Complex Structures of the Abscisic Acid Receptor PYL3/RCAR13 Reveal a Unique Regulatory Mechanism

Xingliang Zhang, Qi Zhang, Qi Xin, Lin Yu, Zheng Wang, Wei Wu, Lun Jiang, Guoqiang Wang, Wenli Tian, Zengqin Deng, Yang Wang, Zhao Liu, Jiafu Long, Zhizhong Gong, and Zhongzhou Chen

1. State Key Laboratory of Agrobiotechnology
2. State Key Laboratory of Plant Physiology and Biochemistry
3. State Key Laboratory of Medicinal Chemical Biology, College of Life Sciences, Nankai University, Tianjin 300071, China
4. The Key Laboratory of Bioorganic Phosphorus Chemistry, Ministry of Education, Tsinghua University, Beijing 100084, China

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SUMMARY

Abscisic acid (ABA) controls many physiological processes and mediates adaptive responses to abiotic stresses. The ABA signaling mechanisms for abscisic acid receptors PYR/PYL/RCAR (PYLs) were reported. However, it remains unclear whether the molecular mechanisms are suitable for other PYLs. Here, complex structures of PYL3 with (+)-ABA, pyrabactin and HAB1 are reported. An unexpected trans-homodimer intermediate observed in the crystal is confirmed in solution. ABA-bound PYL3 greatly promotes the generation of monomeric PYL3, which can excessively increase the efficiency of inhibiting PP2Cs. Structure-guided biochemical experiments show that Ser195 accounts for the key intermediate. Interestingly, pyrabactin binds to PYL3 in a distinct nonproductive mode with gate closure, which sheds light on the design of agonists and antagonists for abscisic acid receptors. According to different conformations of ligand-bound PYLs, the PYLs family can be divided into three subclasses, among which the trans-dimeric subclass, represented by PYL3, reveals a distinct regulatory mechanism.

INTRODUCTION

The phytohormone abscisic acid (ABA) is vital for the regulation of many processes such as plant growth, stomatal aperture, seed maturation, dormancy, and response to abiotic stresses including drought, cold, and salinity (Cutler et al., 2010; Finkelstein et al., 2002; Raghavendra et al., 2010). Plant ABA content significantly increases under abiotic stresses, which changes the expression of related genes and chemical substances and allows the plant to adapt to stress conditions. Therefore, understanding the events in ABA signal transduction has been one of the most important goals in plant research.

The recent identification of the pyrabactin (4-bromo-N-pyridin-2-yl methyl naphthalene-1-sulfonamide) resistance (PYR/PYL)/regulatory component of ABA receptor (RCAR) family (hereafter referred to as PYLs) and their biochemical modes of action represent a major breakthrough in the field (Ma et al., 2009; Park et al., 2009). The structures of apo- and ABA-bound abscisic acid receptors PYL1, PYL2, and PYR1 were solved by several groups (Melcher et al., 2009; Miyazono et al., 2009; Nishimura et al., 2009; Santiago et al., 2009; Yin et al., 2009). Based on these structures, an ABA signaling mechanism was proposed. In these structures, PYL1, PYL2, and PYR1 are cis-homodimers. This term is used to describe two PYL molecules that bind to each other in the same direction (i.e., in a parallel binding mode). Upon binding to ABA or pyrabactin, the gate (Melcher et al., 2009) of PYL undergoes a pronounced conformational change, and the PYL protomers in the cis-homodimer separate slightly, decreasing the area of interface and creating a binding surface for protein phosphatase 2C (PP2C). Upon binding to PP2C, ligand-bound PYL dimers dissociate and form a PYL/PP2C heterodimer via the newly formed surface. In the absence of ABA, the kinase activity of SnRK2 is abrogated by PP2Cs through physical interaction and dephosphorylation (Soon et al., 2012). Therefore, the heterodimer PYL/PP2C occludes the catalytic site of PP2Cs and releases PP2C-mediated inhibition of proteins such as SnRK2 (Fuji et al., 2009). The PYL family (Lytle et al., 2009) contains 14 high-similarity members in Arabidopsis thaliana. It is unclear whether this molecular mechanism is applicable to other abscisic acid receptors. In addition, it is controversial whether apo-PYLs and ABA-bound PYLs exist as a monomer or dimer in solution. Gel filtration coupled to multi-angle laser light scattering and small angle X-ray scattering analysis (SAXS) showed that both PYR1 and PYL2 are dimers either in the presence or in the absence of ABA (Nishimura et al., 2009; Santiago et al., 2009; Yin et al., 2009). However, apo-PYL2 was determined to be monomer in solution by gel filtration and NMR, whereas apo-PYL1 was determined to exist in a monomer–dimer equilibrium (Melcher et al., 2009). Moreover, one important question is whether an intermediate can be detected during the monomer–dimer equilibrium if the monomer exists.

