

REVIEW

Early abscisic acid signal transduction mechanisms: newly discovered components and newly emerging questions

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The plant hormone abscisic acid (ABA) regulates many key processes in plants, including seed germination and development and abiotic stress tolerance, particularly drought resistance. Understanding early events in ABA signal transduction has been a major goal of plant research. The recent identification of the PYRABACTIN (4-bromo-N-[pyridin-2-yl methyl]naphthalene-1-sulfonamide) RESISTANCE (PYR)/REGULATORY COMPONENT OF ABA RECEPTOR (RCAR) family of ABA receptors and their biochemical mode of action represents a major breakthrough in the field. The solving of PYR/RCAR structures provides a context for resolving mechanisms mediating ABA control of protein–protein interactions for downstream signaling. Recent studies show that a pathway based on PYR/RCAR ABA receptors, PROTEIN PHOSPHATASE 2Cs (PP2Cs), and SNF1-RELATED PROTEIN KINASE 2s (SnRK2s) forms the primary basis of an early ABA signaling module. This pathway interfaces with ion channels, transcription factors, and other targets, thus providing a mechanistic connection between the phytohormone and ABA-induced responses. This emerging PYR/RCAR–PP2C–SnRK2 model of ABA signal transduction is reviewed here, and provides an opportunity for testing novel hypotheses concerning ABA signaling. We address newly emerging questions, including the potential roles of different PYR/RCAR isoforms, and the significance of ABA-induced versus constitutive PYR/RCAR–PP2C interactions. We also consider how the PYR/RCAR–PP2C–SnRK2 pathway interfaces with ABA-dependent gene expression, ion channel regulation, and control of small molecule signaling. These exciting developments provide researchers with a framework through which early ABA signaling can be understood, and allow novel questions about the hormone response pathway and possible applications in stress resistance engineering of plants to be addressed.

[**Keywords:** Signal transduction; PYR1; RCAR; stomata; ABA receptor; phytohormone]

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Drought is one of the major abiotic stresses affecting plants; >50% of the Earth's surface area, including the vast majority of agricultural lands, is vulnerable to drought (Kogan 1997). Drought-induced crop losses have a significant economic impact, which is predicted to increase with global climate change (Marris 2008; Battisti and Naylor 2009). The phytohormone abscisic acid (ABA) is the central regulator of abiotic stress resistance in plants, and coordinates a complex regulatory network enabling plants to cope with decreased water availability (Cutler et al. 2010; Kim et al. 2010). Plant ABA content significantly increases under drought or salinity stress conditions, stimulating stomatal closure, changes in gene expression, and the accumulation of osmo-compatible solutes, thus increasing the plant's capacity to cope with stress conditions (Seki et al. 2007; Cutler et al. 2010; Kim et al. 2010). ABA also plays important roles during plant development, including embryo and seed development, and the promotion of seed dormancy (Finkelstein et al. 2008). Given the importance of ABA to plant physiology and development, understanding the signal transduction processes linking the hormone to target responses is a focus of abiotic stress research.

While many intermediate signaling components have been extensively characterized, our understanding of ABA signaling has been hampered by the lack of knowledge regarding the ABA receptor(s). The recent identification of PYRABACTIN (4-bromo-N-[pyridin-2-yl methyl]naphthalene-1-sulfonamide) RESISTANCE (PYR)/REGULATORY COMPONENT OF ABA RECEPTOR (RCAR) proteins (Ma et al. 2009; Park et al. 2009) provided the field with exciting new avenues of research into ABA perception, allowing existing hypotheses to be tested and novel ideas concerning ABA signaling to be generated. This review summarizes the many new findings in early ABA signaling, and also highlights and discusses emerging questions about the signaling network.

The central ABA signaling module

Much recent progress into ABA signal transduction indicates that the earliest events of the signaling pathway

occur through a central signaling module made up of three protein classes: PYR/RCARs, Protein Phosphatase 2Cs (PP2Cs), and SNF1-related protein kinase 2s (SnRK2s) (Fig. 1). In this model, the PYR/RCARs act as ABA receptors, the PP2Cs act as negative regulators of the pathway, and SnRK2s act as positive regulators of downstream signaling (Ma et al. 2009; Park et al. 2009). A double-negative regulatory pathway is established, whereby ABA-bound PYR/RCARs inhibit PP2C activity, and PP2Cs inactivate SnRK2s (Park et al. 2009; Umezawa et al. 2009; Vlad et al. 2009). Thus, in the absence of ABA, the PP2Cs are active and repress SnRK2 activity and downstream signaling. In the presence of ABA, PYR/RCARs interact with PP2Cs and inhibit phosphatase activity, allowing SnRK2 activation and phosphorylation of target proteins (Fig. 1). This newly developed signaling model forms the basis for addressing key discussion questions below, but should continue to be actively investigated.

Identification of ABA receptors

The first stage of hormone signaling must be the specific recognition of the hormone by a receptor. Recent studies identified and confirmed the PYR/RCAR proteins as ABA-binding proteins that interact with the PP2Cs, which function as negative regulators of ABA signaling (Ma et al. 2009; Park et al. 2009). Six of the nine PP2C proteins in the clade containing ABI1 have been shown to be negative regulators of ABA responses (see Table 1; Koornneef et al. 1984; Leung et al. 1994, 1997; Meyer et al. 1994; Rodriguez et al. 1998; Leonhardt et al. 2004; Saez et al. 2004; Kuhn et al. 2006; T Yoshida et al. 2006; Nishimura et al. 2007). Combined cell signaling and genetic analyses provided early evidence that the PP2C ABA-INSENSITIVE 1 (ABI1) functions very early in the ABA signaling pathway, upstream of all known rapid signaling responses—including anion channel activation (Pei et al. 1997), increases in cytosolic free calcium concentration ($[Ca^{2+}]_{cyt}$) (Allen et al. 1999), activation of the SnRK2 OPEN STOMATA 1 (OST1) (Mustilli et al. 2002), Ca^{2+} channel activation, and reactive oxygen species (ROS) production (Murata et al. 2001). Therefore, identifying proteins that interact with PP2Cs was one

strategy used to isolate novel components of early ABA signaling and identify the PYR/RCARs (Ma et al. 2009; Santiago et al. 2009b; Nishimura et al. 2010). RCAR1/PYR1-LIKE 9 (PYL9) was identified in a yeast two-hybrid screen using the PP2C ABI2 as bait (Ma et al. 2009), and a similar strategy using HOMOLOGY TO ABI1 (HAB1) as bait identified PYL5, PYL6, and PYL8 (Santiago et al. 2009b). Independently, an in vivo strategy of ABI1 complex purification from *Arabidopsis* plants led to the identification of nine of the 14 PYR/RCARs as the major in planta interactors of ABI1 (Nishimura et al. 2010). In an alternative approach, chemical genetics identified mutations in the *PYR1* gene based on insensitivity to the synthetic ABA agonist pyrabactin (Park et al. 2009). These multiple independent lines of evidence indicated that the previously uncharacterized PYR/RCAR proteins are major early ABA signaling components. The *Arabidopsis thaliana* genome encodes 14 PYR/RCAR proteins that are highly conserved at the amino acid sequence level (Table 1). PYR/RCARs are small soluble proteins belonging to the START/Bet v I superfamily that contain a central hydrophobic ligand-binding pocket (Iyer et al. 2001). The identification of this new class of ABA signaling proteins has resulted in great excitement within the plant hormone signaling field, providing new avenues of research into ABA signal transduction.

