Hormone interactions in stomatal function

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Abstract Research in recent years on the biology of guard cells has shown that these specialized cells integrate both extra- and intra-cellular signals in the control of stomatal apertures. Among the phytohormones, abscisic acid (ABA) is one of the key players regulating stomatal function. In addition, auxin, cytokinin, ethylene, brassinosteroids, jasmonates, and salicylic acid also contribute to stomatal aperture regulation. The interaction of multiple hormones can serve to determine the size of stomatal apertures in a condition-specific manner. Here, we discuss the roles of different phytohormones and the effects of their interactions on guard cell physiology and function.

Keywords Abscisic acid (ABA) · Auxin · Guard cells · Hormonal cross-talk · Signal transduction · Stomata

Abbreviations

ABA	Abscisic acid
ACC	1-Aminocyclopropane-1-carboxylic acid
BRs	Brassinosteroids
GA	Gibberellins
JA	Jasmonate
1-NAA	1-Napthaleneacetic acid
SA	Salicylic acid

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Introduction

Stomata are natural microscopic pores, each surrounded by a pair of guard cells. Stomata are present throughout the leaf epidermis and are also present on other aerial parts of the plant. Guard cells dynamically regulate the size of stomatal apertures and thereby control gas exchange by the plant. The most important function of stomata is to allow entry of sufficient CO_2 for optimal photosynthesis while conserving water as required by the plant. In addition, these specialized structures also play critical roles in the control of leaf temperature by modulating rates of transpirational water loss, and restrict pathogen invasion via stomatal closure. Multiple environmental factors such as drought, CO_2 concentration, light, humidity, biotic stresses and different plant hormones modulate stomatal apertures (Hirayama and Shinozaki 2007; Israelsson et al. 2006; MacRobbie and Kurup 2007; Neill et al. 2008; Underwood et al. 2007). Opening or closure of stomata is achieved by osmotic swelling or shrinking of guard cells respectively, driven by transmembrane fluxes of K^+, Cl^- and $malate^{2-}$ (Fan et al. 2004; Nilson and Assmann 2007). Reorganization of the cytoskeleton, metabolite production, post-translational modifications of existing cellular proteins and modulation of gene expression are also key components of guard cell biology and determinants of stomatal regulation (Cominelli et al. 2005; Hwang and Lee 2001; Lemichez et al. 2001; Leonhardt et al. 2004; Liang et al. 2005; Shen et al. 1995; Shen and Ho 1995).

A diversity of experimental strategies has uncovered roles for most of the major plant hormones in stomatal regulation. Among these, ABA plays the over-riding role, limiting stomatal apertures under conditions of water stress. In addition, recent work suggests that stomatal function is also regulated by auxin, cytokinin, ethylene,

brassionosteroids, jasmonates, and salicylic acid. It is also evident that these hormones interact during stomatal regulation.

There are recent reviews describing stomatal responses to a number of environmental signals and endogenous components (Hetherington and Woodward 2003; Israelsson et al. 2006; Pandey et al. 2007; Shimazaki et al. 2007; Vavasseur and Raghavendra 2005; Wang and Song 2008). Stomatal regulation by ABA has been particularly well-reviewed (Fan et al. 2004; Hirayama and Shinozaki 2007; Schroeder et al. 2001a, b; Wang and Song 2008). Two reviews describe the participation and interactions between phytohormones in stomatal regulation (Dodd 2003; Pospíšilová 2003). Recently, important progress has been made in elucidating the roles of other hormones in stomatal function. Accordingly, in this article we focus primarily on current understanding concerning regulation of stomatal processes by auxins, cytokinins, ethylene, brassionoster-oids, jasmonates, and salicylic acid. In addition, the effects of cross-talk between and among different hormones on stomatal regulation, particularly the topic of how different plant hormones impact ABA-mediated stomatal control, are also discussed (Fig. 1).

ABA

ABA is a terpenoid that is synthesized from carotenoid precursors (Nambara and Marion-Poll 2005). It has been reported that ABA concentrations can increase up to 30-fold during drought stress (Outlaw 2003), and other

Fig. 1 A proposed model of hormonal interaction in stomatal regulation. See text for details (*Note*: Production of NO in SA-mediated stomatal closure is inferred but has not been directly demonstrated to date)

stresses, such as salinity and cold, also cause ABA biosynthesis and accumulation. Besides acting as a key player in response to drought, ABA plays important roles in plant developmental processes, including cell division, seed maturation, seed dormancy and germination, and post-germination seedling growth (Finkelstein et al. 2002; Leung and Giraudat 1998).