In vivo, the Arabidopsis triple (pyr1;pyl1;pyl4) and quadruple (pyr1;pyl1;pyl2;pyl4) mutants are less sensitive to (+)-ABA inhibition in seed germination and root growth compared to wild-type (Park et al., 2009). However, the quadruple mutant still responded to ABA signals, such as the expression of RD29
in the presence of (+)-ABA. These data imply that other important abscisic acid receptors are required for ABA signaling. According to the data mining from the Arabidopsis expression data, PYR1, PYL1, PYL2, and PYL4 were almost not expressed during seed development (Winter et al., 2007) (Figure S1 available online). On the contrary, PYL3/RCAR13 is primarily expressed in the chalazal seed coat during seed development, especially in the globular stage (Figure S1). In plant ovules, the chalazal is an important tissue in the testa where the connection of the vascular tissues of the maternal funiculus to the seed ends. It is involved in transferring nutrient resources from the mother plant. During early seed development in Arabidopsis, ABA content of seed fluctuates greatly and increases to a maximum between one-third to one-half of the time from seed initiation to maturity (Karssen et al., 1983). Interestingly, ABA was preferentially detected in the nucellus near the chalaza (Peng et al., 2006), and was found to be related to assimilate flow and distribution. In developing seeds, ABA is necessary for inducing the synthesis of reserve proteins and lipids (Finkelstein et al., 2002), as well as for seed physiological dormancy and the desiccation tolerance, to prevent premature germination at the end of the cell division phase of embryogenesis. Considering that PYL3 is highly expressed in the chalazal seed coat from microarray studies, it is speculated to be involved in the complex regulation of ABA signaling pathway, such as assimilates and signal transduction.

The complex structures of PYL1, PYL2, and PYR1 with pyrabactin were also reported (Hao et al., 2010; Melcher et al., 2010; Peterson et al., 2010; Yuan et al., 2010). Pyrabactin bound to PYL1 or PYR1 in productive mode as an agonist whereas to PYL2 in nonproductive mode as an antagonist. Whether the closed state of the gate responding to pyrabactin is an exclusive determinant of agonists/antagonists for abscisic acid receptors (Hao et al., 2010; Melcher et al., 2010; Peterson et al., 2010; Yuan et al., 2010) remains to be elucidated. In addition, microarray analyses have revealed that the transcriptional responses induced by pyrabactin, a synthetic seed germination inhibitor of ABA, are highly correlated with ABA responses in seeds (Park et al., 2009). Although the complex structures of PYR1, PYL1, or PYL2 with pyrabactin were solved recently (Hao et al., 2010; Melcher et al., 2010; Peterson et al., 2010; Yuan et al., 2010), the mechanism underlying that pyrabactin inhibits seed development is not clear.

To explore the aforementioned issues, we focused on the structure–function relationship of PYL3, the most abundantly expressed PYL during seed development. Based on a combination of structural and biochemical methods, high-resolution crystal structures of apo-PYL3 and its structures in complex with (+)-ABA, pyrabactin and HAB1 were determined, respectively. Upon ligand binding, the apo-PYL3 cis-homodimer is converted to a trans-homodimer, in which two ligand-bound PYLs bind to each other in a reverse direction. To our knowledge, this represents a novel intermediate found naturally in the dissociation of PYLs. Meanwhile, Ser195 in PYL3 accounts for the generation of the key intermediates in the presence of ligands. Interestingly, pyrabactin as an antagonist binds to PYL3 with gate closure, which sheds light on the design of agonists and antagonists. According to conformations of ligand-bound PYLs, the PYR/PYL/RCAR family can be divided into three subclasses, which represent different mechanisms of themselves.

