Perception and transduction of abscisic acid signals: keys to the function of the versatile plant hormone ABA

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During the past decade, much progress has been made toward understanding the mechanisms underlying plant hormone activity, from perception to nuclear events. However, the signaling mechanisms for abscisic acid (ABA) have remained largely obscure. Recent breakthroughs identifying FCA, which is an RNA-binding protein, the Mg-chelatase H subunit, and a G protein-coupled receptor as receptors for ABA provide a major leap forward in understanding the initial steps of ABA signaling mechanisms. Recent studies have also revealed the molecular mechanisms of second messenger production, protein modifications such as phosphorylation, and regulatory mechanisms of gene expression in the ABA response. Therefore, the connections between these events are also beginning to be determined. Here, we review recent progress and discuss the overall scheme of the ABA response mechanisms.

ABA response mechanism: unique among plant hormones?
As one of the most important phytohormones known, abscisic acid (ABA) and its action mechanisms have been studied extensively for decades. Recent forward and reverse genetic, cytological and biochemical studies have succeeded in identifying components involved in ABA-related phenomena, including ABA biosynthesis, action (signaling pathway) and degradation. The identification of ABA receptors is vital for understanding the signaling mechanisms. However, despite extensive study, the information on ABA receptors has been restricted to that based on circumstantial evidence. Expectations for the identification of ABA receptors have increased as receptors for other plant hormones have been identified over the past few decades. Recently, three ABA receptors were reported. In addition, there has been substantial progress toward understanding the mode of kinase/phosphatase action, transcriptional regulation, and RNA processing in the ABA response. In this review, we describe these new findings and present a more complete picture of ABA response mechanisms. The current picture is that of a complicated ABA response mechanism with multiple perception sites and a web-like signaling pathway that might be unique among plant hormones. Owing to space restrictions, we will not review the regulatory mechanisms for gene expression, second messenger production, and protein degradation regulation in response to ABA; for these topics, please see other recent reviews [1–6].

Multiple ABA receptors
The most remarkable progress in recent studies of the ABA signaling pathway is the identification of three ABA-binding components: the flowering-time control protein FCA [7], the Mg-chelatase H subunit [8], and the G protein-coupled receptor GCR2 [9]. Studies using ABA conjugated to a protein or chemical have indicated ABA recognition sites at both the cell surface and intracellular space [10]. The identification of multiple ABA receptors seems consistent with these observations.

Using anti-idiotypic antibodies, an ABA-binding protein (ABAP1) was identified in barley [11]. FCA is the closest Arabidopsis homolog of ABAP1. FCA interacts with FY, an RNA processing factor, to form a complex that inhibits the formation of full-length FCA mRNA and the expression of Flowering Locus C (FLC), a key factor in flowering-time control. ABA attaches to FCA with high affinity in vitro, reduces the interaction between FLC and FY, and consequently induces the accumulation of full-length FCA mRNA and FLC mRNA [7]. The identification of an ABA receptor as a component that regulates mRNA stability is intriguing because several ABA-related loci are implicated in RNA metabolism (see below). However, the ABA responses of guard cells and seeds in the feu-1 mutant are normal, suggesting that FCA is not involved in these representative ABA responses [7]. The relationship between FCA and barley ABAP1 is unclear.

Another study of ABA-binding protein from Vicia faba (i.e. ABAR) led to the identification of a putative ABA receptor [12]. ABAR is the Mg-chelatase H subunit previously shown to be involved in chlorophyll synthesis and in plastid-to-nuclear retrograde signaling [8]. ABAR demonstrates stereospecificity and affinity for ABA, underpinning the ABA receptor nature of this protein. In addition, reduced expression or overexpression of the ABAR gene confers reasonable effects on the ABA response in stomatal

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movement, germination and gene expression. Unfortunately, the molecular mechanisms of the action of ABAR as a signal transducer have not yet been clarified. The functions of the Mg-chelatase and ABA receptor are reported to be separable and, therefore, the function of ABAR as an ABA receptor is not involved in this retrograde signaling. However, one of the ABA-related transcriptional factors, ABA INSENSITIVE 4, plays a pivotal role in this retrograde signaling [13,14]. Although the connection between ABAR signaling and plastid-to-nuclear retrograde signaling is obscure, these two signaling pathways might mutually affect plant adaptation to environmental stresses.

