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Abscisic Acid: Emergence of a Core Signaling Network

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Abbreviations
ABA: abscisic acid
ABI: ABA insensitive
ABRE: ABA response element
GPCR: G-protein coupled receptor
GTG: GPCR-type *G* proteins
LEA: late embryogenesis abundant protein
NCED: 9-*cis*-epoxycarotenoid dioxygenase
PP2C: protein phosphatase 2C
PYR/PYL/RCAR: pyrabactin resistant / pyrabactin resistant-like / regulatory component of ABA Receptor
SnRK2: SNF1-related protein kinase

Key Terms
BiFC: Bimolecular fluorescence complementation; a method for monitoring in vivo protein interactions by formation of a functional fluorescent protein
DELLA: family of proteins that function as negative regulators of GA signaling, and are destabilized by GA
infrared thermography: method for viewing infrared light emitted by objects due to their thermal condition; excessive transpiration results in "cool" leaves
osmocompatible solutes: small molecules accumulated by cells to permit osmotic adjustment to a dehydrating environment without interfering with cellular function
pyrabactin: a selective ABA agonist that is not an ABA analog

Abstract

Abscisic acid regulates numerous developmental processes and adaptive stress responses in plants. Many ABA signaling components have been identified, but their interconnections and a consensus on the structure of the ABA signaling network have eluded researchers. Recently, several advances have led to both the identification of ABA receptors and an understanding of how key regulatory phosphatase and kinase activities are controlled by ABA. A new model for ABA action has been proposed in which the soluble PYR/PYL/RCAR receptors function at the apex of a negative regulatory pathway to directly regulate PP2C phosphatases, which in turn directly regulate SnRK2 kinases. This model unifies many previously defined signaling components and highlights the importance of future work focused on defining the direct targets of SnRK2s and PP2Cs, dissecting the mechanisms of hormone interactions (i.e. cross-talk) and defining connections between additional known signaling components and this pathway, and determining how many other pathways control ABA signaling.

Abscisic Acid: A brief history

ABA was discovered in the 1960s. Reviews of its discovery and early chemistry and biology were published in 1969 and 1974 (2, 105). Briefly, ABA was isolated by several groups using activity-guided purification approaches to isolate endogenous growth regulators. Addicott's group at the USDA was searching for compounds isolated from cotton that promote leaf abscission, using a cotyledon abscission assay to guide purification (122). The compound isolated, originally named abscisin II, was also determined to inhibit Avena coleoptile growth (122). ABA's abscission promoting effect was subsequently determined to be partly an indirect consequence of inducing ethylene biosynthesis (26). The Wareing and Cornforth groups in the UK searched for compounds that promote bud dormancy, reasoned that such compounds would be general growth inhibitors, and ultimately isolated dormin as a wheat embryo germination inhibitor present in sycamore leaf extracts. Chemical analyses showed dormin and abscisin II to be the same compound (25), which was ultimately renamed abscisic acid. A third growth inhibitory activity originally isolated from *Aegopodium* tubers in the 1950s and named β-inhibitor (8) was also determined to be ABA (104); thus, the widespread occurrence and importance of ABA as a plant growth regulator was established by the late 1960's. ABA was subsequently documented as an endogenous regulator in some fungi and a variety of animals (for review see: (114, 169)).

Over the past 30 years, molecular genetic, biochemical and pharmacological studies have identified over 100 loci and numerous secondary messengers involved in ABA signaling, including Ca^{2+}, reactive oxygen species (ROS), cyclic nucleotides, and phospholipids. Due to space constraints, we have focused on recent developments linking ABA perception to known signaling elements, and have excluded discussion of second messengers in ABA signaling, an important topic that has been reviewed extensively elsewhere (19, 29, 48, 145).

Major physiological roles of ABA

Many key aspects of ABA's physiological effects were established shortly after its discovery. Wareing's group (159) noted the ability of ABA to antagonize several GA effects, including promotion of seedling growth and a-amylase synthesis. A role for ABA in water relations (specifically guard cell responses) was suggested by the observations that the wilty tomato *flacca* mutant was deficient in ABA, its phenotype could be rescued by exogenous ABA treatment (58, 157), and that ABA applications caused stomatal closure in *Xanthium*(67). Coupled with observations that ABA levels rise substantially after water deprivation, a physiological model for ABA's critical role in guard cell regulation emerged in the early

1970s. The role of ABA in these processes has been extensively studied and reviewed (118, 145, 152). Root growth maintenance during water deficits is also a key adaptive response that maintains adequate water supply. This process involves ABA and is controlled by the concerted action of different hormonal signaling pathways (148). In cases where water uptake and water loss cannot be balanced by primary adaptive responses, different mechanisms may be exploited to avoid and / or tolerate dehydration, which involve regulation of stress-responsive gene expression through ABA and other signaling pathways (183). In particular, the accumulation of osmocompatible solutes and the regulated synthesis of dehydrins and LEA proteins play important roles in both retaining water and protecting proteins and membranes under stress (59, 165). Recently, ABA has been found to affect pathogen responses; its effects range from promoting resistance by inhibiting pathogen entry via stomata to increasing susceptibility by interfering with defense responses mediated by other signaling pathways (reviewed in (160)).

In addition to its role in plant abiotic and biotic stress responses, ABA regulates important aspects of plant growth and development, such as embryo and seed development, promotion of seed desiccation tolerance and dormancy, germination, seedling establishment, vegetative development including heterophylly as well as general growth, and reproduction. For instance, severe ABA-deficient or ABA-insensitive mutants display a stunted phenotype even under well-watered conditions and are severely impaired in seed production (7, 15, 34, 112).

<u>Chemical features necessary for ABA action</u>
Shortly after its discovery, the structure of ABA was deduced by a combination of spectroscopic methods (121) and ultimately confirmed by chemical synthesis (24). The molecular structure of abscisic acid has a number of features that are important for biological activity in plants (Figure 1). One such feature is the side chain of the ABA molecule, which contains two double bonds conjugated to the carboxylic acid; the configuration of the double bond adjacent to the ring is trans and that proximal to the acid group is cis. On exposure to ultraviolet light, biologically active 2-cis,4-trans ABA is reversibly isomerized to the inactive trans form 2-trans, 4-trans ABA. Thus, under low light conditions 2-trans, 4-trans ABA can be employed as an inactive analog for studies to probe biological processes regulated by ABA. Under high light conditions or for long-term studies, the equilibrium between 2-trans, 4-trans ABA and the 2-cis, 4-trans ABA may shift to afford significant quantities of the active form. The active and inactive forms are readily distinguished by HPLC or GC analyses.