RESULTS

PYL3-Ligand Complexes in Crystal Are trans-Homodimers

Recently, it was shown that the ability of PYL3 in the reconstitution of the ABA signaling pathway for SnRK2-mediated phosphorylation in vitro was lowest among PYLs protein (Fujii et al., 2009). To understand the molecular mechanism underlying the functions of PYL3 distinct from other PYLs, we obtained crystal structures of apo-PYL3 and (+)-ABA bound PYL3. The apo-PYL3 structure was solved by heavy atom Pt soaking by SIRAS. There are two PYL3 protomers in the asymmetric unit (Figure 1A and Table 1). One PYL3 protomer consists of four α helices and seven β sheets (Figure S2). It shares a number of unique structures and unique mechanism of ABA receptor PYL3.
conserved residues with other solved PYL family members (Melcher et al., 2009; Miyazono et al., 2009; Nishimura et al., 2009; Santiago et al., 2009; Yin et al., 2009). Moreover, the arrangement of the apo-PYL3 dimer is a cis-homodimer, similar to the solved structures of PYR1, PYL1, or PYL2. This term is used to describe two PYL molecules that bind to each other in the same direction (i.e., in a parallel binding mode).

The complex crystal of PYL3 with (+)-ABA was diffracted to 2.7 Å in the space group I212121 and solved by molecular replacement (Table 1). The complex structure had two PYL3 protomers in each asymmetric unit (Figure 1C). The protomer structure in the PYL3-(+)-ABA complex is similar to that of apo-PYL3, with a root-mean-square deviation (rmsd) of 1.2 Å between their Cα atoms. An electron density appeared in the conserved

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Three crystal experiments for each structure.

*Statistics for highest resolution shell.*
Structures and Unique Mechanism of ABA Receptor PYL3

To determine whether the trans-homodimer conformation exists in other PYL3 complex, we also solved the structure of PYL3 complex with pyrabactin. The PYL3-pyrabactin complex structure was also determined by molecular replacement and further confirmed by the low thermal factors and Fo–Fc differential electron density map (Figures S3B and S3C). There are two PYL3 protomers bound to pyrabactin in each asymmetric unit (Figure S3C). This structure is similar to the PYL-(+)-ABA complex, in a trans-homodimeric conformation (Figure S3C).

Structural Features of the trans-Homodimers
In the above two complex structures, each lid L4 (Figure S2, also known as gate [Melcher et al., 2009] or CL2 [Yin et al., 2009]) is closed, whereas it is open in apo-PYL3 (Figure 2A). Compared with the cis-homodimer (Figure 1A), L4, L5 (also known as latch [Melcher et al., 2009] or CL3 [Yin et al., 2009]), L7, and the ABA binding pocket (Figure 2A) in the trans-homodimer are more exposed to the solvent (Figure 1C). Moreover, their temperature factors are higher, implicating more flexibility in this region, a partial interface for PP2Cs binding. Therefore, this feature implies that the trans-homodimer conformation might promote the binding of PYL3 and PP2C.

Here we elucidate the interactions that stabilize the trans-homodimer. There are two hydrophobic network interaction and several hydrogen bonds in the interface of the trans-homodimer, mostly in loops L4, L2, and x4 (Figure 2B). Notably, two hydrophobic networks (Figure 2B) are formed by Phe81, Leu111, and Val192 in one PYL3 molecule, and by Val202 and Ile203 in the other. As expected, the triple mutant F81A V202A I203A is monomer according to size exclusion chromatography and analytical ultracentrifugation (Figures 3A and 5) and retains ABA binding activity judged from its ability to inhibit HAB1 (Figure 4). However, the inhibitory activity of this mutant is lower than wild-type probably because F81A reduces the binding of ABA (Figure 4). On the contrary, double mutant V202A I203A enhances the inhibitory ability (Figure 4) due to weaker hydrophobic interactions in the interface. Other than the hydrophobic interactions in the interface, two hydrogen bonds are formed between Asn199 in each protomer (Figure 2B).

