Omics Approaches Toward Defining the Comprehensive Abscisic Acid Signaling Network in Plants

Takuya Yoshida, Junro Mogami and Kazuko Yamaguchi-Shinozaki*

Laboratory of Plant Molecular Physiology, Graduate School of Agricultural and Life Sciences, The University of Tokyo, Tokyo, 113-8657 Japan
*Corresponding author: E-mail, akys@mail.ecc.u-tokyo.ac.jp; Fax, +81-3-5641-8009.
(Received March 4, 2015; Accepted April 13, 2015)

ABA is a plant hormone that plays crucial roles in controlling cellular and physiological responses to osmotic stress and in developmental processes. Endogenous ABA levels are increased in response to a decrease in water availability in cells, and ABA sensing and signaling are thought to be mediated according to the current model established in *Arabidopsis thaliana*, which involves pyrabactin resistance 1 (PYR)/PYR1-like (PYL)/regulatory components of ABA receptor (RCAR), protein phosphatase 2C (PP2C) and sucrose non-fermenting-1 (SNF1)-related protein kinase 2 (SnRK2). These core components of ABA signaling have a pivotal role in stress-responsive gene expression and stomatal regulation. However, because a limited number of their upstream and downstream factors have been characterized, it is still difficult to define the comprehensive network of ABA signaling in plants. This review focuses on current progress in the study of PYR/PYL/RCARs, PP2Cs and SnRK2s, with particular emphasis on omics approaches, such as interactome and phosphoproteome studies. Moreover, the role of ABA in plant growth and development is discussed based on recent metabolomic profiling studies.

Abbreviations: ABC transporter, ATP-binding cassette transporter; ABI1/2/5, ABA insensitive 1/2/5; ABRE, ABA-responsive element; AHG3, ABA-hypersensitive germination 3; AIB1, ABA-induced basic helix–loop–helix 1; AKS1, ABA-responsive kinase substrate 1; AREB/ABF, ABRE-binding protein/ABRE-binding factor; bHLH, basic helix–loop–helix; BIN2, brassinosteroid-insensitive 2; CAR, C2-domain ABA-related; CBL, calcineurin B-like; CIPK, CBL-interacting protein kinase; CO, CONSTANS; CPK, calcium-dependent protein kinase; EEL, enhanced Em level; FBH3, flowering bHLH 3; FRET, fluorescence resonance energy transfer; GHR1, guard cell hydrogen peroxide-resistance 1; GSK3, glycogen synthase kinase 3; HAB1, hypersensitive to ABA 1; HAI1, highly ABA-induced PP2C gene 1; ICE1, inducer of CBF expression 1; KAT1, potassium channel in *Arabidopsis thaliana* 1; MAP3K, mitogen-activated protein kinase kinase kinase; MAPK, mitogen-activated protein kinase; MOS3, modifier of snc1, 3; MPK, mitogen-activated protein kinase; PP2C, protein phosphatase 2C; PYR/PYL/RCAR, pyrabactin resistance 1/PYR1-like/regulatory components of ABA receptors; SLAC1, slow anion channel-associated 1; SLAH3, SLAC1 homolog 3; SnRK2, sucrose non-fermenting-1 (SNF1)-related protein kinase 2; SNS1, SnRK2-substrate 1; START domain, steroidogenic acute regulatory protein-related lipid transfer domain; TAF5, TATA-binding protein-associated factor 5; XRN3, exoribonuclease 3.

Introduction

ABA is a plant hormone that regulates diverse processes, including seed maturation and germination, stomatal closure and osmotic stress-responsive gene expression. In the current model established in *Arabidopsis thaliana*, ABA sensing and signaling are mediated by three classes of proteins: pyrabactin resistance 1 (PYR)/PYR1-like (PYL)/regulatory components of ABA receptor (RCAR), protein phosphatase 2C (PP2C) and sucrose non-fermenting-1 (SNF1)-related protein kinase 2 (SnRK2). Under normal growth conditions, in which cellular ABA levels are low, group-A PP2Cs interact with subclass III SnRK2s and inhibit their kinase activities. When ABA accumulates in cells under osmotic stress conditions, such as drought and high salinity, ABA is perceived by PYR/PYL/RCAR receptors that inhibit the phosphatase activities of PP2Cs. Ternary complexes composed of ABA–PYR/PYL/RCAR–PP2C enable the activation of SnRK2s, resulting in the phosphorylation of downstream substrates, such as transcription factors and membrane channel proteins (**Fig. 1**). Thus, major advances have been made toward understanding the molecular basis of the core components of ABA signaling in plants. However, due to the lack of knowledge regarding their upstream and downstream factors, it is still challenging to obtain a comprehensive picture of the ABA signaling network. In this review, we highlight recent progress in the study of PYR/PYL/RCARs, group-A PP2Cs and subclass III SnRK2s in ABA signaling, with particular emphasis on global analyses, including interactome and phosphoproteome profiling. Moreover, the role of ABA in plant growth and development is discussed based on recent studies of metabolomic profiling.

PYR/PYL/RCARs Have Overlapping but Distinct Roles as ABA Receptors

A group of steroidogenic acute regulatory protein-related lipid transfer (START) domain proteins, termed PYR/PYL/RCARs,
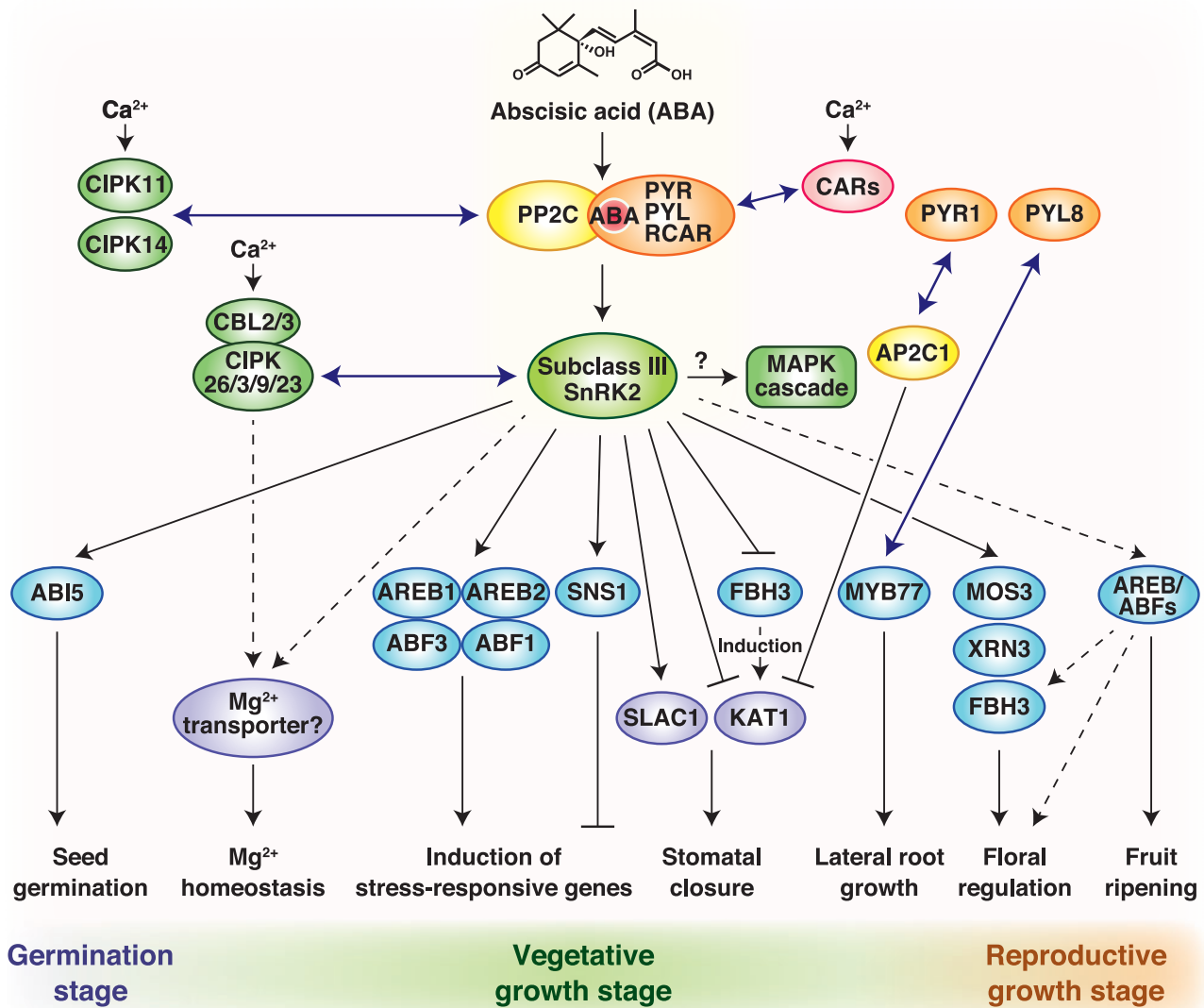
Fig. 1 Schematic model of the ABA signaling pathway, which is mediated by novel signaling components discovered in recent omics studies as well as by the core components PYR/PYL/RCARs, group-A PP2Cs and subclass III SnRK2s. In addition to the core components, several protein kinases/protein phosphatases (depicted as green and yellow ellipses, respectively) are key players in the regulation of ABA-mediated physiological responses during the life cycle of plants. Several PYR/PYL/RCAR proteins (represented by orange ellipses) are also able to regulate ABA responses independent of group-A PP2Cs. C2-domain ABA-related (CAR) proteins are shown as pink ellipses. Downstream targets involved in transcriptional regulation and ion transport are shown as blue and purple ellipses, respectively. Physical interactions identified by interactome analyses are depicted as bidirectional blue arrows. The dashed lines indicate possible but unconfirmed routes. Due to space constraints, not all interacting proteins and/or substrates of the core components are shown. See the main text for details. AREB1, AREB1/ABF2; AREB2, AREB2/ABF4; CIPK3, SnRK3.17/CIPK3; CIPK9, SnRK3.12/CIPK9; CIPK11, SnRK3.22/CIPK11; CIPK14, SnRK3.15/CIPK14; CIPK23, SnRK3.23/CIPK23; FBH3, FBH3/AKS1; PYL8, PYL8/RCAR3; and PYR1, PYR1/RCAR11.