Water deficit promotes ABA biosynthesis, accumulation, and redistribution in the plant body, including transport from the roots to the shoots in the xylem sap. Moreover, drought-induced increases in pH of the apoplast favor extracellular retention of the anionic form of ABA, which may facilitate ABA delivery to the guard cells (Wilkinson and Davies 2002). ABA curtails transpirational water loss by promoting stomatal closure and inhibiting stomatal opening, and this modulation of stomatal apertures is associated with multiple cascades of cellular-biochemical events, including activation of G-proteins (Coursol et al. 2003; Coursol et al. 2005; Wang et al. 2001), production of reactive oxygen species (ROS) (Pei et al. 2000; Zhang et al. 2001), generation of NO (Bright et al. 2006; Desikan et al. 2002; Garcia-Mata et al. 2003; Neill et al. 2002; Sokolovski et al. 2005), elevation of cytosolic pH (Irving et al. 1992), elevation of cytosolic Ca^{2+} via both influx across the plasma membrane and release from cytosolic stores (Allen et al. 2000; Murata et al. 2001; Pei et al. 2000), protein phosphorylation/dephosphorylation (Leung et al. 1994; Li et al. 2000; Merlot et al. 2001; Mustilli et al. 2002), and reorganization of the cytoskeleton (Hwang and Lee 2001; Lemichez et al. 2001). As a result, activities of cation and anion channels at the plasma membrane and tonoplast are altered, resulting in decreases in guard cell concentrations of K^+, Cl^-, and $malate^{2-}$ that drive stomatal closure and inhibit stomatal opening. These components have recently been compiled into a synthetic and predictive model of guard cell ABA signaling (Li et al. 2006). Accordingly, below we focus only on a few key components of the ABA response that have also been studied in the context of guard cell responses to other hormones.

In response to ABA, guard cells generate reactive oxygen species (ROS) (Pei et al. 2000; Zhang et al. 2001). ABA-stimulated ROS production in Arabidopsis guard cells is mediated redundantly by the NADPH oxidase catalytic subunits AtrbohD and AtrbohF, as *atrbohD/F* double mutants show significantly impaired stomatal closure in response to ABA (Kwak et al. 2003). Protein phosphatase 2C proteins, ABI1 and ABI2, are also crucial for ABA-mediated stomatal regulation (Gosti et al. 1999; Leung et al. 1997; Merlot et al. 2001). The dominant negative mutant *abi1-1* shows ABA-insensitive stomatal conductance (Koornneef et al. 1989; Leung et al. 1994), whereas revertant and loss-of-function recessive mutants
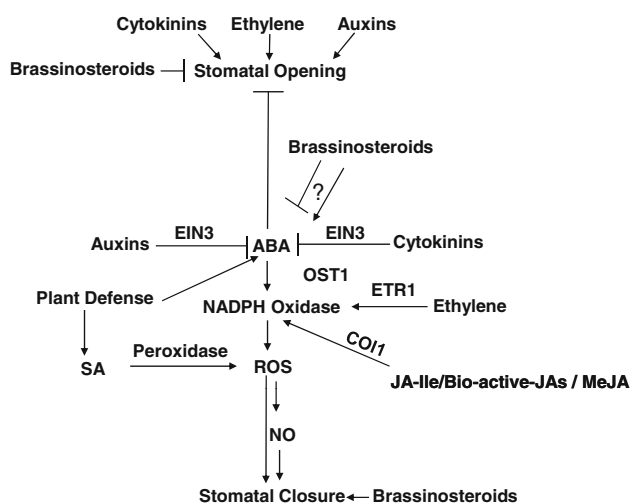
of *ABI1* show hypersensitivity in ABA-mediated stomatal response, leading to the conclusion that ABI1 is a negative regulator of ABA signaling. In response to ABA, dominant *abi1-1* mutants do not generate ROS, but the dominant ABA-insensitive *abi2-1* mutant has the ability to generate ROS. Based on these observations it has been proposed that ABI1 acts upstream of ROS production and ABI2 downstream of ROS production in ABA signaling of guard cells (Murata et al. 2001). Arabidopsis plants harboring mutations in the open stomata1 (OST1) kinase, a guard cell expressed Ser/Thr kinase of Arabidopsis and an orthologue of AAPK in *Vicia faba*, (Assmann 2003; Li et al. 2000; Mustilli et al. 2002), also show impairment of ROS production and stomatal closure in response to ABA. Physical interaction of OST1 with ABI1 has been reported (Yoshida et al. 2006), consistent with the idea that these two proteins are located adjacent to each other in a signaling pathway upstream of AtrbohD/F. Generation of nitric oxide (NO) in guard cells in response to ABA is also critical for stomatal closure (Neill et al. 2002). Arabidopsis guard cells produce NO in response to exogenous treatment with ABA. Exogenous application of NO donors leads to stomatal closure in a variety of plant species (Neill et al. 2002), whereas application of an NO scavenger (e.g. PTIO or cPTIO) inhibits ABA-induced stomatal closure. In Arabidopsis, in addition to nitric oxide synthase-like enzymes, nitrate reductases (NRs) encoded by the genes *NIA1* and *NIA2*, mediate synthesis of nitric oxide (Desikan et al. 2002; Neill et al. 2008). The *nia1 nia2* double mutant exhibits inability to synthesize NO and impaired stomatal closure in response to ABA, suggesting that NO is one of the critical signaling intermediates in ABA-mediated stomatal closure (Desikan et al. 2002; Neill et al. 2002). In Arabidopsis and *Vicia faba*, generation of NO in response to ABA has been shown to reside genetically downstream of ROS production by AtrbohD/F (Bright et al. 2006; Lü et al. 2005). Modulation of pH has been implicated as another signaling event in ABA-mediated stomatal regulation. In guard cells, ABA induces alkalinization of the cytosol which activates the outward K^+ channels that mediate K^+ efflux during stomatal closure, while acidification of the cytosol causes activation of inward K^+ channels (Blatt and Armstrong 1993; MacRobbie 1997; Miedema and Assmann 1996).