To clarify the details of the change in orientation of one PYL3 molecule relative to the other in the trans-homodimer, the angle between the two x4 helices was calculated by MOLMOL (Koradi et al., 1996). Upon ligand binding, one PYL3 protomer rotates by 135° when apo-PYL3 and ligand-bound PYL3 are superimposed. In contrast to PYL2, the two x4 helices and the two PYL3 protomers approach closer after binding ligands (Figure 2B). However, the interface accessible surface area (Krissinel and Henrick, 2007) decreases from 933.64 Å² to 868.09 Å² upon binding to (+)-ABA. This observation suggests that the interaction between the two PYL3 monomers becomes weaker upon ligand binding. The trans-homodimer might easily dissociate to the monomer and promote the formation of PYL3-PP2C heterodimer.

Formation of the trans-Homodimer in Solution
It is controversial whether apo-PYLs or (+)-ABA-bound PYLs exists as a monomer or dimer in solution. To understand how two PYL3 protomers interact with each other, we employed seven methods: gel filtration, crosslinking, disulfide bond engineering, subunit exchange, mass spectrometry, SAXS, and analytical ultracentrifugation, to confirm that the trans-homodimer of PYL3 exists in solution and is not the result of a crystal packing. We first evaluated the influence of ligands on the oligomeric state of PYL3 by gel filtration on a calibrated Superdex-200 HR10/30 column. Wild-type PYL3 was eluted at 15.9 ml, corresponding to a molecular weight of ~44 kDa; in the presence of (+)-ABA, it was eluted at 16.34 ml (~36 kDa) (Figure 3A). To determine whether the 16.34 ml peak corresponded

See alsoFigures S3, S5, and S8.
to the monomer, we performed crosslinking followed by SDS-PAGE to identify the crosslinked products. Using ethylene glycol-bis(succinic acid N-hydroxysuccinimide ester) (EGS), glutaraldehyde, or formaldehyde as crosslinking reagents, the dimer bands increased with the concentration of crosslinking reagents, both in the absence and in the presence of (+)-ABA. Therefore, combination of the crosslinking results and the size exclusion chromatography experiments imply that dimers are the major species in solution (Figures 3B, S4A, and S4B). On the contrary, the dimer band was not observed for the monomeric F81A V202A I203A mutant.

Moreover, analytical ultracentrifugation was used to assess the states of apo- and ligand-bound PYL3 in solution. The sedimentation velocity (SV) results indicate that 3.9% of apo-PYL3 exists as a monomer at 0.2 mM, whereas the proportion of monomer increased to 18.2% in (+)-ABA bound PYL3 (Figure 5). These data support that ligand-bound PYL3 mainly exists as a dimer.

Although we have demonstrated that apo-PYL3 exists mainly as a dimer in solution, it is not clear to which dimer conformation the 16.34 ml peak observed by gel filtration corresponds. To determine the predominate dimer species, we used disulfide bond engineering to study the dimer conformation in solution. Structural analysis shows that the two α4 helices are antiparallel in the PYL3 trans-homodimer, whereas they are parallel in the cis-homodimer. The α4 helices were scanned to find residues that, when mutated to cysteine, could form a disulfide bond. The distances between the Cα atoms of Asn180 and that of Pro208 or Thr209 in two different protomers of the trans-homodimer are 6.11 Å and 6.21 Å, respectively, whereas both of them are >35 Å in the cis-homodimer (Figure 2B). Considering that the residues at the end of the C terminus are flexible and that the Cα–Cα distance between cysteine residues in disulfide bonds is usually ~6.5 Å, we speculated that a disulfide bond would be formed between the above residues under appropriate conditions. To test this hypothesis, Asn180, Pro208, or Thr209 were mutated to cysteine separately or in combination. Samples of the purified proteins were subjected to SDS-PAGE under reducing or nonreducing conditions. The N180C T209C double mutant formed a dimer band only in the presence of (+)-ABA under nonreducing conditions, not in the absence of ligand or under reducing conditions (Figure 3C, i). The N180C P208C T209C triple mutant had similar result (Figure S4C). On the contrary, no dimer band was detected for WT, N180C, T209C mutants under the same conditions, respectively. The ability to form disulfide bonds implicates that the trans-homodimer is formed in solution upon the addition of ligands, but not in the absence of ligands. Interestingly, for an equal molar ratio mixture of N180C mutant and T209C mutant (Figure 3C, iv), the dimer band only appeared with the addition of the (+)-ABA under nonreducing conditions, the same condition as the N180C T209C double mutant. It shows that subunit exchange occurs and a trans-heterodimer forms between the N180C mutant and T209C mutant in the presence of (+)-ABA.