has been demonstrated to act as functional ABA receptors in plants (for reviews, see Cutler et al. 2010, Hubbard et al. 2010, Raghavendra et al. 2010, Weiner et al. 2010). Structural studies (Melcher et al. 2009, Miyazono et al. 2009, Yin et al. 2009) have revealed that PYR/PYL/RCARs harbor binding sites for group-A PP2Cs, which are the key negative regulators in ABA signaling, as well as for ABA. Currently, PYR/PYL/RCARs can be grouped into two subclasses according to their oligomeric states and appear to function via one of two different mechanisms (Miyakawa et al. 2013). For example, PYR1/RCAR11,

PYL1/RCAR12, PYL2/RCAR14 and PYL3/RCAR13, which form homodimers in the absence of ABA, are released as monomers following ABA binding and subsequently interact with group-A PP2Cs. In contrast, PYL4/RCAR10, PYL5/RCAR8, PYL6/RCAR9, PYL8/RCAR3, PYL9/RCAR1 and PYL10/RCAR4 behave as monomers in both the presence and absence of ABA, and these monomers can inhibit group-A PP2Cs regardless of ABA binding. The inhibition of group-A PP2Cs by PYR/PYL/RCARs in the absence of ABA has been observed in vitro and in protoplasts (Fujii et al. 2009, Hao et al. 2011). In addition,

ectopically expressed ABA insensitive 1 (ABI1) fused with yellow fluorescent protein has been shown to interact with PYR/PYL/RCARs in Arabidopsis with or without ABA application (Nishimura et al. 2010). Although it is still unclear whether structural differences among PYR/PYL/RCARs are reflected in variations in their biological functions, the roles of PYR/PYL/RCARs in plants have been extensively studied since they were first identified as ABA receptors.

Given that the protein sequences of 14 members of Arabidopsis PYR/PYL/RCARs are highly homologous, these proteins are thought to have redundant functions in ABA signaling. In fact, studies of multiple mutants of PYR/PYL/RCARs have demonstrated that PYR1/RCAR11, PYL1/RCAR12, PYL2/RCAR14, PYL4/RCAR10, PYL5/RCAR8 and PYL8/RCAR3 have overlapping roles in the regulation of seed germination, stomatal closure and gene expression in ABA signaling (Park et al. 2009, Gonzalez-Guzman et al. 2012). In contrast to the other 13 PYR/PYL/RCARs, PYL13/RCAR7 has three variant amino acid residues in a conserved ABA-binding pocket; therefore, its roles in ABA sensing have remained obscure. Although two prior studies have claimed that PYL13/RCAR7 selectively inhibits PP2Cs in an ABA-independent manner and does not bind ABA (Li et al. 2013, Zhao et al. 2013), a recent study has revealed that it inhibits the phosphatase activities of ABI1, ABI2 and PP2CA/ABA-hypersensitive germination 3 (AHG3), but not that of hypersensitive to ABA 1 (HAB1), at nanomolar ABA levels (Fuchs et al. 2014). Moreover, Arabidopsis plants ectopically expressing PYL13/RCAR7 exhibit increased sensitivity to ABA during seed germination and stomatal closure. Although the ABA sensitivity of the *rcar7* mutant during seed germination is comparable with that of wild-type plants, the *rcar7 rcar9* double mutant shows ABA insensitivity. Taken together, these findings suggest that PYL13/RCAR7 functions as an ABA co-receptor during early seedling development and partners with specific PP2Cs.

In addition, several recent studies have suggested that the role of PYL8/RCAR3 is overlapping with but distinct from that of other PYR/PYL/RCARs. Two large-scale protein–protein interaction studies have found that PYL8/RCAR3 interacts with transcription factors such as MYB77 (Arabidopsis Interactome Mapping Consortium 2011, Lumba et al. 2014). This finding has been further confirmed by a study showing that PYL8/RCAR3, but not other PYR/PYL/RCARs, interacts with MYB77 and its homologs (Zhao et al. 2014). The interaction between PYL8/RCAR3 and MYB77 promotes the DNA binding affinity and transcriptional activity of MYB77. Furthermore, the lateral root growth of the *pyl8* and *myb77* mutants is more strongly inhibited by ABA compared with that of wild-type plants. These data are probably in accordance with an observation that the *pyl8* mutant displays decreased sensitivity to ABA during primary root growth compared with wild-type plants and other *pyr/pyl* single mutants (Antoni et al. 2013). Because MYB77 modulates auxin signaling during lateral root development together with auxin response factors (Shin et al. 2007), PYL8/RCAR3 that is expressed in roots might be involved in auxin signaling, as well as in ABA signaling, by binding to MYB transcription factors (**Fig. 1**).

The identification of proteins that interact with PYR/PYL/RCARs has provided new insight into the regulation of their subcellular localization. A yeast two-hybrid screening assay with PYL4/RCAR10 as bait identified a family of small proteins that contain a lipid-binding C2 domain. These proteins are referred to as C2-domain ABA-related (CAR) proteins (Rodriguez et al. 2014). C2 domains, which are found in myriad eukaryotic proteins, are part of various enzymes that harbor catalytic domains, and they serve as Ca^{2+}-activated modules to promote targeting of catalytic activity to membranes. Although additional catalytic domains are absent in a number of small C2-domain proteins in plants, including CARs, these C2-domain proteins are thought to play roles as calcium sensors. Additionally, many C2-domain proteins exhibit Ca^{2+}-dependent phospholipid binding; indeed, CAR1 and CAR4 bind phospholipid vesicles in response to calcium in vitro (Rodriguez et al. 2014). Moreover, CAR1 and CAR4 mediate phospholipid binding of PYL1/RCAR12, PYL4/RCAR10, PYL6/RCAR9 and PYL8/RCAR3 in a Ca^{2+}-dependent manner. In plant cells, interactions between CARs and PYR/PYL/RCARs were observed in plasma membranes and in the nucleus. Additionally, *car* multiple mutants and Arabidopsis plants overexpressing *CAR* genes display reduced and increased sensitivity to ABA, respectively. Thus, CARs that mediate the interaction of PYR/PYL/RCARs with phospholipid vesicles in calcium signaling might be involved in the regulation of PYR/PYL/RCAR subcellular localization, which affects ABA signaling.

