, soaked, and fresh) on plant water status and rooting capacity under controlled environmental conditions, in combination with planting in soils with different water potential. Results clearly showed that soil moisture had a major effect on rooting. Water-stressed cuttings took a longer time to root and formed fewer roots. Pre-soaking of cuttings had a positive effect on rooting, mainly under the drier soil moisture conditions. Although unrooted hardwood cuttings needed moister soil to initiate rooting, once roots were established, they could tolerate somewhat drier conditions. In good agreement, cutting survival and AR were highest in moister substrate for stem cuttings of juniper (*Juniperus horizontalis*), azalea (*Rhododendron*), and holly (*Ilex crenata*; Rein et al., 1991).

Gas exchange and water relations have also been simultaneously evaluated. Relative water content (RWC) of leaves and osmotic potential increased upon formation of root primordia in *Poinsettia* cuttings (Svenson et al., 1995). Following formation of root primordia, and concurrent with increasing RWC and osmotic

potential, stomatal conductance (g) increased. As roots initially emerged, net photosynthesis and g increased rapidly and continued to increase with further root primordia development and subsequent emergence of adventitious roots. Abscisic acid (ABA) often accumulates under water stress conditions and is a known inhibitor of cell cycle progression (Wolters and Jürgens, 2009). Hence, the level of water stress is a relevant factor for cutting establishment that should be minimized in order to avoid losses and slow establishment of plants.

PHYTOHORMONAL BALANCES: THE SEESAW OF PROMOTION VERSUS REPRESSION

Auxins have a rhizogenic action during the root induction phase (generally from cutting severance up to 96 h) and stimulate cells at the cutting base to engage in the establishment of meristemoids (Garrido et al., 2002). The same phytohormones become inhibitory after 96 h and may arrest or inhibit growth of root primordia (De Klerk et al., 1999). Diaz-Sala et al. (1996), using NPA treatments, showed that the initial 48 h were crucial for auxin-dependent root induction in pine. In addition, mRNA levels of transcription factors possibly related to root meristem fate, as well as cell wall remodeling genes, were increased in presence of exogenous auxin at 24 h (Hutchison et al., 1999; Sánchez et al., 2007; Solé et al., 2008; Vielba et al., 2011).

In general, free IAA endogenous levels have a transient increase during the induction phase, pass through a minimum at the initiation step and resume an increase in the expression phase (Bellamine et al., 1998). The importance of auxin at the induction and expression phases (first and last steps) of the rooting process was demonstrated through the use of anti-auxins, which prevent auxin from exerting its functions. In poplar cuttings, anti-auxins present at one of these phases caused significant inhibition of AR (Bellamine et al., 1998). Moreover, Negishi et al. (2011) compared easy and difficult-to-root lines of *E. globulus* and verified that IAA level was twofold higher in the easy rooting line, confirming the importance of IAA in AR.

A screen for chemicals that cause inhibition of cytochrome P-450 identified one chemical, MA65, which led to an increase in the number of roots of *Arabidopsis* seedlings and twofold higher IAA levels compared to the untreated *Arabidopsis* (Negishi et al., 2011). The observed phenotype was similar to the mutant *superrot2* (*sur2*) which contains high concentrations of free IAA (Delarue et al., 1998) due to a defect in the *SUR2* gene, which encodes the CYP83B1 protein, a cytochrome P450-dependent monooxygenase (Barlier et al., 2000). This increase in IAA production probably happens because cytochrome P450 inhibition blocks the synthesis of indole glucosinolates, providing more substrate (indole-3-acetaldoxime) for the biosynthesis of IAA (reviewed by Bak et al., 2001). The same chemical MA65 was effective for inducing AR in *E. globulus,* but the exact mechanism of action of the chemical in this species awaits further investigation.

The regulation of auxin levels can be done by conjugation of excessive auxin to inactive forms, preventing phytohormone accumulation in the tissue. Auxin degradation, e.g., by peroxidases, is another means of controlling the activity of these regulators. Auxins of different metabolic lability may be conjugated: high stability 1-naphthalene acetic acid (NAA), low stability IAA and moderate stability IBA (De Klerk et al., 1999). IAA can form conjugates with sugars, amino acids, and peptides and these forms are considered resistant to oxidases. IAA can be stored in higher plants as IAA conjugates which might be hydrolyzed depending on the plant demand for free auxin; IBA can also yield IAA by β-oxidation (Woodward and Bartel, 2005). Even if in some cases the conjugation process can be irreversibly inactivated by oxidation (Epstein and Ludwig-Müller, 1993), the most part of auxin conjugates are reversible (De Klerk et al., 1999). When IAA and IBA were exogenously applied to cuttings of *Pisum sativum* L. during adventitious root formation, conjugation of auxins with aspartic acid was the predominant route of metabolism, forming indole-3-acetylaspartic acid (IAAsp) and indole-3-butyrylaspartic acid (Nordström et al., 1991). The authors also verified that the levels of IBA remained high for longer time than those of IAA, indicating higher stability of IBA in rooting solution.

Some gene members of the *GH3* family are involved in the maintenance of auxin homeostasis, contributing to regulation of the auxin pool (Staswick et al., 2005; Chapman and Estelle, 2009). *GH3* genes encode IAA-amide synthetases, which act in the conjugation of physiologically active free IAA excess to amino acids (Staswick et al., 2005). In the moss *Physcomitrella patens*, knock-out of *GH3* genes increased the sensitivity to auxin causing growth inhibition (Ludwig-Müller et al., 2009). Altered auxin sensitivity was also observed in *Arabidopsis thaliana* by overexpression and insertional mutation of *GH3* genes (Staswick et al., 2005). Gutierrez et al. (2012) reported a crosstalk of IAA and JA in which AR-inhibitory JA levels are reduced by conjugation with amino acids through expression of *GH3.3*, *GH3.5*, and *GH3.6* auxin-induced genes, via the action of *ARF6* and *ARF8*, leading to increased number of adventitious roots. GH3 genes would be required for fine-tuning adventitious root initiation in the *Arabidopsis thaliana* hypocotyl, where JA homeostasis is under auxin control (Gutierrez et al., 2012). Curiously, JA accumulation at the cutting base has been shown to be an early, transient, and critical event for rooting of *Petunia* cuttings, and has been discussed to contribute to increasing cell wall invertases and sink strength at the cutting base (Ahkami et al., 2009). Brassinosteroids (BR) have been shown to exert a mild negative regulation of JA-induced inhibition of root growth (Huang et al., 2010). If this applies to AR as well, there could be an additional antagonist crosstalk between JA and BR, regulating the formation phase.

Cytokinins and ethylene have an overall inhibitory effect on induction, but can play a promotive effect during the first 24 h, when cytokinins start to drive cell cycle movement, culminating in mitotic processes (De Klerk et al., 1999; De Klerk, 2002), and ethylene may contribute to auxin transport regulation (Lewis et al., 2011) or to increase the number of auxin-responsive cells (De Klerk and Hanecakova, 2008). Corrêa et al. (2005) observed that kinetin inhibited AR if present during the induction phase in *E. globulus*. The cytokinin type-B response regulator PtRR13, a transcription factor that acts as positive regulator in the cytokinin signaling pathway, has been shown to negatively regulate AR in *Populus*; PtRR13 inactivation upon cutting severance due to the removal of root sources of cytokinin, would alleviate AR inhibition, allowing basipetally transported auxin to accumulate at

cutting base, promoting AR (Ramírez-Carvajal et al., 2009). Ethylene has been shown to promote adventitious root and inhibit lateral root development, predominantly by affecting auxin transport in distinct ways (Negi et al., 2010). Lateral root development inhibition by ethylene was linked to increased expression of PIN3 and PIN7 and auxin transport, preventing auxin accumulation maxima required for pericycle cell activation in roots; in contrast, adventitious root stimulation by ethylene in shoots was due to reduced auxin transport in these organs, favoring auxin accumulation and AR (Lewis et al., 2011). Stimulation of AR in flooded tomato plants was dependent on ethylene accumulation followed by auxin transport increase and allocation to flooded parts of the stem base. Local accumulation of auxin can cause further ethylene production, enhancing the process (Vidoz et al., 2010).

Strigolactones are also involved in adventitious root formation, mostly as repressors, by inhibiting the first divisions of founder cells independently of cytokinins, and perhaps negatively regulating basipetal auxin movement in *Arabidopsis thaliana* and pea (Rasmussen et al., 2012). Upon cutting severance, the content of strigolactones would reduce, since roots are a major source of these phytohormones.

Nitric oxide (NO) has also been proposed as a player in the control of AR. In cucumber, AR was favored by NO, acting downstream of auxin, possibly through different transduction pathways (Lanteri et al., 2009). Auxin-stimulated NO production would increase phosphate cyclic nucleotides cGMP (cyclic guanosine monophosphate) and cADPR (cyclic adenosine 5′-diphosphate ribose), triggering activation of Ca^{2+} channels in the plasmalemma. The release of phospholipids promoted by NO would provide substrates for phospholipases, whose activity and released products could further activate Ca^{2+} release to the cytosol and activate both calcium-dependent protein kinases (CADPKs) and mitogen-associated protein kinases (MAPK). These kinases would in turn lead to cell growth and differentiation associated with AR. NO-promoted AR was also reported for other species, including greenhouse–grown cypress (Lanteri et al., 2009) and *E. grandis* (Abu-Abied et al., 2012). In these studies a co-action of NO and auxin has often become apparent, with NO being induced by auxin. In sunflower, it was suggested that NO could participate with auxin in adventitious root initiation and expression (extension), whereas induction would depend only on auxin (Yadav et al., 2010). Studies on AR of *Tagetes erecta* (marigold; Liao et al., 2009), *Vigna radiata* (mung bean; Li and Xue, 2010), and *Chrysanthemum* (Liao et al., 2010) have suggested that H_2O_2 and NO may act together, possibly as parallel independent pathways dependent on Ca^{2+}, converging on the activation of MAPK cascades leading to AR. A novel interaction of NO and auxin has been shown at the level of NO dependent *S*-nitrosylation of TIR1 auxin receptor, enhancing TIR1-Aux/IAA binding and degradation of the latter, promoting auxin-mediated gene expression (Terrile et al., 2012). The extent of this interesting mechanism in the context of AR is a key research topic to be explored.

Gibberellins (GAs) are generally considered inhibitors of AR. This has been shown, for example, in poplar (Busov et al., 2006). Moreover, lateral root number and growth were promoted in plants with defects in GA production or perception, so that higher root mass and highly branched roots were produced. This inhibitory effect of GA on lateral root development has been partially attributed to changes in polar auxin transport (Gou et al., 2010). In contrast, initiation and elongation of adventitious roots was promoted by GA in deep water rice (Steffens et al., 2006). It is possible that GA may have an AR phase-dependent effect, being inhibitory to root induction and stimulatory to formation. ABA also acts as an inhibitor of lateral root development in *Arachis hypogaea* by blocking cell cycle progression (Guo et al., 2012). Inhibition of adventitious root formation step by ABA was also reported in deep water rice (Steffens et al., 2006).

Polyamines are nitrogen containing, polycationic, low molecular weight aliphatic compounds that can be found in meristematic and actively growing tissues. These metabolites (e.g., putrescine, spermidine, spermine) play various roles, mostly related to control of cell division, development, and stress responses. Because of their positive charges, polyamines are capable of binding to nucleic acids, proteins, and membranes, therefore potentially being able to interfere in processes such as gene expression, cell signaling, membrane stabilization, and modulation of some ion channels (Kusano et al., 2008). Polyamines have been treated as biochemical markers of AR because their concentration peak is consistently associated with the end of the induction phase, similar to auxins. In various unrelated species, AR or promptness to develop adventitious roots is often observed when polyamines peak at the end of adventitious root induction and are metabolized before or at the formation phase (Neves et al., 2002; Arena et al., 2003; Naija et al., 2008).

A tentative model summarizing some of the main data on phytohormonal control of AR is shown in **Figure 1**.

Given the importance of phytohormones, particularly auxins, to the control of AR, the next three sections will examine fundamental aspects of cell cycle control, root tissue differentiation, auxin transport, metabolism, and action. However, it must be emphasized that most of the knowledge presented in these sections is derived from investigations directed to general plant development or development of primary or lateral roots. Although it is clear that these processes are important in AR, their exact contribution in the specific context of the process is far from complete.

CELL CYCLE AND DIVISION – NEW MERISTEMS

Cell divisions in meristems depend on the cell cycle, which involves a mechanism governed by cyclin-dependent kinases (CDKs; Inzé and Veylder, 2006; De Veylder et al., 2007). The association of CDKs and cyclins is required for the induction of cell cycle progression, through phosphorylation of substrates at the transition points of some of its phases (Inzé and Veylder, 2006). G1–S transition is regulated by D-type cyclins (CYCD), which might also be involved in G2–M transition. A-type cyclins (CYCA) are present in S–M, whereas B-type cyclin (CYCB) act in G2–M transition and during M period (De Veylder et al., 2007). G1–S transition may be blocked by abscisic acid, causing inhibition of lateral root primordia initiation in peanut (Guo et al., 2012).

Cyclin-dependent kinases present in plants are A-type (CDKA) and B-type (CDKB), the latter being plant-specific. CDKB

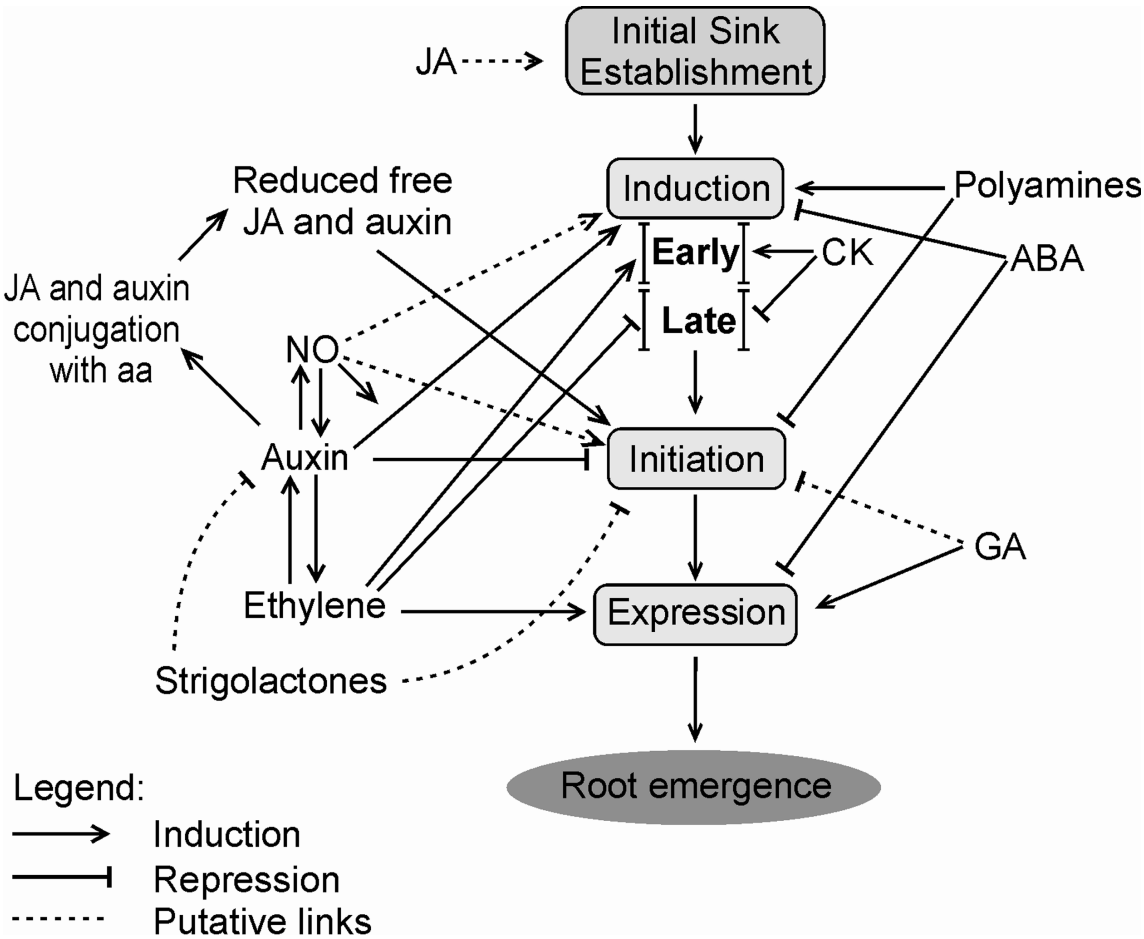

FIGURE 1 | Possible phytohormonal interactions during distinct phases of the adventitious rooting process. JA may promote initial carbohydrate sink establishment before induction or at its early moments. Induction phase is positively regulated by auxin, polyamines and, in early stage, by CK and ethylene. However, during late induction, cytokinin and ethylene act as negative regulators. ABA has a negative effect on AR induction. Initiation phase is inhibited by auxin, polyamines, and GA. JA and auxin are conjugated with aa, so the levels of these phytohormones decrease allowing the progress of the initiation phase. Strigolactones may repress auxin action by reducing its transport and accumulation, or may directly inhibit AR. In contrast, NO is regarded as a stimulator of AR, both during induction and initiation phases. Ethylene increases auxin transport, stimulates expression, but shows a direct repressor effect at induction phase, except perhaps at its early stage, as pointed out above. Auxin may also promote ethylene biosynthesis. Expression phase is induced by ethylene and GA, and suffers repression of ABA. Root emergence is the visible phenotype after the expression phase. Relative positions of phytohormone names within the scheme are not meant to represent differences in importance, but aim at better clarity of the layout. JA, Jasmonic acid; CK, cytokinin; ABA, abscisic acid; GA, gibberellin; NO, nitric oxide; aa, amino acids.

accumulation depends on the cell cycle period, specifically the B1 subclass in the S phase and after G2 until mid-M and B2 subclass, reaching a peak in G2 and M (Boudolf et al., 2006; De Veylder et al., 2007). Moreover, a plant homolog of the tumor suppressor Retinoblastoma (pRb), the RETINOBLASTOMA-RELATED (*RBR*) gene is considered a key cell cycle regulator downstream of the SCARECROW (*SCR*) patterning gene, a member of the GRAS family of transcription factors, acting in the control of cell division, differentiation and cell homeostasis (Wildwater et al., 2005; Borghi et al., 2010). The transcription factors *E2F* and *MYB3R* also take part in the cell cycle control, involved in activation/inactivation of the S-phase and M-phase genes, respectively (De Veylder et al., 2007). Cytokinin and auxin are the main hormones involved with cell proliferation and are indispensable for the progression of the cell cycle (Dewitte and Murray, 2003).

Plant development depends on meristem growth, which happens when cell division predominates over differentiation. The root meristem size is controlled by the balance between cell division and differentiation, where cytokinins and auxins act antagonistically and play important roles (Dello Ioio et al., 2007, 2008; Moubayidin et al., 2009, 2010). In *Arabidopsis*, the *short hypocotyl 2* (*SHY2*) gene acts as a negative regulator of auxin signaling (Tian et al., 2002) by forming heterodimers with *ARF* transcription factors and thus avoiding the activation of auxin-responsive genes. *SHY2* expression is activated by the presence of cytokinins via the route of AHK3 (*Arabidopsis* histidine kinase 3) receptor kinase/cytokinin-responsive *ARR1* transcription factor, and leads to negative regulation of *PIN* genes, involved in the efflux of auxin, which consequently causes a reduction in the root meristem size (Dello Ioio et al., 2008). Moreover, auxins

can cause *SHY2* degradation, and promote the expression of *PIN* genes (Dello Ioio et al., 2008). Furthermore, the transcription factor *ARR12* and GAs also seem to participate in this regulation, *ARR12* inducing a low level of *SHY2* expression and GAs repressing expression of *ARR1* during post-germination meristem growth (Moubayidin et al., 2010).

The root apical meristem is composed of sets of self-renewing and undifferentiated stem cells that allow continued root growth. The quiescent center (QC) takes part in maintaining this condition by supporting meristematic identity of the initial cells around it (Van den Berg et al., 1997; Osmont et al., 2007; Arnaud et al., 2010). The QC cells form part of a region that has a low rate of mitosis, and are histologically distinct from neighboring cells (Doerner, 1998). QC serves as a reservoir of cells for regeneration and ensures the persistence of the apex meristem, as they have self-renewal and self-maintenance capacities. Hormonal activity is important for the QC maintenance and organization (Sabatini et al., 1999; Ortega-Martínez et al., 2007). Reporter genes fused to promoters regulated by auxin were visualized with maximum expression in the position of the QC and root columella (Sabatini et al., 1999). Data obtained by Ortega-Martínez et al. (2007) suggest that ethylene promotes cell division in the QC, indicating that auxin alone would not be sufficient to carry out this function. Surrounding QC, initial cells perform stem cell-like divisions to generate a new initial and a daughter cell, so that the meristem gives rise to all different cell types (Van den Berg et al., 1997).