The states of apo- and ligand-bound PYL3 in solution were further confirmed by mass spectrometry, widely used to determine protein disulfide bond (Barbirz et al., 2000; Gorman et al., 2002). The free thiols in the intact protein were blocked by rapid alkylation with iodoacetamide, followed by proteolytic cleavage by trypsin overnight. From the diagrammatic representation (Figure 6), the specific peptides formed by the proteolysis of N180C T209C mutant in the presence of (+)-ABA were found at m/z 4132.5 and 3871.0, whose disulfide bond were further confirmed by reduction (Figure 3D). The two peptides were reduced to cysteine-containing peptide at m/z 2411.1 with the addition of DTT (Figure 3D). On the contrary, no similar peptides were detected for the N180C T209C mutant without ligands. In addition, the above two m/z values can not be formed for cis-dimer or monomer, theoretically and experimentally. It clearly shows that disulfide bond forms exclusively between residues C180 and C209, and a trans-homodimer exists in the presence of (+)-ABA.
In addition to the aforementioned experiments, the diameter and the shape of the particle observed by SAXS (Figure S5) are consistent with the above results. In summary, these results clearly show that PYL3 trans-homodimer mainly exists in the solution with the addition of (+)-ABA.

S195L Keeps cis-Homodimer in the Presence of (+)-ABA

Another key question is why PYL3 is the only member of subfamily III that adopts the trans-homodimer intermediate. Structural comparison and analytical ultracentrifugation (Figure S6) experiments indicate that the interaction between two PYL3 protomers in apo-PYL3 is weaker than in PYR1, PYL1, or PYL2. Sequence alignment (Figure S2) shows that several amino acids vary within this subfamily. In particular, Ser195 in PYL3 differs greatly from the corresponding leucine in PYR1, PYL1, or PYL2.

Structural analysis shows that the two Ser195 residues in the apo-PYL3 dimer are in the face-to-face conformation and that the distance between the two C\(\beta\) atoms is 4.59 Å. On the contrary, in the trans-homodimer of (+)-ABA-bound PYL3, the two Ser195 residues are in the back-to-back conformation, and the corresponding distance is 7.71 Å. Therefore, mutation of Ser195 to a hydrophobic residue should enhance the dimeric interaction in apo-PYL3, but not in ligand-bound PYL3. To validate this hypothesis, we mutated Ser195 to leucine. As expected from the structural observations, the cis-homodimer state is retained in the PYL3 S195L mutant upon addition of (+)-ABA as determined by analytical ultracentrifugation (Figures 5, S6E, and S6F). Moreover, the N180C S195L T209C triple mutant does not form a dimer band in the presence of (+)-ABA under nonreducing conditions, indicating the inability of this mutant to form the trans-homodimer (Figure 3C, iii). The results indicate that replacement of Ser195 with the hydrophobic leucine greatly enhances the interactions in the cis-homodimer interface, and it is difficult to break these interactions to produce trans-homodimer upon the addition of ligands. Therefore, the single-site mutation of serine to leucine accounts for the distinct mechanism observed in PYL3.