PP2C Phosphatases Form a Complex Network with a Variety of Proteins in ABA Signaling

Biochemical, molecular biological and structural analyses have shown that PYR/PYL/RCAR ABA receptors interact with and inhibit group-A PP2Cs. Consistently, previous genetic and molecular biological studies have revealed that group-A PP2Cs are key negative regulators in ABA signaling (Hirayama and Shinozaki 2007). In the Arabidopsis genome, there are 76 genes encoding PP2C-type serine/threonine phosphatases, nine of which are classified into group-A (Schweighofer et al. 2004). Considering the ABA sensitivities of their mutants and their expression patterns in response to ABA (Yoshida et al. 2006b, Fujita et al. 2009), 6–9 group-A PP2Cs are currently considered to be the core regulators of ABA signaling. As pivotal negative regulators of ABA signaling, group-A PP2Cs have been shown to interact with a variety of proteins, including kinases, transcription factors, metabolic enzymes and chromatin-remodeling complexes (Guo et al. 2002, Himmelbach et al. 2002, Ohta et al. 2003, Miao et al. 2006, Yoshida et al. 2006a, Saez et al. 2008). The signaling network including group-A PP2Cs has been further elucidated by the recent generation of a protein interaction map of ABA-responsive genes (Lumba et al. 2014). ABA-responsive genes identified by whole-genome transcript profiling of an ABA-deficient mutant, *aba2*, have been assessed in yeast two-hybrid assays, resulting in the identification of an interaction network consisting of 512 statistically significant protein–protein

interactions among 138 proteins. The interaction network, which includes three PYR/PYL/RCARs (PYR1/RCAR11, PYL4/RCAR10 and PYL8/RCAR3) and four group-A PP2Cs [ABI1, AHG3, HAB and highly ABA-induced PP2C gene 1 (HAI1)], but no subclass III SnRK2s, has revealed new interactions as well as previously documented interactions. In agreement with the previous literature, PP2Cs have been found to interact with a variety of proteins, including kinases, phosphatases, transcription factors and metabolic enzymes. In addition to subclass III SnRK2s, SnRK3.15, SnRK3.22 and mitogen-activated protein kinase kinase kinase δ4 (MAP3Kδ4) have also been identified as kinases that interact with PP2Cs (**Fig. 1**). Among members of the SnRK3/calcineurin B-like (CBL)-interacting protein kinase (CIPK) families, which are composed of 26 members in Arabidopsis and mediate various signaling pathways through interactions with CBL proteins (Weinl and Kudla 2009), SnRK3.15/CIPK14 and SnRK3.22/CIPK11 are close homologs, and their gain- and loss-of-function mutants exhibit decreased and increased ABA sensitivity during seed germination, respectively (Lumba et al. 2014). Moreover, transcriptome analysis has shown that SnRK3.15/CIPK14 induced by dexamethasone impairs the expression of ABA-inducible genes. In agreement with these results, it has been previously shown that ABI1 and ABI2 interact with several SnRK3/CIPKs (Ohta et al. 2003) and that SnRK3.17/CIPK3 is involved in the induction of gene expression in response to ABA, cold and high salinity (Kim et al. 2003). Collectively, SnRK3/CIPKs might be involved in PP2C-mediated ABA signaling. Interestingly, a subgroup of SnRK3/CIPKs, including CIPK26, SnRK3.12/CIPK9, SnRK3.17/CIPK3 and SnRK3.23/CIPK23, has recently been shown to interact with subclass III SnRK2s (Mogami et al. 2015) (**Fig. 1**). Furthermore, multiple mutants of SnRK3/CIPKs, as well as the *srk2d/e/i* and *aba2* mutants, have exhibited increased susceptibility to high levels of exogenous Mg^{2+}. Although ABA is a key plant hormone that functions under water deficit conditions, the observation that the hypersensitivity of the *aba2* mutant is rescued by ABA application suggests that basal ABA signaling mediated by subclass III SnRK2s has an important role in Mg^{2+} homeostasis. Notably, the increased sensitivity of the *cipk3 cipk9 cipk23 cipk26* quadruple mutant to high Mg conditions was concurrently reported by another group (Tang et al. 2015). Consistent with the interactions of these SnRK3/CIPKs with CBL2 and CBL3 at the vacuolar membrane, whole-vacuole Mg^{2+} currents are impaired in the *cbl2 cbl3* double mutant, which is also hypersensitive to high Mg conditions. Although Mg^{2+} transporters functioning downstream of SnRK2s and SnRK3/CIPKs have not yet been determined, emerging evidence suggests that the phosphorylation network downstream of ABA and calcium signaling is essential for Mg^{2+} homeostasis. As demonstrated by multiple studies, several calcium signaling proteins, such as calcium-dependent protein kinases (CPKs), are involved in ABA signaling via SnRK2s and ABA-responsive element (ABRE)-binding proteins (AREBs)/ABRE-binding factors (ABFs) (for a review, see Fujita et al. 2013). Further studies are needed to reveal the functional significance of the association between core ABA signaling mediated by PP2Cs and

SnRK2s and calcium signaling mediated by SnRK3/CIPKs and CPKs.

In addition to the crucial role of subclass III SnRK2s in ABA-dependent gene expression (Fujita et al. 2009), transcriptional regulation mediated by PP2Cs may also be important for ABA signaling. Indeed, an HD-Zip transcription factor, ATHB6, and bZIP transcription factors, such as AREB/ABFs, have been shown to function downstream of group-A PP2Cs (Himmelbach et al. 2002, Lynch et al. 2012). Moreover, HAB1 may be involved in ABA-inducible gene expression by interacting with SWI3B, which is an Arabidopsis ortholog of the yeast SWI3 subunit of SWI/SNF chromatin-remodeling complexes (Saez et al. 2008). The interactome study has revealed that many transcription factors interact with group-A PP2Cs and that individual interaction partners can be altered in response to different abiotic stresses (Lumba et al. 2014). Each gene involved in the interaction map exhibits similar expression patterns in response to osmotic and salt stress. However, taking into account the co-expression data, several protein–protein interaction pairs are differentially expressed. For example, *HAI1* and its interacting partners exhibit similar co-expression patterns under both osmotic and salt stress conditions, whereas *MAP3Kδ4* and its interacting partners show highly correlated gene expression in response to osmotic stress, but not salt stress. Likewise, the expression of a basic helix–loop–helix (bHLH) transcription factor gene, *ABA-induced basic helix–loop–helix 1 (AIB1)*, is highly correlated with the expression of its interacting proteins under salt stress. Given that transgenic seedlings expressing dexamethasone-inducible *AIB1* show increased sensitivity to salt, but not to mannitol, the function of AIB1 and its interacting proteins may be more closely related to salt stress than osmotic stress. Thus, the integration of the protein–protein interaction map with transcriptome data appears to be a novel approach to determining the factors that function in a stress-specific manner.

ABA has an essential role in stomatal regulation as well as stress-responsive gene expression. Several membrane proteins, including slow anion channel-associated 1 (SLAC1) and potassium channel in *Arabidopsis thaliana* 1 (KAT1), have been demonstrated to function in ABA signaling in guard cells (Joshi-Saha et al. 2011). Another interactome study further advanced the understanding of membrane proteins involved in ABA signaling (Jones et al. 2014b). A split-ubiquitin yeast two-hybrid screen of 3,286 Arabidopsis membrane and signaling proteins identified 12,102 protein interactions among 1,523 proteins. The further application of transcriptomic, genetic and other experimental data to the interaction network allowed for the generation of an ABA-related subnetwork as well as other plant hormone subnetworks. Notably, PYR1/RCAR11 and KAT1 have been found to interact with AP2C1 in the ABA signaling subnetwork (**Fig. 1**). AP2C1 is a member of group-B PP2Cs that has been shown to regulate negatively mitogen-activated protein kinase 4 (MPK4) and MPK6 in response to wounding and pathogens (Schweighofer et al. 2007). Moreover, the stomatal aperture of the *ap2c1* mutant is slightly increased (Brock et al. 2010). Given that the channel activity of KAT1 is completely inhibited by the co-expression of AP2C1 in oocytes

(Jones et al. 2014b), stomatal opening mediated by KAT1 might be negatively regulated by dephosphorylation by AP2C1 as well as phosphorylation by SnRK2 (Sato et al. 2009). Because the *ap2c1* mutant does not exhibit significant changes in ABA sensitivity during seed germination and ABA-inducible gene expression (Brock et al. 2010), further studies are needed to clarify whether ABA signaling mediated by PYR/PYL/RCARs is involved in the regulation of AP2C1. In addition to the interactome study, recent studies have provided new insights into the mechanism by which stomatal closure is mediated by ABA. SLAC1 is a slow anion channel that is activated by subclass III SnRK2s in an ABA-dependent manner. A recent study has identified a receptor-like kinase, guard cell hydrogen peroxide-resistant 1 (GHR1), that mediates the phosphorylation of SLAC1 (Hua et al. 2012). GHR1 is a receptor-like kinase localized to the plasma membrane that interacts with SLAC1 and ABI2, but not ABI1. Whereas SLAC1 activity is activated by GHR1 in oocytes, the co-expression of ABI2, but not ABI1, inhibits this activation. Taken together with the results of genetic studies of the *ghr1* and *ost1* mutants, it is likely that GHR1 phosphorylates SLAC1 independently of SRK2E/SnRK2.6/OST1 and is inhibited by ABI2. Moreover, it has been shown that ABI1 perturbs the interaction between SLAC1 homolog 3 (SLAH3) and CPK21 in the absence of ABA (Demir et al. 2013). SLAH3 is inhibited by ABI1 and activated by CPK21 in ABA signaling mediated by PYL9/RCAR1 (Geiger et al. 2011). Thus, as key negative regulators, group-A PP2Cs might form a complex signaling network with a myriad of proteins to fine-tune ABA signaling.