Some transcription factors, such as *SCR* and *SHORTROOT* (*SHR*), also belonging to the GRAS family of proteins, have crucial role in maintaining the meristematic cells pluripotent identity (Sabatini et al., 2003; Cui et al., 2007). *PLETHORA1* (*PLT1*) and *PLETHORA2* (*PLT2*) are also involved with meristem maintenance, are induced by auxin, and act in parallel with *SHR* and *SCR*, encoding transcription factors *AP2*-like (Aida et al., 2004). *SCR* expression appears to depend of the gene *PDR2* (Ticconi et al., 2009), acting indirectly on QC maintenance. The distribution of *PLT* mRNA is associated with the peak of auxin in stem cells and QC in root meristem (Sabatini et al., 1999). The homeobox transcription factor *WOX5* (*WUSCHEL-RELATED HOMEOBOX 5*), root homologue of the shoot *WUSCHEL* (*WUS*), also has a function in the stem cell maintenance and signaling (Sarkar et al., 2007; Miwa et al., 2009; Stahl et al., 2009).

The involvement of some of these transcription factors in AR in cuttings of tree species has been described. An approach based on cDNA subtractive libraries from rooting competent cuttings of *Pinus radiata* and *Castanea sativa* treated or not with exogenous auxin (Sánchez et al., 2007) yielded data supporting the involvement of clones with homology to *SCR* (*SCR*-like or *SCL*). The content of the corresponding mRNA of these genes increased in both species upon auxin exposure within the first 24 h of the rooting process, coinciding with cell reorganization preceding divisions and establishment of defined root primordia. In *Pinus radiata*, an *SHR*-related clone was identified with an expression pattern similar to that of *SCL*, except for the fact that it was auxin-independent, possibly playing a role in root meristem formation and maintenance, as well as in the cambium zone of hypocotyls (Solé et al., 2008). The expression of *SCL* in *C. sativa* cuttings of juvenile and mature stages was examined in detail (Vielba et al., 2011). A combination of quantitative real time polymerase chain reaction (PCR) and *in situ* hybridization showed that *CsSCL1* was upregulated by auxin, localizing more strongly in the cambium layer and derivative cells in rooting competent shoots, whereas for root incompetent shoots its signal was more diffuse and evenly distributed in the phloem and parenchyma (Vielba et al., 2011). The authors suggest that *CsSCL1* may determine which cells will engage in the root differentiation route, athough they observed that expression of this gene was also present in lateral roots and axillary buds.

Recently, *AINTEGUMENTA LIKE1* (*PtAIL1*), a member of the *AP2* family of transcription factors, has been shown to be associated with cell division and further establishment of adventitious root primordia in *Populus trichocarpa* (Rigal et al., 2012). Transgenic poplar overexpressing *PtAIL1* displayed higher number of adventitious roots, whereas RNA interference (RNAi) downregulation of the same gene transcript resulted in delayed AR. A number of genes were co-regulated with *PtAIL1* based on microarray and comparative analyses of modified poplar lines up or downregulated for the *AP2* transcription factor, included among these additional transcription factors, such as *AGAMOUS-Like6* and *MYB36* (Rigal et al., 2012).

THE CENTRAL ROLE OF AUXINS: TRANSPORT, CONTROL OF LOCAL CONCENTRATION, TIMING, AND METABOLIC DYNAMICS

Auxins are very important for determining pattern in plants. Their spatial distribution is determinant for proper formation of the axis along the plant body. Auxin transport has two main forms: (a) rapid (up to 10 cm per h), often referred to as non-polar, bidirectional transport in the phloem sieve elements, (b) slow (approximately 10 mm per h) or polar, mediated by transporters (Kerr and Bennett, 2007), mostly in vascular parenchyma. Rapid transport in the phloem conducting cells essentially obeys source–sink relations and involves both free IAA and inactive conjugates (Friml and Palme, 2002). Studies with radiolabeled IAA applied to pea leaves indicated that both transport pathways may communicate, at least from the non-polar to the polar system (Cambridge and Morris, 1996). There is also evidence that phloem-based transport may become relatively more important than polar transport, at least in roots, at later stages of seedling development (Ljung et al., 2005).

The polar transport of the major endogenous auxin IAA has specific carriers, which allow intercellular auxin flow and are well-known in *Arabidopsis*. In stems, the transport is active, polar, and basipetal. According to the chemiosmotic model (Raven, 1975), there is a pH gradient between the intra- and extracellular medium, generated by the action of proton pumps in the plasma membrane, which drive protons into the apoplast, making it acidic. In the apoplast, IAA can be found both in anionic and protonated forms, the latter being more lipophilic and capable of easily diffusing through the plasma membrane (Woodward and Bartel, 2005; Zazimalová et al., 2010). On the other hand, the anionic form lacks this capacity and, for it to enter the cell, the action of auxin influx carriers is required. These carriers are amino acid permease-like proteins of the AUX1/LAX family (reviewed in Vieten et al., 2007). These proteins act as H^+/IAA^-

symporters and may participate in lateral root emergence and root hair development (reviewed by Vanneste and Friml, 2009).

Members of the PIN Formed (PIN) protein family are involved with auxin efflux and their asymmetric distribution in the cells is fundamental to the characteristic polar basipetal transport along the stems. The correct localization of PIN proteins is determined by its phosphorylation status, defined by the balance between the kinase protein PID (PINOID) and the phosphatase PP2A. In the case of emerging primordia, the expression of PID is activated, turning PIN protein to a phosphorylated form, leading to its apical localization in the cell. On the other hand, in most situations, PP2A is more active than PID, leading to dephosphorylated PIN protein, resulting in a basal localization in the cell (Michniewicz et al., 2007). Furthermore, the NPA-binding protein and actin filaments of the cytoskeleton also function in the correct positioning of the PIN proteins (Muday and DeLong, 2001). This family of transmembrane proteins has eight members in *Arabidopsis* which are considerably homologous and functionally redundant, being involved in tropisms, embryo development, root meristem patterning, organogenesis, and vascular tissue differentiation (reviewed by Krogan and Berleth, 2007 and Vanneste and Friml, 2009). The Multidrug/P-glycoproteins of the ABCB (ATP-binding cassette B) transporter family (ABCB/MDR/PGP) also contribute to auxin transport, being more closely related to non-polar auxin efflux and maintenance of the main auxin fluxes (Geisler and Murphy, 2006). These transporters may also play a possible role in short-distance lateral auxin movement.

Basipetal auxin transport is also affected by the red/far-red (R:FR) light ratio (Morelli and Ruberti, 2002). In open daylight (high R:FR), auxin moves from the shoot to the root mainly through the central cylinder. However, in shade conditions (low R:FR), a new route, by the outer cell layers, is preferred. This alternative route is less effective and leads to increase in auxin levels in cell layers external to the central cylinder in the stem, enhancing cell elongation in this organ. Consequently, less auxin is transported through the vascular system, decreasing vascular differentiation and the auxin content reaching the root.

Recent findings revealed the function of a new family of putative auxin transporters, the PIN-LIKES (PILS; Barbez et al., 2012; Feraru et al., 2012). These proteins are considered evolutionarily older than PIN proteins and probably preceded the PIN-dependent auxin transport (Feraru et al., 2012), but are similar to PIN family members and also contain the auxin transport domain, predicted to carry out this function. The PILS proteins are localized in the endoplasmic reticulum (ER) and are involved in the intracellular transport of free IAA from cytosol to ER (Barbez et al., 2012; Feraru et al., 2012). According to these findings, PILS activity promotes auxin accumulation in the ER by increasing amide auxin conjugates, reducing free auxin levels. This action could be involved in a compartmentalized-type regulation of auxin metabolism (Barbez et al., 2012). The PIN family member PIN5, which is localized in the ER, is also suggested as an intracellular auxin carrier, stimulating the formation of auxin amino and ester conjugates and their transport to the ER (Barbez and Kleine-Vehn, 2012).

Auxin amino acid and glucose conjugates can also be stored in the vacuole (Ueda et al., 2011). The transport into this cellular compartment has been suggested as an action of the ABC transporter AtMRP5 (*Arabidopsis thaliana* multidrug resistance 5). *Atmrp5-1* mutants, defective in *MRP5* expression, have shown higher free auxin levels and inhibition of root elongation (Gaedeke et al., 2001). This could be due to increased levels of free auxin in the cytoplasm of root cells caused by a disruption in moving auxin conjugates away from the cytoplasm.

Considering other auxins, such as the endogenous IBA and the synthetic auxin (NAA), relatively little is known about transport and metabolism. IBA is more stable than IAA and persists for longer in plant tissues (De Klerk et al., 1999), being basipetally transported in seedling hypocotyls (Rashotte et al., 2003), similarly to IAA. However, IBA seems not to be transported in inflorescences, unlike IAA (Rashotte et al., 2003). Mutations affecting IAA transport did not cause significant effects in IBA transport. The differences between IBA and IAA transport suggest that IBA might use distinct transporters from those used to move IAA (Strader and Bartel, 2011). NAA is more stable than the above auxins and is probably transported by different carriers, as revealed by *aux1* loss-of-function mutants, which respond normally to NAA (Yang et al., 2006).

The formation of auxin gradients, originated by the combined processes of biosynthesis, conjugation, and degradation, as well as inter- and intracellular transport, independently of type, is relevant for both plant morphogenesis and determination of tissue patterns (Vanneste and Friml, 2009; Overvoorde et al., 2010; Simon and Petrasek, 2011). Previous studies of PIN expression and auxin distribution in *pin* mutants showed that PIN proteins are the major players in directional distribution networks that mediate auxin maxima and gradients during different developmental processes (reviewed by Vieten et al., 2007). In the developing embryo, the localization of PIN proteins assumes positions of auxin accumulation along the stages of development and form auxin convergence points, necessary for cotyledon initiation and positioning at the late globular stage (reviewed by Krogan and Berleth, 2007). In shoot apical meristems, auxin promotes *PIN1* expression, which generates auxin accumulation at the sites of leaf primordia formation. These, once established, promote a drain of auxin, which will accumulate at a certain distance from the early primordia, enabling the phyllotactic pattern to be established (reviewed by Berleth et al., 2007).

Recent evidence points to a possible role of APY (apyrases) in regulating auxin transport (Liu et al., 2012). Exogenous ATP is capable of inhibiting auxin transport and gravitropic response in *Arabidopsis*. Apyrases (triphosphate diphosphohydrolases) are enzymes that participate in limiting ATP content. Polar IAA transport in roots and hypocotyls was reduced in *apy2* null mutants when these were suppressed of *APY1* (apyrase 1) expression by an estradiol-induced RNAi. Basal portions of APY-suppressed hypocotyls accumulated less free IAA and morphological defects were seen in roots with the same genetic modification. Problems in gravitropic asymmetry of auxin content were detected by means of DR5::GFP constructs in APY reduced plants, either genetically or treated with APY chemical inhibitors. The relevance of apyrase participation in auxin transport during AR is presently unclear and should be object of further investigation.

Auxin gradients are also very important for root organogenesis and both primary and lateral root formation are issues that

had good advances in the last decades. Studying root outgrowth in *Arabidopsis*, Blilou et al. (2005) concluded that PIN-mediated modulation of auxin distribution controls both cell division and elongation, affecting meristem, elongation zone, and final cell sizes. Dubrovsky et al. (2008) revealed a spatial and temporal correlation of auxin maxima with developmental reprogramming, resulting in lateral root initiation (LRI). The sites and frequency of LRI are controlled by variations in auxin concentration in pericycle cells, which might be correlated with changes in PIN protein localization upon gravistimulation (Benkova and Bielach, 2010). These events will culminate in lateral root primordia formation.

Genetic studies revealed that *pils2pils5* double loss of function mutant had higher free auxin levels, increased hypocotyl growth and presence of lateral roots, which were longer and more abundant than in the PILS5 gain of function phenotype. This evidence suggests that PILS2 and PILS5 could have specific functions in the cellular regulation of root growth (Barbez et al., 2012).

However, relatively little is known about the effects of polar and non-polar auxin transport during adventitious root formation. Using inhibitors of polar auxin transport, various investigations in cuttings or de-rooted seedlings have provided evidence for a significant contribution of this type of transport to AR (e.g., Nordström and Eliasson, 1991; Liu and Reid, 1992; Koukourikou-Petridou and Bangerth, 1997; Guerrero et al., 1999; Garrido et al., 2002; Nicolás et al., 2004). Few studies analyzing the expression of genes encoding auxin carriers during adventitious rhizogenesis were conducted in de-rooted pine seedlings (Brinker et al., 2004), intact rice plants (Xu et al., 2005), carnation cuttings (Oliveros-Valenzuela et al., 2008; Acosta et al., 2009), and mango cotyledon segments (Li et al., 2012). The studies with carnation and mango showed the requirement of increased expression of auxin transporters and increase of polar auxin transport during the induction and formation phase of AR. However, in the case of pine seedling cuttings, increased expression was linked to root formation (Brinker et al., 2004). In rice, the expression of *OsPIN1* was also important during root formation (Xu et al., 2005). Taken together, these findings corroborate the role of auxin in controlling organogenesis, but more studies are necessary to clarify the effects of auxin carriers in AR, mainly in woody species. A summary of mechanisms and factors possibly contributing to transport and local concentration of auxin during AR is illustrated in **Figure 2**.

Considering cuttings used for vegetative propagation, the progressive accumulation and local concentration of auxin in the base of the cuttings seems to be important to generate the peak necessary for starting the rooting process (Acosta et al., 2009) and often this can be facilitated by exogenous application of auxins in recalcitrant species. Meanwhile, recent studies indicate that basipetal auxin transport and auxin accumulation in the rooting zone may be negatively regulated by strigolactones (Rasmussen et al., 2012). This phytohormone class could act reducing auxin levels in the pericycle, decreasing root initiation. This could be a direct effect or via regulation of the amount of local auxin levels, presumably involving impairment of the rooting zone (Rasmussen et al., 2012). Thus, although auxin is the main hormone involved in AR, it clearly does not act alone, since crosstalk between several phytohormones is necessary for the success of this process.

AUXIN RECEPTORS AND ACTION MECHANISMS

Even though auxin is known to play a central role in AR, the specific mechanisms of auxin action in this process are far from being understood. However, considering plant development in general, in the past decade a vast amount of data was reported regarding auxin perception (Mockaitis and Estelle, 2008). At the cellular level, auxin induces various rapid changes in cell physiology, such as membrane depolarization, apoplast acidification, cell wall loosening, activation of plasma membrane ATPases, and control of gene expression (Scherer, 2011). Although many of the signaling pathways leading to the responses mediated by auxin are still to be elucidated, significant knowledge on nuclear receptors for auxin is available. In the recent literature two different proteins are accepted as true auxin receptors, ABP1 and TIR1/AFB (auxin signaling F-box) proteins. The TIR1/AFB-family of F-Box protein members were the first authentic auxin receptors to be discovered (Dharmasiri et al., 2005a; Kepinski and Leyser, 2005). These proteins form nuclear regulatory complexes called SCF-E3-ubiquitin ligases and are responsible for the targeted degradation of a family of transcriptional repressors called AuxIAA proteins (Gray et al., 2001).

AuxIAA proteins are transcriptional repressors that act via dimerization with auxin-responsive transcription factors called ARFs (auxin-responsive factors). Upon binding of auxin to the F-Box (TIR1/AFB) subunit of the SCF TIR1/AFB complexes, their affinity toward the domain II of AuxIAA proteins is greatly enhanced with auxin acting as a "molecular glue" bringing the two proteins together; this binding triggers the ubiquitination of the AuxIAA by the SCF complex leading to its destruction by the 26S proteasome (Tan et al., 2007; Chapman and Estelle, 2009; Maraschin et al., 2009). The degradation of the transcriptional repressor releases the transcriptional activity of ARFs and auxin-responsive genes are expressed (**Figure 3**). The control of AR in intact seedlings of *Arabidopsis* by auxin, for example, involves activation of transcription factors *ARF6* and *ARF8* (Gutierrez et al., 2009). The TIR1/AFB family of auxin receptors is composed of 6 distinct members in *Arabidopsis* (namely TIR1, AFB1, AFB2, AFB3, AFB4, and AFB5), all of which are able to bind auxins specifically and show auxin-enhanced binding to AuxIAA proteins (Mockaitis and Estelle, 2008). Although much of the phenotypes of TIR1/AFB mutants indicate a large degree of redundancy, some specific features have already been identified. For example, TIR1 and AFB2 display a higher affinity for AuxIAA proteins compared to other members.

On the basis of the phenotype of single mutants, TIR1 appears to make the largest contribution followed by AFB2. Both AFB1 and AFB3 contribute to auxin response, but this contribution is only apparent in higher order mutant combinations. The *afb4* and *afb5* mutants are more resistant than *tir1* to picolinate auxins such as picloram, suggesting alternative substrate specificity (Parry et al., 2009). All of the defects observed in *afb4-2* mutant seedlings can be simulated in wild-type seedlings by treatment with auxin, indicating that AFB4 acts as a negative regulator of auxin-dependent processes. The *afb4-2* mutants have shorter roots

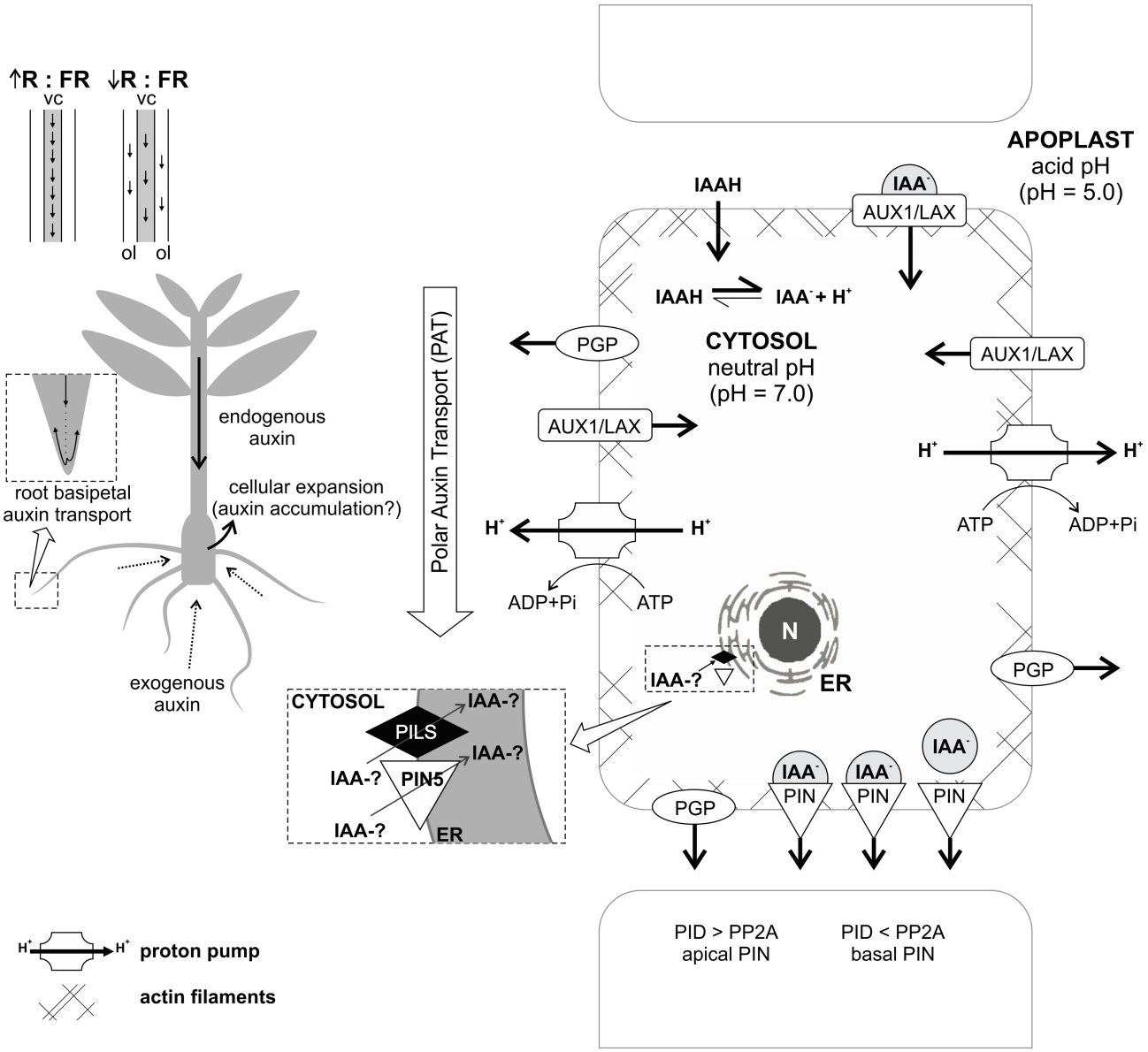

FIGURE 2 | Concept of auxin transport processes probably involved in AR. Active, polar, and basipetal auxin transport contributes to auxin accumulation in the rooting zone of a cutting. Endogenous auxin is transported through the stem in an active, polar, and basipetal way. In daylight, when there is high red:far-red ratio (R:FR), the transport is mainly through the central cylinder (vc). In shade conditions, with low R:FR ratio, a less efficient route by the outer cell layers (ol), is preferred. When exogenous auxin is applied to the medium, it is absorbed by diffusion, which can cause cellular expansion in the basal part of the plant, perhaps due to auxin accumulation. Once adventitious roots are established, they provide a follow up to stem basipetal transport, continuing through the stele as acropetal transport in roots and then basipetal through the subepidermal cell layers of the newly formed organs. Intercellular auxin transport involves specific carriers: AUX1/LAX proteins, related with auxin influx; PGP proteins, related with auxin efflux and lateral transport; and PIN proteins, which have an asymmetrical distribution and allow directed auxin efflux. The PIN correct localization in the cell and the route of the auxin flow is determined by the balance between the kinase protein PID and the phosphatase PP2A. Concerning intracellular transport, auxin can be transported into the endoplasmic reticulum (ER) by the action of PILS proteins and PIN5, which reduce free auxin levels and increase auxin conjugates. It remains to be elucidated if auxin conjugates can be formed into the ER or just in cytosol. For more details, see text. N – nucleus; IAA-? – auxin in free or conjugated form.

and display a higher lateral roots/primary root length ratio than wild-type seedlings, suggesting that AFB4 has a role in anchor or adventitious root production (Greenham et al., 2011).