A membrane-localized ABA receptor, GCR2, was recently identified [9]. GCR2 is predicted to have seven membrane-spanning domains; in fact, the GCR2–YFP fusion protein is localized at the plasma membrane. The T-DNA insertional mutations of GCR2 cause ABA insensitivity in germination, the expression of ABA inducible genes, and stomatal movement. GCR2 protein expressed in Escherichia coli binds to physiologically active ABA with high affinity. Like other G protein-coupled receptors, GCR2 interacts physically with GPA1, the Arabidopsis α subunit of trimeric G protein. ABA disrupts this interaction in yeast cells. These observations are consistent with GCR2 being an ABA receptor. Arabidopsis has two GCR2-related genes, but their functions in ABA responses are unknown. The amino acid sequence of GCR2 is highly similar to that of the peptide-modification enzyme lanthionine synthetase C, which is conserved from bacteria to humans [15], but it is not known whether GCR2 has this enzyme activity. It is also unclear whether GCR2 recognizes intracellular or extracellular ABA, although GCR2 is localized at the plasma membrane.

Second messengers: signaling mediator to signaling components

Upon ABA treatment, second messengers such as Ca2+ and phosphatidic acid (PA) accumulate in cells. Guard-cell movement in response to ABA has been studied as an ideal system for the analysis of the rapid cellular response to ABA. In this response, the increase in intracellular Ca2+ is important. In addition, many other second messengers, including reactive oxygen species (ROS), nitric oxide, and phospholipid-related components such as phosphatidylinositol-3-phosphate and inositol-triphosphate, are involved in regulating Ca2+ levels [2,16,17]. ABA stimulus induces ROS production by NADPH oxidases such as Arabidopsis AtrebD and AtrebF, localized at the plasma membrane [18]. High ROS levels activate Ca2+-permeable channels at the plasma membrane and increase intracellular Ca2+ [19]. ROS are also required for the function of phosphatidylinositol-3-phosphate and nitric oxide in the ABA response in guard cells [20–22]. In addition, ROS regulate ABA INSENSITIVE 1 (ABI1), ABA INSENSITIVE 2 (ABI2), and MAP kinases (see below). These results highlight the importance of ROS in orchestrating second messengers in the ABA response [3].

GPA1 is supposed to play a pivotal role in the production of second messengers. GPA1 is a key factor in responses to several plant hormones during germination [23], sugar sensing [24,25], stomatal movement [26], cell proliferation [27], and the light response [28]. Regarding the response to ABA, the connection between GPA1 and PA is intriguing. PA is produced by phospholipase D and is involved in both the ABA inhibition of stomatal opening and promotion of stomatal closure [29]. An Arabidopsis phospholipase D, PLDα1, is bound to GPA1 GDP (inactive form) and is released from GPA1 GTP (active form) to become active [30]. PA inhibits stomatal opening in the plda1 mutant but not in the gpa1 mutant or gpa1 plda1 double mutant, indicating that PA functions upstream of GPA1 and regulates it directly or indirectly to create a kind of feedback loop in the inhibition of stomatal opening [29]. PA inhibits the action of ABI1, which is a protein phosphatase 2C involved in promoting stomatal closure [29,30] (see below). Another phospholipid derivative, sphingosin-1-phosphate (S1P), is involved in the ABA response in guard cells [31]. Upon ABA treatment, S1P is produced and increases the intracellular Ca2+ level, presumably acting upstream of GPA1 [32]. These findings suggest the important hub-like function of GPA1 in second messenger action in the ABA response.

Protein phosphatase 2C: important hub in the ABA response

Recent studies have revealed connections between second messengers and signaling components in the ABA response. Protein phosphatase 2C (PP2C) is one of the most interesting of these components. The identification of the strong ABA-insensitive loci ABI1 and ABI2, which encode PP2Cs, indicated the importance of PP2C and protein phosphorylation events in the ABA signaling pathway [10]. PA inhibits ABI1 phosphatase activity and recruits ABI1 from the cytoplasm to the plasma membrane [33]. ROS such as H2O2 reduce the PP2C activity of ABI1 and ABI2 in vitro [34,35]. An Arabidopsis glutathione peroxidase, ATGPX3, inhibits the activity of ABI2 and/or ABI1 in an H2O2-dependent manner [36]. The existence of multiple regulators suggests a complex regulatory system for PP2Cs, as well as their importance in hub functions in the ABA response.