While the case for PYR/RCARs acting in ABA signaling is strong, this does not strictly exclude the possibility that other ABA receptors exist (for detailed discussion, see Klingler et al. 2010). Two other unrelated ABA receptors have been proposed: ChlH/GUN5 and GTG1/GTG2. ChlH/GUN5 was identified through homology with an ABA-binding protein from *Vicia faba* (Zhang et al. 2002; Shen et al. 2006), and overexpression of either the full-length protein (Shen et al. 2006) or the C-terminal half of the protein was reported to confer ABA hypersensitivity (Wu et al. 2009). However, the homologous ChlH/GUN5 protein in barley did not bind ABA (Müller and Hansson 2009), and further analyses are required to uncover the significance of this protein class in ABA signaling (Wasilewska et al. 2008). GTG1 and GTG2 are membrane proteins with homology with noncanonical G protein-coupled receptors (GPCRs) with nine transmembrane

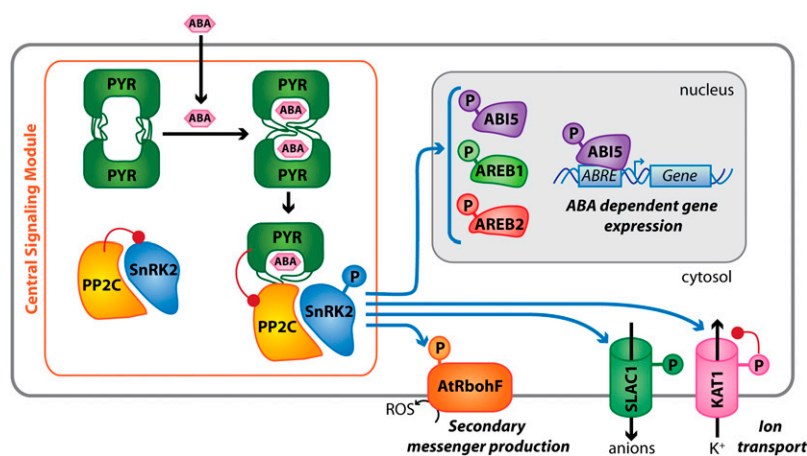


Figure 1. The core ABA signaling pathway. Recent progress in understanding early ABA signal transduction has led to the construction of a PYR/RCAR–PP2C–SnRK2 signal transduction model. In the absence of ABA, PP2Cs inhibit protein kinase (SnRK2) activity through removal of activating phosphates. ABA is bound by intracellular PYR/PYL dimers, which dissociate to form ABA receptor–PP2C complexes. Complex formation therefore inhibits the activity of the PP2C in an ABA-dependent manner, allowing activation of SnRK2s. Several SnRK2 targets have been identified both at the plasma membrane and in the nucleus, resulting in control of ion channels, secondary messenger production, and gene expression. Red connections on left indicate an inhibitory interaction.

Table 1. *The ABA signaling toolkit*

	Protein Family	Locus Number	Gene Name(s)	Example Protein Structural Motifs	References
Core Signaling Module	PYR/RCAR (14) Bet v I domain proteins	At4g17870 At5g46790 At2g26040 At2g38310 At5g53160 At1g01360	PYR1 PYL1 PYL2 PYL4 PYL8/RCAR3 PYL9/RCAR1	PYR1 RCAR1	Ma et al. 2009 Park et al. 2009 Santiago et al. 2009b Nishimura et al. 2010
	PP2C Group A (9) Mg ²⁺ -dependent Ser/Thr protein phosphatase	At4g26080 At5g57050 At1g72770 At1g17550 At3g11410 At5g51760	ABI1 ABI2 HAB1 HAB2 AtPP2CA (<i>ahg3</i>) AHG1	ABI1 HAB1	Koornneef et al. 1984 Meyer et al. 1994 Leung et al. 1994 Leung et al. 1997 Rodriguez et al. 1998 Saez et al. 2004 T Yoshida et al. 2006 Leonhardt et al. 2004 Kuhn et al. 2006 Nishimura et al. 2007
	SnRK2 (10) Ser/Thr protein kinase	At3g50500 At5g66880 At4g33950	SnRK2.2/SnRK2D SnRK2.3/SnRK2I OST1/SnRK2.6/SnRK2E	OST1	Merlot et al. 2002 Yoshida et al. 2002 Fujii et al. 2007 Fujii et al. 2009
Ca ²⁺ -dependent components	CDPK (34) Ca ²⁺ -dependent Ser/Thr kinase	At4g23650 At4g09570 At2g17290 At1g35670 At4g04720 At4g04740 At3g57530	CPK3 CPK4 CPK6 CPK11 CPK21 CPK23 CPK32	CPK6	Choi et al. 2005 Mori et al. 2006 Ma and Wu 2007 Zhu et al. 2007 Geiger et al. 2010
	CaM/CML (57) Calmodulin(-like)	At3g51920 At5g37770	CML9 CML24/TCH2	CML9	Delk et al. 2005 Magnan et al. 2008
	CBL (10) Calcineurin-B like	At4g17615 At5g47100	CBL1/SCABP5 CBL9	CBL1	Guo et al. 2002 Albrecht et al. 2003 Cheong et al. 2003 Cheong et al. 2007 Pandey et al. 2008
	CIPK/SnRK3 (25) Ser/Thr protein kinase	At5g01810 At1g30270	CIPK15/PKS3 CIPK23	CIPK15	Guo et al. 2002 Cheong et al. 2007 Pandey et al. 2008
Others	Rboh (10) NADPH oxidase	At5g47910 At1g64060	AtRbohD AtRbohF	RbohF	Kwak et al. 2003 Suhita et al. 2004
	PP2A Protein phosphatase regulatory subunit	At1g25490	RCN1	RCN1	Kwak et al. 2002

For each gene family, loci associated with ABA signaling in experimental studies are shown. The number in brackets after the gene family name indicates the number of family members encoded in the *A. thaliana* genome. Representative protein structural motifs are depicted for each gene family, with the functional domains indicated. Where mutant names differ from gene names, the alternative mutant name is indicated in brackets. Protein structure information was obtained from UniProt. (NLS) Nuclear localization signal; (PPI) protein phosphatase interaction motif. The CBL–CIPK interaction domain is characterized by the primary amino acid sequence NAF (Albrecht et al. 2001).

domains that hydrolyze GTP (Pandey et al. 2009). Double *gtg1gtg2* mutants retain an ABA response, but have a partially reduced sensitivity to ABA at the level of seed germination and stomatal responses, consistent with the existence of alternative ABA perception pathways (i.e., by PYR/RCARs). A proposed GPCR (GCR2) was also proposed to act as an ABA receptor, but this has been disputed (e.g., Guo et al. 2008), and so will not be discussed further here.