The expression patterns of the TIR/AFB genes are highly overlapped and not auxin-responsive, with the most significant regulation so far described being due to post-translational repression of TIR1, AFB2, and AFB3 by miR393 upon pathogen attack (Navarro et al., 2006). The structural specificity of auxin binding to TIR1 has been investigated to atomic level via X-ray crystallography. The details of this interaction provided valuable information

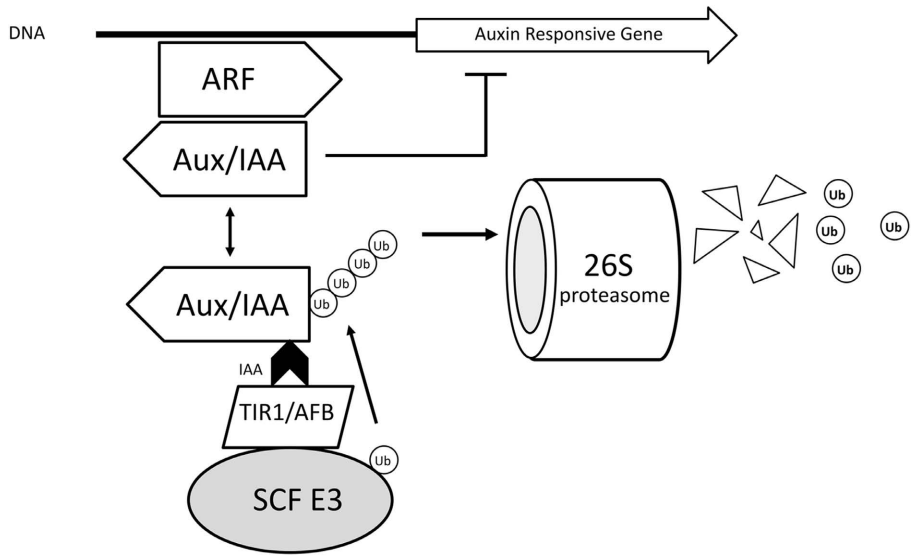

FIGURE 3 | Auxin action mechanism based on TIR1. Upon binding of auxin to the F-Box (TIR1/AFB) subunit of the SCF TIR1/AFB complexes, their affinity toward the domain II of AuxIAA proteins is greatly enhanced with auxin acting as a "molecular glue" bringing the two proteins together; this binding triggers the ubiquitination of the AuxIAA by the SCF complex leading to its destruction by the 26S proteasome. The degradation of the transcriptional repressor releases the transcriptional activity of ARFs and auxin-responsive genes are expressed. E3 – ubiquitin protein ligase.

to understand the mechanism of binding and structural details for active auxins (Tan et al., 2007). Recently, intensive efforts have been successful in designing TIR1-specific auxin antagonists, such as BH-IAA (tert-butoxycarbonylaminohexyl-IAA) and auxinole (Hayashi et al., 2008, 2012). These molecules specifically interact with the auxin-binding pocket on the TIR1 protein, blocking the access to the domain II of AuxIAAs. By testing the effects of blocking TIR1/AFB responses one is able to determine the contribution of TIR1/AFB-dependent transcriptional responses on whole plant phenotypes such as adventitious root formation. Although such inhibitors were designed based on the *Arabidopsis* TIR1 protein, the conservation of the TIR/AFB-AuxIAA mechanism goes all the way to mosses such as *Physcomitrella* sp., broadening the application of chemical tools to investigate physiological events in many unrelated plant species. A scheme on the TIR1 model of auxin action is shown in **Figure 3**.

Auxin-Binding Protein 1 was the first auxin-binding protein discovered, about 40 years ago (Hertel et al., 1972). ABP1 binding to auxin is highly specific and pH-dependent. Null *abp1* mutants are embryo-lethal and the functions of ABP1 on auxin signaling remained obscure since its discovery (Tromas et al., 2010). With the analysis of multiple TIR1/AFB mutants it became clear that nuclear perception of auxin and the degradation of AuxIAAs cannot account for all auxin-dependent cellular responses (Dharmasiri et al., 2005b). It is believed that plasma membrane localized ABP1 acts as an extracellular auxin receptor inducing rapid responses on the membrane and cytosol (Shi and Yang, 2011). The mechanism through which ABP1 is able to transduce the auxin signal to other molecules is still unknown. ABP1 has emerged as the receptor responsible for fast, protein synthesis independent, membrane and cytosolic responses to extracellular auxin concentrations. Many early auxin-dependent responses are attributed to ABP1 signaling: a fast (few milliseconds) drop on plasma membrane polarization, K^+ influxes (0.5 s), rise in cytosolic Ca^{2+} (30 s), phospholipase A activation (2 min), MAPK activation (5 min), among other rapid auxin-triggered responses (Tromas et al., 2010). Recently, it has been demonstrated that auxin binding to ABP1 is able to inhibit clathrin-dependent PIN protein endocytosis at the plasma membrane (Robert et al., 2010). It has been proposed that ABP1 would be the receptor to regulate auxin transport throughout the plant whereas the TIR1/AFB proteins would be the receptors responsible for intracellular auxin transcriptional responses.

The current scenario suggests that ABP1 and TIR1/AFB proteins are components of a two-receptor mechanism for auxin responses (Scherer, 2011) with ABP1 being an early sensor of apoplastic auxin concentrations regulating auxin transport and early, fast, transcriptional-independent, membrane and cytosolic responses, such as apoplast acidification and early elongation. TIR1/AFB would be the receptors responsible for the perception of nuclear and cytosolic auxin concentrations, involved in later, long term developmental responses, triggering transcriptional adaptive responses to the signal input generated by the ABP1-regulated auxin transport (Scherer, 2011). The relative participation of these auxin receptors in AR is currently unclear, but could putatively require sequential and conjunct activity in a rooting phase-dependent fashion. A putative model of ABP1 action and its interaction with TIR1 is shown in **Figure 4**.

miRNA CIRCUITRY

Several miRNAs were reported as involved in root development modulation, reinforcing the growing awareness that miRNAs play pivotal roles in many biochemical or biophysical processes

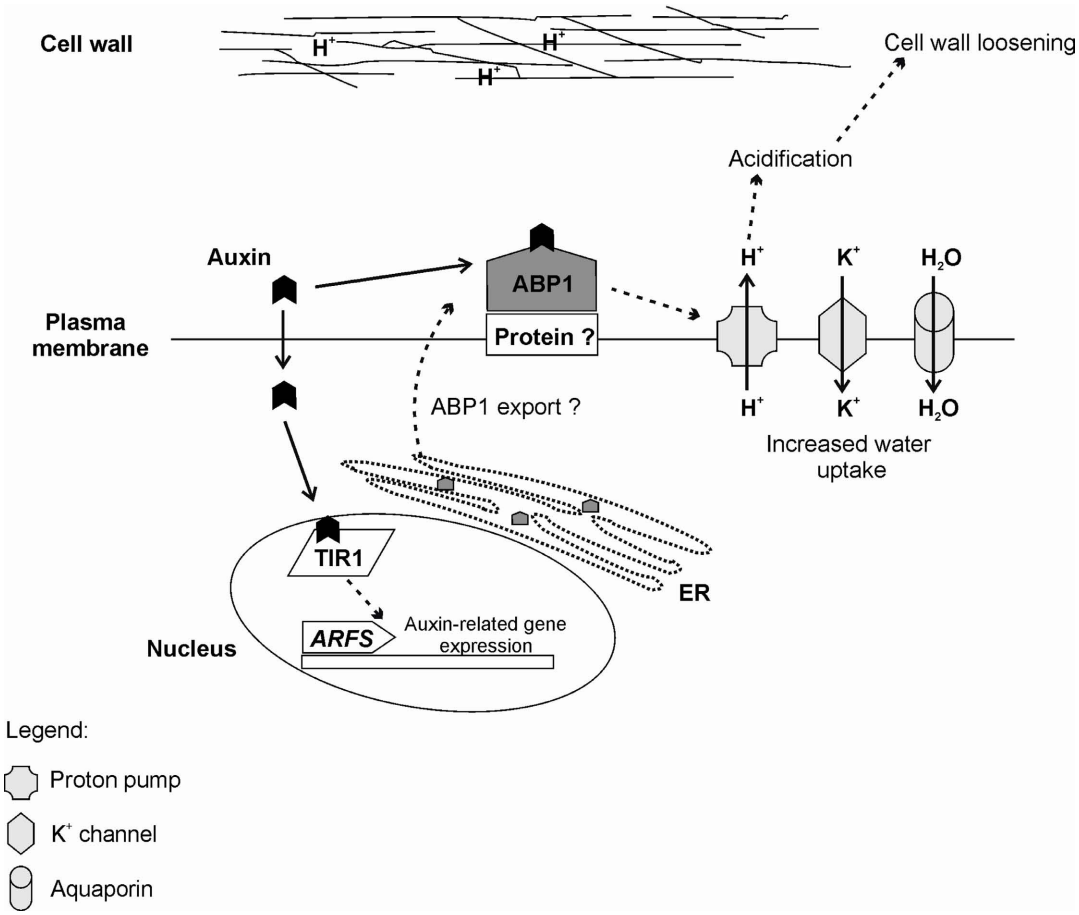

FIGURE 4 | Summary of the auxin perception by ABP1 and TIR1 receptors. Rapid auxin responses are thought to be mediated by ABP1. Auxin is perceived by ABP1 at the outer surface of the Plasma membrane. In this case, ABP1 is anchored by an unknown membrane-associated protein (Protein?). In flowering plants, ABP1 is mainly located at the endoplasmic reticulum (ER) due to an ER retention motif (KDEL). Currently, it is not known how ABP1 is exported to the plasma membrane. Binding of auxin to ABP1 induces several events including activation of proton pumps, which culminates in acidification of the outer space and contributes to cell wall loosening. There is also activation of potassium inward channels, which increase the intracellular K^+ and, consequently, lead to increased water uptake, allowing cell expansion. For slower responses, auxin is perceived by the F-box TIR1, which directs the auxin repressors Aux/IAAs for degradation and releases the auxin response factors (ARFS) to induce auxin-related gene expression.

in planta (Meng et al., 2010). Gutierrez et al. (2009) established that microRNAs miR160 and miR167 were implicated in adventitious root formation through auxin signal further transduced by their downstream ARF targets (Meng et al., 2010). ARF6 and ARF8 targeted by miR167 were shown to be positive regulators of shoot-borne root emergence, whereas ARF17, a target of miR160, was a negative regulator (Gutierrez et al., 2009). ARF17 affects both miR167-dependent and independent regulation of ARF6 and ARF8. Conversely ARF6 represses ARF17 by activating miR160, whereas ARF8 directly represses ARF17. Finally, miR167 and miR160 appear to have opposite roles in controlling the expression of the auxin homeostatic enzyme GH3, which are required for fine-tuning adventitious root initiation in the *Arabidopsis thaliana* hypocotyl, acting by modulating JA homeostasis (Gutierrez et al., 2012). Thus miR160 targets reduce active auxin and AR, whereas miR167 targets act in opposite way (Rubio-Somoza and Weigel, 2011).

ROOT GROWTH AND EMERGENCE THROUGH THE STEM

Adventitious root primordia, with apical meristem and differentiation of the basic root body, are formed and grow through the cortex toward the surface of the stem. Ethylene seems to be important to induce cell wall loosening and facilitate root passage through the stem tissues (Vidoz et al., 2010). Once newly formed roots reach the surface of the stem, a disruption of the epidermis and additional cell wall loosening take place, leading to root emergence. Afterward, the stem itself develops a periderm around the opening of each of the adventitious roots formed, important for protection against microorganism attack and drought (Hatzilazarou et al., 2006). The vascular reconnection between newly formed roots and the shoot is then fully established, allowing root nutrition, hydration, and growth (Hatzilazarou et al., 2006). In this process of vascularization and vascular connection, auxins and cytokinins are relevant for phloem and xylem tissue differentiation. In deepwater rice,

a model has been proposed for phytohormonal interactions regulating root emergence. In this model, ethylene would promote epidermal programmed cell death, root emergence and elongation, and these processes would be co-stimulated by GAs and inhibited by ABA (Steffens et al., 2006).

FINAL REMARKS AND PERSPECTIVES

In spite of a large volume of information on AR accumulated over the last few decades, a complete picture of this key developmental process is far from sight. Phytohormones are certainly at center stage in the conundrum of factors that influence AR. Not surprisingly, their actions involve significant degree of crosstalk, adding to the complexity of the process. In addition, a relevant participation of carbohydrate metabolism and mineral nutrition is evident, frequently modulating phytohormone-based controls. The wound response associated with the typical AR protocols add other players such as JA, H_2O_2, phenolics, and the action of enzymes on phytohormone content.

Faster advances of significant impact (both fundamental and practical) in the field of AR may depend on a number of strategies and scientific decisions for possible consideration by researchers. Although model species are a highly valuable tool for unveiling complex developmental processes, it is probably useful to somewhat diversify research objects, at least a couple of species for each general type of plant material (small herbaceous, monocots and dicots, horticulture/flower like crops, fruit crops, forest species, angiosperms, and gymnosperms) and within these seek for a few genotypes of easier or harder-to-root phenotype, in order to gain a better view of the process. A shift or at least a better balanced focus between research aiming at cuttings and at mother plant status and its implications on subsequent rooting may help achieve a more global understanding/predictable manipulation of AR. The recognition and identification of the main phases of AR should be taken into account in the various materials under investigation, for the process is quite dynamic and requisites and needs change along the process of re-establishing a root system.

From the experimental view point, solid associations must be established between structure and function, with a refinement of sampled cell types and tissues (cell/tissue-specific gene expression, proteomics, and metabolic profiling), always with a kinetic perspective of the successive phases. Another key association in the realm of methodologies is to maintain an open dialog between the basic and applied research with mutual benefits arising from exchanging operational strategies, investigation methods, and process modulation tools. Finally, a conjunct effort to establish clearer boundaries between lateral and adventitious root development and to seek an integrated look at these two processes within the various plant materials investigated may help clarify some of the contradictory data populating the rooting literature.

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The interaction between strigolactones and other plant hormones in the regulation of plant development

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Plant hormones are small molecules derived from various metabolic pathways and are important regulators of plant development. The most recently discovered phytohormone class comprises the carotenoid-derived strigolactones (SLs). For a long time these compounds were only known to be secreted into the rhizosphere where they act as signaling compounds, but now we know they are also active as endogenous plant hormones and they have been in the spotlight ever since. The initial discovery that SLs are involved in the inhibition of axillary bud outgrowth, initiated a multitude of other studies showing that SLs also play a role in defining root architecture, secondary growth, hypocotyl elongation, and seed germination, mostly in interaction with other hormones. Their coordinated action enables the plant to respond in an appropriate manner to environmental factors such as temperature, shading, day length, and nutrient availability. Here, we will review the current knowledge on the crosstalk between SLs and other plant hormones—such as auxin, cytokinin, abscisic acid (ABA), ethylene (ET), and gibberellins (GA)—during different physiological processes. We will furthermore take a bird's eye view of how this hormonal crosstalk enables plants to respond to their ever changing environments.

Keywords: strigolactone, auxin, cytokinin, ethylene, gibberellins, hormone crosstalk, root and shoot architecture, phenotypic plasticity

INTRODUCTION

Plant hormones are small molecules derived from various essential metabolic pathways. They play critical roles during all developmental stages in plants, from early embryogenesis to senescence. Research on plant hormones started as early as the beginning of the last century and has resulted in the discovery of auxins, ethylene (ET), cytokinins (CK), gibberellins (GA), abscisic acid (ABA), brassinosteroids (BRs), jasmonic acid (JA), salicylic acid (SA), and the recently identified strigolactones (SLs). The biosynthetic pathways of these plant hormones have been mostly elucidated, with some minor exceptions, such as some missing steps in SL biosynthesis. Generally, plant hormones exert their effect locally at or near the site of biosynthesis or are mobile between different tissues. The mechanisms of hormone crosstalk can be diverse. Hormone signaling pathways are known to interact at the level of gene expression. A common crosstalk strategy is to control specific key components of signaling pathways of other hormones (Santner et al., 2009; Santner and Estelle, 2009). In this way, hormones might regulate synthesis (hormone levels), sensitivity (hormone response), and transport (hormone distributions) of other hormones.

During the last decade we have witnessed remarkable breakthroughs in plant hormone research, especially with the discovery of the SLs. With this discovery, plant scientists not only got a new tool to study hormonal regulation of plant development but were also triggered to critically assess existing hypotheses on hormone crosstalk mechanisms. SLs were known as host-derived germination stimulants for root parasitic plants such as the witchweeds (*Striga spp.*) and broomrapes (*Orobanche* and *Phelipanche spp.*) since the sixties of last century (Bouwmeester et al., 2003). Their function, as allelochemicals in symbiosis with arbuscular mycorrhizal (AM) fungi, was discovered only recently (Akiyama et al., 2005). SLs promote the establishment of mycorrhizal symbiosis which mainly facilitates the phosphate acquisition from the soil. Later, SLs were found to play a key role in shoot branching inhibition and thus were identified as a new group of plant hormones (Gomez-Roldan et al., 2008; Umehara et al., 2008). Their biological functions were further explored and it was discovered that they also exert their effects on different developmental processes including root development, seed germination, hypocotyl elongation, and secondary growth. Their conserved functions between different plant species are indicative of their indispensability in regulating plant development.

This review will focus on the current knowledge on the SLs and their hormonal crosstalk with other plant hormones such as auxin, CK, ABA, ET, and GA during bud outgrowth, root development, secondary growth, and seeds germination. We will furthermore take a bird's eye view of how this hormonal crosstalk

Abbreviations: P, primordium; SAM, shoot apical meristem; DM, distal meristem; PM, proximal meristem; AM, apical meristem; BM, basal meristem; TZ, transition zone; EZ, elongation zone; DZ, differentiation zone; FC, founder cell; RAM, root apical meristem; PR, primary root; RH, root hairs; LR, lateral root; LRP, lateral root primordia; SL, strigolactone; CK, cytokinin; ET, ethylene; PAT, polar auxin transport.

enables the plant to respond to its ever changing environment, including shade and nutrient deprivation.

SL BIOSYNTHESIS AND PERCEPTION

So far, at least 15 SLs have been structurally identified. They are typically composed of four rings (A–D). The A and B rings vary due to different side groups, while the C and D rings are highly conserved and seem to play an essential role in biological activity (Xie et al., 2010). Like ABA, SLs are also derived from the carotenoid pathway from which they are hypothesized to diverge at β-carotene (Matusova et al., 2005; Lopez-Raez et al., 2008; Rani et al., 2008) (see **Figure 1**). Interestingly, especially considering their common biosynthetic origin, a correlation between ABA levels and SLs production was observed in the ABA mutants *notabilis*, *sitiens*, and *flacca* and in plants treated with AbaminSG, an inhibitor of the ABA biosynthetic enzyme 9-*cis*-epoxycarotenoid dioxygenase (NCED). It was suggested that ABA may regulate SL biosynthesis (Lopez-Raez et al., 2010).