Phosphoproteomic Studies Have Identified Substrates of SnRK2 Kinases

In contrast to the key negative regulatory role of PP2Cs, subclass III SnRK2s are the pivotal activators of ABA signaling. Nine out of 10 Arabidopsis SnRK2 kinases are activated by osmotic stress, and subclass III SnRK2s, SRK2D/SnRK2.2, SRK2E/SnRK2.6/OST1 and SRK2I/SnRK2.3 are also strongly activated by ABA (Boudsocq et al. 2004). Extensive analyses of SnRK2s have been performed, revealing that the *srk2d/e/i* triple mutant exhibits an extreme ABA-insensitive phenotype with regard to seed germination, seedling growth, stomatal regulation and osmotic stress-responsive gene expression (Fujii and Zhu 2009, Fujita et al. 2009, Nakashima et al. 2009). Nonetheless, a limited number of substrates of SnRK2s, such as AREB/ABF transcription factors and the SLAC1 anion channel, were characterized until phosphoproteome studies of the *srk2d/e/i* (alias *snrk2.2/2.3/2.6*) triple mutant were conducted (Umezawa et al. 2013, Wang et al. 2013). A comparison of the phosphoproteomic profile of the *srk2d/e/i* mutant after ABA and dehydration stress treatments with the profile of wild-type plants revealed 32 phosphopeptides that are possible substrates of SnRK2s, including AREB/ABF transcription factors, SnRK2-substrate 1 (SNS1), AtMPK1 and AtMPK2 (Umezawa et al. 2013) (**Fig. 1**). SNS1, the expression of which is constitutive in both the presence and absence of abiotic and biotic stresses, is a protein of unknown function that is conserved in higher plants. Whereas

SNS1 phosphopeptides are increased in wild-type plants after ABA and dehydration stress treatments, SNS1 is not phosphorylated in the *srk2d/e/i* mutant. Moreover, the expression of ABA-responsive genes, such as *RD29B* and *RAB18*, is slightly enhanced under ABA treatment in the *sns1* mutant, which displays increased sensitivity to ABA during the post-germination stages. These findings suggest that SNS1 is a negative regulator that acts downstream of subclass III SnRK2s in ABA signaling. Previous reports have shown that the subgroup C1 mitogen-activated protein kinases (MAPKs) AtMPK1 and AtMPK2 are activated by diverse stimuli, including ABA, jasmonic acid, wounding and high salinity stress (Ortiz-Masia et al. 2007, Hwa and Yang 2008). The peptide identified in the activation loop of AtMPK1 and AtMPK2 has also been demonstrated to be phosphorylated in response to ABA and dehydration; however, it is not phosphorylated in the *srk2d/e/i* mutant (Umezawa et al. 2013). Given that the phosphorylation of the minimal MAPK target motif, [-(pS/pT)-P-], is impaired in the *srk2d/e/i* mutant, subclass III SnRK2s may directly or indirectly activate the phosphorylation states of MAPK cascades, which include AtMPK1 and AtMPK2, in response to ABA and dehydration stress. Although further studies are needed, a growing body of evidence indicates that MAPK pathways play key roles in ABA signaling as well as subclass III SnRK2s (for reviews, see Danquah et al. 2014, Umezawa et al. 2014).

Another phosphoproteomic study of the *srk2d/e/i* triple mutant identified 84 phosphopeptides that are candidate substrates for SnRK2s, most of which have been classified into the nucleotide binding, transcriptional regulation, chloroplast processes and signal transduction categories by gene ontology analysis (Wang et al. 2013). The transcriptional regulators identified correspond to eight proteins, including AREB1/ABF2, AREB3, enhanced Em level (EEL), flowering bHLH 3 (FBH3) and TATA-binding protein-associated factor 5 (TAF5). Three bZIP transcription factors, AREB1/ABF2, AREB2/ABF4 and ABF3, have been well characterized as transcription factors functioning downstream of SnRK2s (Furihata et al. 2006, Fujita et al. 2009) and have been shown to play key roles in ABA-responsive gene expression (Yoshida et al. 2010). Moreover, it has been recently shown that ABF1 is a functional homolog of AREB1/ABF2, AREB2/ABF4 and ABF3, and that a large number of genes functioning downstream of SnKR2s are impaired in the *areb1 areb2 abf3 abf1* quadruple mutant (Yoshida et al. 2015). In contrast to these AREB/ABFs, it has been found that AREB3, EEL, FBH3 and TAF5 are involved in developmental processes rather than osmotic stress-responsive gene expression (Ito et al. 2012, Mougiou et al. 2012, Fujita et al. 2013). Given that transcription factors other than AREB1/ABF2, AREB2/ABF4, ABF3 and ABF1 were not determined to be SnRK2 substrates in the aforementioned phosphoproteomic study (Umezawa et al. 2013), it is likely that osmotic stress-responsive gene expression downstream of SnRK2s is predominantly regulated by the four AREB/ABFs in ABA signaling during the vegetative stages (**Fig. 1**). Although the role of ABA has been well studied in osmotic stress signaling, ABA is also thought to play a role in cold stress signaling. A recent study suggests that SRK2E/SnRK2.6/OST1 is involved in cold signaling through its phosphorylation of

inducer of CBF expression 1 (ICE1), which is a key transcription factor in freezing tolerance (Ding et al. 2015). SRK2E/SnRK2.6/OST1 phosphorylates ICE1 and enhances its stability and transcriptional activity. Moreover, SRK2E/SnRK2.6/OST1 gain- and loss-of-function mutants show altered freezing tolerance. Consistently, the kinase activity of SRK2E/SnRK2.6/OST1 overexpressed in Arabidopsis seedlings is activated by cold stress as well as ABA. However, unlike ABA and osmotic stress treatments, cold stress does not activate SnRK2s in cultured cells (Boudsocq et al. 2004, Yoshida et al. 2006a). Given that ABA levels are modestly decreased during cold stress (Ding et al. 2015), cold stress might activate SRK2E/SnRK2.6/OST1 through ABA-independent mechanisms, which have not yet been determined.

The identification of FBH3 as a possible substrate of SnRK2s has further provided new insights into the role of SnRK2s in ABA signaling. FBH3 and its homologs are bHLH transcription factors that activate *CONSTANS* (*CO*) expression during floral regulation (Ito et al. 2012). A phosphoproteomic study demonstrating that FBH3 and other flowering-associated proteins, such as modifier of snc1, 3 (MOS3) and exoribonuclease 3 (XRN3), are candidate SnRK2 substrates might explain the early flowering phenotype of the *srk2d/e/i* mutant (Wang et al. 2013) (**Fig. 1**). An ABA-deficient mutant, *aba2*, has also been shown to exhibit early flowering (Domagalska et al. 2010), indicating that ABA may have a negative regulatory role in flowering. However, as discussed previously (Finkelstein 2013), ABA is thought to be involved in floral transition and to function in a complex manner. For example, *FBH3* and *CO*, which are induced by drought and ABA, were down-regulated in the *srk2d/e/i* mutant as well as in the *areb1 areb2 abf3 abf1* mutant, which shows late flowering (Yoshida et al. 2015). Thus, FBH3 might be regulated both transcriptionally and post-translationally by SnRK2s. This view is supported by a previous study revealing that ABA-responsive kinase substrate 1 (AKS1), which is identical to FBH3, is a substrate of SRK2E/SnRK2.6/OST1 in guard cells (Takahashi et al. 2013). In the absence of ABA, FBH3/AKS1 induces the expression of *KAT1*, which encodes the major inward-rectifying K$^+$ channel that is involved in stomatal opening, whereas the activity of FBH3/AKS1 is negatively regulated by SRK2E/SnRK2.6/OST1-dependent phosphorylation in response to ABA (**Fig. 1**). Because KAT1 is also negatively regulated by SRK2E/SnRK2.6/OST1-dependent phosphorylation (Sato et al. 2009), dual transcriptional and post-translational regulatory mechanisms governed by subclass III SnRK2s in ABA signaling might tightly control stomatal opening and flowering. Recent findings indicate that photoperiodic flowering regulators, such as CO, play a positive role in stomatal opening (Kinoshita et al. 2011, Ando et al. 2013, Kimura et al. 2015), and further studies of the substrates of SnRK2 will determine the functional consequences of SnRK2-mediated ABA signaling in stomatal and flowering regulation.