Capturing the message—ABA binding and interactions with PP2Cs

The strategies described above showed that PYR/RCARs acted with PP2Cs to confer ABA-induced inhibition of PP2C activity in vitro. Next, it was critical to determine if PYR/RCAR proteins bind ABA directly, and thus act as receptors. Initial evidence for ABA binding of PYR1 was

obtained through heteronuclear single quantum coherence nuclear magnetic resonance studies (Park et al. 2009) and isothermal titration calorimetry analyses (Ma et al. 2009), but whether PYR/RCARs and PP2Cs functioned together as ABA coreceptors remained unknown. Direct ABA binding to PYR/PYLs was subsequently established through the elucidation of PYR1, PYL1, and PYL2 crystal structures in the presence of ABA (Melcher et al. 2009; Miyazono et al. 2009; Nishimura et al. 2009; Santiago et al. 2009a; Yin et al. 2009). The ligand-binding site of PYR/RCAR proteins lies within a large internal cavity. The majority of protein interactions with the ABA molecule are through nonpolar contacts; however, the ring carbonyl, central hydroxyl, and carboxylic acid groups of ABA are held in place through water-mediated hydrogen bonds (Melcher et al. 2009; Miyazono et al. 2009; Nishimura et al. 2009; Santiago et al. 2009a;

Yin et al. 2009). The carboxylate forms a buried salt bridge with an inward-facing lysine side chain amine.

The nonnaturally occurring ABA enantiomer R-(−)-ABA has biological activity (Milborrow 1974), in contrast to many other signaling molecules that are highly stereospecific. ABA is a near-symmetrical molecule, with rotation around the chiral center resulting in a swap of the relative positions of the mono- and dimethyl groups on the ring (Milborrow 1974; Cutler et al. 2010). The crystal structure of PYR1 bound to both the S-(+)-ABA and R-(−)-ABA stereoisomers revealed that the chirality difference is accommodated within the ABA receptor-binding pocket by the flipping of the ABA ring by ~180° (Nishimura et al. 2009). The ability to bind alternative stereoisomers may not translate to equal biological activity in all PYR/RCAR family members; PYR1 interactions with the HAB1 PP2C are promoted by S-(+)-ABA but not R-(−)-ABA in yeast two-hybrid interaction tests (Park et al. 2009). It is worth noting that neither ChlH/GUN5 nor GTG1/GTG2 are able to bind the nonnatural but biologically active R-(−)-ABA isomer (Shen et al. 2006; Pandey et al. 2009), and therefore cannot account for R-(−)-ABA-induced responses. The relative stereo specificity of different PYR/RCAR proteins and their ability to bind alternative ligands could be used to explore the link between biochemical activities and physiological responses, and may also provide useful tools to manipulate ABA signaling in both an experimental and agronomical context.

Crystal structures of PYR1/PYL1/PYL2 in the absence of PP2Cs led to the resolution of both ABA-unbound and ABA-bound structures, thus revealing the protein conformational changes upon ABA binding (Fig. 2; Nishimura et al. 2009; Santiago et al. 2009a; Yin et al. 2009). Two loops flanking the entrance to the ligand-binding pocket, termed the “proline cap” or “gate” (consensus sequence

SGLPA) and the “leucine lock” or “latch” (consensus sequence HRL), will be referred to here as the “cap” and “lock,” respectively. The lock is completely conserved among all PYR/RCAR members, and the cap differs from the consensus sequence in only two of 14 isoforms (PYL12 and PYL13). When ABA binds to the protein, the cap closes over ABA, sealing the hormone within the protein cavity and sequestering it from solvent. The lock rearranges to secure the position of the cap, and an α -helix extends (recoils) in PYR1 to complete lid closure over the ABA molecule. These conformational changes both seal ABA inside the protein and reshape the protein surface, therefore impacting subsequent protein–protein interactions.

One of the interesting findings of the structural studies was that PYR/RCAR proteins are able to dimerize; PYR1, PYL1, and PYL2 all form homodimers within the crystal lattice (Nishimura et al. 2009; Santiago et al. 2009a; Yin et al. 2009). As crystallization may introduce artifacts, PYR1 homodimerization was independently assayed and confirmed in solution (Nishimura et al. 2009; Santiago et al. 2009a). Furthermore, PYR1 homodimerization was found in planta through coimmunoprecipitation experiments (Nishimura et al. 2009). ABA binding also alters the dimer assembly; the relative orientation between the two PYR/RCAR subunits changes (Yin et al. 2009), and the dimer becomes flatter and more compact in solution, as shown by small-angle X-ray scattering (Nishimura et al. 2009). The number of van der Waals contacts and hydrogen bonds between the two proteins decreases in response to ABA binding, which has been suggested to weaken the homodimeric interface (Nishimura et al. 2009; Yin et al. 2009).

The conformational changes in the PYR/RCAR protein upon ABA binding create a new interface on the protein surface, providing a novel site for interaction with PP2C proteins. The PYR/RCAR–PP2C complexes that have been crystallized—PYL1–ABI1 (Miyazono et al. 2009;