Gibberellins (GAs)

Gibberellins (GAs) are diterpine plant hormones with more than one hundred identified structures, of which only a small number are biologically active (Yamaguchi 2008). GAs accelerate many processes of plant development such as germination, stem elongation, growth, leaf expansion and seed development (Sun and Gubler 2004; Yamaguchi 2008). In response to water stress, reduced accumulation of GAs has been observed in some dicots but not in others (Aharoni et al. 1977; Hubick et al. 1986).

Although GAs commonly oppose ABA action, e.g. in seed germination, there is remarkably little evidence for this phenomenon in guard cell biology. Exogenous GA application appears to have little or no effect on stomatal apertures in Arabidopsis (Tanaka et al. 2006). Under darkness, GA application led to transient stomatal opening in *Vicia faba* and *Fritilaria imperialis* (Göring et al. 1990) and promoted stomatal opening in isolated epidermal strips of *Commelina benghalensis* L. (Santakumari and Fletcher 1987). Also, in a study investigating response to water stress, GA-mediated stomatal response has been examined in GA-deficient tomato plants. The leaves of GA-deficient tomato plants did not show significant difference in transpiration in comparison to that of control leaves (Cramer et al. 1995). This suggests that GAs may not be critical players during water stress. The role of GAs in stomatal development has also been studied. GA_3 promotes division of the epidermal cells of Arabidopsis hypocotyls, promoting formation of stomata. In addition, auxin and ethylene enhance GA-mediated formation of stomatal complexes in this organ (Saibo et al. 2003).

Auxins

Auxins are a class of plant hormones consisting of indole-3-acetic acid (IAA) and related molecules with the ability to induce plant responses similar to those induced by IAA (Christian et al. 2008). IAA is one of the predominant naturally occurring forms of auxin in plants. Auxins generally promote cell division, cell elongation, vascular tissue differentiation, stem elongation, and apical dominance, and are essential components in tropic responses (Kepinski 2007). Findings regarding endogenous levels of auxin (IAA) following water stress are contradictory. An increased level of IAA has been reported in leaves of cucumber and in hypocotyls of squash in response to water stress (Sakurai et al. 1985; Zholkevich and Pustovoitova 1993) but in another study, cucumber plants exposed to progressive soil drought, showed a decreased level of IAA until the 4th day, while from the 5th day until the 9th day of drought stress, an increased level of IAA accumulated (Pustovoitova et al. 2003). In tomato, however, no significant change of IAA accumulation was observed in response to drought stress (Schmelz et al. 2003).

