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Regulatory mechanism of abscisic acid signaling

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Abscisic acid (ABA) is a major phytohormone that mediates the adaptation of plants to environmental stresses such as drought and regulates developmental signals such as seed maturation. Studies on ABA signaling have progressed rapidly since the recent discovery of PYR/PYL receptor proteins as soluble ABA receptors. In plant cells, the receptor receives ABA to inhibit the phosphatase activity of type 2C protein phosphatase (PP2C), which is the major negative regulator in ABA signaling. SNF1-related protein kinase 2 (SnRK2) is then released from negative regulation by PP2C, turning on ABA signals by the phosphorylation of downstream factors. Insights into the regulation of PYR/PYL receptor proteins is therefore required in order to control drought-stress tolerance in plants. This article reviews the regulatory mechanism of the ABA receptor by ABA and its selective agonist. Structural analyses of PYR/PYL receptors have clearly elucidated the mechanism of ABA perception of the receptor or the mechanism of interaction with PP2C that leads to inhibition of its phosphatase activity. Moreover, the structures of PYR/PYL receptors complexed with pyrabactin, a selective ABA agonist, have provided the structural basis of ABA agonism and antagonism.

Key words: abscisic acid receptor, allosteric effector, environmental-stress tolerance, pyrabactin, PYR/PYL

Abscisic acid (ABA) is a phytohormone that plays critical roles in adaptive responses to stresses such as drought and high salinity. Under water-stress conditions, ABA accumulates in plant cells, promotes stomatal closure in guard cells, and regulates the expression of many genes whose products help protect vegetative tissues from dehydration or high osmotic pressure. In addition, ABA plays a central role in many developmental stages, such as seed maturation and dormancy. Thus, ABA can be considered a water stress-related phytohormone that contributes to the dehydration and/or desiccation tolerance of cells. Numerous studies have attempted to clarify the cellular and molecular responses to ABA in plants[1].

It was previously reported that type 2C protein phosphatase (PP2C) and SNF1-related protein kinase 2 (SnRK2) are major negative and positive regulators, respectively, in the ABA signaling pathway[1,2]. However, the previous model of ABA signaling was very complicated, because several types of ABA receptor and/or binding protein have been reported, such as G-protein-coupled receptors (GCR2, GTG1/2) and the H-subunit of Mg-chelatase (ABAR), and their relationships to the ABA signaling pathway remain unclear[3–6]. The ABA signaling model was dramatically updated in 2009. A major breakthrough resulted from the discovery of PYR/PYL receptor as a new type of soluble ABA receptor[7,8]. This receptor was linked directly to the ABA signaling pathway through the interaction with PP2C in an ABA-dependent manner.

The importance of ABA signaling to global agriculture is widely accepted, and elucidation of the regulatory mechanism of ABA signaling will be needed in order to develop new techniques, such as the generation of transgenic crops with enhanced ABA sensitivity and the rational design of ABA agonists to tolerate environmental stresses. Several structural studies on PYR/PYL receptors have provided the full mechanistic basis of ABA signaling regulation[9–13]. In this review, we describe recent progress in elucidation of

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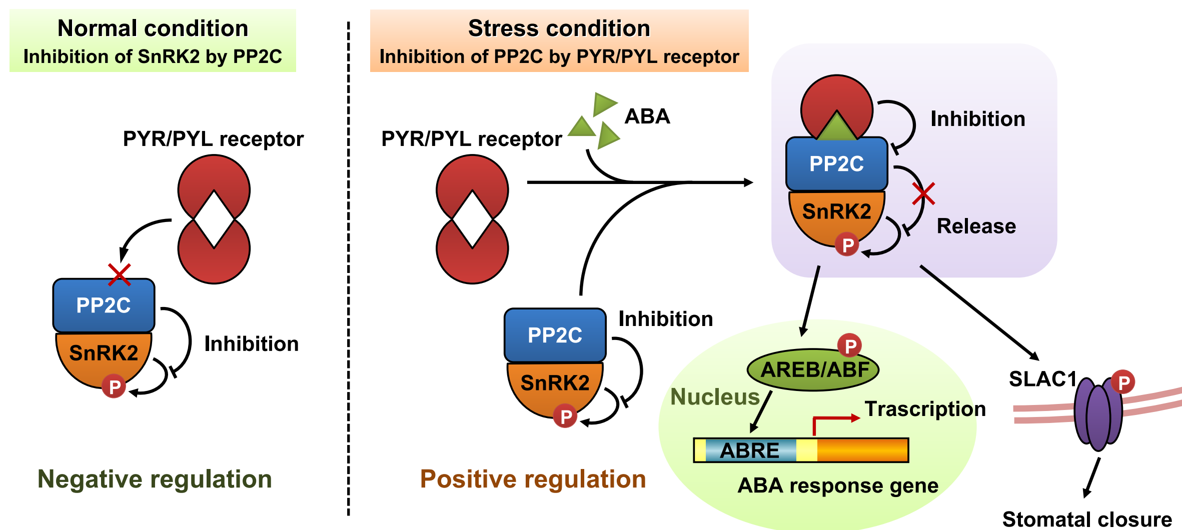