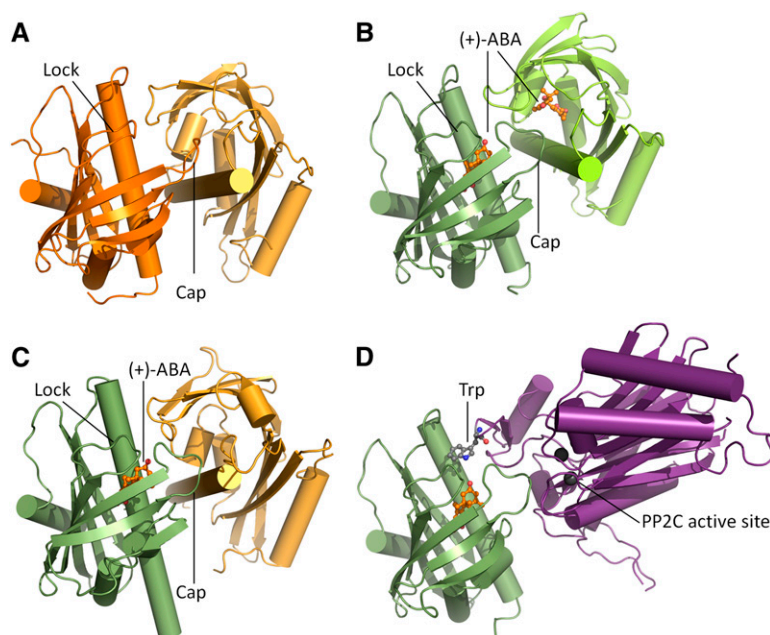


Figure 2. Structural mechanism of ABA–PYR/RCAR–PP2C interactions. The structures of PYR/RCAR proteins in both ABA-unbound conformations (gold) and ABA-bound conformations (green), and in complex with PP2C. The following structures are shown: a PYL2 homodimer in the absence of ABA (A), and a symmetrical PYL2 dimer with both ABA molecules shown in orange (B). Note that the cap and lock have changed position to come in closer contact with the ABA molecule, while reducing dimer interaction. (C) An asymmetrical PYR1 dimer exhibiting “closed” hormone-bound (green) and “open” hormone-free (gold) subunit conformations. (D) A PYL2–HAB1 (PP2C) complex: A tryptophan residue from the PP2C (purple) inserts into the gap between the cap and lock to interact with the ABA molecule. The cap makes contact with the Mg^{2+} -containing active site of the PP2C, therefore preventing phosphatase activity in the presence of ABA. The structures of PYR1 (Nishimura et al. 2009), PYL2, and the HAB1–PYL2 complex (Melcher et al. 2009) are oriented to align PYR/RCAR (shown at left).

Yin et al. 2009) and PYL2–HAB1 (Melcher et al. 2009)—identify a conserved tryptophan residue from the PP2C that inserts between the cap and lock of the PYR/RCAR protein, forming a novel hydrogen bond between the indole ring of the tryptophan and the ketone group of ABA. ABA binding is thus stabilized further by the presence of the PP2C, which is reflected in the ~10-fold increase in PYR/RCAR–ABA binding affinity in the presence of PP2Cs (Ma et al. 2009; Santiago et al. 2009b). For example, the binding affinity of S-(+)-ABA for RCAR1/PYL9 shifts from ~600 nM to 60 nM in the presence of ABI2 (Ma et al. 2009). This may be a function of the ABA off-rate, with PP2C binding favoring PYR/RCAR lid closure, and thus decreasing the rate of ABA dissociation from the protein (Nishimura et al. 2009). The PYR/RCAR proteins occlude access to the catalytic site of the PP2C (Melcher et al. 2009; Miyazono et al. 2009; Yin et al. 2009), therefore providing a direct structural explanation for PYR/RCAR inhibition of PP2C activity (Ma et al. 2009; Park et al. 2009).

The structures provide a framework for mapping known ABA signaling mutations to biological function. The *abi1-1* (G180D) and *abi2-1* (G168D) mutations result in the loss of interaction with PYR/RCARs (Ma et al. 2009; Park et al. 2009) and dominant ABA insensitivity. In the PYR/RCAR–PP2C complex, the G180/G168 residues are in close proximity to the cap; thus, in the mutant protein, the bulkier aspartic acid residue would disrupt hydrogen bonding and introduce a steric constraint to PYR/RCAR and PP2C binding (Yin et al. 2009). Mutations in the PYR/RCAR cap or lock (e.g., PYR1 P88, R116) also reduce ABA sensitivity (Melcher et al. 2009; Miyazono et al. 2009; Nishimura et al. 2009; Park et al. 2009; Yin et al. 2009), underlining the importance of these loops for ABA binding and receptor function. Mutations of residues that make direct contact with the ABA molecule (e.g., PYR1 K59, R116; PYL2 L91) reduce ABA binding and disrupt ABA-induced PYR1 interactions with PP2Cs (Melcher et al. 2009; Nishimura et al. 2009), underlining the importance of ABA-induced conformational changes. ABA binding can, however, be functionally separated from PP2C interaction and inhibition; the PYR1^{P88S} (corresponding to the cap) mutant protein retains ABA binding, but loses ABI1 binding (Park et al. 2009)—a pattern repeated in the PYR1^{H115A} (lock) mutant protein (Melcher et al. 2009). The ABA-dependent root growth phenotype of *Arabidopsis pyr1pyl1pyl2pyl4* seedlings is complemented by overexpression of the wild-type *PYR1* but not *PYR1*^{H115A} (Melcher et al. 2009).

Interestingly, there are biochemical differences in the PYR/RCAR–PP2C interaction, depending on the PYR/RCAR isoform, indicating possible functional specialization among the family members. Some PYR/RCARs interact with ABI1 in the absence of ABA treatment, whereas, for others, the interaction is induced by exogenous ABA (Ma et al. 2009; Park et al. 2009; Santiago et al. 2009b); for PYR1 and PYL1–4, the PYR–PP2C interaction is ABA-dependent, while, for other members of the family (PYL5–12), the interaction is constitutive in yeast two-hybrid analysis (Ma et al. 2009; Park et al. 2009). This pattern was also observed

in *Arabidopsis* plants; without adding exogenous ABA, the most abundant ABI1 interactors were PYL5–12, whereas exogenous ABA treatment increased PYR1/PYL1–4 interactions with ABI1 (Nishimura et al. 2010). Some evidence indicates the possibility that formation of the protein–protein complex between PYR/RCAR and PP2C can be functionally separated from PP2C inhibition: For RCAR1 (PYL9), the interaction with ABI1 and ABI2 is constitutive, but ABA is still required for inhibition of phosphatase activity (Ma et al. 2009).

Handing on the signal—SnRK2s as positive regulators

As PYR/RCARs function through ABA-dependent inhibition of PP2C activity, targets of PP2Cs represent the next stage of the signaling pathway. The SnRK2 Ser/Thr kinase OST1 (OST1/SnRK2.6/SnRK2E) is a known positive regulator of ABA-dependent stomatal movements (Mustilli et al. 2002; Yoshida et al. 2002), and is closely related to the ABA-activated protein kinase (AAPK) of *V. faba* (Li et al. 2000). The *Arabidopsis* genome encodes 10 SnRK2s, of which SnRK2.6/OST1, SnRK2.2, and SnRK2.3 have been associated with ABA signaling. Triple *snrk2.2snrk2.3snrk2.6* mutants are almost completely unresponsive to ABA (Fujii and Zhu 2009; Fujita et al. 2009; Nakashima et al. 2009), indicating that these SnRK2s form a major hub in the ABA signaling network. The C termini of SnRK2.2, SnRK2.3, and SnRK2.6 contain an Asp-enriched domain (Domain II) (Table 1) required for both ABA-specific activation of the kinase (Belin et al. 2006) and interaction with ABI1 (R Yoshida et al. 2006). HAB1 dephosphorylates the kinase within this activation domain to repress kinase activity (Belin et al. 2006; Boudsocq et al. 2007; Vlad et al. 2009). Consistent with this, an unbiased phosphopeptide array screening approach to identify targets of the PP2Cs HAB1, ABI1, and ABI2 isolated OST1 as a PP2C target (Vlad et al. 2009). The PYR/RCAR-mediated inhibition of PP2C activity therefore results in SnRK2 kinase activation, allowing the phosphorylation of downstream targets. Some PP2C–SnRK2 interactions may be constitutive, as ABI1 and SnRK2.3 interact in both the presence and absence of ABA (Umezawa et al. 2009; Nishimura et al. 2010). In contrast, yeast three-hybrid analyses indicate the ABA dependency of ABI2–SnRK2.6 and HAB1–SnRK2.6 interactions with PYL8 and PYL5, respectively (Fujii et al. 2009). More thorough testing in planta may reveal interesting isoform-specific differences in complex assembly.