The importance of ABA's stereocenter and the biological activity of unnatural R-(–)-ABA versus natural S-(+)-ABA has been investigated since the discovery of the plant hormone (reviewed in (88, 178)). In many assays, including stomatal closure, (–)-ABA is weakly active. In seed germination studies in cereals (166) and Arabidopsis (115), applied (–)-ABA has been found to have comparable activity to (+)-ABA (reviewed in (88)). Recent microarray studies in which ABA was supplied to *Arabidopsis* plants have shown that (–)-ABA regulates most (+)-ABA regulated genes (55). In structure/activity studies where stereoisomeric forms of ABA analogs have been compared, the (–)-ABA analogs have been found to be inactive. Genetic studies have shown that (–)-ABA's action in Arabidopsis seeds requires a functional ABA signaling pathway (115). To explain the activity of (–)-ABA, Nambara *et al.* (115) hypothesized the existence of dual selectivity ABA-receptors. Since some members of the recently discovered PYR/PYL/RCAR protein family can bind or respond to both stereoisomers (128, 142), this new protein family contains candidates for the dual selectivity receptors hypothesized by Nambara *et al.*

There are several phenomena that can complicate the interpretation of whole plant structure activity relationship studies. For example, (–)-ABA supplied to plant tissues can trigger biosynthesis of natural (+)-ABA, which can accumulate and cause ABA processes to be induced, as documented in induction of the heterophyllous switch in *Marselia quadrifolia*(88). Furthermore, (–)-ABA is metabolized more slowly than natural (+)-ABA, so the apparent activity of (–)-ABA is magnified. Thus, before drawing conclusions about the physiological mechanism(s) underlying bioactivity of (–)-ABA or other analogs, characterizing their effects on endogenous ABA biosynthesis and / or enlisting the use of mutant strains deficient in ABA biosynthesis should be considered. Because of the confounding effects of metabolism, re-visitation of structure activity relationships using purified receptors in ligand-binding assays should be a productive line of future investigation.

Over 30 years ago, Milborrow proposed a structural hypothesis for the activity of (–)-ABA, based on the near symmetry of ABA (105). The (–)-enantiomer can be rotated about its lengthwise plane to effectively "flip" the positions of its 7' methyl and 8',9' dimethyl ring substitutions (Figure 1) and still leave the relative positions of the other polar functional groups relatively intact within a binding pocket. ABA analogs lacking either the 8', 9' or 7' methyl groups have been synthesized to explore this hypothesis (167). These studies showed that the 7' methyl group is critical to bioactivity. Ultimately, structural investigations of receptors bound to each stereoisomer will be required to resolve this long standing hypothesis for the bioactivity of (–)-ABA.

Additional molecules have been identified that may act on ABA receptors. These include analogs of ABA altered at either the 7', 8' or 9'-carbon atoms (reviewed in (178)), an ABA analog that acts as an ABA antagonist and inhibits expression ABA-induced genes in *Brassica napus* microspore-derived embryos (170) and pyrabactin, a selective ABA agonist that acts through the ABA receptor PYR1 but does not structurally resemble ABA (128) (Figure 1). The structural diversity of these and other ABA signaling modulators raises interesting questions about the nature of the receptor(s)' ABA-binding pocket(s).

ABA Binding Proteins Implicated in Signaling
Microinjection studies and treatments with impermeant ABA analogs in the 1990s suggested that ABA may have both intracellular (3, 147) and extracellular sites of perception (4, 42, 61, 146) and several proteins with the properties of either plasma membrane or intracellular ABA receptors have been described (90, 97, 126, 128, 133, 149). We summarize the current data on these proteins below.

FCA
The first ABA binding protein isolated (ABAP1) was identified in barley aleurone by virtue of its ability to bind an anti-idiotypic ABA antibody (i.e. an antibody against an ABA-antibody) (134). Sequence analysis showed this was related to Arabidopsis FCA, an RNA binding protein with a well documented role in regulation of flowering time (133). However, ABAP1 and FCA differ in several fundamental aspects: ABAP1 was initially described as associated with the plasma membrane, whereas FCA is a nuclear protein with two conserved RNA binding domains not present in ABAP1. Attempts to reproduce the FCA ABA-binding data using radioligand binding assays were unsuccessful (138). Based on these findings, the FCA report was retracted. Risk *et al.* have noted that the filter-based ligand-binding assay employed in the FCA and other receptor studies is prone to artifacts arising from incomplete removal of non-protein bound ABA (137, 138).

ChlH

A second ABA binding protein was isolated from bean epidermal protein preparations using an affinity matrix constructed by linking ABA's carboxylate to an amine-functionalized resin (181), a potentially problematic approach given that ABA's COOH is needed for bioactivity (105). The Arabidopsis relative of the bean protein isolated was named ABAR (for ABA receptor) and shown to possess a K_d of 32 nM. Additionally (R)-(–)-ABA was unable to displace radiolabeled ABA at concentrations >1000 fold above its K_d (149), showing the binding measured was highly stereospecific. Protein sequencing revealed ABAR to be a component of Mg-chelatase, a multisubunit plastid complex which functions to insert Mg^{2+} into protoporphyrin IX, producing Mg-protoporphyrin IX (Mg-proto), a precursor of chlorophylls (99). Modulation of Arabidopsis ABAR function using RNAi or T-DNA insertion alleles induces phenotypes consistent with ABAR playing a role in ABA responses (149).

Mg-proto has been proposed to function as a signal that coordinates nuclear and chloroplast gene expression in Arabidopsis (155) and Chlamydomonas (78). The ABA response locus ABI4, a transcription factor, is a downstream component of this pathway (77). Although the details of retrograde signaling to ABI4 are not yet clear, current data suggests that crosstalk between ABA signaling factors and chloroplast-nucleus communication exists. However it appears likely that the ABA-receptor function ascribed to ChlH is separate from ChlH's role in retrograde signaling (149).

There has been extensive debate about the significance of ChlH to ABA signaling. General concerns about inherent problems with the binding assays used have been raised (138), similar to those raised for FCA. In response to these concerns, alternate ABA-binding assays have been performed, and these suggest that ChlH can bind to ABA immobilized on an affinity column at its carboxylate (172). However, barley's ChlH (whose genetic locus name is XanF) does not bind ABA, and xanF loss-of-function mutants show normal ABA responsiveness, suggesting that barley's ChlH does not function as an ABA receptor (109) or that the monocot and dicot ChlH proteins may differ with respect to ABA binding and signaling. Whatever the case, a molecular explanation for how Arabidopsis's ChlH regulates the myriad ABA-controlled processes reported by Shen et al. (guard cell closure, seed dormancy, gene expression) will be required to fully comprehend the role of this protein in ABA signaling.

G-protein Coupled Receptor candidates
Pharmacological evidence has long suggested the involvement of a G-protein coupled ABA signal transduction pathway in plants (28). Loss-of-function alleles in the sole Arabidopsis G-alpha subunit gene (*GPA1*) show hypersensitivity to ABA at the level of germination and reduced guard cell sensitivity to ABA inhibition of stomatal opening, whereas they exhibit wild-type response to ABA-induced stomatal closure (168). These and other observations (125) suggested that a G-protein coupled receptor (GPCR) might participate in ABA signal transduction. Over-expression of GCR1, the sole classical GPCR encoded by the Arabidopsis genome, reduces seed dormancy (23). but genetic analyses of loss-of-function alleles did not implicate GCR1 in direct ABA perception. The *gcr1* knock-out mutants exhibit ABA-hypersensitivity, however the data also point to pleiotropic roles of GCR1 in other signaling pathways (13).