Several mutants with increased shoot branching phenotype have been identified in several plant species, including *more axillary growth* (*max*) in Arabidopsis (*Arabidopsis thaliana*), *ramosus* (*rms*) in pea (*Pisum sativum*), *dwarf* (*d*) or *high-tillering dwarf* (*htd*) in rice (*Oryza sativa*), and *decreased apical dominance* (*dad*) in petunia (*Petunia hybrida*). All these mutants are defective in SL biosynthesis or signaling. They form the basis for the discovery of genes involved in the SL biosynthetic and downstream signaling pathways. Key catalytic enzymes in the SL biosynthetic pathway include DWARF27 (D27) (Lin et al., 2009; Waters et al., 2012a), CAROTENOID CLEAVAGE DIOXYGENASE 7 and 8 (CCD7 and CCD8), and MAX1 (Booker et al., 2005; Kohlen et al., 2011) (see **Figure 1**). CCD7 and CCD8 are, respectively, encoded by the genes *MAX3*/*RMS5*/*D17(HTD1)*/*DAD3* (Morris et al., 2001; Booker et al., 2004; Zou et al., 2006; Drummond et al., 2009) and *MAX4*/*RMS1*/*D10*/*DAD1* (Foo et al., 2001; Sorefan et al., 2003; Snowden et al., 2005; Arite et al., 2007). Both the F-box protein MAX2/RMS4/D3 (Stirnberg et al., 2007; Yoshida et al., 2012) and the α/β-fold hydrolase D14/D88/HTD2/DAD2 (Arite et al., 2009; Liu et al., 2009; Gaiji et al., 2012; Hamiaux et al., 2012) have been shown to be involved in SL downstream signaling. More aspects about SLs biosynthesis, perception, and signaling as well as structure-function relationships have been nicely addressed and updated in several recent reviews (Janssen

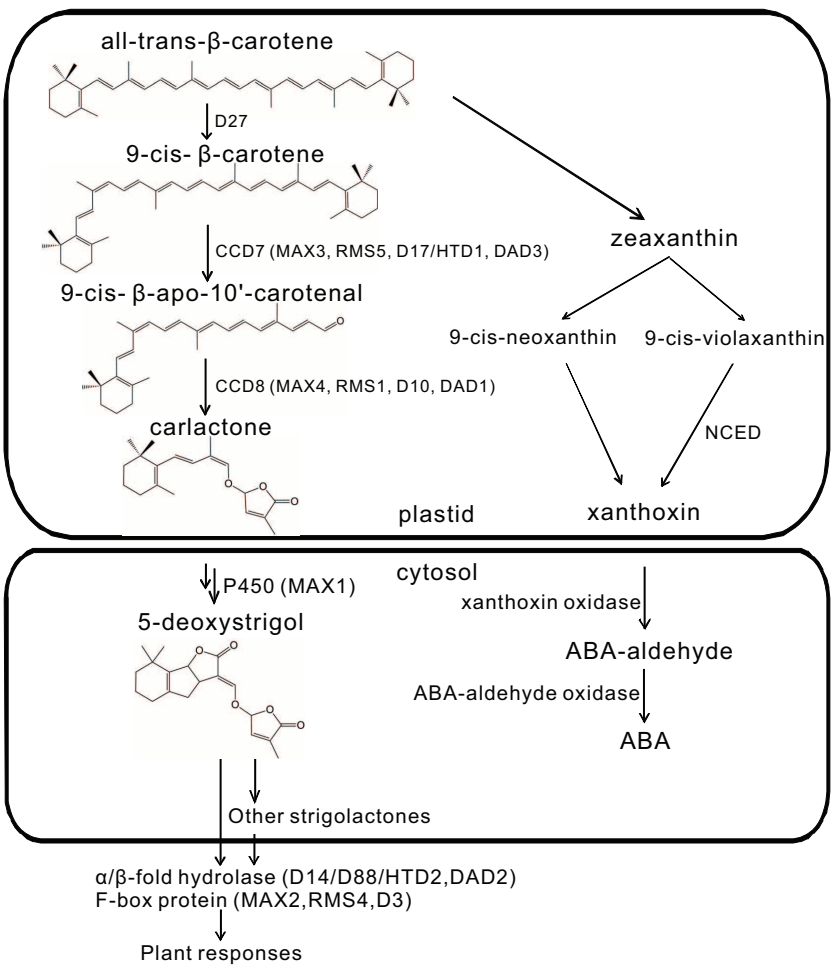

FIGURE 1 | Strigolactone and ABA biosynthetic pathways share a common origin at β-carotene. Adapted and modified from Ruyter-Spira et al. (2013).

and Snowden, 2012; Ruyter-Spira et al., 2013; Zwanenburg and Pospisil, 2013).

INTERACTIONS BETWEEN AUXIN, SL, AND CYTOKININ IN THE CONTROL OF BUD OUTGROWTH

Auxin plays a crucial role in the regulation of bud outgrowth. Auxin is produced mostly in the shoot apex and young leaves (Ljung et al., 2001) and is transported basipetally toward the root apex in the stem through the polar auxin transport (PAT) stream (Petrasek and Friml, 2009) (**Figures 2A–D**). The PINFORMED (PIN) proteins, a family of plasma membrane auxin efflux carriers, determine the direction of this PAT stream. The PINs export auxin out of the cell across the cell membrane into the apoplast from where it is taken up by the next cell after which the whole process is repeated (Galweiler et al., 1998; Wisniewska et al., 2006).

Based on the pioneering work of Sachs (1968), one hypothesis concerning the regulation of bud outgrowth (canalization-based model) proposes that an initial auxin flux from an auxin source (shoot apex or buds) to an auxin sink (root) is gradually canalized into cell files with a large amount of PINs. These cell files will subsequently differentiate into vascular tissue through which auxin will be transported (Sachs, 1981; Domagalska and Leyser, 2011). Auxin export from buds is correlated with the initiation of bud outgrowth and therefore it is believed that buds need to export auxin in order to be activated [reviewed by Muller and Leyser (2011)]. In this model, all buds compete for the release of their auxin into the common main PAT stream in the stem. Auxin exported from active buds (auxin source) reduces the auxin sink strength of the PAT stream in the stem and inhibits other buds from auxin export into the PAT stream (Sachs, 1981; Domagalska and Leyser, 2011). In pea, it was indeed observed that active axillary buds of decapitated stems rapidly triggered PIN1 polarization thus enabling directional auxin export from the buds (Balla et al., 2011). Auxin application on the apex of the decapitated stem inhibited this PIN polarization and also prevented the canalization of laterally applied auxin (simulated as the secondary auxin source) (Balla et al., 2011).

SLs can inhibit shoot branching via its regulation on auxin transport. In Arabidopsis, *max* mutants (*max1*, *max2*, *max3*, *max4*) shown increased transcript levels of the *PIN1/3/4/6* genes and an increased auxin transport capacity in the primary stem when compared to wild type plants (Bennett et al., 2006). Treatment with N-1-naphthylphtalamic acid (NPA), an auxin transport inhibitor, led to a remarkable inhibition of bud outgrowth in *max* mutants in Arabidopsis and *dwarf* mutants in rice (Ishikawa et al., 2005; Bennett et al., 2006; Arite et al., 2007; Lin et al., 2009). Basal application of the synthetic SL GR24 reduced basipetal auxin transport and PIN1 accumulation in the plasma membrane of xylem parenchyma cells in wild type and biosynthetic *max* mutants but not in *max2* (Crawford et al., 2010). These results suggest that SLs dampen the PAT stream in a MAX2-dependent manner (Crawford et al., 2010).

To understand how SLs regulate auxin transport, Leyser's group performed a computer modeling study, in which different processes affecting PAT were simulated. The results from this study suggested that SLs may modulate PIN cycling between the plasma membrane and endosomes (Prusinkiewicz et al., 2009). More recent computer modeling work provided additional support for the canalization-based model for shoot branching control (Shinohara et al., 2013). In this study, the relationship between PIN1 accumulation, auxin transport and shoot branching was explored in three Arabidopsis mutants that show excessive shoot branching: *max2*, *gnom* (*gn*), and *transport inhibitor resistant3* (*tir3*) (Shinohara et al., 2013). Although all three mutants are highly branched, *max2* plants show high PIN:PIN1-GFP levels at the basal plasma membrane of stem parenchyma cells, accompanied by a high PAT capacity, while *tir3* and *gn* mutants show the opposite due to low PIN1 insertion rates at their plasma membranes (Shinohara et al., 2013). SL action was simulated to increase the PIN1 removal rate from the plasma membrane in these three excessive shoot branching mutants (Shinohara et al., 2013). Interestingly, the model predicted that, different concentrations of GR24 treatment can either inhibit or stimulate shoot branching, depending on the auxin transport status and concentration of the treated plant (Shinohara et al., 2013). This was confirmed to occur in *tir3*, in which a low concentration of GR24 promoted shoot branching (10 nM) while a higher GR24 concentration (0.1–1 μM) reduced branching (Shinohara et al., 2013). An explanation for this (maybe unexpected) induced shoot branching resulting from GR24 application is that, assuming that SLs systemically remove PIN1 from plasma membranes, auxin transport capacity is also systemically reduced. A slight reduction in auxin transport in tissue through which auxin is exported from the buds, would still allow bud outgrowth. However, due to this slight decrease, more buds can simultaneously participate in this auxin export process, hereby increasing the number of shoot branches that grow out. The above observation perfectly fits within the canalization theory for the regulation of shoot branching. Finally, the presumed SL mediated reduction in PIN1 endocytosis, used in the computer model, was finally experimentally confirmed and was shown to occur through a clathrin-dependent mechanism (Shinohara et al., 2013).

Consistent with the idea that SLs do not need to directly exert their branching-inhibiting function in the buds, *MAX2* in Arabidopsis is expressed throughout the plant, and particularly high in the vasculature of developing tissues (Stirnberg et al., 2007). Similarly, the other component involved in SL signaling, the α/β-fold hydrolase *D14*, is also expressed in vasculature tissues, especially in xylem parenchyma cells in leaves and stems in close vicinity to axillary buds (Arite et al., 2009). Taken together, depending on auxin transport status, SLs systemically regulate competition between buds to release their auxin into the stem, finally determining how many buds can be activated (Prusinkiewicz et al., 2009; Crawford et al., 2010; Shinohara et al., 2013).

An argument against the above described model is the fact that in Arabidopsis and pea, both wild type and SL biosynthetic mutants rapidly transport additional exogenously applied auxin, suggesting that their auxin transport capacity is not saturated (Brewer et al., 2009). In addition to this, another simulation study recently shown that the increase in auxin transport capacity in the main stem as a result of decapitation occurs too

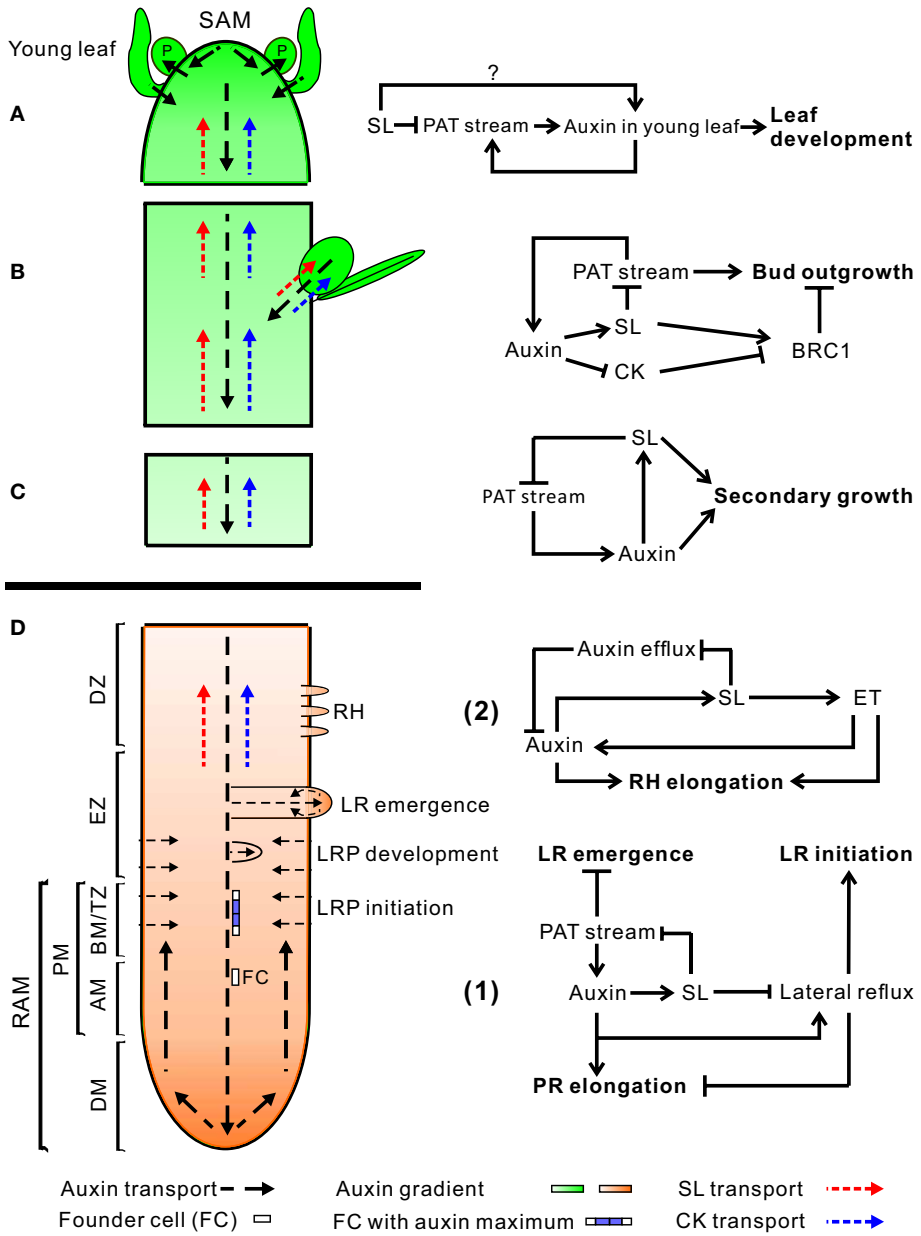

FIGURE 2 | An overview of auxin, SL, and CK transport within the plant (left) and hormone interactions during the regulation of shoot and root development (right). Auxin, strigolactone (SL), and cytokinin (CK) transport are represented by black, red, and blue dotted line, respectively. For hormone interactions (right), arrows represent promotion, while flat-ended lines indicate inhibition. **(A)** Auxin, produced in the shoot apical meristem (SAM) and young leaves, is transported basipetally through the stem in the polar auxin transport (PAT) stream toward the root apical meristem (RAM). Here, but probably also throughout the entire vasculature of the plant, it positively regulates SL biosynthesis (Hayward et al., 2009). As shown by GR24 feeding experiments, SLs transported through the xylem from the root to the shoot down-regulate the free auxin level in young leafs in a MAX2-dependent manner hereby controlling their development (Ruyter-Spira et al., 2011). SLs in the vasculature negatively affect PAT capacity (Crawford et al., 2010), as observed for NPA (Ljung et al., 2001), which negatively feeds back on auxin levels at the sites of biosynthesis. This long distance SL-auxin feedback mechanism, affects plant developmental processes as described below. **(B)** During the regulation of bud outgrowth, SLs reduce the capacity of the PAT stream in the main stem, leading to enhanced competition between buds to release their auxin into the stem (Crawford et al., 2010; Shinohara et al., 2013). On the other hand, SLs and CK are transported acropetally through the xylem and act directly in the buds to control their outgrowth through the joint regulation of TCP transcription factor BRC1 (Braun et al., 2012; Dun et al., 2012). **(C)** SLs have a direct positive effect on secondary growth by activating cell division in the vascular cambium in which they act downstream of auxin.

(Continued)

FIGURE 2 | Continued
The fact that the *max1* mutant still displays some residual cambium activity might point to a SL independent response to auxin. However, this remaining activity could also be due to residual SLs in these mutants (Agusti et al., 2011). **(D)** Hormone interactions during primary root (PR) elongation, lateral root (LR) initiation and development (1) and root hair (RH) elongation (2). (1) Auxin imported from the main PAT stream into the root stimulates SL production. SL export into the xylem and down regulation of the PAT stream feedback on auxin levels in the shoot as described under **(A)**. SL biosynthesis genes are specifically expressed in vascular tissue and the cortex of the proximal meristem of the root, through which the lateral auxin reflux toward the main PAT stream takes place. Therefore it is likely that locally synthesized SLs are controlling the efficiency of this reflux. Primary root elongation and lateral root initiation are determined by the auxin gradient inside the root tip, which is determined by auxin levels imported through the PAT stream, auxin synthesized in the root tip, and local auxin transport, including the auxin lateral reflux. Lateral root development and emergence are controlled by auxin derived from the shoot for which the SL controlled PAT stream capacity and lateral auxin influx into the developing lateral root primordia (LRP) are the main determinants. Although in the flow diagram auxin is depicted as a positive regulator of root growth, auxin displays a dose-response curve with an optimum, such that supra-optimal auxin concentrations will have a negative effect (Ruyter-Spira et al., 2011). (2) The effect of SLs on RH elongation is dependent on both auxin and ethylene (ET) biosynthesis and signaling. It has been suggested that SLs negatively regulate auxin efflux (Koltai et al., 2010). If this would specifically occur in RH cells this would result in increased local auxin levels which stimulates RH elongation. This local action of SLs has not been proven yet. Alternatively, it may be that SLs affect auxin transport in the PAT stream and/or the root tip hereby indirectly affecting the auxin concentration in RH cells. ET acts downstream of SLs and has a direct effect on RH elongation but also interacts with the auxin pathway (Kapulnik et al., 2011b). Abbreviations: P, primordium; DM, distal meristem; PM, proximal meristem; AM, apical meristem; BM, basal meristem; TZ, transition zone; EZ, elongation zone; DZ, differentiation zone; FC, founder cell.

slow to explain the increased bud outgrowth (Renton et al., 2012). Rather, this simulation study suggested that if auxin canalization accounts for bud outgrowth, enhanced auxin levels in the bud itself may be the main driving force (Renton et al., 2012).

SLs as well as CKs are considered acropetally mobile signals that can enter the buds and directly regulate bud activity (second-messenger model) (**Figure 2B**). Controversial to the canalization-based model, this model emphasizes the local action of SLs. Expression patterns of SL biosynthetic genes reveal that SLs are likely synthesized in the vascular tissue of both roots and shoots. Root-derived SLs can be transported acropetally through the xylem sap stream (Kohlen et al., 2011). This is in accordance with grafting studies which already shown that branching-inhibitors can move from the roots to the shoot since the bushy phenotype of SL biosynthesis mutants can be rescued by grafting mutant shoots on wild type roots (Morris et al., 2001; Turnbull et al., 2002; Simons et al., 2007). However, grafting of wild type shoots on SL deficient mutant roots shown that this SL transport is not a prerequisite for branching inhibition, emphasizing the importance of local SL production in the stem. Besides, auxin upregulates the transcription of SL biosynthetic genes such as *CCD7* and *CCD8*, whereas decapitation results in decreased expression of these genes (Sorefan et al., 2003; Johnson et al., 2006; Arite et al., 2007; Brewer et al., 2009; Liang et al., 2010). According to Dun et al. (2013), the GR24 signal was profoundly perceived in the axillary buds rather than adjacent leaves in pea, supporting the direct local inhibitory effect of SLs in axillary buds. They also shown that the inhibitory effect of GR24 was not permanent, which is consistent with SLs' transient signaling role in mediating rapid plant developmental responses (Dun et al., 2013). The recently discovered SL transporter gene, petunia *PLEIOTROPIC DRUG RESISTANCE 1* (*PhPDR1)*, is particularly expressed in the vasculature and nodal tissues near the axillary buds (Kretzschmar et al., 2012), consistent with the fact that cellular transport of SLs is likely needed in this specific region. Indeed, shoot branching in the Petunia *pdr1* mutant is increased compared with the wild type, however not to the extent observed for SL biosynthetic mutants (Kretzschmar et al., 2012). This may point to a SL export-independent bud outgrowth inhibitory process. Considering the co-localization of the expression of *PIN1* and SL biosynthetic genes in vascular parenchyma cells, this SL export-independent process is potentially represented by the SL-mediated inhibition of the PAT capacity. Similar to SL, CKs are mostly synthesized in the roots, albeit with some biosynthesis also occurring in the shoot, and are also transported acropetally through the xylem (Chen et al., 1985; Nordstrom et al., 2004; Tanaka et al., 2006). In contrast to SLs, however, CKs promote bud outgrowth directly and auxin inhibits CK biosynthesis by suppressing the CK biosynthetic gene *IPT* (*ADENOSINE PHOSPHATE-ISOPENTENYL TRANSFERASE*) (Tanaka et al., 2006). Accordingly, decapitation or application of an auxin transport inhibitor led to enhanced expression of CK biosynthetic genes in nodal stem and increased CK levels in pea (Tanaka et al., 2006).