In addition to ABI1 and ABI2, four of the 76 known PP2Cs [AtPP2CA/ABA HYPERSENSITIVE GERMINATION 3 (AHG3), HYPERSENSITIVE TO ABA 1 (HAB1), HYPERSENSITIVE TO ABA 2 (HAB2), and ABA HYPERSENSITIVE GERMINATION 1 (AHG1)] negatively regulate the ABA response in Arabidopsis [37–42]. These six PP2Cs are similar at the amino acid sequence level [37]. The null mutants of these PP2Cs have similar ABA-hypersensitive phenotypes, of different strengths in germinating or adult plants. Transgenic plants possessing the abi1-1-type mutation in AtPP2CA/AHG3 or HAB1 have strong ABA insensitivity, similar to that of abi1-1 and abi2-1 [40,43]. These observations suggest that these PP2Cs have largely overlapping functions; however, they also have distinct properties, different expression profiles, and different preferences for interacting proteins. The PA binding site of ABI1 is unique among these six PP2Cs [29]. Overlapping but distinct properties of these PP2Cs might constitute a complex PP2C-substrate network for the elaborate ABA signaling pathway.
The *abi1-1* type mutation, which changes the Gly residues conserved among PP2Cs to Asp, reduces phosphatase activity *in vitro*, but causes strong dominant ABA insensitivity *in planta*. The molecular mechanism underlying the effect of this mutation is unknown. According to the predicted three-dimensional structure of HAB1 based on the human PP2Ca structure, this Gly residue is located on the surface that interacts with substrates [43], indicating that this mutation affects the interaction of this enzyme with its substrate. To date, information on the native substrates of these PP2Cs is extremely limited [44,45]. The identification and biochemical characterization of the native substrates should provide information regarding the molecular basis of the action of PP2Cs, as well as the nature of this type of mutation.

**Protein kinases in the ABA signaling pathway: pivotal components of the ABA signaling network**

Recent studies have revealed a connection between PP2C and SNF1-related kinases (SnRKs), particularly SnRK2- and SnRK3-type kinases. Genetic and biochemical studies have demonstrated the pivotal functions of SnRK2s in stomatal movement, ABA-inducible gene expression, and ABA and stress responses in germination [46–50]. Of the ten SnRK2s encoded by the genome, SnRK2.2, SnRK2.3, SnRK2.6, SnRK2.7, SnRK2.8 in *Arabidopsis* and SAPK8, SAPK9 and SAPK10 in rice are activated by ABA stimulus [51–53].

An intriguing connection between PP2C and SnRK2 was indicated by the finding that OPEN STOMATA 1 (OST1)/SRK2E/SnRK2.6, which is a key factor in the stomatal ABA response in *Arabidopsis*, requires normal ABI1 function for its ABA-dependent activation [48,49]. In addition, OST1/SRK2E/SnRK2.6 interacts directly with ABI1 protein but only weakly with the *abi1-1* mutant protein through the C-terminal domain of the kinase (Figure 1) [54], which is important for ABA-dependent activation [53]. Although the physiological significance of this interaction has not been elucidated, it might link the ABA-activating kinases and ABA-implicated PP2Cs. The phosphorylation of several Ser/Thr residues in the P-loop of SnRK2s is required for their activation [55–57], suggesting that some other kinases might function upstream of SnRK2s in the ABA signaling pathway. Alternatively, given that all these residues might be auto-phosphorylated [56], some stimuli might induce auto-phosphorylation and activate these kinases, and PP2Cs might regulate the phosphorylation status of these residues.