The third putative ABA receptor isolated, GCR2, has been proposed to be a G-protein coupled receptor (90). Currently, there is controversy regarding this factor's role in ABA signaling (39, 46) and its definition as a G-protein coupled receptor (57, 66). The protein also shows greatest similarity to soluble bacterial enzymes in the LanC superfamily (66). Additionally subsequent measurements have been unable to detect ABA binding to GCR2

(137). Because of the multiple levels of uncertainty surrounding the role of GCR2 in ABA signal transduction, it will not be described further.

Reasoning that a divergent GPCR could be the missing ABA-perceiving component of the Arabidopsis G-protein regulatory pathway, Pandey et al. searched the Arabidopsis genome for candidate GPCRs by bioinformatics and identified *G*PCR-*t*ype *G* proteins (GTG)1 and GTG2 based on topological similarity to GPCRs (126). The Arabidopsis GTGs contain nucleotide binding and GTPase activating domains, which makes them unlike other GPCRs. The closest human homolog of the GTGs, called GPR89/GPHR, was originally annotated as an orphan GPCR but was identified in a forward genetic screen as a factor necessary for protein transport through the ER and shown to be an ion-transporter involved in Golgi acidification (98); in addition, GPR89 does not contain nucleotide binding or GTPase activating domains (126).

Binding experiments using GTG protein reconstituted in the presence of phosphatidyl choline showed stereospecific binding to (+)-ABA (i.e. receptor bound +/-ABA could not be displaced with >1000 fold excess of (−)-ABA); however it should be noted that only 1% of the recombinant protein assayed binds ABA (22, 137). Pandey *et al.* attribute the stoichiometry of binding to the notorious difficulties associated with refolding membrane proteins into functionally active forms (126). Interestingly, the GTGs' ABA-binding is stimulated by GDP, suggesting that the GDP-bound form of the receptor is the high-affinity binding state. Observations of GFP-GTGs in protoplasts near the periphery of the cell, along with microsomal sedimentation of GTGs, suggest they are plasma membrane localized proteins. Pandey et al. conclude that GTGs are plasma membrane localized GPCRs that control ABA signaling.

Consistent with GTG regulation of G-Protein signaling, direct physical interactions between GPA1 and GTGs were observed and it was additionally determined that GPA1 functions to inhibit the intrinsic GTPase activity of the GTGs, but not their ABA-binding properties. Moreover, the GTPase activity of GTGs is negatively regulated by GTP-bound GPA1, which has been suggested to be the major form of GPA1 *in vivo* (65). When in the GDP-bound (inactive) form, GPA1 has also been shown to bind and inhibit the phospholipase PLDα1. ABA signaling activates GPA1, permitting PLD release and production of phosphatidic acid, which promotes ABA-induced stomatal closure and gene expression, as well as other stress responses, by multiple feedback mechanisms involving binding to a variety of targets including protein phosphatases, protein kinases, and metabolic enzymes (reviewed in (87)).

Arabidopsis *gtg1/gtg2* double mutants display reduced ABA sensitivity in seed germination, root growth, stomatal response and gene expression ABA-response assays (126). In contrast to the *gpa1* phenotype, gtg1/gtg2 shows wild-type response for ABA inhibition of stomatal opening. The single *GTG* loci mutants reveal no obvious phenotypes, suggesting the GTGs are functionally redundant in ABA signaling. Since the loss-of-function GPA1 phenotype may be either increased or decreased ABA response, depending on the tissue type, the observation that GPA1 could regulate the GTGs' ABA-binding implies that the ABA-related phenotypes of *gpa1* mutants may be partly due to effects attributable to action through the GTGs. The GTG data imply that the G-alpha subunit GPA1 is not involved in signal transduction downstream of the GTG receptors, which makes this GPCR signaling system unprecedented. An interesting avenue of future investigation will be to identify the direct downstream targets of the GTGs and link their action to other factors involved in ABA signaling.

PYR/PYL/RCAR Receptors

Four separate research groups (97, 128, 143) identified the most recently reported class of ABA binding proteins, the PYR/PYL/RCAR proteins. The characterization of a synthetic selective ABA agonist called pyrabactin (128) led to the connection between PYR1 and ABA signaling. Genetic analyses showed that PYR1 is necessary for pyrabactin action *in vivo*, but loss-of-function alleles lack detectable ABA related phenotypes due to genetic redundancy (described further below). A yeast two hybrid screen was employed in an attempt to understand how pyrabactin and PYR1 agonize ABA signaling. This revealed that PYR1 binds to the group A protein phosphatases (PP)2Cs ABA-insensitive (ABI)1, ABI2 and Homology to ABI1 (HAB)1 in response to ABA and pyrabactin. Park *et al.* have called this the PYR/PYL family, based on the necessity of PYR1 (*pyrabactin resistance 1*) and members of its 13 PYR1-like (PYL) relatives for proper ABA signal transduction. Conversely, using the ABA signaling protein phosphatase ABI2 as bait in a yeast two hybrid screen, Ma et al. identified RCAR1 (regulatory component of ABA Receptor), which corresponds to PYL9. Similarly, Santiago et al. (142) identified PYL5 through its constitutive interaction with HAB1. Nishimura et al. identified several PYR/PYL proteins by virtue of their constitutive interactions with YFP-ABI1 purified from transgenic plants (JI Schroeder, personal communication). PYR/PYL/RCAR proteins are members of the large super family of soluble ligand binding proteins named the START family by Iyer et al. (60) and more recently the Bet v I-fold superfamily in recognition of a conserved domain originally identified in the major pollen allergen of white birch (*Betula verrucosa*) (132).

RCAR1 binds to (S)-(+)-ABA with a K_d of 660 nM; interestingly, its K_d for (+)-ABA is enhanced ~10-fold ($K_d = 64$ nM) by inclusion of ABI1 in binding assays. Similar cooperative interactions (i.e. PP2C enhancement of measured K_ds) have been observed between PYL5 and HAB1, suggesting PP2Cs may stabilize ABA binding to PYR/PYL proteins. Systematic investigations of the 14 PYR/PYL/RCAR family members suggest that, with the exception of PYL13, the entire family is capable of activating ABA-signaling in response to ABA using a newly developed protoplast assay system (Jian Kang Zhu, personal communication).

The binding of ABA to RCAR1 shows a strong preference for the natural (+)-stereoisomer, while the K_d of PYL5 for (−)-ABA is ~20 fold lower than its K_d for (+)-ABA (1.1 vs. 19.1 μM). In yeast two hybrid assays, PYL2, PYL3 and PYL4 interact with HAB1 in response to either 10 μM (S)-(−)-ABA or (R)-(+)-ABA. Collectively, these observations suggest that multiple members of the protein family bind (−)-ABA; this new receptor family may therefore explain the pervasive bioactivity of (−)-ABA noted in many studies. The differential selectivities for pyrabactin and (−)-ABA in comparison to (+)-ABA suggests that the ligand binding pockets of PYR/PYL/RCAR proteins are likely to contain non-conserved or variable residues that can be exploited for selective receptor activation. More generally, the successful isolation of the synthetic selective ABA agonist, pyrabactin, demonstrates that the ABA signaling pathway can be controlled by compounds unrelated to ABA, which opens the door for controlling ABA signaling by simple synthetic small molecules. Of particular value in the future would be agonists and antagonists for multiple members of the PYR/PYL/RCAR family. Such probes would enable pharmacological investigation of defined PYR/PYL/RCAR functions in non-model system organisms and complement genetic studies in model systems.