In parallel with the identification of SnRK2 substrates, global changes in phosphoproteins in response to ABA and osmotic stress have been investigated by quantitative isotope-assisted mass spectrometric methods (Kline et al. 2010, Stecker et al.

2014), enabling the identification of novel phosphoproteins and allowing for improved understanding of the phosphorylation networks involved in ABA signaling. Subclass III SnRK2s, which are activated by osmotic stress as well as ABA, have been suggested to be essential for both ABA-dependent and ABA-independent signaling under osmotic stress conditions (Fujita et al. 2013). However, previous in-gel kinase assays, which have demonstrated that osmotic stress directly activates SnRK2s in an ABA-independent manner (Yoshida et al. 2006a, Boudsocq et al. 2007), indicate the existence of SnRK2 activation mechanisms that are not mediated by PYR/PYL/RCAR–PP2C ABA receptor complexes. In agreement with this possibility, quantitative phosphoproteomic analysis has shown that SRK2D/SnRK2.2, SRK2E/SnRK2.6/OST1 and SRK2I/SnRK2.3 are highly phosphorylated following short-term ABA treatment but not short-term osmotic stress (Stecker et al. 2014). However, short-term osmotic stress rapidly increases the levels of phosphopeptides corresponding to kinases and signaling-related proteins, including subclass I SnRK2s (SRK2A/SnRK2.4, SRK2B/SnRK2.10, SRK2G/SnRK2.1 and SRK2H/SnRK2.5), RAF18, MAP4Kα1 and Vac14. These proteins might play significant roles in early signaling events in response to osmotic stress. Although the results of several biochemical and structural studies have suggested that the kinase activities of subclass III SnRK2s depend on the autophosphorylation and/or phosphorylation of the activation loop by a glycogen synthase kinase 3 (GSK3)-like kinase, brassinosteroid-insensitive 2 (BIN2) (Belin et al. 2006, Umezawa et al. 2009, Ng et al. 2011, Cai et al. 2014), the molecular mechanism underlying the ABA-independent activation of SnRK2s remains to be elucidated.

ABA Signaling is Involved in Metabolic Regulation

In addition to its critical role in the stress response, ABA is also involved in growth and developmental processes, such as seed maturation and the regulation of seed germination. As evidenced by its increased levels in seeds, ABA is one of the key plant hormones in seed maturation, and the molecular mechanism underlying the mediation of ABA signaling by transcription factors such as ABI5 has been extensively studied (Finkelstein 2013). However, little is known about the manner by which ABA regulates plant growth and development in vegetative tissues. Integrated transcriptome and metabolome analyses have revealed the roles of ABA-dependent and ABA-independent signaling in the metabolic changes occurring under osmotic stress conditions in Arabidopsis and rice (Urano et al. 2009, Maruyama et al. 2014). Moreover, the growth phenotypes and gene expression patterns of six Arabidopsis accessions grown under mild drought stress conditions have been recently analyzed, and it has been shown that ABA signaling, proline metabolism and cell wall adjustments promote a robust response to drought stress that occurs in different genetic backgrounds (Clauw et al. 2015). Cytokinin signaling is reportedly down-regulated by dehydration stress in Arabidopsis and rice (Maruyama et al. 2014) and is suggested

to play a key role in osmotic stress signaling together with ABA (Ha et al. 2012). In addition, transcriptome and metabolome profiling of developing Arabidopsis leaves grown under mild osmotic stress conditions has shown that cell proliferation and expansion are mainly regulated by ethylene and gibberellin signaling in leaves (Skirycz et al. 2010). Thus, the cross-talk that occurs between ABA signaling and other plant hormone pathways could be exploited to control rigorously the tolerance and growth of plants exposed to osmotic stress conditions.

Recent studies have shown that the key regulators in ABA signaling are involved in metabolomic regulation in various plant species. Arabidopsis SnRK1 kinases are essential for energy and stress signaling (Baena-González and Sheen 2008). SnRK1.1 has recently been shown to interact with and be inhibited by group-A PP2Cs, such as ABI1 and PP2CA/AHG3 (Rodrigues et al. 2013). In addition to the finding that *pp2c* mutants and Arabidopsis overexpressing SnRK1.1 were hypersensitive to sugar, genes induced by SnRK1.1 activation were found to overlap with ABA-inducible genes. Thus, group-A PP2Cs might play a role in processes involved in both ABA and energy signaling. Moreover, a role for ABA in fruit development has been postulated by several research groups (**Fig. 1**). Consistent with ABA accumulation during tomato fruit ripening (Zhang et al. 2009), metabolomic profiling has shown that SlAREB1, which is a close ortholog of Arabidopsis AREB/ABFs that functions in ABA signaling, regulates primary metabolism in tomato fruit (Bastías et al. 2014). Similarly, a grape ortholog of Arabidopsis AREB/ABFs, *VvABF2*, which is highly expressed in the berry, has been shown to be involved in phenolic compound synthesis and cell wall softening, as determined by transcriptome analysis of cultured cells overexpressing *VvABF2* (Nicolas et al. 2014). Additionally, strawberry orthologs of Arabidopsis PYR1/RCAR11, ABI1 and SRK2E/SnRK2.6/OST1 have been suggested to be involved in fruit ripening (Chai et al. 2011, Jia et al. 2013, Han et al. 2015). Although plant hormones such as ethylene, gibberellin and auxin have been extensively studied, these recent findings suggest that ABA signaling also has a substantial role in fruit development and ripening.

Conclusions and Future Perspectives

In this review, we focused on recent progress made toward greater understanding of the comprehensive ABA signaling network, in which PYR/PYL/RCARs, group-A PP2Cs and subclass III SnRK2s serve as the core components. Global analyses, such as interactome and phosphoproteome studies, have revealed that group-A PP2Cs and subclass III SnRK2s function as hubs in ABA signaling via a variety of substrates and interacting proteins (**Fig. 1**). In addition, metabolomic profiling has been performed to elucidate the manner in which ABA mediates plant growth and development. Our understanding of plant molecular biology is rapidly increasing due to technological advances that facilitate comprehensive analyses, including hormonome analysis (Kojima et al. 2009), lipidomic profiling (Bromke et al.

2015) and the construction of an artificial microRNA library (Hauser et al. 2013). In fact, genome-wide genetic screening using this library was performed to identify functionally redundant genes involved in ABA signaling (Hauser et al. 2013). In addition to the identification of ABA signaling components, the identification of ABA transporters has broadened our knowledge of the manner by which ABA is transported in plants (Umezawa et al. 2010). Recently, a study of the ATP-binding cassette (ABC) transporter ABCG25, which is an ABA exporter, showed that ABA that is synthesized in specific cells, such as phloem companion cells in vascular tissues, can be transported to guard cells (Kuromori et al. 2014). Furthermore, the fluorescence resonance energy transfer (FRET)-based reporters that have been developed enable the monitoring of the distribution and dynamics of ABA in plants (Jones et al. 2014a, Waadt et al. 2014). Thus, the future application of these omics approaches and technologies to the study of various plants will enable a deeper understanding of the mechanisms of ABA synthesis, metabolism, transport and signaling. This knowledge may be useful for the manipulation of drought stress tolerance in crops. In addition to numerous attempts to engineer the genes involved in ABA synthesis and signaling, an important step toward the maintenance of crop growth and yield during drought stress has recently been achieved using an engineered PYR1/RCAR11 ABA receptor in combination with an agrochemical (Park et al. 2015). Expression of a modified form of PYR1/RCAR11 caused sensitivity to the agrochemical mandipropamid at nanomolar concentrations and altered stomatal responses and drought stress tolerance in transgenic Arabidopsis and tomato. A chemical screening approach has also proven to be useful for the identification of chemicals that manipulate ABA responses in plants (Okamoto et al. 2013). Although a sulfonamide ABA agonist, quinabactin, inhibits the activity of HAB1 specifically through dimeric PYR/PYL/RCARs, drought stress tolerance in Arabidopsis and soybean was efficiently enhanced by application of quinabactin as well as ABA. Based on these studies, promising combinations of PYR/PYL/RCARs and agrochemicals that can maintain crop growth and yield might be identified in further investigations. Despite the considerable gap between achievements under controlled conditions and the improvement of agronomic traits in the field, our knowledge of the nature of ABA in plants should be useful for increasing crop production, which is a global concern.

Funding

Acknowledgments

Disclosures

References

Ando, E., Ohnishi, M., Wang, Y., Matsushita, T., Watanabe, A., Hayashi, Y. et al. (2013) TWIN SISTER OF FT, GIGANTEA, and CONSTANS have a positive but indirect effect on blue light-induced stomatal opening in Arabidopsis. *Plant Physiol.* 162: 1529–1538.