Consistent with the second-messenger model, SLs and CK, mediated by auxin, act antagonistically and locally in the buds to control bud outgrowth (Brewer et al., 2009; Ferguson and Beveridge, 2009; Dun et al., 2012). Based on decapitation and girdling experiments, it was hypothesized that growing axillary branches/buds affect auxin sink strength and also bud responsiveness to SLs (Ferguson and Beveridge, 2009). Auxin levels in the stem negatively regulate bud outgrowth by maintaining local high SL and low CK levels (Ferguson and Beveridge, 2009). Once buds are activated, auxin is exported into the stem to allow vasculature development (Ferguson and Beveridge, 2009). Recent research suggests that both SLs and CK can interact directly in buds to control bud outgrowth, converging at a common target in the bud, possibly a TCP transcription factor, BRANCHED1 (BRC1) (Dun et al., 2012). In eudicots such as Arabidopsis and pea, BRC1 has been suggested to be expressed in axillary buds and act downstream of SLs signaling during shoot branching inhibition (Aguilar-Martinez et al., 2007; Braun et al., 2012; Dun et al., 2012). The expression of the pea *PsBRC1* mostly occurred in the axillary buds and was up-regulated by application of GR24 and down-regulated by CK treatment (Braun et al., 2012; Dun et al., 2012). However, overexpression of *BRC1* ortholog *FC1* (*FINE CULM 1*) in rice could only partially rescue the tillering phenotype of the SL signaling mutant *d3* (Minakuchi et al., 2010). GR24 treatment did not significantly affect the expression

of *FC1* whereas CK treatment did down-regulate its expression (Minakuchi et al., 2010). In maize, it seems that *BRC1* ortholog *TB1* (*TEOSINTE BRANCHED 1*) has evolved independent from SL signaling which may be explained by the fact that maize domestication is associated with a gain-of-function mutation in the *TB1* gene (Guan et al., 2012). Further research is still needed to clarify the regulatory mechanisms of the *BRC1* gene family and to find out whether additional factors in the axillary bud are involved in the regulation of bud outgrowth. Recent findings have shed some light on how other factors interact with FC1 in rice, targeting D14 to control shoot branching (Guo et al., 2013). Their results shown that OsMADS57, which is one of the transcription factors from the MADS-domain family, directly suppressed D14 transcription to control rice tillering, while FC1 could disturb this inhibitory effect of OsMADS57 on D14 by binding to the OsMADS57 (Guo et al., 2013).

Although second-messenger and canalization-based models look controversial, they can also be compatible since both local and systemic action of SL signaling are needed for adaptive plant responses. **Figure 2** presents an overview of auxin, SLs and CK transport within the plant (left) and interactions between these hormones during the regulation of shoot and root development (right).

STRIGOLACTONE INTERPLAY WITH OTHER HORMONES IN REGULATING ROOT DEVELOPMENT

Plant root system displays a large plasticity which is required to guarantee resource acquisition in response to changing environments. Most dicot species have a typical allorhizic root system with a primary (tap) root (PR) and several orders of lateral roots (LR) (Osmont et al., 2007). Adventitious roots (AR) are initiated from non-root tissues such as the hypocotyl or stem. Most monocot species are characterized by a secondary homorhizic root system including the embryonic PR, post-embryonic shoot-borne crown roots, and LRs (Osmont et al., 2007). On a micro scale, the root system architecture also includes root hairs (RH) that expand the root surface area and hence the capacity of plants to withdraw nutrients and water from the soil (Gilroy and Jones, 2000).

PRIMARY ROOT DEVELOPMENT

PR growth is mainly determined by the activity of the root apical meristem (RAM). This is a complex region of the root tip including a stem cell niche (SCN), a proximal meristem (PM), and a distal meristem (DM) (**Figure 2D**). Cell division, elongation, and differentiation in the RAM are tightly controlled by plant hormones. In this process, auxin is the main player. Different levels of cellular auxin have a different effect on gene expression, which determines cell fate. In roots, high auxin levels tend to stimulate cell division whereas lower levels favor cell expansion (Doerner, 2008). Auxin is mostly synthesized in the young leaves at the shoot apex (Ljung et al., 2001) and directionally transported through the vascular cambium of the shoot toward the RAM (Blilou et al., 2005; Petrasek and Friml, 2009). In roots, auxin is particularly accumulated in the quiescent center (QC), the columella initials and lateral root cap where auxin maxima are formed (Blilou et al., 2005; Petersson et al., 2009; Petrasek and Friml, 2009; Brunoud et al., 2012). Besides the auxin that is imported from the shoot, local auxin biosynthesis in the root also contributes to auxin homeostasis in the root tip (Chen and Xiong, 2009; Petersson et al., 2009). A major determinant of root growth is the auxin concentration gradient which is formed along the longitudinal axis of the root meristem. This concentration gradient is established due to the directional action of auxin transporters including auxin influx carriers such as AUXIN RESISTANT1(AUX1) and LIKE-AUX1 family and efflux carriers such as PINs and ATP-BINDING CASSETTE (ABC) transporters (Blilou et al., 2005; Kleine-Vehn et al., 2006; Grieneisen et al., 2007; Zazimalova et al., 2010). The directionality of the auxin flux is determined by the polar subcellular localization of these auxin efflux proteins (Sauer et al., 2006; Wisniewska et al., 2006; Petrasek and Friml, 2009). In the primary root, basally localized PIN1, PIN3, and PIN7 in the stele facilitate the acropetal auxin transport toward the root apex (Petrasek and Friml, 2009) (**Figure 2D**). In the columella, PIN3 and PIN7 redirect the auxin flow laterally toward the epidermis and the lateral root cap. PIN2 then facilitates the auxin flow from there upwards to the elongation zone (Petrasek and Friml, 2009). In addition, PIN2 in the cortex is also functional and fine-tunes both the rootward and shootward auxin flux, thus helps maintain auxin maxima at the root tip (Rahman et al., 2010). Finally, in the elongation zone, auxin is transported back into the main PAT stream through a lateral auxin reflux in the endodermis/cortex [as reviewed in Petrasek and Friml (2009)] (**Figure 2D**).

SLs are suggested to modulate the auxin gradient in the PR tip. The PR length of SL biosynthesis mutants (*max1*, *max3*, and *max4*) and SL signaling mutant (*max2*) is shorter than in wild-type plants (Ruyter-Spira et al., 2011). Application of GR24 (2.5 μM) rescued the short root phenotype of SL-deficient mutants but not of SL-insensitive mutant *max2* (Ruyter-Spira et al., 2011). The increased PR length was associated with an expansion of the meristem and transition zone sizes, through a higher number of smaller cells in both zones (Ruyter-Spira et al., 2011). Previously, modeling in which a reduction of the lateral auxin reflux was simulated shown a similar cellular patterning in the primary root tip (Grieneisen et al., 2007). This suggests that SLs may reduce the efficiency of the auxin lateral reflux into the main PAT stream which would affect auxin levels in both meristem and transition zones (Ruyter-Spira et al., 2011). Also consistent with these results, it has been demonstrated that expression of MAX2 under endodermis-specific SCARECROW (SCR) promoter in *max2* led to a wild-type level concerning meristem cell number, LR density, and RH elongation (Koren et al., 2013). Since PIN3-mediated auxin transport through the endodermis plays an important role in LR initiation (Marhavy et al., 2013), SLs' effects on PR growth and LR formation may indeed act through mediating auxin flux in the root tip (Koren et al., 2013). Interestingly, there was also evidence showing that SHORT HYPOCOTYL 2 (SHY2), which is the central mediator between auxin-CK antagonistic interaction in balancing cell differentiation with cell division in the meristem (Dello Ioio et al., 2008; Perilli et al., 2012), may be involved in endodermal SL signaling to regulate meristem size (Koren et al., 2013). Thus, SHY2 seems the converging point for auxin, CK as well

as SLs. SLs may regulate PIN-based auxin flux via MAX2 and/or SHY2 (Koren et al., 2013); however, it is still not clear how SLs regulate SHY2. Besides, both *max2* and *shy2-31* mutants shown reduced sensitivity to CK treatment, suggesting that MAX2 and SHY2 participate in CK signaling in the root (Koren et al., 2013).

It has been suggested that the regulatory role of SLs in PR growth is mediated through their inhibitory effect on auxin-efflux carriers (Koltai et al., 2010; Ruyter-Spira et al., 2011; Koren et al., 2013). As mentioned in the previous part, SLs signaling has recently been found to rapidly trigger PIN1 depletion from plasma membrane of xylem parenchyma cells. However, compared to the shoot, the effect of SLs on PIN1 depletion in root is less drastic and less specific. No obvious short-term effect of GR24 on PIN1 accumulation was observed in the root tip even within 2 d (Shinohara et al., 2013). Only in the longer term (6 d), the inhibitory effect by GR24 treatment could be detected in the provascular region (Ruyter-Spira et al., 2011). This could be explained by SLs' feedback inhibition on auxin biosynthesis in young leaves and auxin transport capacity in the stem, which would lead to reduced auxin supply to the root (Ruyter-Spira et al., 2011). However, if the short term inhibitory effects of SLs on PINs are only expected to specifically occur in the endodermis cells of the transition zone (TZ), visualization of this process is technically challenging.

LATERAL ROOT INITIATION AND DEVELOPMENT

LR originates from a few auxin primed pericycle founder cells (FCs) located opposite of the xylem poles in the basal meristem (BM) of the parental root (Peret et al., 2009) (**Figure 2D**). LR formation is subsequently initiated through a series of anticlinal and periclinal cell divisions—controlled by auxin—in the primed FC. This process is promoted by the auxin reflux in the TZ (Casimiro et al., 2001; De Smet et al., 2007; Dubrovsky et al., 2008; Marhavy et al., 2013). Particularly, PIN3, which is transiently induced in the endodermis during early stages of LR initiation, enables proper auxin gradient for transition from FC to LR initiation (Marhavy et al., 2013). LR initiation is followed by tightly regulated cell divisions leading to subsequent LR primordial (LRP) development and finally LR emergence (Peret et al., 2009; De Smet, 2012) (**Figure 2D**). As LRP develop, auxin efflux carriers promote the accumulation of auxin in the tips of the multilayered LRP. The formation of a proper auxin maximum is a crucial event during LR development (Petrasek and Friml, 2009) (**Figure 2D**). The accumulated auxin in developing LR tips also serves as a local signal to remodel adjacent cells by inducing the expression of auxin influx carrier LAX3 (LIKE AUX1 3) in cortical and epidermal cells, which leads to cell separation in LRP overlaying tissues, thus enabling LR emergence (Swarup et al., 2008).

While LR initiation is dependent on auxin which is circling inside the root tip (and is derived from both the shoot and the root) (Reed et al., 1998; Casimiro et al., 2001; Marchant et al., 2002; Wu et al., 2007), subsequent LR development is solely sustained by shoot derived auxin transported to the parent root and into the LRP through the PAT stream (Casimiro et al., 2001; Bhalerao et al., 2002; Chhun et al., 2007; Wu et al., 2007). Inherent to these different auxin sources, the regulatory mechanisms controlling LR initiation and subsequent development are also different; however in both cases the control of PINs plays an important role.

SLs act as regulators for LR initiation and LRP development (**Figure 2D**). SL-deficient (*max3* and *max4*) and SL-insensitive (*max2*) mutants shown increased density of LRs compared with wild type (Kapulnik et al., 2011a). Treatment of Arabidopsis seedlings with increasing concentrations of GR24 shown that LR density is reduced when 2.5 μM GR24 is applied, however LR initiation is only reduced with 5 μM GR24 (Ruyter-Spira et al., 2011). Therefore it was concluded that the reduction in LR density observed with 2.5 μM GR24 results from a delay in LR development (Ruyter-Spira et al., 2011). Indeed, a LR developmental study shown a specific accumulation of LR stage V primordia according to the LR developmental scale of Malamy and Benfey (1997). The arrested primordia displayed reduced levels of auxin reporter DR5-GUS and pPIN1-PIN1-GFP, suggesting that reduced auxin levels inside LRP are responsible for their delayed development or arrest (Ruyter-Spira et al., 2011). Auxin is provided to the developing primordia by a PIN1-dependent auxin influx from the PAT stream in the stem into the LRP interior toward the LR cap. It has been shown that GR24 application to the roots of Arabidopsis reduced auxin levels in young leaves (Ruyter-Spira et al., 2011). Possibly, the SL-mediated reduction in auxin transport in the PAT stream temporarily increases auxin levels in vascular tissue throughout the plant, which negatively feeds back on auxin production in young leaves (or positively on auxin degradation), similar to what has been observed upon application of the auxin transport inhibitor NPA (Ljung et al., 2001). The role of SL signaling in lateral root development may also involve *SHY2* (Koren et al., 2013), which has been suggested to suppress LR initiation but promotes LR development by mediating PIN activity and auxin homeostasis (Goh et al., 2012). Endodermis-specific expression of *SCR:MAX2* in *max2* background restored LR density to a wild-type level. As PIN3-dependent auxin reflux between endodermis and pericycle has a critical function in LR initiation (Marhavy et al., 2013), the fact that MAX2-mediated endodermal SL signaling is sufficient to confer sensitivity to LR formation implies that SL signaling may regulate LR formation via modulating auxin flux in the elongation zone (Koren et al., 2013).

Hence the mechanism underlying the GR24 mediated reduction of LR initiation is likely similar to the one described above for PR growth, i.e., a reduction in auxin reflux through the transition zone. In addition, the above described reduction in shoot derived auxin likely also contributes to the reduction in both PR growth and LR initiation (**Figure 2D**).

ROOT HAIR ELONGATION

RHs are tip-growing, tube-like outgrowths that help to anchor roots in the soil and assist in the uptake of nutrients and water (Gilroy and Jones, 2000). In the differentiation zone (DZ) of the root, RH emerge at the base of the epidermis cells. RH development can be divided into two stages: determination of hair/non-hair cells and hair morphogenesis (Lee and Cho, 2008). A cell in contact with two cortex cells will develop into a hair cell.

RH initiation has been suggested to be directly mediated by optimal auxin levels and signaling, whereas ET's effect is indirect and likely to act through regulating intracellular auxin levels (Muday et al., 2012). RH elongation requires an optimal intracellular auxin level which is regulated by auxin efflux and influx carriers. Auxin efflux PIN2 facilitates auxin supply through basipetal auxin transport from the root apex to the RH differentiation zone (Cho et al., 2007). PIN2 in the cortex has recently been shown to fine-tune both the rootward and shootward auxin flux (Rahman et al., 2010). Modeling of the auxin flow suggests that auxin influx carrier AUX1-dependent transport through non-hair cells can maintain auxin supply for developing hair cells and sustain RH outgrowth (Jones et al., 2009). ET also plays a positive role in regulating RH elongation (Tanimoto et al., 1995; Rahman et al., 2002). Both the Arabidopsis *ein2* (*ethylene insensitive 2*) mutant and ET-resistant mutant *aux1* exhibited decreased RH length (Rahman et al., 2002). Application of a low concentration of 1-naphthaleneacetic acid (NAA) (10 nM) could restore RH length of ET-resistant mutant *aux1* (Rahman et al., 2002). However, a much higher level of NAA (100 nM) was needed to recover RH length of *ein2* to the wild-type level, suggesting that the loss of ET signaling makes roots less sensitive to auxin (Rahman et al., 2002). SLs interact with auxin and ET in regulating RH elongation (**Figure 2D**). In tomato, a high dose of exogenous GR24 (27 μM) resulted in shorter and fewer RH than in the control (Koltai et al., 2010). The authors suggested that the effect of SLs is mediated via an effect on auxin efflux carriers (Koltai et al., 2010). In *Arabidopsis*, treatment with a low dose of GR24 increases the RH length in WT and in *max3* and *max4* mutants but not in *max2*, indicating the positive regulatory role of SLs in RH elongation, mediated via the MAX2 protein (Kapulnik et al., 2011b). Concerning RH elongation, SL signaling mutant *max2* has a similar sensitivity to ET precursor ACC as wild type, whereas ET signaling mutants *ein2-1* and *etr1-1*(*ethylene resistant1-1*) show reduced sensitivity to GR24, suggesting that SL signaling is not necessary for the ET response but ET signaling is involved in the SL response (Kapulnik et al., 2011b). Furthermore, SL application stimulates expression of ET biosynthetic genes (Kapulnik et al., 2011b). Taking together, these results suggest that ET biosynthesis is necessary for SLs to have an effect on RH elongation and that ET acts downstream of SLs (**Figure 2D**). The relationship between SLs and auxin in RH formation was also explored by the same authors. RH elongation upon IAA application in *max2* was similar to that of wild type, suggesting that SL signaling is not necessary for the auxin response. In contrast, auxin perception mutant *tir1-1* exhibited a reduced response to GR24 compared with the wild type, implying that auxin perception is needed for the SL response (Kapulnik et al., 2011b). However, the reduced sensitivity of *tir1-1* to GR24 may also be due to its reduced response to ET since *tir1-1* also shows reduced sensitivity to ACC. Moreover, the double mutant *aux1-7ein2-1* (insensitive to auxin and ET) shows reduced sensitivity to GR24 compared with the wild type upon RH elongation. Therefore, the effect of SLs on RH elongation is dependent on both auxin and ET biosynthesis and signaling while ET signaling also directly interacts with the auxin pathway (Kapulnik et al., 2011b) (**Figure 2D**).

As mentioned above, RH initiation and elongation takes place in epidermis cells (Lee and Cho, 2008). Endodermal SL signaling, mediated by MAX2, is still sufficient to confer sensitivity for RH elongation, suggesting the effect of SLs on RH elongation is likely to occur in a non-cell-autonomous manner (Koren et al., 2013).

ADVENTITIOUS ROOT FORMATION

ARs are post-embryonic roots that arise from non-root tissues. They can be induced by direct organogenesis from differentiated cells or from callus formed upon mechanical damage such as a cutting (Li et al., 2009). The formation of ARs in tomato occurs in the lower part of the hypocotyl as well as from the shoot-root junction. IAA application enhances AR formation in tomato hypocotyls in a dose-dependent manner (Negi et al., 2010). In rice calli, overexpression of auxin biosynthetic gene *YUCCA1* (*YUC1*), results in increased numbers of ARs (crown roots) as well as active crown root formation in the elongated node of the stem, suggesting that increased auxin production promotes AR development from both callus and stem (Yamamoto et al., 2007). Interestingly, in the stem, *OsYUC1-GUS* is expressed in the parenchyma cells surrounding the vascular bundles, suggesting local auxin biosynthesis in the vasculature of the stem (Yamamoto et al., 2007). In addition, AR emergence and development in rice are significantly suppressed in *OsPIN1* RNAi lines (Xu et al., 2005), suggesting an essential role of PIN1-dependent PAT during the process of AR initiation and development. Since SLs have been found to trigger PIN1 depletion from xylem parenchyma cells in the stem (Shinohara et al., 2013), it is also plausible to predict their inhibitory effect on PAT and thus AR development.

Indeed, studies on Arabidopsis and pea (*Pisum sativum*) show that SLs negatively regulate AR formation (Rasmussen et al., 2012a,b). SL biosynthetic and signaling mutants of both species displayed increased number of AR compared with wild type. It was suggested that SLs suppress AR formation by inhibiting the very early divisions of FCs (Rasmussen et al., 2012b). When *MAX2* is expressed in *max2* under the control of a xylem-specific promoter *NST3* (*NAC SECONDARY WALL THICKENING PROMOTING FACTOR3*), the AR formation is restored to the wild type level. This is consistent with the fact that *MAX2* is expressed in vasculature tissues throughout the plant. The authors suggest that SL signaling in the xylem is sufficient to mediate the formation of pericycle-derived AR. Interestingly, etiolation is known to induce AR formation in hypocotyls and this process is stimulated in all *max* mutants. The expression of *MAX3* and *MAX4* in wild type hypocotyls is induced upon light exposure, suggesting that local SL biosynthesis is involved in the regulation of AR formation during the process of de-etiolation (Rasmussen et al., 2012b). SL treatment of Arabidopsis wild type and *max* biosynthesis mutants (but not the signaling mutant *max2)*, results in a reduction in AR number even in the presence of elevated auxin levels (such as in *35S: YUC1* plants). The auxin response mutant *auxin resistant 1 (axr1)* and the *axr1max1-4* double mutants hardly form ARs. Auxin application (although not all concentrations) increases the number of ARs in *max* mutants (Rasmussen et al., 2012b). These findings indicate that SLs can at least partially revert the positive effect of auxin on AR formation and *AXR1* functions upstream

of SLs in the early stages of AR initiation (Rasmussen et al., 2012b). The authors also investigated possible crosstalk between SLs and CK in regulating AR development as CK are known to suppress AR formation. CK responsiveness is not impaired in the SL mutants and CK mutants are also SL-responsive, indicating that SLs and CK act independently in AR formation (Rasmussen et al., 2012b).