In contrast to PYR1 and PYL1 to PYL4, RCAR1 and PYL5 show constitutive binding-interactions with PP2Cs in both yeast two hybrid assays and *in planta* bimolecular fluorescence complementation (BiFC) experiments (97, 128, 142). These observations coupled to the cooperative effects of PP2Cs on K_ds have led to the currently unresolved questions: does ABA bind to a PYR/PYL/RCAR-PP2C co-receptor, or does ABA bind first to the PYR/PYL/RCAR receptors followed by secondary PP2C interactions that stabilize

ligand binding? It is possible that the signaling mechanism may vary for different family members, paralleling their differing interactions with PP2Cs observed in yeast two hybrid assays. Ultimately, structural studies of PYR/PYL/RCAR proteins in their apo, ABA-bound and PP2C complexed forms will be required to fully resolve these points.

As described in detail below, extensive genetic evidence has shown that group A PP2Cs are negative regulators of ABA signaling. Since PYR1 is necessary for the action of pyrabactin, an activator of signaling, Park et al. hypothesized that the function of PYR1 was to inhibit PP2C activity, which would relieve the negative input into the signaling pathway provided by the PP2Cs. Numerous lines of evidence from several labs are consistent with this model, which is detailed in the phosphatase section of this review below. The point mutant, PYR1^{P88S} severely impairs the physical interaction between PYR1 and HAB1, but not ABA binding to PYR1, showing that ABA binding can be uncoupled from PP2C inhibition (128). Additionally, the dominant / hypermophic *abi1-1* and *abi2-1* encoded mutant proteins (ABI1 ABI2) do not bind PYR1 in response to ABA, which has led to a model that these proteins are hypermorphic because they cannot be inhibited by PYR/PYL proteins in response to ABA.

Genetic evidence for the role of the PYR/PYL proteins in ABA signaling comes from multiple sources. Triple *pyr1;pyl1;pyl4* and quadruple *pyr1;pyl1;pyl2;pyl4* mutants were constructed (128) and both mutant lines show reduced sensitivity in germination and root growth responses to (R)-(+)-ABA. The quadruple mutant is also impaired in ABA-induced stomatal closure (JI Schroeder, personal communication) and displays reduced sensitivity to ABA induction of RD29a, NCED3 ad P5CS1 mRNAs (128). Importantly, the ABA-induced activation of SNF1-related kinases (SnRK2s) is reduced in the quadruple mutant, which has led to a new model for ABA signaling described in a separate section below. Over-expression of PYL5 in Arabidopsis confers drought resistance (142), which is an important result that clearly connects this new receptor family to stress tolerance physiology. Overexpression of RCAR1 and RNAi with RCAR1 caused ABA hyper and hyposensitivity respectively. Collectively, the genetic analyses strongly support a role for PYR/PYL/RCAR proteins in controlling many aspects of ABA signaling.

Protein Phosphatases involved in ABA signaling
Protein phosphatases are divided according to substrate specificity into Ser/Thr, Tyr and dual-specificity classes (16). Depending on their biochemical and structural features, plant Ser/Thr phosphatases are further divided into PP1, PP2A and PP2C groups (16). Genetic evidence indicates that both PP2A and PP2C are involved in ABA signaling. Pharmacological approaches also suggest that Tyr and dual-specificity phosphatases participate in ABA signaling, however. However, because of space constraints, we will focus mainly on the role of PP2A and PP2Cs in ABA signaling.

PP2A is a holoenzyme composed of three subunits: a catalytic subunit C complexed with a scaffolding subunit A to form an AC core enzyme, which binds a regulatory subunit B. The Arabidopsis mutant *roots curl in npa (rcn)1* is affected in a scaffolding subunit A, it shows reduced levels of PP2A activity and was initially isolated as an auxin transport mutant (40). Indeed, PP2A regulates auxin fluxes in the root through regulation of PIN proteins (103). Additionally, disruption of RCN1 also leads to enhanced sensitivity to ethylene and reduced sensitivity to ABA (80, 81); in particular, rcn1 shows ABA-insensitive stomatal response because of lack of ABA activation of anion channels. Regarding PP2A catalytic (PP2Ac) enzymes, five subunits (PP2Ac1-5) are present in the Arabidopsis genome and a recessive mutation in the catalytic subunit PP2Ac-2 leads to enhanced sensitivity to ABA in different

processes (129) suggesting that PP2Ac-2 is a negative regulator of ABA responses, which contrasts with the apparent positive role of RCN1. Whereas PP2CAc-2 is a catalytic subunit with an apparently specific role in ABA signaling, RCN1 exhibits pleiotropic phenotypes in different hormonal pathways, which might explain this apparent paradox. Interestingly, pp2ac-2 partially suppresses the ABA-insensitive phenotype of abi1-1, which suggests that both PPs may act in the same ABA signaling pathway (129).

PP2Cs are usually described as Mg++/Mn++ -dependent monomeric enzymes. Whereas the cation requirement is a fundamental aspect of these phosphatases, the monomeric nature of the enzyme should be revisited according to recent breakthroughs, since regulation of the catalytic PP2C by the PYR/PYL/RCAR family of ABA receptors has emerged as a critical aspect of PP2C function ((97, 128, 142). Numerous lines of genetic evidence suggest that the PP2Cs are negative regulators of ABA signaling and this function is conserved from moss to Arabidopsis (76). At least six Arabidopsis PP2Cs from group A act as negative regulators of the pathway (44, 79, 83, 102, 120, 139, 140, 177). It appears that the site of action of PP2Cs may be in the nucleus, in spite of the localization in both cytosol and nucleus (107). A certain hierarchy in their function can be established according to gene expression levels, tissue expression patterns and analysis of ABA response in different combinations of *pp2c* knockout lines (139). A salient feature of the PP2C regulatory system is that combined inactivation of at least three relevant members leads to partial constitutive ABA-response (139). Therefore, the PP2Cs are key repressors of the signaling pathway, and can be considered analogous to the DELLAs, Aux/IAAs, JAZ or CTR proteins that negatively regulate gibberellin, auxin, jasmonic acid or ethylene signaling, respectively. A common feature of these signaling pathways is that a mechanism of derepression, either through regulation of activity or proteolytic degradation, is employed to control signaling. In the case of ABA signaling, inhibition of PP2C function is orchestrated by the PYR/PYL/RCAR family of receptors that connect ABA perception directly to release of the PP2C physiological braking system (97, 128, 142). Secondary messengers can also regulate PP2C activity: phosphatidic acid binds directly to ABI1, resulting in both decreased phosphatase activity and tethering to the plasma membrane, thereby limiting access to nuclear factors (reviewed in (87)). In addition to the role ABA plays in stress responses, it also plays a key role in regulating plant growth and development and constitutive activation of the pathway leads to growth defects. Therefore, proper control (i.e. repression of the pathway) is required to avoid a constitutive response to endogenous ABA that might be deleterious for plant growth.