Antoni, R., Gonzalez-Guzman, M., Rodriguez, L., Peirats-Llobet, M., Pizzio, G.A., Fernandez, M.A. et al. (2013) PYRABACTIN RESISTANCE1-LIKE8 plays an important role for the regulation of abscisic acid signaling in root. *Plant Physiol.* 161: 931–941.

Arabidopsis Interactome Mapping Consortium. (2011) Evidence for network evolution in an Arabidopsis interactome map. *Science* 333: 601–607.

Baena-González, E. and Sheen, J. (2008) Convergent energy and stress signaling. *Trends Plant Sci.* 13: 474–482.

Bastías, A., Yañez, M., Osorio, S., Arbona, V., Gómez-Cadenas, A., Fernie, A.R. et al. (2014) The transcription factor AREB1 regulates primary metabolic pathways in tomato fruits. *J. Exp. Bot.* 65: 2351–2363.

Belin, C., de Franco, P.O., Bourbousse, C., Chaignepain, S., Schmitter, J.M., Vavasseur, A. et al. (2006) Identification of features regulating OST1 kinase activity and OST1 function in guard cells. *Plant Physiol.* 141: 1316–1327.

Boudsocq, M., Barbier-Brygoo, H. and Laurière, C. (2004) Identification of nine sucrose nonfermenting 1-related protein kinases 2 activated by hyperosmotic and saline stresses in Arabidopsis thaliana. *J. Biol. Chem.* 279: 41758–41766.

Boudsocq, M., Droillard, M.J., Barbier-Brygoo, H. and Laurière, C. (2007) Different phosphorylation mechanisms are involved in the activation of sucrose non-fermenting 1 related protein kinases 2 by osmotic stresses and abscisic acid. *Plant Mol. Biol.* 63: 491–503.

Brock, A.K., Willmann, R., Kolb, D., Grefen, L., Lajunen, H.M., Bethke, G. et al. (2010) The Arabidopsis mitogen-activated protein kinase phosphatase PP2C5 affects seed germination, stomatal aperture, and abscisic acid-inducible gene expression. *Plant Physiol.* 153: 1098–1111.

Bromke, M.A., Hochmuth, A., Tohge, T., Fernie, A.R., Giavalisco, P., Burgos, A. et al. (2015) Liquid chromatography high-resolution mass spectrometry for fatty acid profiling. *Plant J.* 81: 529–536.

Cai, Z., Liu, J., Wang, H., Yang, C., Chen, Y., Li, Y. et al. (2014) GSK3-like kinases positively modulate abscisic acid signaling through phosphorylating subgroup III SnRK2s in Arabidopsis. *Proc. Natl Acad. Sci. USA* 111: 9651–9656.

Chai, Y.M., Jia, H.F., Li, C.L., Dong, Q.H. and Shen, Y.Y. (2011) FaPYR1 is involved in strawberry fruit ripening. *J. Exp. Bot.* 62: 5079–5089.

Clauw, P., Coppens, F., De Beuf, K., Dhondt, S., Van Daele, T., Maleux, K. et al. (2015) Leaf responses to mild drought stress in natural variants of Arabidopsis thaliana. *Plant Physiol.* 167: 800–816.

Cutler, S.R., Rodriguez, P.L., Finkelstein, R.R. and Abrams, S.R. (2010) Abscisic acid: emergence of a core signaling network. *Annu. Rev. Plant Biol.* 61: 651–679.

Danquah, A., de Zelicourt, A., Colcombet, J. and Hirt, H. (2014) The role of ABA and MAPK signaling pathways in plant abiotic stress responses. *Biotechnol. Adv.* 32: 40–52.

Demir, F., Horntrich, C., Blachutzik, J.O., Scherzer, S., Reinders, Y., Kierszniowska, S. et al. (2013) Arabidopsis nanodomain-delimited ABA signaling pathway regulates the anion channel SLAH3. *Proc. Natl Acad. Sci. USA* 110: 8296–8301.

Ding, Y., Li, H., Zhang, X., Xie, Q., Gong, Z. and Yang, S. (2015) OST1 kinase modulates freezing tolerance by enhancing ICE1 stability in Arabidopsis. *Dev. Cell* 32: 278–289.

Domagalska, M.A., Sarnowska, E., Nagy, F. and Davis, S.J. (2010) Genetic analyses of interactions among gibberellin, abscisic acid, and brassinosteroids in the control of flowering time in Arabidopsis thaliana. *PLoS One* 5: e14012.

Finkelstein, R. (2013) Abscisic acid synthesis and response. *Arabidopsis Book* 11: e0166.

Fuchs, S., Tischer, S.V., Wunschel, C., Christmann, A. and Grill, E. (2014) Abscisic acid sensor RCAR7/PYL13, specific regulator of protein phosphatase coreceptors. *Proc. Natl Acad. Sci. USA* 111: 5741–5746.

Fujii, H., Chinnusamy, V., Rodrigues, A., Rubio, S., Antoni, R., Park, S.Y. et al. (2009) In vitro reconstitution of an abscisic acid signalling pathway. *Nature* 462: 660–664.

Fujii, H. and Zhu, J.K. (2009) Arabidopsis mutant deficient in 3 abscisic acid-activated protein kinases reveals critical roles in growth, reproduction, and stress. *Proc. Natl Acad. Sci. USA* 106: 8380–8385.

Fujita, Y., Nakashima, K., Yoshida, T., Katagiri, T., Kidokoro, S., Kanamori, N. et al. (2009) Three SnRK2 protein kinases are the main positive regulators of abscisic acid signaling in response to water stress in Arabidopsis. *Plant Cell Physiol.* 50: 2123–2132.

Fujita, Y., Yoshida, T. and Yamaguchi-Shinozaki, K. (2013) Pivotal role of the AREB/ABF–SnRK2 pathway in ABRE-mediated transcription in response to osmotic stress in plants. *Physiol Plant* 147: 15–27.

Furihata, T., Maruyama, K., Fujita, Y., Umezawa, T., Yoshida, R., Shinozaki, K. et al. (2006) Abscisic acid-dependent multisite phosphorylation regulates the activity of a transcription activator AREB1. *Proc. Natl Acad. Sci. USA* 103: 1988–1993.

Geiger, D., Maierhofer, T., Al-Rasheid, K.A., Scherzer, S., Mumm, P., Liese, A. et al. (2011) Stomatal closure by fast abscisic acid signaling is mediated by the guard cell anion channel SLAH3 and the receptor RCAR1. *Sci. Signal.* 4: ra32.

Gonzalez-Guzman, M., Pizzio, G.A., Antoni, R., Vera-Sirera, F., Merilo, E., Bassel, G.W. et al. (2012) Arabidopsis PYR/PYL/RCAR receptors play a major role in quantitative regulation of stomatal aperture and transcriptional response to abscisic acid. *Plant Cell* 24: 2483–2496.

Guo, Y., Xiong, L., Song, C.P., Gong, D., Halfter, U. and Zhu, J.K. (2002) A calcium sensor and its interacting protein kinase are global regulators of abscisic acid signaling in Arabidopsis. *Dev. Cell* 3: 233–244.

Ha, S., Vankova, R., Yamaguchi-Shinozaki, K., Shinozaki, K. and Tran, L.S. (2012) Cytokinins: metabolism and function in plant adaptation to environmental stresses. *Trends Plant Sci.* 17: 172–179.

Han, Y., Dang, R., Li, J., Jiang, J., Zhang, N., Jia, M. et al. (2015) FaSnRK2.6, an ortholog of Open Stomata 1, is a negative regulator of strawberry fruit development and ripening. *Plant Physiol.* 167: 915–930.

Hao, Q., Yin, P., Li, W., Wang, L., Yan, C., Lin, Z. et al. (2011) The molecular basis of ABA-independent inhibition of PP2Cs by a subclass of PYL proteins. *Mol. Cell* 42: 662–672.

Hauser, F., Chen, W., Deinlein, U., Chang, K., Ossowski, S., Fitz, J. et al. (2013) A genomic-scale artificial microRNA library as a tool to investigate the functionally redundant gene space in Arabidopsis. *Plant Cell* 25: 2848–2863.

Himmelbach, A., Hoffmann, T., Leube, M., Höhener, B. and Grill, E. (2002) Homeodomain protein ATHB6 is a target of the protein phosphatase ABI1 and regulates hormone responses in Arabidopsis. *EMBO J.* 21: 3029–3038.