SL AND AUXIN ACTION DURING SECONDARY GROWTH

Plant growth initiated by apical meristems leads to development of primary tissues such as epidermis, vascular bundles and leaves. In addition to primary growth, plants, especially tree species, also display secondary growth during which they expand their growth axes laterally. Secondary growth depends on the activity of the vascular cambium which originates from the procambium and parenchyma cells (Ye, 2002). The vascular cambium has the capacity to divide and form a continuous ring of meristem cells located between the primary xylem and the phloem in the vascular bundles (Ursache et al., 2013). The cylindrical layer of cambium undergoes cell division, resulting in new xylem on the inside and new phloem on the outside (Ye et al., 2002; Ursache et al., 2013). There is strong evidence that procambium patterning is regulated by PIN1-dependent PAT (Scarpella et al., 2004, 2006). Also secondary xylem differentiation was shown to be associated with reduced PAT. The Arabidopsis *interfascicular fiber* mutant (*ifl1*) displays reduced secondary growth (Zhong and Ye, 2001). The authors shown that reduced expression of auxin efflux carriers and the resulting reduced PAT along the inflorescence stems and hypocotyls in this mutant lead to a block of vascular cambium activity (Zhong and Ye, 2001).

SLs have recently been proven to positively regulate secondary growth (**Figure 2C**). SL biosynthetic and signaling mutants all displayed reduced cambium activity compared with wild type. Local application of GR24 stimulates cell division in the interfascicular cambium in wild type and all Arabidopsis SL biosynthetic *max* mutants and to a lesser extent in the *max2* signaling mutant (Agusti et al., 2011). Remarkably, the *max2* mutant is still slightly responsive to GR24 which is not consistent with its complete insensitivity in other processes such as shoot branching and root development. This suggests that there may also be other factors involved in the transduction of the SL signal in this particular physiological process (Agusti et al., 2011). In this study of Agusti et al. (2011), shoot branching is not affected by GR24 application showing that the effect of SLs on cambium development in inflorescence stems is mechanistically independent from the effect they have on shoot branching (Agusti et al., 2011). Interestingly, although the *max1* mutant displays reduced secondary growth, its auxin concentration, signaling and transport are enhanced. This suggests that the effect of SLs on secondary growth is direct and independent of auxin accumulation (Agusti et al., 2011). In addition to this, local NPA application, which reduces the initially enhanced auxin transport capacity observed in the *max* mutants, does not restore secondary growth, suggesting that SL biosynthesis and signaling are required for auxin to stimulate cambium activity. This conclusion is supported by the fact that GR24 application to the auxin insensitive *axr1-3* mutant results in a similar increase in cambial activity as observed for wild type and the *max* mutants. Collectively, these results suggest that SLs function downstream of auxin in the regulatory pathway of secondary growth in Arabidopsis (Agusti et al., 2011). However, the observed remaining cambium activity in *max1* cannot be ignored. It would suggest that either auxin also has a direct effect or that residual SLs are still present in the *max1* mutant background.

SL AND OTHER HORMONES DURING SEEDS GERMINATION

SLs have been identified as germination stimulants for seeds of parasitic plants *Orobanche spp*. and *Striga spp*. These parasitic plants seeds are usually dormant in soil and germinated only when they are close to host roots. Previous studies shown that ABA levels decrease during seeds pre-conditioning of *O. minor* (Chae et al., 2004). Still, seed dormancy release depends on an additional reduction of ABA levels which was recently shown to be mediated through ABA catabolism which is triggered by GR24 application (Lechat et al., 2012). Other hormones such as CK and ET can promote parasitic plant seeds germination in the absence of SLs (Logan and Stewart, 1991; Babiker et al., 1993, 1994; Sugimoto et al., 2003), suggesting that they may act downstream of SLs; whereas CK promotes germination by enhancing ET biosynthesis (Babiker et al., 1993). Furthermore, GA is necessary but not sufficient to trigger *Striga* seeds germination (Toh et al., 2012).

Currently, model plant Arabidopsis is also being used to explore hormone interactions, including SLs, during seed germination. Based on thermoinhibition experiments, a positive role of SLs in Arabidopsis seeds germination was revealed (Toh et al., 2012). Both SLs biosynthetic and signaling mutants shown enhanced sensitivity to high temperature which is a constraint for normal germination (Toh et al., 2012). GR24 could not only alleviate thermoinhibition by decreasing ABA levels and increasing GA levels, but also break secondary dormancy in Arabidopsis. Nice comparisons were made between hormone interactions occurring during the alleviation of thermoinhibition in parasitic and non-parasitic seeds germination (Toh et al., 2012). In both cases, SLs reduce the ABA:GA ratio, leading to enhanced germination activity. To trigger Striga seed germination, SLs also positively regulate CK which contributes to ET production (not proven for Arabidopsis yet) (Toh et al., 2012). However, as expected when considering the difference in germination behavior between parasitic plants and Arabidopsis, differences between hormone signaling networks were also reported. GA, for instance, is sufficient to counteract thermoinhibition in Arabidopsis seeds but is not sufficient to do so in parasitic plants seeds (Chae et al., 2004; Toh et al., 2012). Besides, parasitic plants seeds are very sensitive to SLs that are exuded from host plants, suggesting their evolutionary dependence on hormone interaction (Toh et al., 2012). Light signaling related topics concerning seeds germination will be discussed in the following The Response to Light section. Interestingly, a smoke-derived compound, karrikin, has similar effects on seed germination in a MAX2-dependent manner (Nelson et al., 2011). The *kai2* (karrikin insensitive 2) mutant seeds are insensitive to GR24. It was suggested that there is a butenolide-based signaling mechanism via KAI2 which is distinct from SL signaling, providing an adaptive response to smoke (Waters et al., 2012b).

HORMONE INTERACTIONS IN RESPONSE TO ENVIRONMENTAL STIMULI

Plants, unlike animals, are sessile organisms and hence require phenotypic plasticity, which is the ability of a certain genotype to produce different phenotypes in response to varying environmental conditions (Pfennig et al., 2010). Meristem development is of vital importance for the adaptation of plants to changes in the environment. Regulation of axillary meristem outgrowth, for example, is one of the major strategies that plants adopt to adjust their body plan, leading to changes in shoot branching. Another mechanism to modify the body plan is to alter secondary growth of stems and roots by regulating development of lateral meristem tissue, especially the vascular cambium (Agusti and Greb, 2013), allowing plants to regulate root and shoot thickness. Collectively, all plant meristems are closely coordinated to face environmental challenges during plant development. In the following paragraphs we will elaborate on how SLs and other plant hormones are involved in the regulation of two different environmentally regulated physiological processes, the response to light and the response to nutrient shortage.

THE RESPONSE TO LIGHT

Light is a highly variable environmental factor affecting plant growth and development. Changes in light quality and intensity affect multiple processes in plants, such as intensively studied shade avoidance syndrome (SAS). During this response, plants are able to detect a decrease in the R:FR and initiate morphological changes that help plants to compete with their neighbors (Franklin, 2008), such as elongation of internodes, hypocotyls, and petioles, reduced shoot branching and leaf development, inhibited root growth, early flowering, and reduced seed set in the long term (Ruberti et al., 2012). The stimulation of the elongation responses can be as rapid as a few minutes and the process is reversible. The photoreceptors responsible for the response to changes in light quality in the red and far-red regions are the phytochromes, including PhyA to PhyE in higher plants.

Light also affects the levels of plant hormones and in turn, plant hormones affect the photoreceptor signal transduction (Wang et al., 2013). Shade has been reported to induce a rapid increase in auxin levels, its PIN-based transport (i.e., PIN1 and PIN3) and auxin signaling, resulting in enhanced elongation growth (Tao et al., 2008; Keuskamp et al., 2010; Hornitschek et al., 2012). Notably, it has been shown that *PIN1* expression was regulated by the photomorphogenesis repressor COP1 (CONSTITUTIVE PHOTOMORPHOGENIC 1), which is suppressed by light-activated PHYB. COP1 not only controlled the transcription of *PIN1* and the capacity of the PAT stream in the hypocotyls but also affected PIN1 and PIN2 intracellular distribution in the root tip thus affecting root elongation. This suggests that COP1 efficiently coordinates both root and shoot growth under changing light conditions (Sassi et al., 2012).

SLs were shown to be essential components of the low R:FR mediated reduction of bud outgrowth. In Arabidopsis it was shown that both *BRC1* and the SL biosynthetic and downstream signaling genes *MAX4* and *MAX2* were needed to suppress branching during low R:FR conditions (Finlayson et al., 2010). In addition to this, functional *AXR1*, was also essential for the control of shoot branching under low R:FR conditions, confirming that auxin signaling is important during shade avoidance reactions (Tao et al., 2008) and is probably needed to induce SL biosynthesis. Indeed, auxin was shown to induce SL biosynthetic gene expression under normal light condition (Hayward et al., 2009). It's very likely that it's the similar case under shade: auxin levels and PAT stream are promoted under shade, which may enhance SL biosynthesis, leading to reduced bud outgrowth.

A low R:FR and/or inactive PHYB also induce an elongation response in branches. Interestingly, the Arabidopsis *max2* mutation inhibited the elongation response of rosette branches in the presence of the *phyB* mutation, while *axr1-12* and *max4* maintained the elongation response of branches in the *phyB* mutant (Finlayson et al., 2010). Also for other light regulated plant growth characteristics, such as decreased hypocotyl growth and de-etiolation, MAX2 dependency has been observed while the SL biosynthetic mutants did not display the corresponding photomorphogenic phenotypes. For instance, while *max2* is hyposensitive to red, far-red, and blue light, leading to longer hypocotyls (Stirnberg et al., 2002; Shen et al., 2007; Nelson et al., 2011), this was not the case for *max1*, *max3*, and *max4* (Shen et al., 2012). Therefore, it was suggested that MAX2 regulates photomorphogenesis in a SL-independent manner, and may form complexes consisting of different ligands and/or substrates. In this respect it is intriguing that not only the response to SLs, but also to smoke derived compounds called karrikins, requires MAX2 (Nelson et al., 2011). An alternative explanation could be that the SL biosynthetic mutants tested in these studies are leaky, and still produce sufficient SLs to result in different phenotypes when compared to the signaling mutant. Based on altered expression patterns of GA and ABA biosynthesis and catabolic genes in Arabidopsis *max2* seeds, in combination with a *max2* specific germination phenotype, it was hypothesized that MAX2 would also affect photomorphogenesis by modulating hormonal levels in a non-SL dependent manner (Shen et al., 2012). However, again, it could be that the hormonal levels in the SL biosynthetic mutants are not enough reduced to result in a phenotype. It would therefore be interesting to include SL biosynthetic double or triple mutants in these experiments. A direct link between SLs and photomorphogenesis has been suggested (Tsuchiya et al., 2010). It was shown that SLs inhibit hypocotyl elongation in the dark. However, it must be noted that non-physiological levels of GR24 (50 μM) were applied. A mechanistic explanation for the MAX2/SL role in photomorphogenesis was provided with the discovery that GR24 (10 μM) mediates nuclear exclusion of COP1, which leads to the stabilization of HY5 (ELONGATED HYPOCOTYL 5) and reduced hypocotyl elongation (Tsuchiya et al., 2010). This led to the intriguing conclusion that SL application can mimic light under dark conditions (Tsuchiya et al., 2010). However, in contrast to above results (Tsuchiya et al., 2010), it was recently found that HY5 is not necessarily required for MAX2-dependent SL regulation of hypocotyl growth (Waters and Smith, 2013). It was proposed that HY5 and MAX2 act in separate signaling pathways during early light-mediated seedling

development and that they may subsequently interact, in later developmental stages, downstream of auxin and light signaling (Waters and Smith, 2013).

THE RESPONSE TO NUTRIENT DEPRIVATION

Nutrient deprivation is another important abiotic stress frequently encountered by plants. Phosphorus (P), for example, is one of the essential macronutrients required by plants but only the inorganic phosphate (Pi) is the phosphorus form which is accessible for plants. As roots are the main site for Pi acquisition, plant roots usually cope with Pi-limiting conditions by investing more energy into root growth, resulting in reduced shoot/root ratio (including inhibited shoot branching), inhibited PR elongation and enhanced LR and RH growth (Williamson et al., 2001; Linkohr et al., 2002; Niu et al., 2012). It has been shown that the root tip is involved in sensing low Pi (Svistoonoff et al., 2007).

In Arabidopsis, the *phosphorus starvation-insensitive* (*psi*) mutant, displaying reduced inhibition of PR growth and reduced LR and RH growth under Pi-limited conditions, shown less sensitivity to auxin and enhanced ability to sustain auxin response in the root tip than wild type plants under low Pi, suggesting that low Pi can increase the sensitivity of roots to auxin (Wang et al., 2010). The enhanced auxin sensitivity induced by Pi deprivation is conferred by an increased expression of *TIR1*, which accelerates the degradation of AUX/IAA proteins (Perez-Torres et al., 2008).

In addition to auxin, SLs are also important regulators of root architecture under Pi-limiting conditions. SL production in roots is promoted by Pi starvation (Yoneyama et al., 2007; Lopez-Raez et al., 2008; Jamil et al., 2011). Interestingly, while LR development in Arabidopsis SL biosynthetic and signaling mutants was increased during normal Pi conditions, LR outgrowth was decreased during Pi starvation (Ruyter-Spira et al., 2011). Similarly, in rice, crown root elongation in wild type was increased in Pi-deficient media while *d10* and *d14* mutant plants did not show such response (Arite et al., 2012). Particularly the results in Arabidopsis suggest that the increase in SL production under Pi-limited conditions is necessary for the expansion of the root system, allowing the plant to explore a larger area of the soil for nutrients. That this is due to an interaction with auxin is suggested by the results of an experiment in which GR24 was applied to Arabidopsis plants growing on medium also containing auxin (NAA) which resulted in a more rapid elongation of lateral roots than in the absence of GR24 (Ruyter-Spira et al., 2011). Moreover, GR24 application to plants grown with sufficient Pi caused a more severe reduction in lateral root number compared with plants grown under Pi starvation (Ruyter-Spira et al., 2011). Because Pi starvation increases auxin sensitivity (Perez-Torres et al., 2008; Koltai, 2012) and GR24 application was shown to decrease auxin levels in the leaves, it is likely that the final effect of GR24 (or SLs in general) in the low Pi response depends on the auxin status of the plant, as affected by the environment (Pi level) of the plant.

The effect of SL on Pi starvation-mediated changes in RH density also sheds light on the mechanism by which SL affect auxin signaling. Arabidopsis SL biosynthetic and signaling mutants shown a remarkably lower RH density, than wild type plants and only the response of the SL biosynthetic mutant *max4*, not that of *max2,* could be rescued by exogenous treatment with GR24 (Koltai, 2012; Mayzlish-Gati et al., 2012). These results could be explained by the absence of low Pi mediated induction of TIR1 in *max2* while TIR1 expression is induced in wild type plants. This would render SL mutant plants less sensitive to auxin during Pi starvation. Moreover, this SL-mediated RH response to low Pi was suggested to be independent or downstream of the ET signaling pathway, while only auxin, and not ET was able to restore the relatively low RH density in the *max2* mutant (Koltai, 2012; Mayzlish-Gati et al., 2012).

The expression of SL exporter *PDR1* is also induced by Pi deprivation. PDR1 is localized in the plasma membrane of subepidermal cells of roots, facilitating SL exudation into the rhizosphere and promotes the symbiotic interaction with AM fungi and hence Pi uptake by the plant (Kretzschmar et al., 2012). SL production in the root is relatively high. A part of this SL pool is transported upwards to the shoot. It has been shown in Arabidopsis and tomato that under low Pi, increased levels of SLs travel through the xylem (Kohlen et al., 2011). This systemic mode of action allows SLs to rapidly regulate aboveground architecture by altering PIN accumulation (Shinohara et al., 2013), thus facilitates nutrient re-allocation. However, under Pi deficiency, transcript levels of SL biosynthetic genes were also slightly increased in the shoot (Umehara et al., 2010), suggesting that local SL biosynthesis in the shoot also contributes to the branching inhibition observed during low Pi conditions. However, currently it is not known to what extent this local production is sufficient, and if it is, why SLs are transported to the shoot through the xylem. One explanation could be that long-distance transport of SLs provides a feedback mechanism for auxin levels (through production and/or degradation) in auxin producing tissues in the shoot, as was demonstrated to occur upon GR24 application in Arabidopsis seedlings (Ruyter-Spira et al., 2011). In conclusion, SLs play multiple roles in the response of plants to low Pi conditions. They not only improve Pi acquisition by improving AM fungi symbiosis but also act as long-distance signal to optimize shoot architecture in a nutrient-limited environment and regulate root architecture in such a way that Pi uptake can be improved.

In summary, plants have evolved multiple adaptive mechanisms to achieve phenotypic plasticity, not only by regulating whole plant architecture, but also by balancing nutrient allocation among different organs in response to changing environments. Plant hormones play a crucial role in these adaptive responses and their intricate interaction enables fine-tuned responses to many different changes in the environment.

PERSPECTIVE

Plants exhibit a high degree of plasticity, which is defined by their ability to adjust their development to changes in the environment. Hormone interactions can fine-tune the plant response and determine plant architecture when plants are challenged by environmental stimuli such as nutrient deprivation and canopy shade. One of the essential nutrients plants strongly respond to is phosphate. Modern agriculture is highly dependent on its application, and its finite resource is worrying and deserves immediate

attention. Future strategies need to focus on lower phosphate fertilizer application accompanied by improved phosphate use efficiency (PUE) by agricultural crops. Improved PUE is a highly desirable trait to which also root architecture contributes. Since SLs are involved in different plant developmental processes leading to plant architectural changes, including root architecture, more knowledge about their role, particularly under phosphate limiting conditions, is highly desirable. This includes the low phosphate mediated regulation of SL transport within the plant and the exudation to the rhizosphere as well as the local regulation of SL biosynthesis and transport in close vicinity to the buds.

SL crosstalk with other plant hormones is still a research area in its infancy, certainly at the cellular and genetic level. As we have pointed out in this review, a common target for many plant hormones is the regulation of auxin levels and gradients through their effect on PINs. The exact mechanism of how SLs do this however still needs to be resolved. Because different hormonal and environmental signals also interact with each other this is very complex. Computational modeling and simulations may facilitate the interpretation of complicated datasets, leading to predictions or the establishment of new models.

Finally, the intriguing structural diversity in SLs observed in plants and its relevance for differential regulation of various plant developmental processes is of great interest. Improved knowledge about SL perception and downstream signaling mechanisms will shed more light on the biological relevance of this structural diversity. The discovery of genetic variation and favorable alleles of genes involved in SL diversification and downstream signaling processes would be an interesting asset to future breeding programs as it will help to fine-tune SL action in such a way that maximum benefit is obtained in agriculture (improved PUE, better crop architecture, etc.), without negative side effects (germination of parasitic weeds).

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Cytokinin cross-talking during biotic and abiotic stress responses

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As sessile organisms, plants have to be able to adapt to a continuously changing environment. Plants that perceive some of these changes as stress signals activate signaling pathways to modulate their development and to enable them to survive. The complex responses to environmental cues are to a large extent mediated by plant hormones that together orchestrate the final plant response. The phytohormone cytokinin is involved in many plant developmental processes. Recently, it has been established that cytokinin plays an important role in stress responses, but does not act alone. Indeed, the hormonal control of plant development and stress adaptation is the outcome of a complex network of multiple synergistic and antagonistic interactions between various hormones. Here, we review the recent findings on the cytokinin function as part of this hormonal network. We focus on the importance of the crosstalk between cytokinin and other hormones, such as abscisic acid, jasmonate, salicylic acid, ethylene, and auxin in the modulation of plant development and stress adaptation. Finally, the impact of the current research in the biotechnological industry will be discussed.

Keywords: cytokinin, stress, hormonal crosstalk, salicylic acid, abscisic acid

INTRODUCTION

During their lifespan, plants are exposed to continuously changing environmental conditions and pathogen threats. Various abiotic and biotic stresses, such as heat, cold, drought, high salinity, or pathogen attacks, can severely affect plant development, growth, fertility, and productivity. To survive, plants must be able to react rapidly to various stress signals, activate efficient defense responses, and adapt to new conditions. Plant hormones are key components of these defense and adaptation mechanisms. To mediate the responses and adaptations to stresses, different hormonal pathways are upregulated or downregulated. Modifications in the hormonal abundance and signaling will usually impact on the degree of resistance or susceptibility to the various stresses.