Finally, although not a protein phosphatase, the inositol polyphosphate 1-phosphatase SAL1/FIERY1, which functions in the catabolism of inositol 1,4,5-trisphosphate (IP3), acts as negative regulator of both ABA and stress signaling in Arabidopsis (174). *fry1/sal1* mutants accumulate more IP3 in response to ABA treatment, which genetically connects phosphoinositide turnover and ABA signaling. Recent results also reveal increased ABA content in a novel *sal1* allele (named *alx8*) as well as connection of *SAL1* with an ABA-independent stress response pathway (171). It is currently unclear however how IP3 levels control ABA signaling, which will be important to address in future studies.

Targets of PP2Cs
Given the central role of PP2Cs in ABA signaling and their close connection with ABA perception, a complete inventory of their targets will likely be necessary to completely understand ABA signal transduction. Steps toward this goal have been made over the past years by using yeast two hybrid screens to identify binding partners. Among the proteins that have been identified are protein kinases implicated as positive regulators of ABA signaling. ABI1 binds to the transcription factor ATHB6, which appears to negatively regulate some

aspects of ABA signaling (49). Yeast two hybrid interactions have been described for ABI1 and OPEN STOMATA (OST)1 (176), different PP2Cs and SnRK2.2 (128) as well as ABI2 and a CBL-INTERACTING PROTEIN KINASE (PKS3/CIPK15), (47). Recent work from two groups has taken these observations much further and demonstrated that, in the absence of ABA, PP2Cs directly inactivate SnRK2 kinases by dephosphorylating multiple residues in the kinase activation loop (Umezawa et al 2009; Vlad et al; both in press). Thus, a critical role of the PP2Cs is to dephosphorylate and inactive the SnRK2s, which are essential positive signaling components in ABA signaling (as described below).

Additionally, PP2Cs may function as a hub that connects ABA perception with the control of different effectors involved in stress tolerance. For instance, ABI2 interacts with SALT OVERLY SENSITIVE (SOS)2, a kinase in the SnRK3 family that plays a critical role in Arabidopsis salt tolerance (123) via its regulation of the plasma membrane localized Na^+ / H^+ antiporter encoded by SOS1. It is conceivable that ABA signaling through the PYR/PYL/RCAR protein might enable ABA regulation of cation homeostasis via the SOS pathway. Similarly, the interaction of PP2CA and the AKT2 K^+ channel has been documented, which might connect ABA to control of K^+ transport and membrane polarization during stress situations (18). These links are at present speculative, however they highlight the critical importance of defining new PP2C targets and the power of the newly proposed PYR/PYL/RCAR signaling network for generating testable mechanistic hypotheses about ABA's action within the cell.

Finally, the interaction of HAB1 and SWI3B, a putative component of SWI/SNF chromatin remodelling complexes, provides a potential link between ABA signaling and regulation of plant transcriptional response on the chromatin template (141). The observed interaction of ABI2 and the preprotein of fibrillin (a plastid-associated lipid binding protein) may point to a mechanism for ABA-mediated control of light stress-triggered photoinhibition via direct PP2C regulation (175), however the localization of these two proteins to different compartments complicates this model.

Protein kinases involved in ABA signaling
The reversible phosphorylation of proteins is a fundamental mechanism by which living organisms modulate signal transduction events. Among the kinases that have been implicated in ABA signaling, both calcium-independent (SnRK2s) and calcium-regulated enzymes (SnRK3s/CIPKs and CDPKs/CPKs) have emerged as important factors. The first report of a SnRK2 involved in the ABA signaling pathway was wheat PKABA1, which is transcriptionally upregulated by ABA, phosphorylates the transcription factor TaABF1 (a member of the ABF/AREB family of TFs that recognize ABRE sequences) and mediates ABA-suppression of GA-induced gene expression in cereal grains (43). Subsequently, the *Vicia faba* protein AAPK (ABA-activated serine-threonine protein kinase), a SnRK2 family member, was shown to be involved in the regulation of ABA-induced stomatal closure (86). This guard-cell specific kinase was identified as an ABA-activated kinase using in-gel kinase assays (86). A dominant negative version of AAPK renders stomata insensitive to ABA-induced closure by eliminating ABA activation of plasma membrane slow anion channels (86). The putative AAPK ortholog in Arabidopsis was identified genetically as the locus OST1 (Open Stomata 1)/SnRK2.6 in a screen that used infrared thermography to identify plants with transpiration defects (110). Perturbation of AAPK or OST1 function does not affect stomatal regulation by light or CO_2, suggesting they are involved specifically in ABA signaling. The ABA-activated kinases SnRK2.2 and SnRK2.3 are closely related to OST1, but a double *snrk2.2;snrk2.3* mutant shows only modest defects impaired in ABA-mediated stomatal control (33). The double mutant shows strong ABA-insensitive phenotypes in seed

germination and root growth inhibition as well as reduced expression of ABA-inducible genes (33). A triple *snrk2.2;2.3;ost1* mutant has been constructed and the severity of its phenotypes suggests that the 3 kinases are global positive mediators of ABA signaling (34, 112).

SnRKs subgroup 3 (SnRK3s/CIPKs/PKSs) interact with calcium binding proteins such as SOS3/SCaBPs/CBL proteins and some members regulate ABA signaling in a calcium-dependent manner. For instance, the calcium binding protein SCaBP5/CBL1 and its interacting protein kinase PKS3/CIPK15 function as negative regulators of germination and stomatal ABA responses (47). The transient increase in cytosolic calcium induced by ABA, in addition to being perceived by calcium sensors that are positive regulators of ABA signaling (see below CDPKs/CPKs), might also be perceived by SCaBP/PKS3, leading to suppression of the PKS3 repression on ABA signaling (47). CIPK3/PKS12 is also involved in negative regulation of ABA signaling in seed germination, although ABA-induced stomatal closure was not affected in a loss-of-function *cipk3* mutant (69). Additionally, CIPK3 appears to play a general in modulating cold- and salt-induce gene expression but not drought-induced gene expression. The loss-of-function *cipk23* mutant exhibits reduced transpirational water loss and enhanced response to ABA-mediated stomatal closure and inhibition of stomatal opening, but ABA-response during seed germination was not affected (17). Again, as reported for PKS3/CIPK15 and CIPK3/PKS12, CIPK23/PKS17 plays a negative regulatory function in ABA signaling.

In contrast a positive role is played by another family of calcium-dependent PKs (CDPKs/CPKs), which are distinguished by a structural arrangement in which a calmodulin-like regulatory domain is located at the C-terminal end of the enzyme (54). Thus, CDPKs/CPKs have both kinase and calcium sensor domains in a single polypeptide, and therefore, they can be directly activated by calcium. The first evidence for a CDPK to be involved in ABA signaling was provided through constitutive expression of CPK10/CDPK1 and CPK30/CDPK1a, which activated an ABA-inducible promoter in maize leaf protoplasts (Sheen et al., 1996). Later on, it was shown that constitutive overexpression of CPK32 resulted in ABA-hypersensitive inhibition of seed germination (21). The use of gene knock-out mutations affecting CPK3 and CPK6 served to identify these proteins as positive regulators of ABA-regulated stomatal aperture, but the mutants lacked phenotypes in ABA-response during seed germination or early seedling growth (108). Finally, *cpk4* and *cpk11* mutants showed a pleiotropic ABA-insensitive phenotype in the former responses as well as stomatal movement, indicating they are positive regulators in the calcium-CDPK mediated ABA signaling pathway (184).