Figure 1 Schematic diagram of a double-negative regulation system in ABA signaling.

our understanding of the mechanism by which PYR/PYL receptors recognize and bind ABA to inhibit the phosphatase activities of PP2C, and we discuss the structural basis of ABA agonism and antagonism.

Outline of the ABA signaling pathway

The core components of the ABA signaling pathway are the PYR/PYL receptor, PP2C, and SnRK2. Among the PP2C family members in plants, group A PP2Cs act as global regulators of ABA signaling. Some of the loss-of-function mutants of group A PP2Cs show significant ABA sensitivity, indicating that they are major negative regulators of ABA signaling[1]. On the other hand, the SnRK2 family was identified as a major positive regulator. There are 10 SnRK2 members in *Arabidopsis*, and they are categorized into subclasses labeled I, II, and III[14]. Among these, subclass III contains three kinases—SRK2D/SnRK2.2, SRK2E/OST1/SnRK2.6, and SRK2I/SnRK2.3—that are strongly activated by ABA. ABA-induced protein kinase activities are largely impaired in the *srk2dei* triple mutant[15,16]. In addition, the mutant lacks most ABA responses, including seed maturation and dormancy, ABA-responsive gene expression and stomatal movements. These findings indicate that subclass III SnRK2s act as central hubs in ABA signaling.

The core components form the signaling complex, which offers a double-negative regulation system in ABA signaling (Fig. 1)[17]. Normally, PP2C inactivates SnRK2 by dephosphorylation and the ABA signals are silent. Once ABA is induced by environmental conditions or developmental cues, the ABA-bound PYR/PYL receptor interacts with PP2C and inhibits its phosphatase activity. SnRK2 is then released from negative regulation by PP2C, turning on ABA signals by the phosphorylation of downstream factors such as AREB/ABF bZIP transcription factors, SLAC1 slow anion channels, and others. Phosphorylated AREB/ABF activates numerous downstream genes in response to water stress, including many late embryogenesis abundant (LEA) class genes and other transcription factors[18]. SLAC1, which has a central role in guard cells, induces rapid stomatal closure to prevent water loss by perspiration[19,20]. Therefore, the ABA signaling pathway consists of just four steps to gene expression or another output, and its switching point is ABA perception of the PYR/PYL receptor.

ABA perception of the PYR/PYL receptor with structural changes

Structural analyses of PYR/PYL receptors clearly elucidated the mechanism of ABA perception[9–13]. Figure 2a shows the structure of ABA-bound PYL1, a PYR/PYL family member in *Arabidopsis*. The receptor exhibits a helix-grip fold similar to those of the START protein superfamily, and binds an ABA molecule in its internal cavity. This structure defines the binding mode of ABA and explains its stereoselectivity (Fig. 2b). The carboxyl group of ABA forms an ion pair with K86 and a water-mediated hydrogen bond network with five other polar residues. This network is required for the binding of ABA to PYL1. All other residues in the cavity are hydrophobic and form van der Waals contacts with a 2,6,6-trimethyl-cyclohexene ring. PYR/PYL receptors show a higher affinity to the (+)stereoisomer of ABA than to the biologically less active (–)stereoisomer (Fig. 2c, d)[9,10]. The flipped dimethyl group in the (–)ABA would cause steric hindrance between the dimethyl group and the narrow pocket that accommodates the monomethyl group[9]. Thus, the arrangement of the hydrophobic residues surrounding the cyclohexene moiety defines the stereoselectivity of ABA isomers.