Structure of the PYL3-(+)-ABA-HAB1 Complex and the Inhibitory Mechanism of PP2C by PYL3

The major players in ABA signaling include a subclass of Mg\(^{2+}\)- and Mn\(^{2+}\)-dependent serine-threonine PP2Cs. HAB1 plays a major role and is a negative regulator of ABA signaling at an early stage in the pathway. HAB1 is primarily expressed in meristematic tissues, guard cells, embryos, and siliques. Therefore, to study the role of ABA receptors on seed development, we focused on studying the interaction between PYL3 and HAB1.

The PYL3-(+)-ABA-HAB1 complex structure contains one PYL3 and one HAB1 (Figure 7A). The indole ring of W385 of HAB1 inserts into the ABA binding pocket of PYL3. The structure of PYL3-(+)-ABA in the PYL3-(+)-ABA-HAB1 complex is very similar to PYL3-(+)-ABA complex with an rmsd of 0.7 Å calculated by the Dali server (Holm et al., 2008). However, there are several unexpected conformational changes between them. The most drastic changes are found in L4; in particular Pro112 moves closer to W385 of HAB1 and locks tightly into the pocket. Moreover, the side chain of Arg140 becomes visible in the electron density of the PYL3-(+)-ABA-HAB1 complex and is involved in binding to W385.

The overall structure of the PYL3-(+)-ABA-HAB1 complex is similar to the PYL2-(+)-ABA-HAB1 complex (Melcher et al., Structure 20, 780–790, May 9, 2012 ©2012 Elsevier Ltd All rights reserved 785
2009), and the overall rmsd of the C-α atoms for PYL and HAB1 are 1.2 Å and 0.6 Å, respectively. However, there are several obvious differences in the binding interface. First, only one Mn²⁺ is found in the active center of HAB1, whereas there are three Mg²⁺ ions in the PYL2-(+)-ABA-HAB1 complex (Melcher et al., 2009). Second, due to the smaller side chains of Ser106 and Val108 in PYL3 compared to that of PYL1, PYL2, and PYR1 (Figures S2 and S3D), Leu111 moves more deeply to the binding pocket in order to bind (+)-ABA, which will make the binding of HAB1 weaker. Moreover, the electron density of several side chains in the HAB1 active site is not visible, indicating that the active site of HAB1 is more flexible than in other PP2C structures. Third, in contrast to the PYL2-(+)-ABA-HAB1 complex structure, the conformation of residues E138 and H139 in PYL3 (latch) is not changed. Finally, the hydrophilic residue Ser195 greatly weakens the hydrophobic interaction (Figure 7B) in the heterodimeric interface of PYL3-(+)ABA-HAB1, which is strengthened by Leu172 in PYL2 (corresponding to Ser195 in PYL3) together with Phe81 and Tyr404 in HAB1. However, the PYL3 S195L dimer is more difficult to disassociate as mentioned above. Taking these two opposite effects into account, S195L mutant slightly enhances the binding ability to HAB1 (Figure S7A) and decrease the dissociation of dimeric S195L mutant (Figures 4, S6E, S6F, and S7C). Therefore, these differences can explain why the inhibitory activity of PYL3 is lower than that of other members in subfamily III (Clade A), which entirely contain a leucine at the corresponding site.

In the PYL3-(+)ABA complex structure, the ABA binding pocket is closed and exposed to solvent. This raises the question of whether PP2C can directly bind to the ligand-bound PYL3 without the disassociation of the trans-homodimer. To test this hypothesis, we engineered a disulfide bond within helices α₄ that locks the protein in the trans-homodimer conformation. The activity experiments show that PP2C is inhibited by 70% in the N180C P208C T209C triple mutant (Figure S4D). Compared to wild-type, the introduction of a disulfide bond will secure the trans-homodimer conformation and block the formation of the PYL3-HAB1 heterodimer. We further confirmed the
interaction using a yeast two-hybrid. The yeast two-hybrid assays were performed using PYL3 or mutants as the bait and HAB1 as the prey with and without (+)-ABA. We found that the yeast grew faster for the PYL3 F81A V202A I203A mutant in SD medium lacking Leu, Trp, His, and Ade (SD-4) in the presence of (+)-ABA (Figure S7A). These data show that monomeric PYL3 interacts with HAB1 more readily than the trans-homodimer. It is consistent with the recent report that monomeric receptors have a competitive advantage than dimeric receptors (Dupoux et al., 2011). Therefore, the monomeric conformation is indispensable for the inhibitory activity of PYL3.