SnRK3-type kinases [SnRK3/CBL-INTERACTING PROTEIN KINASES (CIPK)/PKS] are implicated in the ABA response and also interact with PP2Cs. The reduced expression of *PKS3* and *SOS3-LIKE Ca^{2+} SENSOR/
BINDING PROTEIN 5, the gene encoding the Ca\(^{2+}\)-binding regulator of PSK3, similarly enhances the ABA-sensitive phenotype in Arabidopsis [58]. In addition, the knockout mutant of CIPK3 is hypersensitive to ABA and abiotic stress [59]. Conversely, increased or decreased PSK18 activity causes an enhanced or reduced ABA sensitivity, respectively [60], whereas increased or decreased PK3 and CIPK3 activity causes the opposite response, suggesting their distinct functions in the ABA response. Interestingly, several SnRK3s interact with ABI1 and ABI2 (Figure 1) [58,60,61]. The physiological function of SnRK3/CIPK/PKS is dependent upon which Ca\(^{2+}\)-binding regulator is attached [62], indicating the complex network of SnRK3/CIPK/PKS, Ca\(^{2+}\)-binding regulatory components, and PP2Cs in the ABA response.

The interaction between SALT OVERLY SENSITIVE 2 (SOS2) and ABI2 requires the amino acid sequence located near the abi2-1 mutation site [61]. As this region is predicted to be spatially close to the active site, the ABI2–SOS2 interaction might be the enzyme–substrate recognition mechanism. From this perspective, it is intriguing that the target motif in SOS2 (named PPI) contains a sequence similar to the consensus target site (R/K-X-X-T/S) for protein kinases such as Ca\(^{2+}\)-dependent protein kinase (CDPK), and that changes in these amino acid residues diminish the binding activity [61]. Hence, these kinases are thought to be substrates of PP2Cs, although the phosphorylation of these sites has not yet been demonstrated and the functional relationship between the kinases and PP2Cs is not clear. SnRK3/CIPK/PKS is regulated by Ca\(^{2+}\) through its regulator protein.

Given the importance of Ca\(^{2+}\) as a second messenger in the ABA response, the function of CDPK in the ABA response has been studied extensively [63,64]. Recent studies have succeeded in identifying the functioning of CDPK in the ABA response. AtCPK32 regulates the ABA-related transcription factor ABRE BINDING FACTOR (ABF) 4/ABSCISIC ACID RESPONSIVE ELEMENT BINDING PROTEIN (AREB) 2 [65]. Both CPK3 and CPK6 have pivotal roles in the ABA response in guard cells [66]. Arabidopsis also activates mitogen-activated protein kinase (MAPK) cascades. ABA treatment of plant tissues induces the activation of kinases with molecular masses of 42–46 kDa, which are consistent with the sizes of putative plant MAP kinases. An analysis of the disruption mutation of AtMPK3 revealed its function in the ABA response in germination [67]. However, these MAPKs were also stimulated by many other signals. Both ABA and H\(_2\)O\(_2\) tend to activate the same MAPKs and, thus, it is possible that ABA activates MAPKs through the accumulation of endogenous H\(_2\)O\(_2\) [68]. RPK1, a receptor-type kinase of Arabidopsis, appears to be involved in the ABA response because its antisense gene or disruption mutation reduces ABA sensitivity in germination and stomatal response [69]. Interestingly, the overexpression of the putative extracellular domain of RPK1 causes weak ABA insensitivity, implying that RPK1 is somehow involved in the early steps of the ABA signaling pathway, although a receptor-like function has not yet been demonstrated.

As described above, many protein kinases function in the ABA response. Although kinase-interacting proteins involved in the ABA response have been isolated, their physiological relevance is not yet clear. By contrast, the results of extensive studies of many transcription factors involved in the ABA and stress responses indicate that the phosphorylation of their specific residues has a crucial role in their function [1]. Recently, several studies have reported remarkable connections between ABA-responsive element binding proteins (AREBs/ABFs) and SnRK2s, such that SnRK2s interacts directly with AREBs/ABFs and/or phosphorylates them in vitro [46,50,55,70]. In addition, changing all five phosphorylation residues (Ser/Thr) of AREB1 to aspartic acid and, thus, mimicking phosphorylation, confers the constitutive activation of ABA-responsive genes [70]. The multiple phosphorylation sites suggest that this transcription factor might function as an integration point for multiple signals. These findings demonstrate a direct link between signaling components and transcriptional regulators in the ABA response.