Other protein kinases from different families have been implicated in ABA signaling, although in general the downstream factors regulated by them are not known. For instance, the receptor-like kinase 1 (RPK1) is a positive regulator of ABA signaling according to the ABA-insensitive phenotypes of knockout and antisense-RPK1 transgenic plants (124). ABA induces the activation of a MAPK in barley aleurone protoplasts (72), but genetic evidence using loss-of-function lines is lacking.

Given the multiplicity of kinases implicated in ABA signaling, the extremely ABA-insensitive phenotype reported for the triple *snrk2.2/2.3/2.6* mutant is noteworthy. This triple mutant can germinate and grow on 50 μM ABA, which is an order of magnitude higher than other ABA-insensitive mutants such as *abi1-1*(34). It is therefore critical to assess the relative contributions of the various kinases (and other factors) implicated in ABA signaling by studying the phenotypes of their loss-of-function mutants. The near complete elimination of ABA responses in the triple *snrk2.2/2.3/2.6* mutant marks a milestone and indicates that the

direct phosphorylated targets of these 3 SnRK2s are likely to be a critical part of the future understanding of ABA signaling. High throughput screens are being developed to assist in identifying new substrates of these kinases (Vlad et al. in press) and extensive evidence has shown that the SnRK2s can directly phosphorylate members of the ABF/AREB/ABI5 clade of bZIP transcription factors (33, 37, 64, 73). Thus, at least one class of physiologically relevant targets of the SnRK2s appears to be DNA binding proteins involved in gene activation in response to ABA.

Additionally, it is currently unknown if SnRK2s require activating kinases *in vivo* or if their documented autophosphorylation is sufficient for activation. In *in vitro* assays using SnRK2 immunoprecipitates, SnRK2s are sensitive to the broad-spectrum kinase inhibitor staurosporine (11). However treatment of protoplasts with staurosporine, does not block SnRK2 activation by ABA. It has therefore been hypothesized that an upstream SnRK2-activating kinase may exist (11). Such factors (possibly CDPKs?) could provide a positive regulatory input that acts in opposition to the negative SnRK2 regulation mediated by PP2Cs.

ABA regulated gene expression
As with most signal transduction pathways, ABA response eventually leads to changes in gene expression, which may involve changes in transcription, transcript processing and stability. The specific changes depend on cell type and developmental stage, such that there is not a universal set of ABA regulated genes, but they are generally thought to contribute to tolerance of dehydrating conditions. These may be developmentally imposed, as in seed or pollen maturation, or in response to environmental stresses such as drought, salinity or low temperatures.

In recent years, extensive transcriptome data sets have been produced, made publicly available and re-analyzed by numerous researchers. Comparisons of transcriptomes for Arabidopsis and rice exposed to ABA and various abiotic stresses have shown changes affecting 5 to 10% of the genome; more than half of these changes were common to drought, salinity and ABA treatments (113, 150). The ABA regulated genes in Arabidopsis seedlings include slightly over 10% of the genome, split fairly evenly between induced and repressed genes; this is two to six times as many genes as are regulated by most of the other plant hormones (117). Comparison with transcriptome data from imbibing seeds shows that less than two-thirds of the genes characterized as ABA-induced in seedlings meet the same criteria in seeds, although many of the same classes of genes are induced in both. The ABA-induced genes are enriched for those encoding proteins involved in stress tolerance, such as dehydrins and enzymes that detoxify reactive oxygen species, enzymes of compatible solute metabolism, a variety of transporters, regulatory proteins such as transcription factors, protein kinases and phosphatases, and enzymes involved in phospholipid signaling. ABA-repressed gene products are enriched for proteins associated with growth including cell wall, ribosomal, plasma membrane, and chloroplast proteins. Similar classes of genes have been shown to be ABA-regulated in genera ranging from Arabidopsis to the moss *Physcomitrella* (27), and at least some of their regulators are conserved as well (76, 135). Consistent with the effects on gene expression predicted to affect metabolism, recent metabolome analyses have identified ABA-dependent drought-induced changes in synthesis of glucose, branched-chain amino acids, saccharopine, proline and polyamines (163).

In addition to effects on known protein coding genes, recent transcriptome analyses using Arabidopsis genome tiling arrays have shown that nearly 8000 unannotated transcriptional units are present in the "intergenic" regions, and 5 to 10% of these units are also regulated by ABA (100, 179). The vast majority of these are antisense transcripts for coordinately-regulated genes, and may be derived from sense RNA templates since intron-exon junctions are generally conserved and many lack any known ABA-responsive elements upstream of their 5' ends.

Cis- and trans-acting factors mediating ABA-regulated gene expression

The regulatory elements responsible for mediating these ABA-induced changes in protein coding gene expression have been identified by both biochemical and genetic approaches (reviewed in (30, 50, 113). The most common classes of regulatory sequences conferring ABA inducibility are the "G-box" ABA Response Elements (ABREs) recognized by members of the basic leucine zipper (bZIP) transcription factor family. In addition, many ABA-regulated genes contain binding sites for proteins of the MYB and MYC families. Also present are GC-rich sequences found in Drought Response Elements (DREs) or Coupling Elements (CEs) that are bound by APETALA2 (AP2) family proteins such as the DRE-Binding proteins (DREBs)/C-repeat binding factors (CBFs), or the RY/Sph element bound by B3 domain proteins such as ABA-INSENSITIVE3/VIVIPAROUS1 (ABI3/VP1). However, some of these motifs are correlated with ABA-independent stress signaling (163) or developmentally regulated expression (45) of these same genes.

Each of these transcription factor families is composed of dozens of members, so identification of specific factors regulating individual genes has required genetic analyses with mutants and/or ectopic expression. Candidates within each family have been chosen based on either *in vitro* binding to the conserved DNA sequences or their own ABA- or stress-inducible expression. Additionally, ABA and stress-regulated members of the homeodomain–leucine zipper (HD-ZIP) (5), No Apical Meristem/ATAF/CUP-Shaped Cotyledons (NAC) (35), WRKY (63, 173), non-MYC basic helix-loop-helix (bHLH) (85), and Zn-finger classes of transcription factors participate in some ABA responses. In one example, over 200 transcription factors representing at least 20 protein families were characterized as ABA-regulated at a single developmental stage (117), but the specific roles of most of these are still unknown.

Some of the best characterized activators of ABA- or stress-responsive genes are the subfamily of bZIP factors including ABI5 and the ABA Response Element Binding Factors (AREBs/ABFs/DPBFs). Although *ABI5* has been identified in multiple forward genetic screens (12, 32, 79, 92, 115), the AREB and ABF proteins were initially identified on the basis of binding to ABREs in yeast one-hybrid screens (20, 162) and loss of function lines display extremely weak phenotypes. Reverse genetics studies have shown some redundant functions in ABA and/or stress responses, cross-regulation of expression within the family, and modification of activity by formation of specific heterodimers (9, 31, 36).