ABA perception by a PYR/PYL receptor induces struc-

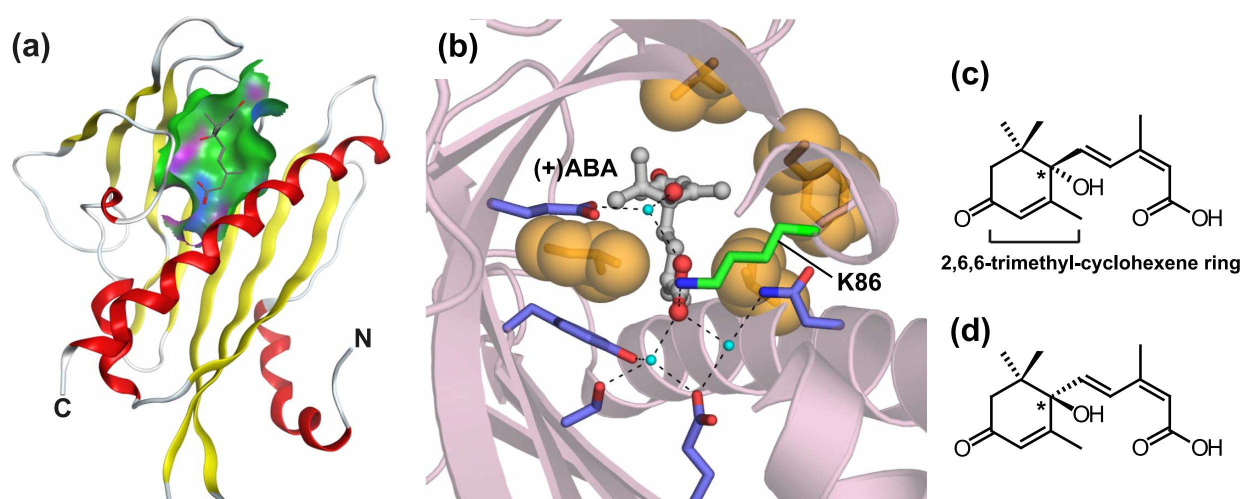

Figure 2 ABA-binding mode of the ABA receptor. (a) Overall structure of PYL1 complexed with ABA. Stick and ribbon diagrams show ABA and PYL1, respectively. An ABA molecule binds in an internal cavity, which is shown by a surface diagram. Red, blue and green surfaces represent polar, charged and hydrophobic residues, respectively. (b) Structural basis of (+)ABA stereoselectivity. Polar residues (blue) and K86 (green) form a hydrogen bond network mediated by water molecules (cyan). Hydrophobic residues (orange) are located around the 2,6,6-trimethyl-cyclohexene ring of ABA. Panels (a) and (b) were created using Protein Data Bank (PDB) coordinates of ABA-bound PYL1 (3JRS). (c, d) Chemical structures of ABA stereoisomers: (c) (+)ABA; (d) (−)ABA. An asterisk shows the position of asymmetric carbon.

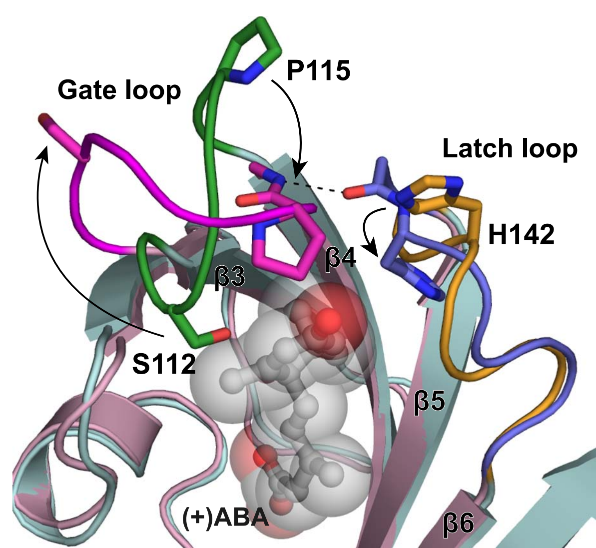

Figure 3 Open-to-closed gating mechanism of PYL1. Structures of apo- (green and orange) and ABA-bound (magenta and blue) states are superposed on each other. The gate and latch loops move to a closed conformation upon ABA binding, as shown by arrows. Two closed loops form a hydrogen bond, which is represented by a dashed line. This figure was created using the PDB coordinates of apo-PYL1 (3KAY) and ABA-bound PYL1 (3JRS).