Emerging questions relating to the central signaling module

The central PYR/RCAR–PP2C–SnRK2 signaling module provides an elegant model of early events in ABA signal transduction at a molecular and structural level. However, to understand the early signaling pathway more fully, the following important questions should be addressed.

Does PP2C binding dissociate the PYR1/PYL1,2 dimer?

Structural and mutational analyses indicate significant overlap between PYR1/PYL1,2 residues located in the

dimer interface and those participating in the PYR1/PYL1,2-PP2C complex, suggesting that PYR1/PYL1,2 homodimers and PYR1/PYL1,2-PP2C complexes are mutually exclusive (Fig. 2). The model therefore implies that formation of PYR1/PYL1,2-PP2C complexes is correlated with dissociation of PYR1/PYL1,2 homodimers, but does not directly distinguish whether dimer dissociation precedes PP2C complex formation or vice versa. As described above, binding of ABA energetically weakens the homodimeric interface between the PYR1/PYL1,2s; however, ABA-bound PYR1/PYL1,2 dimers are stable enough to form both in concentrated solutions of purified proteins and when over-expressed in plant tissues (Nishimura et al. 2009; Santiago et al. 2009a; Yin et al. 2009). The protein and hormone concentrations could tune the association/dissociation equilibria for homodimers and PP2C inhibition complexes, to appropriately respond to the plant's environmental conditions.

Do PYR/RCAR heterodimers occur and regulate ABA sensitivity?

The PYR/RCAR family proteins in *Arabidopsis* are very closely related in sequence and structure, suggesting the possibility of heterodimer formation. Different PYR/RCAR isoforms have different ligand and protein affinities; for example, PYR1 interacts with HAB1 only in the presence of (+)-ABA, while PYL2 and HAB1 interact in the presence of both the natural and unnatural ABA isomers (Park et al. 2009). PYR/RCAR heterodimers may further modulate ABA sensitivity, and provide increased flexibility in the signaling cascade.

How is ABA dependency of PYR/RCAR-PP2C complexes achieved?

As described earlier, PYL5–12 (excluding PYL8) form constitutive complexes with PP2Cs (Ma et al. 2009; Park et al. 2009), yet RCAR1 (PYL9)-mediated inhibition of ABI1 and ABI2 is ABA-dependent (Ma et al. 2009). Constitutive PYL5–PYL12 (excluding PYL8) interaction with PP2Cs suggests that these PYR/RCARs may not energetically favor receptor-receptor dimer formation. The crystal structures indicate that PYR/RCAR-PP2C interactions occur at the surface created by the closing of the ligand-binding pocket, which raises the question of how ABA can access the receptor protein if binding of the PP2C has already closed the cap and lock loops. All structural studies thus far have considered PYR/RCAR proteins of the same subfamily (PYR1–PYL2); structure studies of PYL5–12 isoforms in the presence and absence of ABA and in complex with PP2Cs should help to resolve this uncertainty in mechanism.

Which PYR/RCAR and PP2C isoforms form functional receptor complexes in planta?

The vast majority of research on PYR/RCAR-PP2C interactions has been done in vitro or through heterologous expression in tobacco. Under these conditions, multiple combinations of PYR/RCAR-PP2C can form complexes;

for example, ABI1 interacts with PYR1, PYL1, PYL8 (RCAR3), and RCAR1 (PYL9) (Ma et al. 2009; Miyazono et al. 2009; Park et al. 2009; Yin et al. 2009; Szostkiewicz et al. 2010). However, the diversity of interactions indicated in vitro does not necessarily occur in intact plants. In vitro analysis indicates that the specific PYR/RCAR-PP2C isoforms in a complex determine its biochemical parameters; for example, half maximal inhibition of PP2C activity occurs at a lower [ABA] in a PYL9-ABI1 complex than a PYL9-ABI2 complex (Szostkiewicz et al. 2010). Determination of the major interactions in vivo is therefore required to relate biochemical and physiological data. Identification of PYR/RCAR-ABI1 interactions in *Arabidopsis* leaves indicates that several combinations of interactions may occur at any one time (Nishimura et al. 2010). This question needs to be considered in a cell- or tissue-specific manner for full characterization of the physiologically relevant pathways. Similarly, identification of the most relevant in planta PP2C-SnRK2 interactions is required.

How are SnRK2s activated?

Given that PP2C-mediated inhibition of OST1 occurs through dephosphorylation, the origin of the activating phosphorylation should be considered. Two models of SnRK2 activation can be envisaged: autophosphorylation or phosphorylation by upstream kinases. In-gel analysis indicates that SnRK2s autophosphorylate (Belin et al. 2006; Boudsocq et al. 2007; Fujii et al. 2009; Vlad et al. 2009), yet OST1 can be activated by osmotic stress in the *abi1-1* and *abi2-1* backgrounds, indicating a role for an unknown protein kinase (R Yoshida et al. 2006). The current model predicts that SnRK2s are constitutively active and lose their ABA dependency in the absence of PP2Cs. Consistent with this, in an *abi1hab1pp2ca* mutant, SnRK2.2 and SnRK2.3 (and SnRK2.6, to a lesser extent) activity is up-regulated (Fujii et al. 2009). SnRK2.6/OST1 is reported to mediate ABA-independent responses, indicating additional possible regulation mechanisms (Zheng et al. 2010). Testing the model that inhibition of PP2C activity alone is sufficient for SnRK2 activation in planta (Fujii et al. 2009) is important; additional components might reduce the energetic cost incurred by continual removal of activating phosphates by PP2Cs.

Do PP2Cs target proteins other than SnRK2s?