RNA metabolism

In recent years, accumulating evidence has suggested that the ABA response involves mRNA metabolism. In addition, the identification of FCA (which is involved in the regulation of mRNA stability) as one of the ABA receptors has attracted much attention to studies of the regulatory mechanism of the ABA response in mRNA metabolism.

Defects in the components of RNA processing, including splicing, transportation from the nucleus to the cytoplasm and degradation, affect the ABA response. Cap binding complex (CBC) binds the 5’ terminal cap structure of mRNA, protects it from decapping enzymes, enhances splicing, and promotes first-round translation. Defects in CBC components induce ABA-hypersensitive phenotypes [71,72]. One such mutation, ABA hypersensitive 1 (abh1), affects the expression of some genes [71,73,74]. Similarly, a defect in an Lsm protein, SUPERSENSITIVE TO ABA AND DROUGHT 1, which is involved in RNA splicing and/or mRNA decapping, causes an enhanced stress response [75]. A DEAD-box RNA helicase, LOW EXPRESSION OF OSMOTICALLY RESPONSIVE GENES 4, is required for cold stress and ABA responses [76]. The accumulation of polyA RNA in the nuclei in this mutant suggests that this RNA helicase functions in mRNA export from the nucleus (Figure 2). There is also a link between miRNA-mediated gene regulation and the ABA response. HYPONASTIC LEAVES 1 (HYL1), which is involved in miRNA processing, is required for several phenomena, including the ABA response [77,78]. An impaired function of polyA-specific ribonuclease (PARN) ABA HYPERSENSITIVE GERMINATION 2 (AHG2)/AtPARN, which is one of the three eukaryotic deadenylases involved in the first step of the mRNA degradation process, causes an abnormal ABA response [79]. A defect in a component for nonsense-mediated mRNA decay LOW BET A-AMYLASE 1 (LBA1)/UPF1 affects ABA and sugar responses [80]. Nonsense-mediated decay is an mRNA surveillance system for removing mRNA with premature termination codons (Figure 2). These observations indicate that the ABA response requires proper mRNA processing.

ABA also controls RNA metabolism. AAPPK INTERACTING PROTEIN 1 (AKIP1), a heterogeneous nuclear
ribonucleoprotein, interacts with the SnRK2 kinase AAPK from *Vicia faba*. ABA treatment activates the RNA-binding activity of AKIP1 and induces its relocation into nuclear speckles [81], presumably through phosphorylation by AAPK. Similar observations were reported for an *Arabidopsis* AKIP1 counterpart, UBA2a [82]. Although the role of nuclear speckles is unclear, they appear to function as storage areas for transcription or splicing factors [83]. Thus, ABA presumably induces dynamic changes in nuclear events not only in gene activation but also in the splicing mode by changing the composition of splicing factors [84].

These observations emphasize the importance of mRNA processing in the ABA response. However, the question
arises as to why defects in so many different components of RNA metabolism result in abnormal ABA-response phenotypes. Indeed, some mutants such as abh1, hyl1, ahg2 and lba1 show pleiotropic phenotypes. One possible explanation is that a multi-step post-transcriptional regulation is required to control the complicated network of the ABA response. The identification of FCA as an ABA receptor seems to support this idea. A recent highly sensitive transcriptome analysis indicated that ABA treatment modulates the expression of the more abundant genes [85]. To exert such a drastic change in gene expression, the ABA response might require fine regulation of mRNA processing. Therefore, any malfunctions in gene expression processes and post-transcriptional regulation might affect the execution of the ABA response. Related to this assumption, it should be noted that protein degradation events, particularly those related to transcriptional factors, are also deeply involved in the ABA response [5,6]. Such sophisticated systems might be required to regulate gene products to achieve fine regulation of the ABA response.