tural changes essential for ABA signal transduction. Superposition of the apo- and ABA-bound structures of PYL1 shows the conformational differences in the two conserved loops (Fig. 3)[9,10]. One of the two loops connects β-strand 3 with β-strand 4 (referred to as the gate loop), and the other loop connects β-strand 5 with β-strand 6 (referred to as the latch loop). Upon ABA binding, P115 on the gate loop moves toward the 2,6,6-trimethyl-cyclohexene ring of ABA to close the gate on the cavity, whereas S112 is flipped out of the ABA-occupied cavity. In addition, H142 on the latch loop turns into the cavity to form van der Waals contacts with ABA. The latch loop locks the closed gate loop by a hydrogen bond and van der Waals contacts. Multiple conformations of these loops are observed in the ABA-bound structures of the receptors. Most residues on these loops very weakly contribute to ABA binding[10]. Hence, the closed conformations of the gate and latch loops are expected to be insufficiently stabilized even if ABA is trapped in the cavities of its receptors[9,10].

Structural changes by ABA perception appear also in the homodimer assembly of several PYR/PYL receptors[13]. The dimerization of the receptors can be observed in their crystal structures and confirmed in solution by small-angle X-ray scattering (SAXS)[11] and size-exclusion chromatography[13], The relative orientation of the two protomers is modified by conformational changes in the gate loop upon ABA binding[13]. The contact between protomers seems to stabilize the gate loop with conformational plasticity in the open or closed state. Recently, it was reported that some PYR/PYL receptors behave as monomers in the apo state and constitutively bind to PP2C in the same manner as ABA-bound receptors[21]. Therefore, the fixation of the gate loop in the dimer interface prevents the binding of PP2C to the loop of the apo receptor via an induced fit mechanism. The homodimer assembly is required to strictly regulate the ABA-dependent switching of signal transduction by the PYR/PYL receptor.

ABA-induced inhibitory mechanism of PP2C

The inhibitory mechanism of the ABA-bound receptors is well defined by the structure of the ternary complex with PP2Cs[9,10,13]. Figure 4 shows the interface between PYL1 and ABI1, a PP2C family member in *Arabidopsis*. The β-hairpin of ABI1 provides the major binding interface with the ABA-bound PYL1[10]. There are two crucial residues for binding the closed gate and latch loops of the receptor. W300 inserts its indole ring between the two closed loops of the ABA-bound receptor. The indole imine group forms a water-mediated hydrogen bond with the two closed loops and the carbonyl group of ABA. In addition, R304 seems to hold down the gate loop of the receptor by stacking between its guanidinium group and P115 on the loop of PYL1. These interactions further lock the gate loop in the closed conformation, which properly situates the gate loop into the active site of PP2C.

The catalytic site of ABI1 is sealed by the closed gate loop of PYL1. S112 on the gate loop exposed upon ABA binding forms a hydrogen bond between its side chain and E142, one of the conserved catalytic residues. Thus, the ABA-bound receptor is capable of competitively inhibiting the phosphatase activity of PP2C by using the gate loop like a plug. The serine residue also contacts G180 on the active site loop of ABI1 through the hydrogen bond with a backbone amine. The mutation to an aspartic acid residue at this position is known as an *abi1-1* mutation, a typical gain-of-function mutation[22–24]. This structural observation explains why the mutation hinders contact between the gate loop of the ABA-bound receptor and the active site of PP2C[9,10,13].