Auxin typically plays a positive regulatory role in stomatal opening although high exogenous auxin concentrations can inhibit stomatal opening (Lohse and Hedrich 1992). In response to auxin, activation of the plasma membrane H^+-ATPase occurs in guard cells, as in other

cell types (Lohse and Hedrich 1992). Proton extrusion via the H$^+$-ATPase leads to hyperpolarization of the membrane, which in turn facilitates K$^+$-uptake. Low auxin concentrations promote the activity of the inward K$^+$ channels that mediate K$^+$ influx during stomatal opening, while higher auxin concentrations inhibit these channels and promote the activity of the outward K$^+$ channels (Blatt and Thiel 1994), consistent with the bimodal effects of auxin on stomatal apertures.

There is evidence that auxin-induced acidification of the guard cell cytosol and the auxin binding protein1, ABP1, participate in stomatal responses to auxin (Gehring et al. 1998; Irving et al. 1992; Thiel et al. 1993). In guard cells of *Vicia faba*, addition of a peptide consisting of the 12 C-terminal amino acid residues of maize ABPzm1 inactivates K$^+$ influx channels and activates K$^+$ efflux channels via elevation of cytosolic pH (Thiel et al. 1993). Similarly, in *Paphiopedilum tonsum*, the peptide induces cytoplasmic alkalization and stomatal closure (Gehring et al. 1998). However, according to our knowledge, to date there is no published report using a functional genetics approach to confirm a role of ABP1 in stomatal function.

Antagonistic stomatal regulation has been observed between ABA and auxin. Auxin represses stomatal closure in response to ABA in epidermal peels of *Commelina communis* (Snaith and Mansfield 1982) and a similar oppositional role of auxins was observed when NAA was applied in combination with ABA to opened stomata in epidermal peels of Arabidopsis (Tanaka et al. 2006).

Cytokinins

Cytokinins are adenine-derivative molecules with diverse active forms. Zeatin, dihydrozeatin, and isopentyladenine are important cytokinins found in higher plants (Dello Ioio et al. 2008). Cytokinins play positive roles in germination, root and shoot development, and nodulation, and oppose leaf senescence and pathogen invasion (Sakakibara 2006; To and Kieber 2008).

Plants maintain their shoot-water status by decreasing stomatal apertures in response to drying soil. Stomata respond to chemicals produced by dehydrating roots even when leaf water status is kept constant (Davies and Zhang 1991; Gowing et al. 1990). It has been shown that increased cytokinin concentration in the xylem sap promotes stomatal opening and simultaneously decreases sensitivity to ABA (Wilkinson and Davies 2002). Water stress leads to reduced synthesis of cytokinin in roots and its transport to shoot (Pospíšilová 2003; Pustovoitova et al. 2003). Conversely, transgenic tobacco plants overexpressing the *ZOG1* gene, encoding trans-zeatin o-glucosyltransferase, and exhibiting an increased level of total cytokinin content (total content of

O-glucosides of *trans*-zeatin and *cis*-zeatin), show a delayed decrease of stomatal aperture in response to water deficit (Havlova et al. 2008).

Stomatal response to exogenous application of cytokinin depends on the concentration and cytokinin species. Both synthetic and natural cytokinins can cause stomatal opening in the grass *Anthephora* (Jewer and Incoll 1980), and inhibition of stomatal closure was observed in the *amp1-1* cytokinin overproducing mutant of Arabidopsis (Tanaka et al. 2006), while in the monocot *Commelina*, inhibition of stomatal opening in response to a high concentration of cytokinin has been reported (Blackman and Davies 1983). Exogenous cytokinins, like auxins, can inhibit ABA-induced stomatal closure in diverse species (Blackman and Davies 1983; Das et al. 1976; Jewer and Incoll 1980; Stoll et al. 2000; Tanaka et al. 2006). Recently it has been shown that in darkness, cytokinin induces stomatal opening by decreasing H$_2$O$_2$ levels and NO levels within guard cells (She and Song 2006; Song et al. 2006).