HORMONES AND ABIOTIC STRESSES

Plants can perceive and respond to environmental changes. For instance, seasonal variations in day/night length or in temperature might directly affect the reproductive cycle, flowering, and fruit set. However, unpredicted changes, such as flooding, extreme temperature, heavy metals, drought, or high salt levels, will be perceived as stress conditions and might have a strongly negative impact on grain yield, grain weight, and plant biomass. Likewise, the root system architecture will adapt in terms of growth and branching as a reaction to different stresses. Among the various stress conditions, salinity and drought are currently the major problems. Saline soils represent a total of 323 million hectares worldwide (Brinkman, 1980), whereas drought affects 1–3% of the land surface and is predicted to increase to up to 30% by 2090 (Burke et al., 2006). To cope with these stresses, plants modify the levels of the different phytohormones directly or indirectly. This altered hormonal balance also affects the plant development, with a direct impact on seed development, seed germination, dormancy, and overall plant growth (Finkelstein et al., 2002).

ABSCISIC ACID –THE ABIOTIC STRESS HORMONE

In response to abiotic stresses, such as drought and salinity, endogenous abscisic acid (ABA) levels increase rapidly, activating specific signaling pathways and modifying gene expression levels (Seki et al., 2002; Rabbani et al., 2003; Kilian et al., 2007; Goda et al., 2008; Zeller et al., 2009). In fact, up to 10% of protein-encoding genes are transcriptionally regulated by ABA (Nemhauser et al., 2006).

Abscisic acid is one of the most studied phytohormone because of its rapid response and prominent role in plant adaptation to abiotic stresses. In the meantime, the key components of the ABA signaling pathway have been characterized (Sreenivasulu et al., 2007; Cutler et al., 2010; Hirayama and Shinozaki, 2010; Raghavendra et al., 2010; Debnath et al., 2011; Fujita et al., 2011). In *Arabidopsis thaliana*, the pyrabactin resistance1 (PYR1)/PYR1-LIKE (PYL)/regulatory components of ABA receptor (RCAR) proteins have been proposed as the main intracellular ABA receptors (Ma et al., 2009; Park et al., 2009; Santiago et al., 2009a; Nishimura et al., 2010). Multiple ABA receptor loss-of-function mutants, such as *pyr1/pyl1/pyl4, pyr1/pyl1/pyl2/pyl4,* and *pyr1/pyl1/pyl2/pyl4/pyl5/pyl8* are insensitive to ABA, even at concentrations as high as 100 μM (Park et al., 2009; Gonzalez-Guzman et al., 2012). Particularly, the quadruple and sextuple mutants were less sensitive to the ABA-mediated inhibition of seed germination, root growth, stomata closure, and expression of ABA responsive genes (Park et al., 2009; Nishimura et al., 2010; Gonzalez-Guzman

et al., 2012). Accordingly, *PYL5* overexpression resulted in high drought resistance and an enhanced response to ABA (Santiago et al., 2009b).

In the presence of ABA, the PYR/PYL/RCAR proteins form a ternary complex that via direct interaction inhibit clade A protein phosphatase 2C (PP2C), including ABA-INSENSITIVE 1 (ABI1), ABI2, and hypersensitive to ABA 1 (HAB1) (Nishimura et al., 2007; Santiago et al., 2009a; Szostkiewicz et al., 2010). Similarly to the receptor mutants, mutants in the PP2C activity, such as *abi1-1*, are also insensitive to ABA (Fujii and Zhu, 2009; Cutler et al., 2010). PP2C repression activates downstream targets, such as the protein kinases belonging to the sucrose non-fermenting 1-related subfamily2 SnRK2.2/D, SnRK2.3/I, and SnRK2.6/OST1/E, which trigger ABA-dependent gene expression and signaling (Umezawa et al., 2009; Vlad et al., 2009). Accordingly, the *snrk2.2*/*snrk2.3*/*snrk2.6* triple mutant is highly insensitive to ABA and severely affects plant growth and seed yield (Fujii and Zhu, 2009).

CYTOKININ IN ABIOTIC STRESS RESPONSES

Besides ABA, other hormonal pathways, including cytokinin (CK), are activated when a plant is exposed to stress. The CK-dependent modulation of stress responses has been studied at various levels. The alteration of endogenous CK levels in reaction to stress suggests that this hormone is involved in stress responses. For instance, in response to drought, the *in planta* concentration and transport of *trans*-zeatin riboside decreases drastically, whereas the ABA levels increase (Hansen and Dörffling, 2003; Davies et al., 2005). Interestingly, when the partial root zone-drying approach was applied, the CK concentration decreased, not only in roots, but also in leaves, buds, and shoot tips, along with increased ABA levels (Stoll et al., 2000; Kudoyarova et al., 2007). These observations demonstrate that the local stress exerted on the root might trigger changes in the CK levels in various plant organs, including the shoot, and, consequently, in developmental processes, such as the apical dominance (Hansen and Dörffling, 2003; Schachtman and Goodger, 2008). Typically, reduced CK levels would enhance the apical dominance, which, together with the ABA regulation of the stomatal aperture, aids to adapt to drought stress.

The negative CK-regulatory function in plants exposed to drought has been demonstrated in genetic studies in which the endogenous CK levels were modified, either by loss of the biosynthesis genes isopentyl transferase (IPT) or by overexpression of cytokinin oxidase (CKX)-encoding degradation genes (Werner et al., 2010; Nishiyama et al., 2011; Wang et al., 2011b). A reduced CK content in the *ipt1/ipt3/ipt5/ipt7* quadruple and *ipt8* single mutants or overexpression of *CKX1* and its homologs correlates with an increased resistance to both salt and drought stresses.

In agreement with the increased abiotic stress resistance at low CK levels, mutants lacking the functional CK receptors are more resistant to abiotic stresses (Tran et al., 2007; Jeon et al., 2010; Kang et al., 2012). For example, the *Arabidopsis histidine kinase* (*AHK*) loss-of-function mutants *ahk2/ahk3* and *ahk3/ahk4* were significantly more resistant to freezing temperatures than the wild type (Jeon et al., 2010). Similarly, all *ahk* single and multiple mutants, with the exception of *ahk4*, showed an enhanced resistance to dehydration (Kang et al., 2012). Furthermore, like the CK-metabolic mutants *ipt1/ipt3/ipt5/ipt7*, *ipt8,* and the *CKX1*-overexpressing plants, the *ahk* mutants affected dramatically the ABA sensitivity (Tran et al., 2007) and were hypersensitive to ABA treatments.

Downstream of the AHK receptors, the *Arabidopsis* histidine phosphotransfer (AHP) proteins mediate stress signaling (Hwang and Sheen, 2001; Hutchison et al., 2006; To and Kieber, 2008; Hwang et al., 2012). AHP proteins translocate into the nucleus and activate the type-B *Arabidopsis* response regulator (ARR) factors that trigger the transcription of specific genes in response to CK. A negative feedback loop is provided by type-A ARRs that inhibit the activity of type-B ARRs by a still unknown mechanism (**Figure 1**). Of all ARRs, type-A ARRs are the only ones of which the expression is altered under stress, e.g., *ARR5*, *ARR6*, *ARR7*, and *ARR15* are upregulated upon cold stress (Jeon et al., 2010; Jeon and Kim, 2013); *ARR5*, *ARR7*, *ARR15,* and type-C *ARR22* are upregulated in response to dehydration (Kang et al., 2012); and *ARR5* expression increases in response to salt stress (Mason et al., 2010). Stimulation of *ARR5*, *ARR6*, *ARR7*, and *ARR15* expression in response to cold stress requires the activity of several components of the CK signaling pathway, including AHP2, AHP3, and AHP5, and also ARR1 (Jeon and Kim, 2013). Likewise, in response to salt stress, *ARR5* upregulation depends on *ARR1* and *ARR12* (Mason et al., 2010). Furthermore, the negative regulatory role of AHP2, AHP3, and AHP5 during drought stress has been described recently (Nishiyama et al., 2013).

Despite the clear indications that CK and the CK signaling components function in stress responses (Hwang et al., 2012), the high degree of redundancy in the CK signaling pathway, including three CK receptors, six AHPs, 10 type-A ARRs, and 11 type-B ARRs, makes it difficult to dissect the role of each specific component (Hwang et al., 2012). Interestingly, although CK levels are reduced, the type-A ARRs that belong to the early CK-responsive genes are upregulated (Jeon et al., 2010; Mason et al., 2010; Kang et al., 2012; Jeon and Kim, 2013). Furthermore, a quadruple type-A ARR loss-of-function mutant *arr3/arr4/arr5/arr6* is resistant to salt stress, which is unexpected because to type-A ARRs act as CK signaling repressors (Mason et al., 2010). These observations imply that in stress responses the role played by the CK signaling pathway is more complex. In this context, AHKs might function as stress sensors that would activate the CK signaling pathway independently of CK levels (Urao et al., 1999; Tran et al., 2007; Jeon et al., 2010). In fact, another member of the histidine kinase family, AHK1, is able to sense and transduce changes in osmolarity to trigger downstream signaling pathways (Urao et al., 1999; Tran et al., 2007). However, unlike the CK receptors AHK2, AHK3, and AHK4, AHK1 positively regulates stress responses. Thus, it remains to be elucidated whether AHK2, AHK3, or AHK4 can sense abiotic stresses independently of CK, or whether AHK1 might crosstalk with a downstream CK signaling cascade.

Besides core components of the CK transduction cascade, downstream targets in stress responses have been disclosed as well. The cytokinin response factor (CRF) transcription factors of the APETALA2 (AP2) family have been identified as early CK response genes of which the expression is rapidly induced after CK application (Rashotte et al., 2006). Interestingly, the *CRF6* homolog is also highly responsive to various abiotic stress

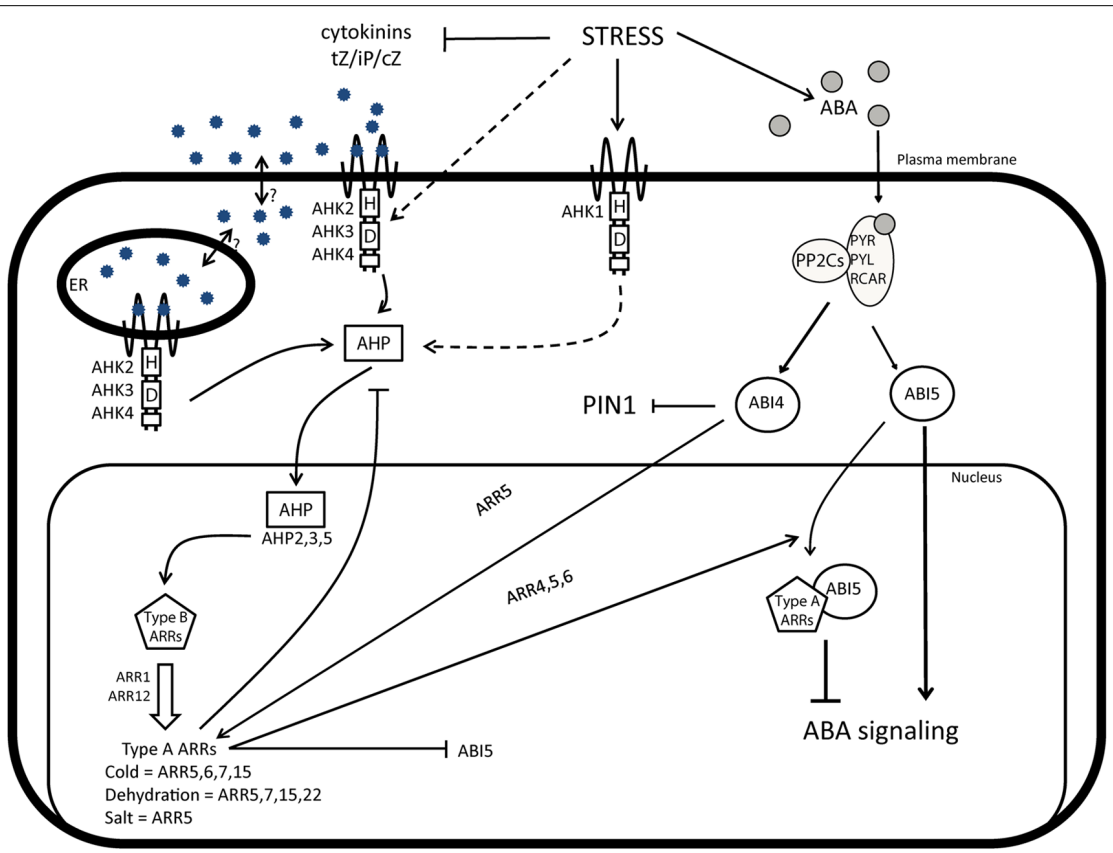

FIGURE 1 | CK and crosstalks during abiotic stress responses. Under non-stress conditions, CK activates signaling mediated through AHK receptors, AHPs, and type-B response regulators ARRs. Type-B ARRs stimulate the expression of the early CK response genes, including type-A *ARR* genes that provide a negative feedback loop of the CK signaling. Besides this negative feedback loop, type-A ARRs also repress the expression of *ABI5* and interfere with the ABA signaling, through the physical interaction with ABI5. In response to stress, ABA levels increase and, simultaneously, CK levels decrease. The recognition of ABA by the receptors PYR/PYL/RCAR promotes the interaction with PP2C proteins that will activate downstream responses through signaling components including ABI5 and ABI4. At the same time, ABA interferes with the activity of CK and auxin and via ABI4 attenuates the expression of the *PIN1* auxin efflux carrier and enhances the transcription of the CK signaling repressor *ARR5*. Interestingly, type-A ARRs, such as ARR5, are upregulated, despite the low CK levels, probably because of the indirect activation of the CK signaling pathway by alternative receptors of the histidine kinase family, such as AHK1.

treatments (Zwack et al., 2013) and, recently, its regulatory role has been characterized in leaf senescence control (Zwack et al., 2013).

HORMONAL CROSSTALKS AND ABIOTIC STRESS RESPONSES

The altered ABA sensitivity in plants with modified CK levels and signaling (Tran et al., 2007; Werner et al., 2010; Nishiyama et al., 2011; Wang et al., 2011b) hints at a crosstalk between ABA and CK. Interestingly, ARR4, ARR5, and ARR6 have been found to interact with ABI5 and also to regulate its expression levels. ABI5 is a basic leucine zipper protein that positively regulates the ABA signaling. The interaction with type-A ARRs attenuates the ABI5 activity and suppresses the ABA signaling (**Figure 1**; Wang et al., 2011b). Thus, type-A ARRs might, in addition to their regulation of the CK signaling, also control ABA signaling.

New insights into the ABA-CK crosstalk have been gained from the functional analysis of ABI4 (Shkolnik-Inbar and Bar-Zvi, 2010), that belong to the AP2 family of transcription factors. Similar to ABI5, ABI4 is also a positive regulator of the ABA signaling (Wind et al., 2013) and of the type-A *ARR5* expression that represses the CK signaling. Simultaneously, ABI4 attenuates the expression of the *PIN-FORMED 1* (*PIN1*) gene, an auxin efflux carrier that is an essential component of the polar auxin transport machinery (Shkolnik-Inbar and Bar-Zvi, 2010). Thus, ABI4 might represent an important crosstalk point on the interface of ABA, CK, and auxin pathways (**Figure 1**), in agreement with observations demonstrating that both the levels of CK and auxin, as well as of the PIN3 and PIN7 auxin efflux carriers, are suppressed when the ABA level increases (Hwang and Sheen, 2001; Wang et al., 2011a). Altogether, the strong impact of stress on plant development might result from the combined activities of several hormonal pathways, such as ABA and development-related hormones, such as CK and auxin.

The hormonal pathway of ethylene (ET) contributes also to the complexity of the hormonal network underlying plant responses to stresses. ET has been studied both in a developmental and stress context (Cary et al., 1995; Chae et al., 2003; Dietz et al., 2010; Kushwah et al., 2011; Beguerisse-Díaz et al., 2012;

Vanstraelen and Benková, 2012; Zhai et al., 2013) and, recently, its role as a negative regulator of freezing tolerance has been demonstrated (Shi et al., 2012). The ET activity in stress responses is mediated by the downstream transcription factor of the ET signaling cascade, ethylene-insensitive 3 (EIN3). EIN3 suppresses the expression of the *C-repeat/dehydration response element-binding factor 1* (*CBF1*), *CBF2,* and *CBF3* genes, which mediate the response to cold stress, and also of the CK signaling repressors *ARR5*, *ARR7,* and *ARR15* by direct binding to their promoters (Shi et al., 2012). Although ET interferes with the CK signaling output, its pathway is also affected by CK. Indeed, CK stabilizes 1-aminocyclopropane-1-carboxylate synthase 5 (ACS5) and ACS9 (Vogel et al., 1998; Chae et al., 2003; Hansen et al., 2009) that convert *S*-adenosyl-methionine to 1-aminocyclopropane-1-carboxylic acid (ACC), the rate-limiting step in the ET biosynthesis. This stabilization might lead to an ET accumulation and, consequently, affect plant growth processes, such as root growth (Cary et al., 1995; Růžička et al., 2007). The complexity of the hormonal regulatory network underlying stress responses has been suggested (Lehotai et al., 2012) by the activation of both CK and ET signaling in response to selenite-induced stress by means of the ARR5 and ACS8 markers and decrease in the auxin levels.

Interestingly, the CK-ET and CK-ABA interactions exhibit tissue-specific features. CK treatments have been demonstrated to promote the ABA accumulation in shoots, but not in roots, in contrast to ET that accumulates predominantly in roots in response to high CK levels (Žďárská et al., 2013).

PLANT HORMONES IN RESPONSES TO BIOTIC STRESSES

Hormones also tightly regulate plant responses against pathogens. The networks that control the immune responses in plants are highly complex and have been extensively reviewed (Feys and Parker, 2000; Broekaert et al., 2006; Robert-Seilaniantz et al., 2007; Nishimura and Dangl, 2010). The best characterized hormones that play a role in pathogen response/defense are salicylic acid (SA), jasmonate (JA), and ET. Depending on the lifestyle of the pathogens, a different response will be triggered by the plant. Against biotrophic pathogens, the resistance largely depends on SA-mediated responses and the principal defense strategy is programmed cell death (apoptosis) that restricts the biotrophic pathogen to the infection site, preventing its proliferation, and further spreading in the plant (Dangl and Jones, 2001; Jones and Dangl, 2006; Nishimura and Dangl, 2010; An and Mou, 2011). In contrast, for necrotrophic pathogens that feed on death tissue only, cell death is beneficial. These pathogens induce defense responses that depend on JA and ET to prevent cell death and that trigger the secretion of antimicrobial compounds and the accumulation of proteins with antimicrobial and antifungal activity, such as plant defensins (Overmyer et al., 2000; Andi et al., 2001; Alonso and Stepanova, 2004; Broekaert et al., 2006; Balbi and Devoto, 2008; Fonseca et al., 2009; Gfeller et al., 2010). Because of their difference in the nature of the defense strategy, the JA–ET interaction tends to antagonize the SA responses (Peña-Cortés et al., 1993; Doares et al., 1995; Petersen et al., 2000; Kloek et al., 2001), so that the stress-activated JA–ET signaling might suppress the SA-mediated resistance and vice versa. However, these two pathways might synergistically interact and be considered a fine-tuning mechanism to respond to biotic stresses (Cui et al., 2005; Mur et al., 2006; Truman et al., 2007).

Once the pathogens or microbes have gained access to the plant tissues, they are sensed in each cell by pattern recognition receptors present in the plasma membrane of the host plant cells and bind to microbe-associated molecular patterns (MAMPs; Gómez-Gómez, 2004; Zipfel et al., 2006), the mechanism designated basal resistance or MAMP-triggered immunity (MTI). To overcome MTI, pathogens secrete effectors into the plant cytosol. In this manner, these proteins interfere with the plant immune responses (Chisholm et al., 2006) and modify the host proteins to evade detection and, hence, enhance their virulence, which is referred to as effector-triggered susceptibility. However, the coevolution of plants and microbes has led to the acquisition of the R proteins that specifically recognize these pathogen effectors or avirulence (avr) proteins in a characterized response known as gene-for-gene resistance or effector-triggered immunity (ETI) (Flor, 1971). This specific resistance response is noticeable by localized cell death at the infection site and is known as the hypersensitive response (Hammond-Kosack and Jones, 1996; Greenberg and Yao, 2004).