Hirayama, T. and Shinozaki, K. (2007) Perception and transduction of abscisic acid signals: keys to the function of the versatile plant hormone ABA. *Trends Plant Sci.* 12: 343–351.

Hua, D., Wang, C., He, J., Liao, H., Duan, Y., Zhu, Z. et al. (2012) A plasma membrane receptor kinase, GHR1, mediates abscisic acid- and

hydrogen peroxide-regulated stomatal movement in Arabidopsis. *Plant Cell* 24: 2546–2561.

Hubbard, K.E., Nishimura, N., Hitomi, K., Getzoff, E.D. and Schroeder, J.I. (2010) Early abscisic acid signal transduction mechanisms: newly discovered components and newly emerging questions. *Genes Dev.* 24: 1695–1708.

Hwa, C.-M. and Yang, X.-C. (2008) The AtMKK3 pathway mediates ABA and salt signaling in Arabidopsis. *Acta Physiol. Plant.* 30: 277–286.

Ito, S., Song, Y.H., Josephson-Day, A.R., Miller, R.J., Breton, G., Olmstead, R.G. et al. (2012) FLOWERING BHLH transcriptional activators control expression of the photoperiodic flowering regulator CONSTANS in Arabidopsis. *Proc. Natl Acad. Sci. USA* 109: 3582–3587.

Jia, H.F., Lu, D., Sun, J.H., Li, C.L., Xing, Y., Qin, L. et al. (2013) Type 2C protein phosphatase ABI1 is a negative regulator of strawberry fruit ripening. *J. Exp. Bot.* 64: 1677–1687.

Jones, A.M., Danielson, J.A., Manojkumar, S.N., Lanquar, V., Grossmann, G. and Frommer, W.B. (2014a) Abscisic acid dynamics in roots detected with genetically encoded FRET sensors. *Elife* 3: e01741.

Jones, A.M., Xuan, Y., Xu, M., Wang, R.S., Ho, C.H., Lalonde, S. et al. (2014b) Border control—a membrane-linked interactome of Arabidopsis. *Science* 344: 711–716.

Joshi-Saha, A., Valon, C. and Leung, J. (2011) A brand new START: abscisic acid perception and transduction in the guard cell. *Sci. Signal.* 4: re4.

Kim, K.N., Cheong, Y.H., Grant, J.J., Pandey, G.K. and Luan, S. (2003) CIPK3, a calcium sensor-associated protein kinase that regulates abscisic acid and cold signal transduction in Arabidopsis. *Plant Cell* 15: 411–423.

Kimura, Y., Aoki, S., Ando, E., Kitatsuji, A., Watanabe, A., Ohnishi, M. et al. (2015) A flowering integrator, SOC1, affects stomatal opening in Arabidopsis thaliana. *Plant Cell Physiol.* 56: 640–649.

Kinoshita, T., Ono, N., Hayashi, Y., Morimoto, S., Nakamura, S., Soda, M. et al. (2011) FLOWERING LOCUS T regulates stomatal opening. *Curr. Biol.* 21: 1232–1238.

Kline, K.G., Barrett-Wilt, G.A. and Sussman, M.R. (2010) In planta changes in protein phosphorylation induced by the plant hormone abscisic acid. *Proc. Natl Acad. Sci. USA* 107: 15986–15991.

Kojima, M., Kamada-Nobusada, T., Komatsu, H., Takei, K., Kuroha, T., Mizutani, M. et al. (2009) Highly sensitive and high-throughput analysis of plant hormones using MS-probe modification and liquid chromatography–tandem mass spectrometry: an application for hormone profiling in Oryza sativa. *Plant Cell Physiol.* 50: 1201–1214.

Kuromori, T., Sugimoto, E. and Shinozaki, K. (2014) Intertissue signal transfer of abscisic acid from vascular cells to guard cells. *Plant Physiol.* 164: 1587–1592.

Li, W., Wang, L., Sheng, X., Yan, C., Zhou, R., Hang, J. et al. (2013) Molecular basis for the selective and ABA-independent inhibition of PP2CA by PYL13. *Cell Res.* 23: 1369–1379.

Lumba, S., Toh, S., Handfield, L.F., Swan, M., Liu, R., Youn, J.Y. et al. (2014) A mesoscale abscisic acid hormone interactome reveals a dynamic signaling landscape in Arabidopsis. *Dev. Cell* 29: 360–372.

Lynch, T., Erickson, B.J. and Finkelstein, R.R. (2012) Direct interactions of ABA-insensitive (ABI)-clade protein phosphatase (PP)2Cs with calcium-dependent protein kinases and ABA response element-binding bZIPs may contribute to turning off ABA response. *Plant Mol. Biol.* 80: 647–658.

Maruyama, K., Urano, K., Yoshiwara, K., Morishita, Y., Sakurai, N., Suzuki, H. et al. (2014) Integrated analysis of the effects of cold and dehydration on rice metabolites, phytohormones, and gene transcripts. *Plant Physiol.* 164: 1759–1771.

Melcher, K., Ng, L.M., Zhou, X.E., Soon, F.F., Xu, Y., Suino-Powell, K.M. et al. (2009) A gate–latch–lock mechanism for hormone signalling by abscisic acid receptors. *Nature* 462: 602–608.

Miao, Y., Lv, D., Wang, P., Wang, X.C., Chen, J., Miao, C. et al. (2006) An Arabidopsis glutathione peroxidase functions as both a redox transducer and a scavenger in abscisic acid and drought stress responses. *Plant Cell* 18: 2749–2766.

Miyakawa, T., Fujita, Y., Yamaguchi-Shinozaki, K. and Tanokura, M. (2013) Structure and function of abscisic acid receptors. *Trends Plant Sci.* 18: 259–266.

Miyazono, K., Miyakawa, T., Sawano, Y., Kubota, K., Kang, H.J., Asano, A. et al. (2009) Structural basis of abscisic acid signalling. *Nature* 462: 609–614.

Mogami, J., Fujita, Y., Yoshida, T., Tsukiori, Y., Nakagami, H., Nomura, Y. et al. (2015) Two distinct families of protein kinases are required for plant growth under high external Mg^{2+} concentrations in Arabidopsis. *Plant Physiol* 167: 1039–1057.

Mougiou, N., Poulios, S., Kaldis, A. and Vlachonasios, K. (2012) Arabidopsis thaliana TBP-associated factor 5 is essential for plant growth and development. *Mol. Breed.* 30: 355–366.

Nakashima, K., Fujita, Y., Kanamori, N., Katagiri, T., Umezawa, T., Kidokoro, S. et al. (2009) Three Arabidopsis SnRK2 protein kinases, SRK2D/SnRK2.2, SRK2E/SnRK2.6/OST1 and SRK2I/SnRK2.3, involved in ABA signaling are essential for the control of seed development and dormancy. *Plant Cell Physiol.* 50: 1345–1363.

Ng, L.M., Soon, F.F., Zhou, X.E., West, G.M., Kovach, A., Suino-Powell, K.M. et al. (2011) Structural basis for basal activity and autoactivation of abscisic acid (ABA) signaling SnRK2 kinases. *Proc. Natl Acad. Sci. USA* 108: 21259–21264.

Nicolas, P., Lecourieux, D., Kappel, C., Cluzet, S., Cramer, G., Delrot, S. et al. (2014) The basic leucine zipper transcription factor ABSCISIC ACID RESPONSE ELEMENT-BINDING FACTOR2 is an important transcriptional regulator of abscisic acid-dependent grape berry ripening processes. *Plant Physiol.* 164: 365–383.

Nishimura, N., Sarkeshik, A., Nito, K., Park, S.Y., Wang, A., Carvalho, P.C. et al. (2010) PYR/PYL/RCAR family members are major in-vivo ABI1 protein phosphatase 2C-interacting proteins in Arabidopsis. *Plant J.* 61: 290–299.

Ohta, M., Guo, Y., Halfter, U. and Zhu, J.K. (2003) A novel domain in the protein kinase SOS2 mediates interaction with the protein phosphatase 2C ABI2. *Proc. Natl Acad. Sci. USA* 100: 11771–11776.

Okamoto, M., Peterson, F.C., Defries, A., Park, S.Y., Endo, A., Nambara, E. et al. (2013) Activation of dimeric ABA receptors elicits guard cell closure, ABA-regulated gene expression, and drought tolerance. *Proc. Natl Acad. Sci. USA* 110: 12132–12137.

Ortiz-Masia, D., Perez-Amador, M.A., Carbonell, J. and Marcote, M.J. (2007) Diverse stress signals activate the C1 subgroup MAP kinases of Arabidopsis. *FEBS Lett.* 581: 1834–1840.