Ethylene

The gaseous plant hormone ethylene generally promotes senescence of plant organs, leaf abscission, fruit ripening, root hair development, stem elongation of aquatic plants, and adventitious root formation. Ethylene retards stem elongation and promotes stem thickening in terrestrial plants. Ethylene also plays a critical signaling role during biotic stress responses (Bleecker and Kende 2000; Guo and Ecker 2004). In three dicot species, common bean (*Phaseolus vulgaris* L.), cotton (*Gossypium hirsutum* L.) and miniature rose (*Rosa hybrida* L., cv Bluesette), it has been shown that the rate of ethylene production is not affected during progressive soil drying (Morgan et al. 1990), although the imposition of rapid drought stress leads to ethylene production in wheat (Narayana et al. 1991). In maize plants with compromised ability for ABA-production, higher ethylene production has been shown in response to moderate water stress but not in response to severe water deficits (Voisin et al. 2006). In non-aquatic plants, both stomatal closure and increased biosynthesis of ethylene occur during flooding, suggesting that ethylene (along with ABA) could be one of the causative agents of stomatal closure under these conditions (Dat et al. 2004; Jackson 2002). Taken together, these studies suggest that the interplay between water stress and ethylene production is highly species-dependent.

Ethylene causes extra stomatogenesis in both Arabidopsis leaves (Serna and Fenoll 1996) and cucumber hypocotyls (Kazama et al. 2004). As discussed below, ethylene has been linked to promotion of both stomatal closure (Pallas and Kays 1982) and stomatal opening

(Levitt et al. 1987; Madhavan et al. 1983; Merritt et al. 2001).

Exogenous application of ethylene gas, ethephon (an ethylene-releasing compound), or ACC (the immediate precursor of ethylene) all promote stomatal closure in Arabidopsis leaves (Desikan et al. 2006). Ethylene-induced stomatal closure is inhibited by 1-methylcyclopropane (1-MCP), a competitive inhibitor of the ethylene receptor, and is reduced or absent in *etr1* ethylene receptor mutants, as well as in the ethylene signaling mutants *ein2-1* and *arr2* (Desikan et al. 2006). As discussed previously, H_2O_2 is one of the key signaling molecules in ABA-induced stomatal closure, and ethylene-mediated stomatal closure is dependent on H_2O_2, generated by the NADPH oxidase AtrbohF (Desikan et al. 2006).

Unexpectedly, given the above data, ethylene or its precursor, ACC, also have been shown to oppose stomatal closure, specifically, ABA-stimulated stomatal closure in isolated epidermal peels of Arabidopsis (Tanaka et al. 2005; Tanaka et al. 2006). ABA induction of stomatal closure is also suppressed in plants of the ethylene-overproducing mutant *eto1-1* (Tanaka et al. 2005). Ethylene biosynthesis/signalling is also required for the opposing effects of both cytokinins and auxins on ABA-induction of stomatal closure, since these opposing effects are not observed in the *ein3-1* ethylene-insensitive mutant, or in the presence of 1-MCP (Tanaka et al. 2006). Ethylene does not affect dark-induced stomatal closure suggesting that its inhibitory role in guard cells is specific to the ABA response.

Because ethylene alone promotes stomatal closure whereas ethylene in concert with other hormones opposes stomatal closure, unique and as yet little-understood cross-talk mechanisms must be activated in the presence of multiple hormonal stimuli.

Brassinosteroids (BRs)

Brassinosteroids are growth-promoting polyhydoxylated steroidal plant hormones which positively influence seed germination, stem elongation, vascular differentiation and fruit ripening (Clouse and Sasse 1998; Steber and McCourt 2001; Symons et al. 2006), pollen tube growth (Hewitt et al. 1985), and leaf epinasty (Heping and Shankun 1995; Schlagnhaufer and Arteca 1985). In cucumber, a role for BRs in promoting photosynthesis has been reported (Yu et al. 2004). Also, BRs have been implicated in the activation of the V-ATPase, which possibly plays a role in hypocotyl elongation (Schumacher et al. 1999). In addition, BR-stimulated plasma membrane hyperpolarization and cell expansion in Arabidopsis suspension cells have been demonstrated (Zhang et al. 2005).

Whether water stress causes changes in BR levels has not been investigated in many plant species. Recently, in pea, it has been shown that the level of castasterone, a biologically active BR, is not significantly altered by water stress (Jager et al. 2008), suggesting that the response of plants during water stress is not regulated by endogenous BR levels. In addition, in pea, by using BR-deficient mutants and BR-perception mutants, Jager et al. (2008) showed that endogenous BRs or perception of same are not required for plants to respond to water stress. However, exogenous application of BRs induces water stress tolerance in diverse plant species, including cucumber (Pustovoitova et al. 2001), wheat (Sairam 1994), *Phaseolus vulgaris* (Upreti and Murti 2004), Arabidopsis, and *Brassica napus* (Kagale et al. 2007; Krishna 2003), raising the question of whether exogenous vs. endogenous BRs have different roles in water stress, or whether the roles of BRs are species-specific.