### ABA synthesis and degradation pathway

Our understanding of the ABA biogenesis and degradation pathways has increased in recent years. The genes for several enzymes involved in the crucial steps of ABA biogenesis, for example, zeaxanthin epoxidase, 9-cis epoxycarotenoid dioxygenase, and aiscisic aldehyde oxidase, were identified in Arabidopsis and other plants [86,87]. Active ABA levels are downregulated by two pathways: a degradation process and conjugation with sugar. In the degradation process, ABA is first hydroxylated at C-8', predominantly producing 8'-hydroxy ABA, which is subsequently converted to phaseic acid. The Arabidopsis P450 CYP707 family catalyzes this step [88,89]. Analysis of the CYP707 genes indicates that their expression is regulated by stress or development [90,91]. Therefore, total ABA levels seem to be regulated at the gene expression level for both de novo biogenesis and degradation.

ABA is conjugated with glucose by ABA glucosyl transferase, forming physiologically inactive ABA glucosyl ester [92], and is released by β-glucosidase [93]. AtBG1, an Arabidopsis β-glucosidase, is involved in the regulation of endogenous ABA levels [94]. A loss-of-function mutation of AtBG1 decreases endogenous ABA levels and impairs growth; the overexpression of AtBG1 confers the opposite phenotypes. Interestingly, stress treatments rapidly activate AtBG1 through polymerization. Although the polymerization mechanism is unknown, it is interesting that AtBG1 is localized in the endoplasmic reticulum, where many types of cellular stress are monitored. The ABA release by AtBG1 is sufficient to induce the expression of stress-responsive genes and the accumulation of extracellular ABA, suggesting that AtBG1 contributes to the increase of both intra- and extracellular ABA levels [94].

### The ABA signaling network

We now have information on three putative ABA receptors with different structures, different subcellular localizations (i.e. nuclear, plastid and plasma membrane), and presumably different downstream pathways. The most important implication is that ABA acts simultaneously and independently at multiple sites in the cell and evokes different responses at each site (Figure 3). Such a mode of signal perception might represent a complicated multi-input signaling pathway. Moreover, in the ABA signaling pathway, second messengers and signaling components constitute nonlinear, web-like signaling networks in which many components directly or indirectly affect each other (Figure 3). These features contrast with the actions of other known plant hormones. For example, ethylene and cytokinin signals are received by receptors coupled with histidine kinases (or related proteins) and are transduced through a linear signaling pathway to modify gene expression [95,96]. TRANSPORT INHIBITOR RESPONSE 1 and GIBBERELLIN-INSENSITIVE DWARF 1 recognize auxin and gibberellin, respectively, and both are directly involved in the degradation of transcription regulators [97–99]. ABA is deeply involved in responses to many abiotic stresses. The multiple perception sites of ABA reflect the broadness and versatility of its physiological functions. Owing to the sessile and autotrophic nature of plants, the regulation of chloroplast development and maintenance of photosynthetic machinery, and the control of the transition to reproductive growth are crucial for plant survival, as well as adaptation to abiotic environmental stresses. The integration of the ABA signal into chloroplast–nuclear communication and flowering-time control provides a great advantage in adapting to environmental stresses. A web-like network with many nodes is suitable for gathering information and allowing cross-talk with other signaling pathways, and it contributes to the efficient propagation and fine tuning of the signal. Presumably, the ABA signaling system has developed in complexity because it enables plants to respond to and adapt to stresses efficiently.

### Future directions

Studies are needed to provide more detailed information on the molecular functions of ABA receptors, including the structural requirements for ABA receptors and the way in which ABA modulates receptor activity. The search for additional receptors is also a worthy task. The number of components known to be involved in the ABA response is increasing. Over the next several years, the mission in ABA research will be to connect these components as nodes of the ABA network. Some nodes will have multiple connectors, such as phosphorylation sites and interaction motifs, and some nodes will also have connectors in other dimensions linked to gene expression, RNA metabolism, and protein degradation mechanisms. Further identification of the components will exponentially increase the complexity of the network. Moreover, the large number of family proteins such as multiple kinases, PP2Cs, and transcriptional factors makes it difficult to specify these components as individual nodes in the ABA signaling network. To elucidate the complexity of both cross-talk with other plant hormone responses and the number of family proteins, detailed analyses of the physiological and biochemical functions of each component are necessary. Comprehensive analytical approaches, including proteomic analysis of phosphorylated proteins, interactomes and metabolomes, combined with systems biology approaches...
(e.g. Ref. [100]) will be powerful tools for integrating the network components to elucidate the larger picture of the ABA response mechanism.

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