The identification of SnRK2s as direct targets of PP2Cs reveals one downstream pathway for ABA signaling through PP2Cs, but does not rule out others. Genetic analyses indicate both independent and overlapping functions for the closely related PP2Cs ABI1 and ABI2 (e.g., Gilmour and Thomashow 1991; Gosti et al. 1995; Pei et al. 1997; Murata et al. 2001; Rubio et al. 2009). Phosphopeptide array studies (Vlad et al. 2009) identified additional HAB1 targets, including enzymes involved in primary metabolism and proton pumps. Other PP2C-interacting proteins identified include the chromatin remodeling factor SWI3 (Saez et al. 2008), several calcineurin-B-like protein (CBL)-interacting protein kinases (CIPK) (Guo et al.

2002; Ohta et al. 2003), and the calcium-dependent protein kinase 21 (CPK21) and CPK23 (Geiger et al. 2010). PP2Cs may therefore have a broad range of substrates, and, as such, may represent the first major branchpoint in the ABA signaling network; these alternate targets should therefore be considered in more detail.

Location of ABA perception

The identification of the PYR/RCAR proteins as ABA receptors provides clear evidence that ABA perception occurs primarily intracellularly through small soluble proteins. The site of ABA perception has been debated for several years, with evidence for both intracellular and extracellular receptors. Early studies suggested the presence of a transmembrane ABA receptor; ABA binding to proteins in *V. faba* is abolished in the presence of trypsin, suggesting an ABA-binding site on the external face of the membrane (Hornberg and Weiler 1984). An extracellular model of ABA perception was also suggested through experiments using ABA–protein conjugates that were unable to enter the cell, but were able to induce gene expression in rice suspension cells (Schultz and Quatrano 1997) and barley aleurone cells (Gilroy and Jones 1994), and ion channel activity in cultured *Arabidopsis* suspension cells (Jeannette et al. 1999); in some studies, microinjection of ABA failed to stimulate responses (Anderson et al. 1994; Gilroy and Jones 1994). However, the extracellular pH dependence of ABA responses also indicated an intracellular site of ABA action in one of these studies (Anderson et al. 1994). More directly, several independent studies in guard cells have suggested that ABA action can be intracellular. Patch-clamp analysis of guard cells showed that cytosolic ABA application is sufficient for rapid regulation of ion channels (Schwartz et al. 1994; Schwarz and Schroeder 1998; Levchenko et al. 2005). Microinjection of either ABA or photolysable ABA stimulated stomatal closure (Allan et al. 1994; Schwartz et al. 1994), again suggesting an intracellular site of ABA perception. Some of the conflicting data may be resolved with the recent identification of two ABC transporters that mediate transmembrane ABA flux. AtABCG40 and AtABCG25 are both members of the ABCG subfamily, with AtABCG40/PDR12 being a full-length transporter and AtABCG25 being a half-size ABC transporter. AtABCG40 increases the rate of ABA uptake into cells when expressed heterologously in either yeast or cultured tobacco BY2 cells (Kang et al. 2010), whereas AtABCG25 mediates ABA efflux (Kuromori et al. 2010). AtABCG40 is expressed primarily in guard cells and AtABCG25 is expressed primarily in the leaf vasculature, suggesting that the two transporters act coordinately to control ABA flux in mature plants, with AtABCG25 mediating ABA efflux from the site of ABA synthesis, and AtABCG40 allowing ABA uptake at the guard cell plasma membrane. Lack of effects of microinjected ABA may therefore be explained through the cellular export of ABA.

Potential alternative ABA receptors have different cellular locations than the PYR/RCARs, which may contribute to the diversity in predicted ABA response

sites. GTG1 and GTG2 are plasma membrane proteins (Pandey et al. 2009), and therefore might be responsible for ABA perception at the plasma membrane. ChlH/GUN5, being part of the chlorophyll biosynthesis pathway, is located within the chloroplast. As ABA is synthesized from intermediates in the carotenoid biosynthesis pathway, which is also chloroplast-located, there is the potential for a plastid-specific ABA perception mechanism that may therefore link light perception to ABA responses.

How does the central signaling module interact with other ABA-dependent processes?

As described above, ABA activates downstream signaling through the PYR/RCAR-mediated inactivation of PP2Cs, resulting in the activation of SnRK2s. Several targets of SnRK2.6/OST1 have been identified—including the ion channels SLOW ANION CHANNEL-ASSOCIATED 1 (SLAC1/RCD3) (Geiger et al. 2009; Lee et al. 2009; Vahisalu et al. 2010) and the inward-rectifying K⁺ CHANNEL IN *ARABIDOPSIS THALIANA* (KAT1) (Sato et al. 2009), the ROS-generating enzyme RESPIRATORY BURST OXIDASE HOMOLOG F (RbohF) (Sirichandra et al. 2009), and the bZIP transcription factors ABI5 (Nakashima et al. 2009) and ABA-RESPONSIVE ELEMENT-BINDING FACTOR 2 (ABF2) (Fujii et al. 2009). While many of the targets identified contain the SnRK2 consensus target sequence R-X-X-[S/T] (Furihata et al. 2006; Vlad et al. 2008), SLAC1 and KAT1 are phosphorylated at nonconsensus sites (Geiger et al. 2009; Sato et al. 2009), suggesting greater diversity of SnRK2 targets than indicated previously. The identification of both ion channels and transcription factors as targets of OST1 connects ABA to terminal responses through a PYR–PP2C–SnRK2 pathway, reflected in the rapid activation of stomatal closure after ABA application (Siegel et al. 2009).

Regulation of ion channels

Stomatal aperture is regulated by the coordinated actions of ion channels at the plasma membrane and tonoplast (for review, see Schroeder et al. 2001). ABA induces a large depolarization at the plasma membrane that is controlled through (1) the inhibition of the plasma membrane H⁺-ATPase (Goh et al. 1996), (2) inhibition of the K⁺ influx channel (Schroeder and Hagiwara 1989; Lemtiri-Chlieh and MacRobbie 1994), and (3) activation of the slow (S-type) anion channel (Schroeder and Hagiwara 1989). This depolarization activates K⁺ efflux from the cell, resulting in a net loss of solutes and a decrease in turgor. SnRK2 phosphorylation is now associated with two of these major regulatory events at the plasma membrane; namely, the inhibition of K⁺ influx channels and the activation of S-type anion channels. Thus, the central signaling module interfaces with membrane proteins required for stomatal closure.