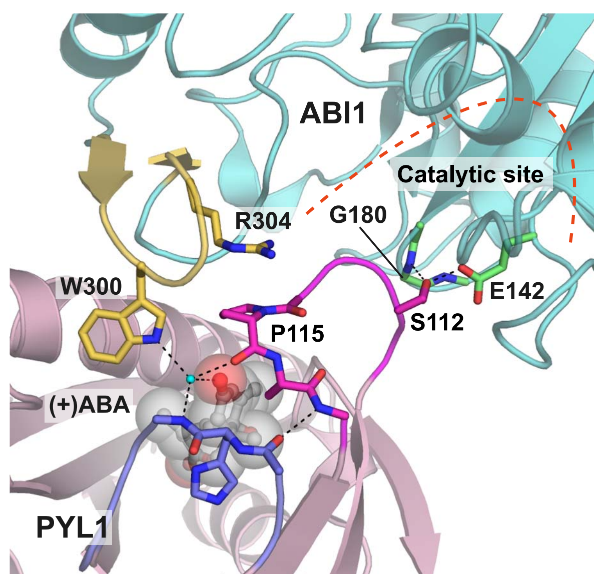

Figure 4 ABA-induced interaction between PYL1 and ABI1. The gate (magenta) and latch (blue) loops contact the β-hairpin of ABI1 (yellow), which also indirectly interacts with ABA mediated by a water molecule (cyan). This figure was created using PDB coordinates of the complex of ABA-bound PYL1 and ABI1 (3JRQ).

Structural basis of ABA agonism and antagonism

Pyrabactin is a synthetic ABA agonist that has been used to identify PYR/PYL receptors[8]. The chemical structure of pyrabactin (Fig. 5a) is not similar to that of (+)ABA (Fig. 2c). ABA activates all PYR/PYL receptors to inhibit PP2C, whereas pyrabactin activates several receptors, such as PYR1 and PYL1, and inhibits others, such as PYL2[8]. In other words, pyrabactin acts as a selective agonist for PYR1 and PYL1. As a result of pyrabactin's selectivity, it inhibits seed germination but shows no transcriptional activation of an ABA-responsive gene in vegetative tissues[8]. Therefore, a robust framework is necessary for the design of new agonists capable of regulating ABA signaling globally. Several groups have reported the structures of ABA receptors bound to pyrabactin[25–28], providing new insights into ABA agonism and antagonism to aid the rational design of ABA agonists.

Structural analyses have demonstrated that PYL1 binds to pyrabactin in a manner similar to that of ABA[25,27]. Figure 5b shows the binding mode of pyrabactin in the ABA-binding cavity of PYL1. The pyridyl nitrogen of pyrabactin forms a water-mediated hydrogen bond with K86 in the cavity. The water molecule is located in the same position as the carboxylate oxygen in ABA, which directly contacts K86. This structural observation well explains why the pyridyl nitrogen is a key function group for binding to PYL1[8]. In addition, the naphthalene ring of pyrabactin mimics the function of the 2,6,6-trimethyl-cyclohexene ring of ABA. These two rings would be the major driving force that pulls the hydrophobic residues of the gate loop over to achieve a closed conformation. Moreover, the bromine group of pyrabactin forms a water-mediated hydrogen bond with two closed loops of PYL1. The structure of pyrabactin-bound PYL1 and ABI1 shows that pyrabactin-induced conformational changes in two loops are required in order to provide the binding interface with PP2C to inhibit its activity[25]. Thus, the agonism of pyrabactin is quite similar to that of ABA, although their chemical structures are quite different.

In contrast to PYL1, pyrabactin does not induce change in the gate loop of PYL2 in a closed conformation (Fig. 5c)[25,26,28]. Pyrabactin binds to the ABA-binding cavity of PYL2; however, its binding mode to PYL2 is quite different from that to PYL1. PYL2 K64, which corresponds to PYL1 K86, forms a water-mediated hydrogen bond with the sulfonamide group of pyrabactin. Moreover, the naphthalene ring is located far from the gate loop of PYL2, which is not capable of inducing conformational changes in the gate loop. Thus, pyrabactin acts as an ABA antagonist to PYL2. The activity of pyrabactin as a selective ABA agonist is defined by inducing conformational changes in the gate loops of ABA receptors.