Park, S.Y., Fung, P., Nishimura, N., Jensen, D.R., Fujii, H., Zhao, Y. et al. (2009) Abscisic acid inhibits type 2C protein phosphatases via the PYR/PYL family of START proteins. *Science* 324: 1068–1071.

Park, S.Y., Peterson, F.C., Mosquna, A., Yao, J., Volkman, B.F. and Cutler, S.R. (2015) Agrochemical control of plant water use using engineered abscisic acid receptors. *Nature* 520: 545–548.

Raghavendra, A.S., Gonugunta, V.K., Christmann, A. and Grill, E. (2010) ABA perception and signalling. *Trends Plant Sci* 15: 395–401.

Rodrigues, A., Adamo, M., Crozet, P., Margalha, L., Confraria, A., Martinho, C. et al. (2013) ABI1 and PP2CA phosphatases are negative regulators of Snf1-related protein kinase1 signaling in Arabidopsis. *Plant Cell* 25: 3871–3884.

Rodriguez, L., Gonzalez-Guzman, M., Diaz, M., Rodrigues, A., Izquierdo-Garcia, A.C., Peirats-Llobet, M. et al. (2014) C2-domain abscisic acid-related proteins mediate the interaction of PYR/PYL/RCAR abscisic acid receptors with the plasma membrane and regulate abscisic acid sensitivity in Arabidopsis. *Plant Cell* 26: 4802–4820.

Saez, A., Rodrigues, A., Santiago, J., Rubio, S. and Rodriguez, P.L. (2008) HAB1–SWI3B interaction reveals a link between abscisic acid signaling and putative SWI/SNF chromatin-remodeling complexes in Arabidopsis. *Plant Cell* 20: 2972–2988.

Sato, A., Sato, Y., Fukao, Y., Fujiwara, M., Umezawa, T., Shinozaki, K. et al. (2009) Threonine at position 306 of the KAT1 potassium channel is

essential for channel activity and is a target site for ABA-activated SnRK2/OST1/SnRK2.6 protein kinase. *Biochem. J.* 424: 439–448.

Schweighofer, A., Hirt, H. and Meskiene, I. (2004) Plant PP2C phosphatases: emerging functions in stress signaling. *Trends Plant Sci.* 9: 236–243.

Schweighofer, A., Kazanaviciute, V., Scheikl, E., Teige, M., Doczi, R., Hirt, H. et al. (2007) The PP2C-type phosphatase AP2C1, which negatively regulates MPK4 and MPK6, modulates innate immunity, jasmonic acid, and ethylene levels in Arabidopsis. *Plant Cell* 19: 2213–2224.

Shin, R., Burch, A.Y., Huppert, K.A., Tiwari, S.B., Murphy, A.S., Guilfoyle, T.J. et al. (2007) The Arabidopsis transcription factor MYB77 modulates auxin signal transduction. *Plant Cell* 19: 2440–2453.

Skirycz, A., De Bodt, S., Obata, T., De Clercq, I., Claeys, H., De Rycke, R. et al. (2010) Developmental stage specificity and the role of mitochondrial metabolism in the response of Arabidopsis leaves to prolonged mild osmotic stress. *Plant Physiol.* 152: 226–244.

Stecker, K.E., Minkoff, B.B. and Sussman, M.R. (2014) Phosphoproteomic analyses reveal early signaling events in the osmotic stress response. *Plant Physiol.* 165: 1171–1187.

Takahashi, Y., Ebisu, Y., Kinoshita, T., Doi, M., Okuma, E., Murata, Y. et al. (2013) bHLH transcription factors that facilitate K^+ uptake during stomatal opening are repressed by abscisic acid through phosphorylation. *Sci. Signal.* 6: ra48.

Tang, R.J., Zhao, F.G., Garcia, V.J., Kleist, T.J., Yang, L., Zhang, H.X. et al. (2015) Tonoplast CBL–CIPK calcium signaling network regulates magnesium homeostasis in Arabidopsis. *Proc. Natl Acad. Sci. USA* 112: 3134–3139.

Umezawa, T., Nakashima, K., Miyakawa, T., Kuromori, T., Tanokura, M., Shinozaki, K. et al. (2010) Molecular basis of the core regulatory network in ABA responses: sensing, signaling and transport. *Plant Cell Physiol.* 51: 1821–1839.

Umezawa, T., Sugiyama, N., Mizoguchi, M., Hayashi, S., Myouga, F., Yamaguchi-Shinozaki, K. et al. (2009) Type 2C protein phosphatases directly regulate abscisic acid-activated protein kinases in Arabidopsis. *Proc. Natl Acad. Sci. USA* 106: 17588–17593.

Umezawa, T., Sugiyama, N., Takahashi, F., Anderson, J.C., Ishihama, Y., Peck, S.C. et al. (2013) Genetics and phosphoproteomics reveal a protein phosphorylation network in the abscisic acid signaling pathway in Arabidopsis thaliana. *Sci. Signal.* 6: rs8.

Umezawa, T., Takahashi, F. and Shinozaki, K. (2014) Phosphorylation networks in the abscisic acid signaling pathway. *Enzymes* 35: 27–56.

Urano, K., Maruyama, K., Ogata, Y., Morishita, Y., Takeda, M., Sakurai, N. et al. (2009) Characterization of the ABA-regulated global responses to dehydration in Arabidopsis by metabolomics. *Plant J.* 57: 1065–1078.

Waadt, R., Hitomi, K., Nishimura, N., Hitomi, C., Adams, S.R., Getzoff, E.D. et al. (2014) FRET-based reporters for the direct visualization of abscisic acid concentration changes and distribution in Arabidopsis. *Elife* 3: e01739.

Wang, P., Xue, L., Batelli, G., Lee, S., Hou, Y.J., Van Oosten, M.J. et al. (2013) Quantitative phosphoproteomics identifies SnRK2 protein kinase substrates and reveals the effectors of abscisic acid action. *Proc. Natl Acad. Sci. USA* 110: 11205–11210.

Weiner, J.J., Peterson, F.C., Volkman, B.F. and Cutler, S.R. (2010) Structural and functional insights into core ABA signaling. *Curr. Opin. Plant Biol.* 13: 495–502.

Weinl, S. and Kudla, J. (2009) The CBL–CIPK Ca(2+)-decoding signaling network: function and perspectives. *New Phytol.* 184: 517–528.

Yin, P., Fan, H., Hao, Q., Yuan, X., Wu, D., Pang, Y. et al. (2009) Structural insights into the mechanism of abscisic acid signaling by PYL proteins. *Nat. Struct. Mol. Biol.* 16: 1230–1236.

Yoshida, R., Umezawa, T., Mizoguchi, T., Takahashi, S., Takahashi, F. and Shinozaki, K. (2006a) The regulatory domain of SRK2E/OST1/SnRK2.6 interacts with ABI1 and integrates abscisic acid (ABA) and osmotic stress signals controlling stomatal closure in Arabidopsis. *J. Biol. Chem.* 281: 5310–5318.

Yoshida, T., Fujita, Y., Maruyama, K., Mogami, J., Todaka, D., Shinozaki, K. et al. (2015) Four Arabidopsis AREB/ABF transcription factors function predominantly in gene expression downstream of SnRK2 kinases in abscisic acid signalling in response to osmotic stress. *Plant Cell Environ.* 38: 35–49.

Yoshida, T., Fujita, Y., Sayama, H., Kidokoro, S., Maruyama, K., Mizoi, J. et al. (2010) AREB1, AREB2, and ABF3 are master transcription factors that cooperatively regulate ABRE-dependent ABA signaling involved in drought stress tolerance and require ABA for full activation. *Plant J.* 61: 672–685.

Yoshida, T., Nishimura, N., Kitahata, N., Kuromori, T., Ito, T., Asami, T. et al. (2006b) ABA-hypersensitive germination3 encodes a protein phosphatase 2C (AtPP2CA) that strongly regulates abscisic acid signaling during germination among Arabidopsis protein phosphatase 2Cs. *Plant Physiol.* 140: 115–126.

Zhang, M., Yuan, B. and Leng, P. (2009) The role of ABA in triggering ethylene biosynthesis and ripening of tomato fruit. *J. Exp. Bot.* 60: 1579–1588.

Zhao, Y., Chan, Z., Xing, L., Liu, X., Hou, Y.J., Chinnusamy, V. et al. (2013) The unique mode of action of a divergent member of the ABA-receptor protein family in ABA and stress signaling. *Cell Res.* 23: 1380–1395.

Zhao, Y., Xing, L., Wang, X., Hou, Y.J., Gao, J., Wang, P. et al. (2014) The ABA receptor PYL8 promotes lateral root growth by enhancing MYB77-dependent transcription of auxin-responsive genes. *Sci. Signal.* 7: ra53.