The regulatory sequences for any given gene contain binding sites for a variety of factors, and ABA response generally depends on the presence of at least one ABRE and either Coupling Elements or additional ABREs. One exception to this is the MYB- and MYC-regulated RD22 gene, which lacks any ABREs (1). However, all of these arrangements allow for regulation by varying combinations of transcription factors whose identities are determined by the availability of specific regulators and binding sites. Several of the transcription factor families, such as bZIPs and NACs, form both homo- and heterodimers within their families (62). In addition, synergistic interactions between specific members of distinct families include those for bZIP and B3 domain factors (31, 52), bZIP and DREB factors (116), and NAC and Zn finger Homeodomain factors (161). Some of these reflect direct physical interactions between transcription factors (52, 111, 161), whereas others may be indirect due to interactions with additional members of a protein complex or binding of adjacent cis-acting sequences (116).

Despite the large number of genes that are down-regulated by ABA, the mechanisms of repression are less well characterized than those for induction. Some transcription factors, such as VP1 and ABI4, have been demonstrated to have either activator or repressor functions on distinct gene targets (10, 53). Alternatively, repression may involve early induction of factors such as the NAC factor ATAF1 that eventually down-regulate ABA responsive genes (96), analogous to DELLA-dependent repression of GA signaling (180). Another mechanism of down-regulation depends on transcriptional repressors, e.g the VP1/ABI3-Like (VAL) subfamily of B3 domain proteins or trihelix proteins that bind the same or overlapping sequences as the B3 or bZIP domain activators (38, 45, 156), or bZIPs that form inactive heterodimers with the activators, thereby blocking activation (9), reminiscent of the AUX/IAA repression of ARF activity in auxin signaling. However, the latter mechanisms are more important for repressing ABA-induced genes than mediating ABA-repression of other genes.

Transcription factor binding requires accessible or "open" chromatin. Recent studies have shown that ABA can also modify access to DNA via chromatin remodeling. ABA induces rapid changes in histone modification (153), but the gene specificity of these changes has not been characterized. Several of the ABI-related PP2Cs can interact directly with a homolog of the SWI3 component of SWI/SNF chromatin-remodeling complexes, and this interaction blocks full induction of a subset of ABA-regulated genes (141). For at least one family member, HAB1, ABA releases this repression by inhibiting the phosphatase activity that is required for interaction with SWI3. Conversely, three histone chaperones designated NUCLEOSOME ASSEMBLY PROTEIN 1 (NAP1), repress plant response to abscisic acid (ABA) such that triple mutants are slightly hypersensitive to ABA (91), which suggests that NAP1 may play a negative role in ABA signaling.

Regulating activity and stability of the regulators

Many of the AREB/ABF genes are themselves ABA- or stress-induced, but the proteins are inactive until phosphorylated by specific SnRK2s or CPKs (21, 33, 74, 130, 184). Potential phosphorylation sites are present in four conserved domains outside the bZIP domain, such that partially redundant kinases regulate partially redundant transcription factors at multiple developmental stages. Consistent with this redundancy, transcriptome analyses of Arabidopsis mutants defective in 3 SnRK2 genes have shown that these kinases and bZIPs regulate overlapping subsets of genes (34, 113). Similar redundancy of kinases and of bZIPs occurs in rice, and their regulatory relationships are conserved (74).

Additional interactions have been identified between multiple classes of transcription factors and proteins likely to mediate formation of protein complexes. For example, specific 14-3-3 proteins interact with multiple members of the ABF family of bZIPs and possibly VP1 (144). This interaction depends on phosphorylation of at least one conserved site in the bZIP and promotes activation of an ABA-responsive promoter by co-expressed bZIPs and VP1. Recently, an arm repeat protein initially identified as interacting with the bZIP ABF2 (ARIA) (70) was also found to interact with an AP2 domain protein (82); all three of these proteins are positive regulators of ABA response.

The phosphorylation-based activation might be reversible by appropriate phosphatases, or the proteins may be inactivated by additional modifications or destroyed when ABA signaling wanes. Proteasomal degradation has been documented for the bZIP protein ABI5 (93) and the B3 domain protein ABI3 (182). In the case of ABI5, stress or ABA treatments result in stabilization of ABI5 in young seedlings. A variety of interacting proteins affecting ABI5 accumulation or activity have been identified, including the RING E3 ligase KEEP ON GOING (154), the SUMO E3 ligase SIZ1 which stabilizes but inactivates ABI5 by sumoylation (106) and the AFP family of proteins of unknown function (41, 94), but these interactions are not limited to ABI5 or ABA signaling. For example, *SIZ1* is the sole locus encoding a SUMO E3 ligase in Arabidopsis and has also been implicated in regulation of response to phosphate deficiency, drought response, thermotolerance (both heat and cold), innate immunity, and flowering (101). Protein localization may also be regulated; the *SUPERSENSITIVE TO ABA AND DROUGHT (SAD) 2* locus represses ABA signaling, is homologous to proteins involved in importing cargo proteins through nuclear pores and may control nuclear localization of as-yet-unidentified regulators (165).

Regulation of RNA processing and stability

The transcription factors discussed so far are important for the selective activation of specific genes, but some basic aspects of RNA metabolism have surprisingly specific effects in stress and ABA response. RNA polymerase II is regulated directly by the phosphorylation state of its C-terminus, which affects the balance between transcription initiation and elongation, as well as various aspects of pre-mRNA processing, in yeast and animal cells (51). Mutations in a C-terminal domain (CTD) phosphatase-like protein (CPL3), expected to dephosphorylate this domain, result in overexpression of ABA-inducible genes (75). A related protein, CPL1, was independently identified as FIERY2 (FRY2) in a screen for mutants that hyper-induced a stress-responsive promoter driving luciferase production. FRY2/CPL1 is a double-stranded RNA binding protein that regulates expression of several DREB/CBF class transcription factors and their targets, possibly through interactions with structured RNA, or with MYB or NAC transcription factors (6). The *SAD1* locus encodes a member of a small nuclear ribonucleoprotein (snRNP) complex implicated in splicing, export, and degradation of RNAs (174). A point mutation in this locus is sufficient to confer hypersensitivity to ABA, but the specific targets of SAD1 regulation have not been reported.

Processing of the 5' and 3' ends of transcripts have also been implicated in ABA response. Two mRNA 5' cap–binding proteins (CBPs) appear to participate in mRNA processing of negative regulators of ABA signaling: ABA Hypersensitive (ABH)1 *(56)*, whose animal homolog is called CBP80, and CBP20 *(127)*. Mutants at these loci are hypersensitive to ABA and consequently drought tolerant. Despite the central nature of CAP-binding in RNA processing, only a small number of genes are misexpressed in the *abh1* mutant, including some encoding signaling molecules. For example, PP2CA transcripts are reduced in *abh1* mutants, consistent with the ABA hypersensitivity of these mutants *(56)*. At the 3' end of transcripts, the poly(A) tail is processed by ABA HYPERSENSITIVE AT GERMINATION (AHG)2, a poly(A)-specific ribonuclease that appears to destabilize transcripts induced in response to ABA, abiotic stress or salicylic acid *(119)*.