Brassinosteroids regulate stomatal development and function, and provide another example of a hormone that cross-talks with ABA during stomatal regulation. Increased stomatal density has been observed in the Arabidopsis BR biosynthetic mutant *bul1/dwf7* in comparison to wild type plants (Catterou et al. 2001). Jackpine seedlings pretreated with homobrassinolide show delayed stomatal closure in response to water stress (Rajasekaran and Blake 1999). In contrast, the specific BR, brassinolide (BL), promotes stomatal closure and inhibits stomatal opening in epidermal peels of *Vicia faba* (Haubrick et al. 2006). In addition, the role of BL has been studied in ion channel regulation in guard cell protoplasts of *Vicia faba*, where it has been shown to inhibit the inwardly rectifying K^+ channels that mediate K^+ uptake during stomatal opening.

In *Vicia faba*, co-application of BL did not alter the extent of stomatal response to a given concentration of ABA, while in whole-plant physiological observations in sorghum, co-application of epibrassinolide and ABA resulted in an increased drought-protective effect over that observed with ABA alone (Xu et al. 1994a, b). Conversely, a BR-deficient mutant of Arabidopsis, *sax1*, was reported to show enhanced stomatal closure in response to ABA (Ephritikhine et al. 1999). In Arabidopsis, early induction of drought-inducible genes *RD29A*, *ERD10* and *RD22* in response to epibrassinolide has been shown (Kagale et al. 2007). This suggests possible cross-talk between ABA and BR signaling. Taken together, these observations suggest that interactions between BRs, ABA, and guard cell output may occur in a species-specific manner (Haubrick and Assmann 2006; Haubrick et al. 2006).

There may also be interactions between BRs and ethylene. An inductive effect of BRs on expression of ACC synthase in mungbean and Arabidopsis has been reported (Vert et al. 2005; Yi et al. 1999), and it has been suggested

that exegenous application of BRs promotes tomato ripening by increasing endogenous ethylene levels (Vardhini and Rao 2002). It would be interesting to assess whether ethylene acts as a signaling intermediate during BR-mediated stomatal regulation.

Jasmonates

Jasmonates are lipid-derived plant hormones regulating vegetative and reproductive growth, and defense responses against abiotic stresses (UV light and ozone), insects and necrotrophic pathogens (Katsir et al. 2008a). The coronatine-insensitive1 (COI1) gene encodes an F-box protein that is required for many jasmonate-mediated responses (Benedetti et al. 1995; Feys et al. 1994; Xie et al. 1998). The jasmonate ZIM-domain (JAZ) proteins are transcriptional repressors and play a critical regulatory role in jasmonate-mediated signaling (Staswick 2008). Methyl esters (MeJA), glycosyl esters and amide-linked amino acid conjugates (JA-Ile, JA-Leu, JA-Val and JA-Phe) are some of the different varieties and derivatives of jasmonic-acid-related compounds found in plants (Kramell et al. 1995). MeJA had been considered as one of the most potent forms of JA in signaling, but recently it has been established that JA-Ile is the most active derivative of JA (Guranowski et al. 2007; Staswick and Tiryaki 2004; Staswick 2008; Thines et al. 2007). JA-Ile promotes the interaction of COI1 and JAZ1. This complex subsequently causes degradation of JAZ1 proteins via the 26S proteasome, and this degradation promotes JA signaling. JA-Val, JA-Leu and JA-Ala are bioactive JA-derivatives (Katsir et al. 2008b). In contrast, MeJA does not promote COI1-JAZ1 interaction, indicating MeJA may not be an active JA derivative. It is yet to be determined whether MeJA promotes interaction of COI1 with any other specific member of JAZ family proteins or whether MeJA is metabolically converted to bio-active jasmonate derivatives in order to transduce signals (Thines et al. 2007).