Electrophysiological analyses established that S-type anion channels are dependent on ATP and sensitive to the Ser/Thr kinase inhibitor K252a, providing early evidence that phosphorylation is required for ABA responses and

channel activation in guard cells (Schmidt et al. 1995). Consistent with this, activation of S-type anion channels is abolished in *abi1-1* and *abi1-2* (Pei et al. 1997). SLAC1, identified recently as the S-type anion channel, is a 10-transmembrane domain protein with an extended cytosolic N-terminal region (Negi et al. 2008; Vahisalu et al. 2008) that includes an OST1 phosphorylation site required for channel activity (Geiger et al. 2009; Lee et al. 2009; Vahisalu et al. 2010). Expression of SLAC1 and OST1, but not SLAC1 alone, resulted in channel activity (Geiger et al. 2009; Lee et al. 2009). OST1 needed to be constitutively bound to SLAC1 via split YFP (bimolecular fluorescence complementation [BiFC]) constructs for strong anion channel activation (Geiger et al. 2009). The addition of ABI1, ABI2, or PP2CA to the system abolished channel activity (Geiger et al. 2009; Lee et al. 2009), demonstrating that the PP2C–SnRK2 regulatory module can be reconstituted. Expression of RCAR1/PYL9 conferred ABA-dependent phosphorylation of the SLAC1 N terminus when coexpressed with ABI1 and OST1 (Geiger et al. 2010). ABA-dependent activation of the channel in this heterologous system remains to be demonstrated.

Guard cell K^+ uptake is also SnRK2-dependent. K_{in}^+ currents are inhibited by protein phosphatase inhibitors (Li et al. 1994; Thiel and Blatt 1994), indicating that inward-rectifying K^+ uptake channels KAT1 (Schachtman et al. 1992) and KAT2 are targets of a phosphorylation-dependent pathway. Both KAT1 and KAT2 facilitate K^+ influx in guard cells, with dominant-negative mutations in both channel proteins inhibiting K^+ uptake (Kwak et al. 2001; Lebaudy et al. 2008). Following previous conflicting reports arguing for (Mori et al. 2000) and against (Li et al. 1998) phosphorylation of KAT1 by the OST1 homolog AAPK in *V. faba*, OST1 has been shown recently to phosphorylate the C terminus of KAT1 in *Arabidopsis*; mutation of the phosphorylated residue inhibits both K^+ conductance and complementation of a K^+ -deficient yeast strain, indicating that this site is critical for channel activity (Sato et al. 2009).

ROS and Ca^{2+} signaling

ABA-dependent stomatal closure is associated with small signaling molecules (also known as secondary messengers), including nitric oxide (Garcia-Mata et al. 2003), ROS (Pei et al. 2000), and cytosolic free calcium (McAinsh et al. 1990). ABA-induced stomatal closure is dependent on two plasma membrane NADPH oxidases (RbohD and RbohF) (Kwak et al. 2003) that generate ROS in the cell wall space. ABA-induced increases in ROS (Pei et al. 2000; Mustilli et al. 2002; Suhita et al. 2004) are abolished in the *ost1-2* mutant (Mustilli et al. 2002; Suhita et al. 2004), indicating that SnRK2s function upstream of ROS production (Fig. 1). A recent study showed that RbohF is a direct target of OST1; RbohF and OST1 interact physically in BiFC assays, and OST1 phosphorylates two sites in the extended N terminus (Sirichandra et al. 2009). One of the target motifs (R-X-X-S) is conserved between all *Arabidopsis* Rboh isoforms, suggesting that SnRK2 activation may be a widespread mechanism of Rboh regulation.

Analysis of ABA-induced stomatal closure in *Arabidopsis* under conditions in which increases in $[Ca^{2+}]_{cyt}$ are inhibited indicates that $[Ca^{2+}]_{cyt}$ -dependent mechanisms are responsible for ~70% of stomatal closure (Siegel et al. 2009), and several Ca^{2+} -associated proteins are required for normal stomatal closure, including CPKs, CBLs, and CIPKs (Table 1; for review, see Dodd et al. 2010; Kim et al. 2010; Kudla et al. 2010). However, the central PYR/RCAR–PP2C–SnRK2 signaling module is not thought to be Ca^{2+} -dependent; ABI1 contains a Ca^{2+} -binding EF hand domain, suggesting Ca^{2+} sensitivity of the phosphatase (Leung et al. 1994; Meyer et al. 1994), but expression of truncated ABI1 provides tentative evidence that the EF hand may not have physiological significance (Sheen 1998). This raises the question: How is Ca^{2+} sensitivity of stomatal closure achieved if the seemingly Ca^{2+} -independent central signaling module directly targets channels responsible for ion efflux? The identification of RbohF as an OST1 phosphorylation target provides a possible mechanistic link between the central signaling module and $[Ca^{2+}]_{cyt}$ -dependent processes. H_2O_2 stimulates Ca^{2+} -permeable channels (I_{Ca}) at the plasma membrane, and H_2O_2 -induced stomatal closure requires external Ca^{2+} , showing that the ROS– I_{Ca} – $[Ca^{2+}]_{cyt}$ pathway is involved in ABA signaling (Pei et al. 2000; Murata et al. 2001; Kwak et al. 2003; Suhita et al. 2004). Ca^{2+} -dependent and Ca^{2+} -independent pathways can function in parallel to regulate cellular components; for example, the N terminus of SLAC1 is phosphorylated by both SnRK2.6/OST1 (Geiger et al. 2009) and the Ca^{2+} -dependent kinases CPK21 and CPK23 (Geiger et al. 2010).

Transient increases in $[Ca^{2+}]_{cyt}$ are observed in guard cells under resting or nonstimulated conditions (for example, see Young et al. 2006), which might appear to contradict a model of $[Ca^{2+}]_{cyt}$ being required for ABA-induced stomatal closure. However, recent studies suggest that physiological stimuli such as ABA and CO_2 increase or “prime” the sensitivity of Ca^{2+} -dependent processes (Young et al. 2006). Notably, in the presence of the stimulus, ABA or CO_2 , downstream target signaling proteins are more Ca^{2+} -responsive (Young et al. 2006). For example, $[Ca^{2+}]_{cyt}$ -dependent activation of S-type anion channels and K_{in}^+ channel down-regulation are induced by pre-exposure to ABA (Siegel et al. 2009; Chen et al. 2010), and also by pre-exposure to high extracellular $[Ca^{2+}]$ (Allen et al. 2002). This novel Ca^{2+} sensitivity priming model could produce specificity in plant $[Ca^{2+}]_{cyt}$ signaling, in light of the diversity and large number (>200) of Ca^{2+} sensors encoded in the *Arabidopsis* genome (Day et al. 2002). How priming functions at a mechanistic level is an important unresolved question, but several nonmutually exclusive mechanisms can be envisaged, including subcellular relocation of Ca^{2+} sensors, post-translational modification, protein–protein interactions, coincidence detection of parallel signaling pathways, and transcriptional reprogramming (Fig. 3; for review, see Kim et al. 2010). Further analysis of potential priming mechanisms is required to fully understand the mechanisms of $[Ca^{2+}]_{cyt}$ -dependent ABA signaling.