Although the features of the ABA-binding cavity are well

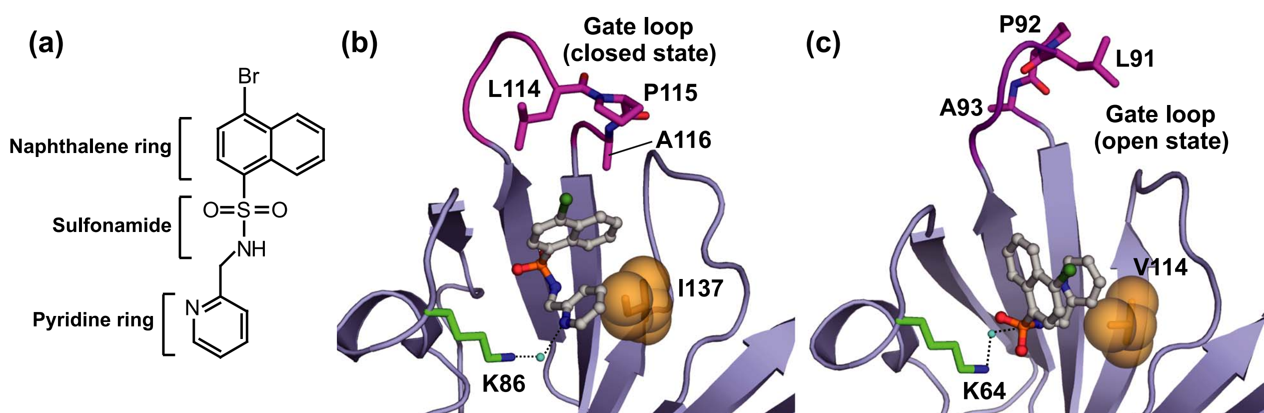

Figure 5 Structural basis of pyrabactin selectivity. (a) Chemical structure of pyrabactin. (b) Pyrabactin-induced gate closure of PYL1 (ABA agonism). The arrangement of pyrabactin in the ABA-binding cavity is defined by a contact between the pyridine ring and PYL1 I137 and a water-mediated hydrogen bond between the pyridyl nitrogen and PYL1 K86. The closed gate loop contacts the naphthalene ring of pyrabactin by van der Waals attractions. This panel was created using PDB coordinates of pyrabactin-bound PYL1 (3NEF). (c) Pyrabactin-binding mode of PYL2 with an open gate loop (ABA antagonism). Pyrabactin is located away from the gate loop by a water-mediated hydrogen bond between the sulfonamide group and PYL2 K64 in the ABA-binding cavity, which makes the gate loop stay in an open conformation. This panel was created using PDB coordinates of pyrabactin-bound PYL2 (3NS2).

conserved between PYL1 and PYL2, pyrabactin binds differently to PYL1 than to PYL2. A possible reason for the pyrabactin selectivity has been explained by a single residue variation between valine and isoleucine[25,28]. These residues differ from each other by only one methyl group. In the ABA-binding cavity, the residue corresponding to PYL1 I137 is substituted to a valine residue in PYL2 (PYL2 V114). The side chain of V114 contacts the pyridine ring of pyrabactin, and hence the V114I mutation would cause steric hindrance between the additional methyl group and the pyridine ring. To avoid such a steric clash, pyrabactin would be rotated 90 degrees in the cavity of PYL1[28]. Mutation analyses have in fact shown that the swapping of the isoleucine and valine residues switched the pyrabactin selectivity of PYL1 and PYL2[25,28]. Thus, the arrangement of hydrophobic residues in the ABA-binding cavity influences not only the stereoselectivity of ABA but also the selective agonism of pyrabactin.

Summary

Recent structural investigations have led to significant progress in understanding the regulation of ABA signaling. ABA induces conformational changes in the PYR/PYL receptor, creating a continuous binding interface with PP2C, and does not directly bind to PP2C. These findings show that the PYR/PYL receptor is the direct ABA receptor and that ABA regulates the receptor protein as an "allosteric effector." In addition, pyrabactin allosterically regulates several PYR/PYL receptors as a synthetic ABA agonist in the same manner as ABA. This common concept is required for the development of effective ABA agonists. Moreover, if we take advantage of variations in each PYR/PYL receptor, the design of global ABA agonists or alternative ABA agonists with valuable selectivities can be developed.

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