It has been proposed that JA could be an important player for stomatal closure during drought stress based on its accumulation during drought (Creelman and Mullet 1997) and its positive regulatory role in stomatal closure (Gehring et al. 1997; Munemasa et al. 2007; Suhita et al. 2003, 2004). Water-stressed soybean leaves showing a 15% loss of fresh weight accumulated 5-fold more JA within 2 h but the level of JA declined to that of control plants by 4 h (Creelman and Mullet 1995). Also, endogenous JA levels can increase more than 50-fold in pathogen-inoculated leaves, while a greater than 2-fold induction of JA levels has been shown in systemic leaves of Arabidopsis plants in response to infection by Alternaria brassicicola

(Penninckx et al. 1996). The stomatal physiology of the response to pathogen-induced JA in plants is not known.

MeJA-mediated stomatal closure has been associated with cytoplasmic alkalinization in guard cells, production of ROS (via AtrbohD/F) and NO, and activation of K^+ efflux channels (Evans 2003) and slow anion channels (Gehring et al. 1997; Munemasa et al. 2007; Suhita et al. 2003; Suhita et al. 2004). These effects are similar to those of ABA, suggesting an overlapping use of signaling components for stomatal closure. This idea is supported by observations of MeJA hyposensitivity of stomatal closure in the ost1 (ABA hyposensitive) mutant, and reduced ABA-mediated stomatal closure in the jar1 (MeJA insensitive) mutant (Suhita et al. 2004).

coi1 mutants do not show stomatal closure, ROS or NO production, or activation of slow anion channels or Ca^{2+} permeable channels in response to MeJA, but do so in response to ABA, suggesting that COI1 is required for MeJA signaling but not ABA signaling in guard cells (Munemasa et al. 2007). MeJA-induced stomatal closure has also been studied in the ABA-insensitive protein phosphatase 2C mutant, abi2-1. In this mutant, stomatal closure is not observed in response to either MeJA or ABA, but production of ROS and NO in response to both MeJA and ABA are retained. Taken together, these results indicate that COI1 functions upstream of ROS and NO in MeJA but not ABA signaling, while ABI2 functions downstream of ROS and NO after the MeJa- and ABA-signaling pathways have converged.

As discussed in previous sections, drought can alter the balance of several different hormones. To specifically address the role of ABA in JA-mediated stomatal regulation at the whole plant level, the JA-mediated stomatal response has been studied in ABA-biosynthetic mutant sitiens tomato plants. When the petioles of sitiens were incubated in JA, they did not show stomatal closure as assessed by gas-exchange measurements; however, when petioles were pre-incubated with ABA, they showed stomatal closure in response to JA (Herde et al. 1997). This suggests that, in tomato, the JA-mediated stomatal response requires ABA. In soybean, exogenous application of MeJA did not affect endogenous ABA levels, but water-stressed barley seedlings pretreated with JA showed more than 4-fold accumulation of ABA in comparison to water stressed-barley seedlings not pretreated with JA. This indicates a role for JA in ABA biosynthesis under water stress conditions (Bandurska et al. 2003). Many drought-responsive genes are regulated by MeJA (Huang et al. 2008) and several MeJA-regulated, drought-responsive genes are also regulated by ABA with similar expression kinetics (Huang et al. 2008; Nemhauser et al. 2006). These data support overlapping use of signaling components by ABA and MeJA.

Salicylic acid (SA)

Salicylic acid (SA) is a phenolic compound with key roles in pathogen defense, thermogenesis and flowering (Dempsey et al. 1999; Raskin 1992). While a 2-fold accumulation of SA has been reported in water-stressed tomato leaves (this change, however, is not statistically significant) (Schmelz et al. 2003), in whole-plant physiological observations in Zea mays, exogenous application of SA suppressed drought tolerance (Németh et al. 2002). Nevertheless, it has been reported that SA plays a positive regulatory role in stomatal closure. Application of SA induces production of ROS (Dong et al. 2001; Mori et al. 2001) and leads to stomatal closure in Vicia faba and Commelina communis (Lee 1998; Manthe et al. 1992; Mori et al. 2001). It has been suggested that SA mediates ROS production not via NADPH oxidases but rather via a peroxidase-catalyzed reaction. SA-mediated production of ROS may lead to elevation of cytosolic Ca^{2+}, thereby promoting stomatal closure (Mori et al. 2001) and deterring pathogen invasion via stomatal openings (Melotto et al. 2006).