SALICYLIC ACID IN BIOTIC STRESSES

During the hypersensitive response, different signal transduction pathways are activated. Tissues distal from the infection site develop an enhanced broad-spectrum resistance to secondary infections that is the systemic acquired resistance (SAR; Yarwood, 1960; Ross, 1961). Before SAR is triggered in remote leaves, SA, which is crucial for this defense strategy, accumulates (Malamy et al., 1990). When transgenic *Arabidopsis* plants express the bacterial SA hydroxylase gene *nahG* that disables the SA accumulation because of its fast turnover to catechol, they cannot develop SAR and induce the pathogen resistance (*PR*) gene expression (Gaffney et al., 1993; Delaney et al., 1994). Furthermore, lipid transfer proteins and SA-binding proteins might be involved in the SA accumulation-triggering signaling in SAR (Park et al., 2007). The non-expresser PR1 (NPR1) protein acts downstream of SA and transduces the signal to promote the *PR* gene expression (Durrant and Dong, 2004). During SAR induction, an oxidative burst occurs, followed by an increase in antioxidants to neutralize the harmful effects of reactive oxygen species. This reducing environment can then convert NPR1 from its inactive oligomeric form into its activated monomeric form that can be transported from the cytosol to the nucleus and activate transcription factors (Kanzaki et al., 2003; Mou et al., 2003), via protein-protein interactions between NPR1 and the TGACG sequence-specific (TGA) transcription factors (Zhang et al., 1999).

JASMONIC ACID AND ETHYLENE IN BIOTIC STRESSES

The defense response to an attack by necrotrophic pathogens and chewing insects is mediated through the JA pathway that commonly acts together with ET to mount a coordinated defense response. One of the best characterized components of the JA signaling pathway is the coronatine insensitive (COI1) receptor (Devoto et al., 2002; Xu et al., 2002). COI1 is part of the Skp1/Cullin/F-box (SCF) E3 ubiquitin-ligase protein degradation complex SCF^{COI1}. High JA levels promote the interaction of the SCF^{COI1} complex with the JA ZIM (JAZ) domain repressors

and activate the transcription of JA-responsive genes. The *coi1* mutants that lack the functional JA receptor are more susceptible to infections by insects and necrotrophic pathogens, such as *Botrytis cinerea*, *Pythium irregulare*, or *Alternaria brassicicola* (van Wees et al., 2003; Adie et al., 2007; Ferrari et al., 2007; Ye et al., 2012). Likewise, mutations that stabilize the JAZ proteins (JAZ1Δ3A) increase the susceptibility against herbivores, such as *Spodoptera exigua* (Chung et al., 2008), further supporting the significance of a functional JA signaling pathway in plant defense responses.

The JA-mediated responses against pathogens is strengthened by the ET activity. Ethylene is perceived in plants by the receptors ethylene resistant1 (ETR1), ETR2, ethylene-insensitive4 (EIN4), ethylene response sensor1 (ERS1), and (ERS2) that belong to a histidine kinase family (Bleecker et al., 1988; Chang et al., 1993; Hua et al., 1995, 1998; Sakai et al., 1998). Mutations in these receptors not only confer ET insensitivity, but also increase susceptibility to necrotrophic pathogens (Geraats et al., 2003). Downstream from these receptors, the Raf-like kinase constitutive triple response 1 (CTR1) is active, which is a negative ET response regulator. In the presence of ET, the *CTR1* repression activates EIN2 (Guzmán and Ecker, 1990; Kieber et al., 1993; Chao et al., 1997) and, subsequently, stimulates the EIN3/EIL-like (EIL) transcription factors, whereas mutations in *EIN2* confer ET insensitivity, in addition to an increased susceptibility to necrotrophic pathogens (Geraats et al., 2003).

Although both JA and ET contribute jointly to the plant's fight against pathogen attacks, the molecular mechanisms of their crosstalk are not well understood, but new insights into the molecular mechanisms underlying their interactions have been provided (Zhu et al., 2011). The JAZ repressors of the JA signaling interact physically with the EIN3/EIL1 transcription factors and attenuate their ability to activate genes (Zhu et al., 2011). This interaction has a striking developmental impact, because it enables JA to contribute to the ET response regulation. Thus, besides the classical mechanism in which ET induces the EIN3/EIL1 stabilization (Guo and Ecker, 2003; Potuschak et al., 2003), EIN3/EIL1 is released from repression by JA through JAZ degradation, thereby triggering ET responses (Zhu et al., 2011).

The hormonal interplay between pathways that depend on JA–ET and SA is particularly important when plants are exposed to multiple pathogens of both biotrophic and necrotrophic types. Under such conditions, an effective defense requires only one of these pathways, but still they need to be tightly balanced with each other. This very complex crosstalk between JA and SA has been reviewed thoroughly (see Beckers and Spoel, 2006; Thaler et al., 2012).

CYTOKININ AND ITS CROSSTALK WITH SALICYLIC ACID

One of the first indications on the involvement of CK in biotic stress came from tobacco (*Nicotiana tabacum*) plants in which the *S*-adenosyl-homocysteine hydrolases (SAHHs) were downregulated. Originally, SAHHs have been studied in mammals because of their role in the regulation of transmethylation and mRNA 5′ capping during viral replication (De Clercq, 1998). Interestingly, the tobacco plants with low *SAHH* expression not only exhibited an enhanced resistance against the tobacco mosaic virus (TMV), cucumber mosaic virus, potato virus X, and potato virus Y (Masuta et al., 1995), but also increased CK levels and CK-related developmental defects.

In attacked plants, the CK levels are coregulated with the SA levels (Kamada et al., 1992; Sano et al., 1994, 1996; Masuta et al., 1995). Tobacco plants that overexpressed the Ras-related small GTP-binding protein 1 (RGP1)-encoding gene exhibited higher levels of SA and of the acidic pathogenesis-related 1 (*PR-1a*) gene than those of wild-type plants, in correlation with an enhanced resistance against TMV infection. Interestingly, these transgenic plants also showed phenotypes typical for a high endogenous CK activity, such as reduced apical dominance and increased tillering (Kamada et al., 1992), as was, indeed, confirmed later (Sano et al., 1994, 1996). Furthermore, in both wild-type and *RGP1*-overexpressing plants, the CK perception inhibited by the use of the competitive inhibitor 2-chloro-4-cyclohexylamino-6-ethylamino-s-triazine interfered with the expression of the SA-dependent *PR-1a* and the basic JA-dependent *PR-1* after wounding (Sano et al., 1996), thereby suggesting that CK contributes to the defense responses mediated by SA and JA.

As mentioned, the recognition of the pathogen Avr effector proteins by the resistance (R) proteins is an important part in plant defense responses. This interaction triggers ETI, which is characterized by the production of SA and the subsequent induction of *PR* genes and SAR. A dominant-positive mutant of the coiled-coil nucleotide-binding leucine-rich-repeat (CC-NB-LRR) protein UNI (*uni-1D*) that constitutively activates ETI (Igari et al., 2008) exhibits an enhanced expression of *PR-1, PR-5*, and of the type-A ARR CK-signaling repressors and increased endogenous CK levels, with phenotypic alterations typical for high CK activity as a consequence (**Figure 2**; Igari et al., 2008). In *uni-1D* plants, CK levels decreased by the *CKX1* induction reduces both the *PR-1* and of type-A *ARR* gene expression. However, in these *uni-1D* plants, overexpression of the bacterial SA hydroxylase-encoding *nahG* gene prevents SA accumulation and interferes with the *PR-1* expression, but without effect on the type-A *ARR* gene induction and the CK-like phenotypes (Igari et al., 2008). A similar CK-related phenotype has been observed in the knockdown mutant *rin4K-D* of the resistance to *Pseudomonas syringae* pv. *maculicola* (RPM1)-interacting protein 4 (RIN4), which is a negative regulator of R proteins. In *rin4K-D plants,* the R proteins Resistant to *P. syringae* 2 (RPS2) and RPM1 are constitutively active and trigger ETI, whereas both *PR-1* and *ARR5* transcript levels are upregulated and the phenotypic alterations are typical for high CK activity (**Figure 2**; Igari et al., 2008).

Another indication of the crosstalk between CK and SA has emerged from the characterization of the CRF 5 (**Figure 2**; Liang et al., 2010). Indeed, the *CRF5* expression is upregulated in response to *Pseudomonas syringae* pv. *tomato* DC3000 (*Pst* DC3000) and the transcript levels of SA-induced *PR-1*, *PR-3*, *PR-4*, and *PR-5* are increased in the *CRF5*-overexpressing lines (Rashotte et al., 2006; Cutcliffe et al., 2011). This crosstalk mechanism between CK and SA has been elucidated (Choi et al., 2010) by showing that pretreatment of *Arabidopsis* plants with CK significantly increased the resistance against *Pst* DC3000 infection. Correspondingly, mutants defective in CK perception and signaling, such as *ahk2/ahk3* and *arr2,* or plants with reduced

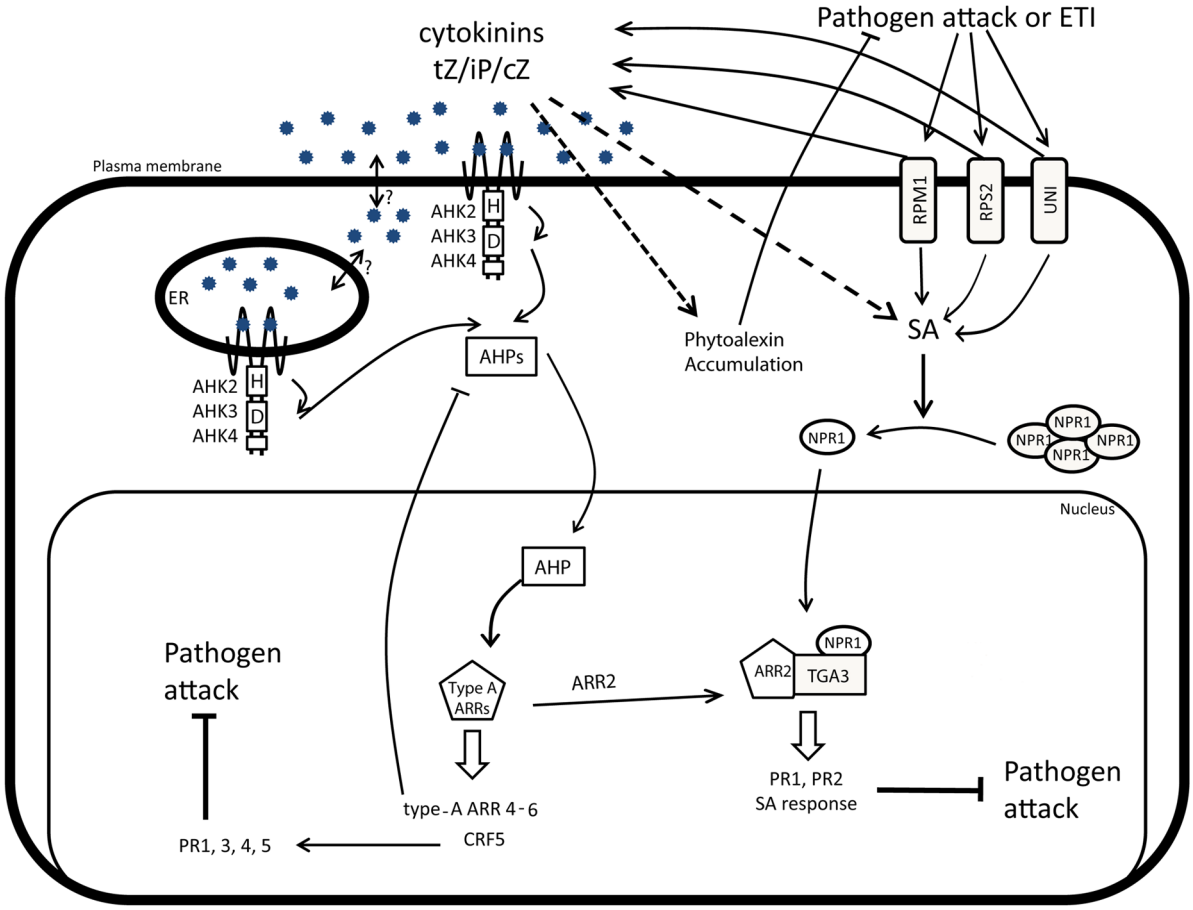

FIGURE 2 | CK and hormonal crosstalks during biotic stress responses. Pathogen attacks stimulated by PAMP-triggered immunity (PTI) and effector-triggered immunity (ETI) correlate with a dramatic production of SA and CK. The accumulation of CK will induce the production and accumulation of phytoalexins in a SA-independent manner and also enhance the SA-dependent immunity. In response to pathogens, NPR1 monomerizes and translocates to the nucleus where it interacts with TGA3. The NPR1-TGA3 activity is further regulated through interaction with the type-B ARR2 response regulator, a component of the CK signaling pathway. The TGA3-NPR1-ARR2 complex is required to induce the SA-mediated resistance and to trigger the expression of *PR1* and *PR2*. High CK levels, induced after pathogen attacks, can activate the *CRF5*-mediated branch of the CK signaling pathway and contribute to the regulation of the *PR1*, *PR3*, *PR4*, and *PR5* expression.

endogenous CK levels, such as *35S::CKX2* and *35S::CKX4*, were more susceptible to *Pst* DC3000. In contrast, the plant resistance to Pst DC3000 was enhanced by high endogenous CK levels due to overexpression of the CK biosynthesis (*IPT*) genes or by CK signaling promoted by increased *ARR2* expression (Choi et al., 2010). Therefore, CK has been proposed to affect priming, a defense-related response activation and might assist plants to cope with infections through the induced SA signaling and increased *PR* expression levels (Igari et al., 2008; Choi et al., 2010; Liang et al., 2010). This scenario is strongly supported by the findings that ARR2 interacts directly with the SA response factor TGA3, which binds the promoter regions of *PR-1* and *PR-2*, and that this interaction is essential for the enhanced resistance of the *35S::ARR2* lines. Altogether, both the SA-triggered translocation of NPR1 into the nucleus and the formation of a complex with TGA3-ARR2 are seemingly necessary for the development of a full SA-mediated defense response (Choi et al., 2010, 2011). The impact of CK on the plant defense has been characterized in the *Pst* DC3000-*Arabidopsis* interaction model with the *SA induction deficient 2 (sid2)* mutant that fails to accumulate SA (Naseem et al., 2012). The increased susceptibility of *sid2* toward *Pst* DC3000 can only be partially recovered by CK treatment (Naseem et al., 2012), thereby supporting that CK treatments enhance the immunity in an SA-dependent manner (Naseem and Dandekar, 2012).

Recently, the CK-promoted protection against pathogenic infections has been suggested to be involved in SA-independent mechanisms (Großkinsky et al., 2011). In the *P. syringae* pv. *tabaci*-tobacco interaction model, higher CK levels before infection increase the resistance of tobacco against *P. syringae* pv. *tabaci* and this resistance depends on increases phytoalexin levels, such scopoletin and capsidiol, which accumulate in the presence of CK (Großkinsky et al., 2011). Thus, the mechanism underlying the CK-mediated resistance of tobacco differs from that in *Arabidopsis* that is based on an SA-dependent transcriptional control. In the solanaceous plant species, CK appears to promote primary defense responses through an increase of the phytoalexin-pathogen ratio

in the early infection phases that then efficiently restricts the pathogen development.

CYTOKININ AND ITS CROSSTALK WITH JASMONIC ACID

Even though there is not much evidence for an interplay between JA and CK, these hormonal pathways might be linked directly (Ueda and Kato, 1982; Dermastia et al., 1994; Sano et al., 1996) and their interaction might be antagonistic (Naik et al., 2002; Stoynova-Bakalova et al., 2008). Typically, in wounded plants, the JA levels increase significantly, whereas the SA levels remain unchanged, but both CK applications and high endogenous CK levels accelerate the defense response to reach a faster maximum release of JA and methyl jasmonate (MeJA) than in control plants (Sano et al., 1996; Dervinis et al., 2010). In potato (*Solanum tuberosum*), JA treatments can induce the accumulation of CK ribosides (Dermastia et al., 1994), whereas they might strongly inhibit the CK-induced callus growth (Ueda and Kato, 1982). These observations hint at a very complex and unexplored interplay, in which the outcome probably depends not only on the CK-JA ratio, but also that of other hormones as well.

CYTOKININ AND ITS CROSSTALK WITH AUXIN

Crosstalk between CK and auxin has been widely studied over the years, particularly in a developmental context in which their interaction is primarily antagonistic (Bishopp et al., 2011; Vanstraelen and Benková, 2012), although a number of recent studies undoubtedly point toward a role of auxin in stress responses. Various pathogens can produce auxins or modulate auxin levels *in planta* to enhance the plant susceptibility to infection (Chen et al., 2007; An and Mou, 2011). In *Arabidopsis* plants lacking the functional *RPS2* gene, the expression of the *P. syringae* type III effector *AvrRpt2* decreased the resistance against *Pst* DC3000, and also show altered auxin levels and auxin-related phenotypes (Chen et al., 2007). This direct correlation between sensitivity and auxin levels implies that auxin promotes plant susceptibility. Also, a recent study in which *PR1* was used as a marker gene in the *Pst* DC3000–*Arabidopsis* interaction revealed that, whereas the immunity was positively promoted by CK and SA, it was negatively regulated by auxin, JA, and ABA (Naseem et al., 2012). Interestingly, the positive effect of CK pretreatments on the plant immunity can be repressed by a combined CK and auxin treatment (Naseem et al., 2012). Based on this evidence, CK and auxin might play a highly possible antagonistic role in plant defense responses, but the specific mechanisms that modulate this crosstalk are still unknown.

A model for the CK–auxin interplay in plant defense has been proposed (Naseem and Dandekar, 2012). After infection, pathogens will modulate the auxin levels and the signaling that will diminish the responses mediated by SA and CK, whereas CK pretreatments will prevent the auxin-based susceptibility, due to the known effect of CK on auxin transport and signaling.

CONCLUSIONS AND FUTURE PERSPECTIVES

Nowadays, one of the major objectives of plant biologists is to improve plant performances under less favorable environmental conditions. By enhancing plant defense responses against biotic and abiotic stress, non-cultivable land might be used, the losses due flooding and infections be decreased, and the amount of applied fertilizers and pesticides in the fields be reduced. However, because the crosstalk between stress-related and developmental hormones is largely unknown, and uncharacterized, usually unforeseen problems occur when the stress resistance is modified. Ideally, plants with enhance resistance to stress or pathogen attacks should not be affected in growth or developmentally hampered. In this context, it is crucial to understand the hormonal crosstalks underlying plant responses to various stresses, because the modification of one single hormonal pathway will very probably alter the activity of other hormonal pathways as well.

The complexity of the impact of hormones on the resistance to stress can be nicely illustrated with examples of plants with altered CK levels. Due to the importance of CK in stress responses, several genes involved in the regulation of CK levels have been proposed as possible targets to enhance stress resistance, such as the *IPT* and *CKX* genes (Werner et al., 2010; Nishiyama et al., 2011; Wang et al., 2011b). However, the benefit of the stress-tolerant phenotype of the *IPT* loss-of-function mutants or of *CKX*-overexpressing plants was counteracted by developmental defects caused by low bioactive CK levels, such as N6-(Δ2-isopentenyl)adenine and *trans*-zeatin (Nishiyama et al., 2011). To overcome this drawback, it is necessary to control the CK activity either in an organ or in a tissue-specific manner, an approach that has already been used in several species (McCabe et al., 2001; Sýkorová et al., 2008; Ghanem et al., 2011; Qin et al., 2011). For instance, as a consequence of downregulated CK levels in root tissues only (Werner et al., 2010), root length, branching, and biomass increased and the plants were also more resistant to abiotic stress treatments, such as severe drought or heavy metal contaminations (Werner et al., 2010). Furthermore, modulation of CK-mediated defense to stress might at the same time attenuate the input provided by other signaling pathways, such as ABA (Wang et al., 2011b). A reduced CK content leads to a decrease in ABA content and hypersensitivity to ABA treatments (Nishiyama et al., 2011), in contrast to the stressed plants in which the ABA levels are upregulated (Stoll et al., 2000; Hansen and Dörffling, 2003; Kudoyarova et al., 2007). Correspondingly, overexpresssion of *IPT8* results in insensitivity to ABA treatments and prevents the induction of *ABI1* and *ABI5* in seedlings (Wang et al., 2011b). These examples clearly show that a good knowledge of the molecular mechanisms underlying the hormone-mediated responses and of the mutual communication among hormonal pathways might be very rewarding in the targeted modulation of specific hormonal pathways and, hence, in the effective plant adaptation to concrete environmental conditions.

Extended studies on the genes that mediate the crosstalk between CK and other developmental and stress-related hormones might identify novel targets for the stress tolerance improvement of crop species. Importantly, the identification of molecular components and mechanisms that mediate the phytohormonal interplay might enable us to dissect the stress-related from the developmental functions.

Finally, to increase the plant resistance against various stresses, new alternative approaches should take in account the specific features of the plant species and the distinct mechanisms that underlay their stress responses (Choi et al., 2010; Großkinsky et al., 2011). A nice example of such a strategy is the enhanced

drought stress tolerance of alfalfa (*Medicago sativa*) by means of CK-overproducing *Sinorhizobium meliloti* without impact on nitrogen fixation (Xu et al., 2012).

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