**Gate Closure and Nonproductive Binding in the PYL3-Pyrabactin Complex**

In the PYL3-pyrabactin complex, pyrabactin is stabilized in the PYL3 pocket by hydrophobic interaction networks and several hydrogen bonds. In Mol B of the PYL3-pyrabactin complex, there are two sulfate ions. One sulfate ion binds in the binding pocket and forms a salt bridge with Lys79 (Figure S3F). Another sulfate ion is located between L4 and L5, forms several salt bridges with Arg140 and makes loop L5 shift, which is the distinct conformational change of Latch (L5) among all PYL complex structures (Figure 2A). Compared with other PYL-pyrabactin complexes (Hao et al., 2010; Melcher et al., 2010; Peterson et al., 2010; Yuan et al., 2010), the orientation of the naphthalene and pyridine ring of pyrabactin in PYL3 is rotated by 80° (Figures S8G and S8H). Moreover, the position of the sulfonamide group moves to F81 and do not form a hydrogen bond with Lys79, which is greatly different from other PYLs-pyrabactin complexes. Last but not least, the conformation of pyrabactin is the most compact compared to that of other PYLs-pyrabactin complexes, which might decrease the binding affinity to PYL3 because the contact surface between them is reduced.

It was reported that PYL2–4 were the only pyrabactin-insensitive PYLs and lid conformation was thought to be the key factor (Melcher et al., 2010; Peterson et al., 2010; Yuan et al., 2010). However, the lid L4 in the PYL3-pyrabactin complex is in the closed conformation, which is completely different from that in the PYL2 or PYL2 mutant pyrabactin complex (Figure 2A). These data raise the question of whether the closed conformation of L4 in PYL3 can bind to PP2C and inhibit the activity of PP2C. To test this hypothesis, the inhibitory activity of PYL3 on HAB1 was tested with the ligands (+)-ABA and pyrabactin. PYL3 can inhibit the phosphatase activity of HAB1 with an IC$_{50}$ of 0.317 µM in the presence of (+)-ABA. In the presence of pyrabactin, the IC$_{50}$ increases to 627 µM (Figure S8A). PYL3 only inhibited 44% of the phosphatase activity of HAB1 in the presence of 500 µM pyrabactin. This 2,000-fold difference in selectivity of different ligands reflects a change in the ability of PYL3 to bind to HAB1. Moreover, isothermal titration calorimetry does not detect obvious binding between PYL3 and pyrabactin due to extremely low binding affinity, whereas the binding affinity (K$_d$) is 7.7 µM for (+)-ABA (Figures S8B and S8C). Interestingly, V134I mutant increases the inhibitory activity by 60% compared to wild-type (Figure S8D). This mutant might change the position and orientation of pyrabactin in the binding pocket and enhance the inhibiting activity.

Compared to the PYL3-(+)-ABA-HAB1 complex, lid L4 in the PYL3-pyrabactin complex moves 1.63 Å toward L4 and tightly closes the latch. In addition, due to the smaller side chains of Ser106 and Val108 in PYL3 compared to that of PYL1, PYL2, and PYR1 (Figures S2 and S3G), Leu111 binds to pyrabactin tighter, which gives rise to lid L4 too close toward the pocket compared to that in PYL1, PYL2, or PYR1. There is not enough space between gate L4 and latch L5 for the insertion of Trp385 of HAB1. Therefore, the interaction between pyrabactin-bound PYL3 and PP2C is seriously crippled (Figure S3I). Moreover, residues Pro112, Leu141, Pro177, and Thr185 in the PYL3-pyrabactin complex would clash with the side chain of Trp385 of HAB1 when superimposed with the PYL3-(+)-ABA-HAB1 complex. Therefore, the inhibitory ability of PYL3 on HAB1 is weak when binding to pyrabactin. Taken together, pyrabactin can work as an antagonist for PYL3 during the development of seed.