Stomatal closure in response to bacterial pathogens is compromised in transgenic NahG plants (deficient in SA) and in the SA biosynthetic mutant eds16-2, indicating that SA is required for stomatal defense (Melotto et al. 2008). It has been proposed that it is the combinatorial action of SA and ABA signaling pathways that mediates stomatal closure in response to bacterial pathogens (Melotto et al. 2006). A role for ABA in defense-evoked stomatal closure has also been confirmed: the ABA-insensitive ost1 mutants do not show stomatal closure in response to flg22, a Pathogen Associated Molecular Pattern (PAMP) elicitor, and the ABA-deficient aba3-1 mutant does not show stomatal closure in response to the bacterial pathogen Pst DC3000 (Melotto et al. 2006). In response to pathogens, endogenous SA levels can be increased 10-to-100-fold in local leaves and up to 10-fold in systemic leaves (Dempsey et al. 1999; Wildermuth et al. 2001). It is yet to be discovered whether basal levels of SA are sufficient for stomatal closure in response to PAMP elicitors or whether these elevated levels are required. The status of stomata during activation of systemic-acquired resistance (SAR) is also as yet unknown. It would be interesting to discover (at the whole-plant level), how stomata behave during activation of SAR, especially since exogenous treatment with ABA suppresses the induction of SAR in Arabidopsis, while activation of SAR suppresses induction of ABA biosynthesis-related genes (Yasuda et al. 2008). It is well known that SA is one of the important players for activation of SAR (Delaney et al. 1994; Gaffney et al. 1993). Although both ABA and SA play positive roles for stomatal closure in response to PAMP elicitors, activation of SAR negatively regulates ABA signaling (Yasuda et al. 2008). This shows condition-specific positive/negative interaction among ABA and SA.

Conclusions and perspectives

In summary, ABA, JAs, BRs and SA are positive regulators of stomatal closure, while auxins and cytokinins are generally positive regulators of stomatal opening. In contrast, ethylene plays a dual regulatory role on stomatal apertures in a condition- and species-specific manner. Interaction of auxin, cytokinin, or ethylene with ABA inhibits ABA-mediated stomatal closure. Interaction of ABA and SA positively regulates stomatal closure, and impedes invasion of bacterial pathogens, while genetic analyses of components involved in stomatal regulation by ABA and JAs have identified commonalities in the signaling pathways of these two hormones. In addition to ABA, the hormones JA, auxin, cytokinin, ethylene, BRs and gibberellins all modulate expression of drought-related genes (Huang et al. 2008; Nemhauser et al. 2006), suggesting cross-talk by different signaling pathways during drought stress, but the effects of such cross-talk on the guard cell transcriptome have yet to be assessed.

The roles of many hormones in stomatal function have been studied by exogenous application of specific hormones, which may not always mimic the effects of changes in endogenous hormone levels. Furthermore, exogenous vs. endogenous effects may vary between tissue types and species. Simultaneous quantification of different phytohormones in guard cells during open and closed conditions would give a more realistic view concerning their roles in stomatal functions. It also would be interesting to study stomatal function more extensively in different hormone biosynthetic mutants; a study such as this might uncover the regulatory roles played by specific hormones.

Despite recent progress on hormonal control of stomatal function, many questions remain unanswered. For example, ethylene plays a positive regulatory role in stomatal closure but acts antagonistically to exogenous ABA: what is the molecular mechanism? While either ABA or JAs promote stomatal closure and the JA-mediated stomatal response requires exogenous ABA in ABA-deficient tomato, stomatal behavior in response to co-application of these hormones has not yet been studied in wild type plants: will ABA and JAs act synergistically, additively or otherwise to achieve maximal stomatal closure?

Many plant hormonal responses are developmental in nature, whereas hormonal regulation of stomatal apertures is a reversible, non-developmental process. Are the subcellular targets of hormones discovered in guard cells (e.g. ion channels) also cellular targets of hormonal regulation in

irreversible developmental processes, and in specific cell types other than guard cells? Experiments in years to come are anticipated to answer these and other questions through the concerted application of a range of molecular, genetic, cell biology, and biochemical techniques. A final key question is whether stomatal regulatory mechanisms, which to date have been explored primarily in dicot species, prevail in other species as well, and in particular whether they occur in the graminaceous species with their unique guard cell morphology and dominance in agroecosystems. Thus, the ultimate challenge is a comparative analysis of hormonal regulation of stomatal function among the diverse plant species that feed and fuel the planet.

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