In addition to binding mRNA caps, CBP80 and CBP20 bind to the caps of primary microRNA (miRNA) transcripts and are required to complete their processing to miRNAs *(71)*. Another double-stranded RNA–binding protein, HYPONASTIC LEAVES 1 (HYL1), is important in miRNA production *(164)*. The *hyl1* mutation has pleiotropic effects, including ABA hypersensitivity, that can be partially explained by overaccumulation of the ABI5 transcription factor resulting from altered MAP kinase signaling *(95)*.

Numerous small RNAs have been implicated in stress- or ABA-signaling *(151)*, some of which affect ABA response via effects on transcription factor levels. ABA signaling via ABI3 in stressed seedlings induces miR159 production, which promotes cleavage of transcripts for two MYB factors that promote ABA response, thereby attenuating the response *(136)*. The targets of miR160 are a set of auxin response factors (ARFs); over-production of a miRNA-resistant form of one of these ARFs results in ABA hypersensitivity at germination, reflecting cross-talk between auxin and ABA signaling *(89)*. Although miRNAs and siRNAs are generally thought of as inhibitors of their targets, precedent from animal systems indicates that under stress conditions they may function as activators instead *(84)*.

Several RNA processing enzymes appear to have conflicting effects on ABA response, such that mutants have opposite effects on physiological and molecular responses to ABA. For example, a subunit of the Elongator complex, ABO1/ELO2, appears to decrease ABA-induced seedling growth inhibition and stomatal closure, and even modulates stomatal density, yet appears to positively regulate some ABA-induced genes including the regulators *ABF2/AREB1* and *ABI1 (14)*. Conversely, mutations affecting two DEAD-box RNA helicases, STRESS RESPONSE SUPPRESSOR (STRS)1 and STRS2, result in slightly decreased ABA sensitivity at germination but increased tolerance of abiotic stresses and increased expression of DREB/CBF class transcription factors and some stress-regulated genes *(68)*. DEAD-box RNA helicases affect diverse aspects of RNA metabolism, so the mechanism of these effects are still unknown, but the phenotypes suggest that attenuation of stress signaling is required for full ABA response.

Cross-talk with other signaling pathways

All organisms must integrate information provided via multiple signals and it appears that many of the loci identified in ABA signaling act as nodes connecting multiple pathways responding to other hormones, developmental signals, nutrient levels, and environmental signals. For example, we are learning of regulatory mechanisms that coordinate ABA vs. GA antagonisms mediating seed responses to light, chilling, and water availability via cross-regulation of multiple transcription factors and their target genes *(131)*, Studies of cross-talk with sugar signaling have identified cross-regulation of transport and metabolism, with transcription factors such as ABI4 acting as nodes (reviewed in {Gibson, 2004 #240; Dekkers, 2008 #241; *(158)*. As more specific factors are tested genetically, and network modeling strategies are applied, our understanding of these linkages should continue to improve.

Summary: PYR/PYL/RCARs -| PP2Cs -| SnRK2s, A New Model for ABA signaling

As detailed in this review, a multitude of signaling factors have been connected to ABA responses. The interrelationships of these factors have often times been poorly defined, leaving many factors unconnected to the larger picture of ABA signaling. The identification of PYR/PYL/RCAR receptors and the regulatory pathway that transmits signal through to transcription factors via PP2Cs and SnRK2s presents an important step forward in our current understanding of ABA signal transduction. The current model for ABA action through the PYR/PYL/RCAR receptors is as follows (Figure 2). PYR/PYL/RCAR proteins bind to ABA and inhibit the action of group A PP2C proteins (97, 128, 142) The PP2Cs dephosphorylate SnRK2s on their activation loop and prevent accumulation of active SnRK2s (Vlad et al; Umezawa et al. in press). The SnRKs in turn are involved in the phosphorylation of bZIP transcription factors, which is thought to be necessary for their ability to promote ABA-induced gene expression (33, 37, 64, 73). Thus, a negative regulatory pathway controls ABA-mediated transcriptional responses. This model fails to explain the role of many important regulators of ABA physiology, including second messengers. Thus, a future challenge for researchers in the field will be to ascertain the relationship between known factors and the PYR/PYL/RCAR or other networks. Additionally, the relatively large number of proteins found to interact with the group A PP2Cs suggests that possibility of other paths of information may flow downstream of the PP2Cs.

Summary Points

- The recent isolation of new ABA receptors will enable molecular insight into the selectivity and bioactivity of numerous chemical ABA modulators.
- GTG1/2, atypical GPCRs, bind (+)-ABA and participate in ABA signaling through an uncharacterized mechanism.
- PYR/PYL/RCAR proteins bind ABA and inhibit group A PP2Cs, negative regulators of ABA signaling.
- Whether PYR/PYL/RCARs function as direct receptors, or co-recepors with PP2Cs (or possibly both) is currently unresolved.
- Group A PP2Cs are negative regulators of ABA signaling that inhibit SnRK2 kinases.
- SnRK2 kinases positively regulate ABA-induced gene expression by directly phosphorylating bZIP transcription factors (i.e. ABFs/AREBs).
- Both the PP2Cs and SnRKs are likely to have many more substrates and / or interaction partners than currently identified.
- There is tremendous redundancy and feedback at every level of the ABA signaling network.

Future Issues

- How does ABA receptor structure permit dual specificity binding to (+) and (–) ABA?
- Can new selective agonists and antagonists of various PYR/PYL/RCAR members be obtained?
- What is the molecular mechanism used by PYR/PYL/RCARs and GTGs to regulate guard cell electrophysiology?
- Are there other targets of the SnRKs besides bZIPs?
- Does ABA's antagonism of GA action operate via the PYR/PYL/RCAR pathway?
- Does ChlH integrate with the PYR/PYL/RCAR pathway?
- What is the mechanism of PP2C inhibition by PYR/PYL/RCAR proteins?
- Are there kinases that directly activate SnRK2s, or is their autophosporylation sufficient to explain activation *in vivo*?

Related Resources

Plant Hormone Profiling
ABA Signaling
Rodriguez Lab
Cutler Lab

Figure Legends

Fig 1. Chemical structures discussed in this review. At bottom is an illustration of the ability of ABA stereoisomers to be rotated along its lengthwise plane to maintain positioning of polar functional groups, which Millborrow (105) suggested could explain (-)-ABA's pervasive bioactivity and also points to the possible importance of receptor contacts with the ring methyl groups for stereoselectivity.

Fig 2. Summary of ABA Signaling Factors. (A) Signaling through PYR/PYL/RCARs. In the absence of ABA (left), PYR/PYL/RCARs are not bound to PP2Cs PP2C activity is high, which prevents SnRK2 activation. In the presence of ABA, PYR/PYL/RCARs bind to and inhibit PP2Cs, which allows accumulation phosphorylated SnRK2s and subsequent phosphorylation of ABFs. *(B) Model of ABA interactions with current receptor classes.* ABA receptors identified include the plastid localized ChlH, plasma membrane localized GTGs and nucleocytoplasmic PYR/PYL/RCARs. These proteins all mediate effects on gene expression and guard cell electrophysiology (represented as A- channel for simplicity). The interconnections of these various factors are not currently known. Solid lines indicate direct interactions and dotted lines indicate unkown interactions. Positive interactions are noted by an arrow; bars indicate repression.

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