Transcriptional responses

ABA treatment induces changes in gene expression in more than ~10% of the *Arabidopsis* genome (Sánchez et al. 2004; Seki et al. 2007; Yang et al. 2008; Zeller et al. 2009), resulting in the increased expression of stress-associated and signaling component transcripts. The major ABA-dependent *cis*-regulatory element associated with expression of abiotic stress-responsive genes is the ABA response element (ABRE; ACGTGT) (for review, see

Yamaguchi-Shinozaki and Shinozaki 2005). Analysis of synthetic promoters suggests that, although a single ABRE may be insufficient, two copies of the ABRE or a single ABRE combined with the related ABRE-coupling element (ABRE-CE; ACGCGT/G/C) renders a promoter ABA-sensitive (Hobo et al. 1999; Zhang et al. 2005). Several bZIP transcription factors—including SnRK2 phosphorylation targets ABI5, AREB1/ABF2, and AREB2/ABF4 (Johnson et al. 2002; Furihata et al. 2006; Fujii et al. 2009; Nakashima et al. 2009)—bind the ABRE and induce ABA-dependent gene expression. Correspondingly, ABA-induced gene expression is compromised in *snrk2.2snrk2.3snrk2.6* mutants (Fujii and Zhu 2009; Nakashima et al. 2009). However, transcriptome analysis indicates that, despite significant overlap between genes misregulated in *snrk2.2snrk2.3snrk2.6* mutants and *abi5* mutants, some transcripts are misregulated in the *snrk2* triple mutant and not *abi5*, and vice versa (Nakashima et al. 2009). Thus, a full description of the complexity of ABA-induced transcriptional regulation may also include the action of the central signaling module on alternative transcriptional activators binding to other ABA-related *cis*-elements. One possible class of alternate transcriptional regulators is the Ca^{2+} -regulated CALMODULIN-BINDING TRANSCRIPTIONAL ACTIVATORS (CAMTAs) (Bouché et al. 2002; Finkler et al. 2007), which bind the ABRE-CE (Doherty et al. 2009), and therefore may contribute to ABA-dependent transcriptional regulation or function in a parallel stress signaling pathway. An additional layer of complexity in ABA-induced control of gene expression is provided by links to chromatin remodeling. HAB1 interacts with the *A. thaliana* homolog of the yeast SWI3 subunit of SWI/SNF chromatin remodeling complexes, and *swi3b* knockout plants have reduced sensitivity to ABA in seed germination and growth assays (Saez et al. 2008).

Conclusions

Recently, a revolution has occurred in our understanding of ABA perception and signaling, allowing signaling cascades from ABA binding to target responses to be mapped out. As with all major discoveries, the identification of the PYR/RCAR–PP2C signaling mechanisms

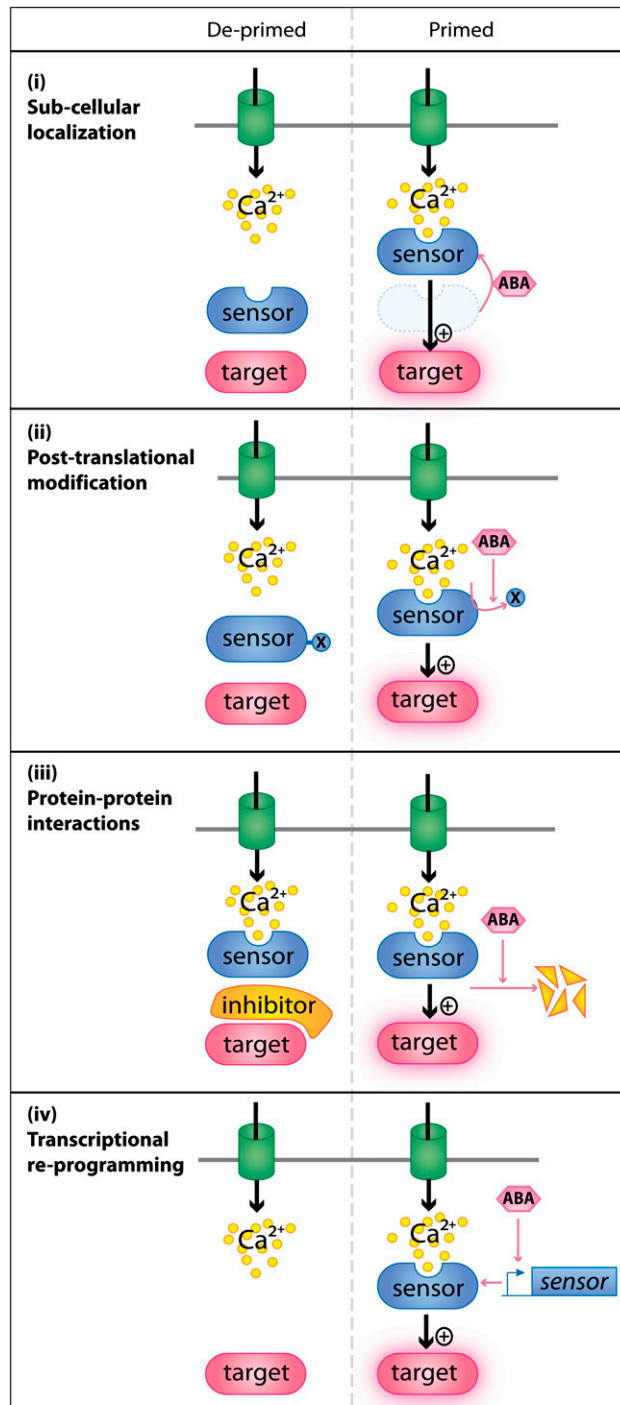


Figure 3. Possible mechanisms of Ca^{2+} sensitivity priming by ABA. Exposure to ABA enhances the Ca^{2+} sensitivity of the signaling pathway through several potential mechanisms; a calcium sensor is shown as the point of priming for clarity, but the priming mechanism may potentially involve downstream components. (i) ABA allows the appropriate subcellular localization of the sensor protein. (ii) ABA treatment causes the appropriate post-translational protein modifications for activity; removal of a negatively regulating modification is shown, but addition of a positively regulating modification is equally possible. (iii) ABA treatment provides the appropriate additional components of a protein complex to associate with the sensor; removal of a negative regulator is indicated, but ABA may also recruit positive interactors. (iv) ABA-stimulated transcriptional changes include expression of the sensor protein.

raises interesting new questions. ABA signaling issues still needing to be resolved include the detailed biochemical and structural mechanisms of dimer dissociation and PP2C association, and ABA-receptor responses in those PYR/RCARs that interact constitutively with PP2Cs. The considerable evidence reviewed here suggests that ABA signaling is mediated by a complex protein network, so other known and potentially unknown components remain to be integrated with PYR/RCAR receptor signaling. To complement the biochemical and structural details, the central signaling module also needs to be considered in a physiological context, as it is likely that cell and tissue specificity exists within the network. In addition to enabling new approaches to understanding the fundamental biology of ABA signaling, the identification of PYR/RCARs may enable new strategies for the development of drought-resistant crops.

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