**DISCUSSION**

In our study, apo-PYL3 cis-homodimer generates a distinct conformational arrangement and becomes a trans-homodimer upon ligand binding, which more easily dissociates into two monomers. The monomeric PYL3 plays the role in physical interaction with PP2Cs and the trans-homodimer is an important intermediate. The formation of the trans-homodimer in the
A. thaliana are ABA receptors (Fujii et al., 2009). From the phylogram of proteins (Table S1a) and exists in limited species. Interestingly, the PYL/PYR/RCAR family, PYL3 is one of the most distinctive except in mostly exists as dimers with a dissociation constant (Kd) of formed on the apo-PYL3 and apo-PYL2 confirms that they dimer and the lower binding affinity to PP2C. properties of PYL3 may justify the formation of the isoelectric point (PI) is alkaline (Table S1b). Thus, the distinct among all the active members of the PYL/PYR family whose ligands. In turn, the trans-homodimer dissociates to monomer and forms a trans-homodimer occurs after apo-PYL3 recognizes and binds to ligands. In turn, the trans-homodimer dissociates to monomer more easily and binds to PP2C more conveniently than the cis-homodimer. Noteworthy, only the appropriate gate closure can induce PP2C binding.

See also Figures S7 and S8 and Table S1.

presence of ligands exposes a partial area of interface for PP2Cs binding (Figures 1C and 7B). The newly exposed area in trans-dimeric PYL3 is favorable for PP2C binding, which ingenuously orchestrate the dissociation of homodimeric PYL3 to monomer and the formation of heterodimer of PYL3-(+)-ABA-PP2C. Combining our findings, we propose a specific mechanism for PYL3 (Figure 8).

The PYL family, one of the star-related lipid-transfer (START) protein families (Lytle et al., 2009), contains 14 members in Arabidopsis. Except for PYL13, which is inactive, all the PYLs are ABA receptors (Fuji et al., 2009). From the phylogram of the PYL/PYR/RCAR family, PYL3 is one of the most distinctive proteins (Table S1a) and exists in limited species. Interestingly, all the residues corresponding to S195 in PYL3 were not serine among the found members of PYL/PYR/RCAR family in plants, except in Arabidopsis lyrata. In addition, PYL3 is the only protein among all the active members of the PYL/PYR family whose isoelectric point (pI) is alkaline (Table S1b). Thus, the distinct properties of PYL3 may justify the formation of the trans-homodimer and the lower binding affinity to PP2C.

Analytical ultracentrifugation sedimentation equilibrium performed on the apo-PYL3 and apo-PYL2 confirms that they mostly exists as dimers with a dissociation constant (Kd) of 7.76 µM and 0.95 µM, respectively (Figures S6A and S6C). After the addition of (+)-ABA, the Kd of PYL3 increased almost six times, whereas the corresponding value for PYL2 is 3.32 µM (Figures S6B and S6D). As expected, two protomers of the mutant PYL3 S195L homodimerize more strongly with Kd of 1.16 µM and 7.23 nM with or without (+)-ABA, respectively (Figures S6E and S6F). These results show that the PYL3 trans-homodimer more easily disassociates to the monomer compared to the PYL3 mutant cis-homodimer and PYL2 cis-homodimer in the present of (+)-ABA. The measured Kd value of PYL3 trans-homodimer is a little higher than expected, but there are several reasons to explain it. First, the above Kd had to be measured at 4°C due to the protein instability under long duration of experiments at room temperature. However, Arabidopsis grows at the room temperature. The Kd value is estimated at the µM level from the sedimentation velocity analytical ultracentrifugation at 20°C (Figure 5). Second, maybe are some undiscovered partner components or special physiological environment for PYL3 in vivo to lower the Kd value. In fact, we do not get entangled with the measured Kd value in vitro as unexpected. PYL3 can be excessively expressed and enriched at some specific tissues and several stages, which makes it possible that the local high concentration of PYL3 satisfies the demand of Kd value as high as measured in vitro. The expression level of PYL3 in the chalazal seed coat at the globular stage is 1,000 times higher than in the other tissues (e.g., the peripheral endosperm). Therefore, it is reasonable to speculate that PYL3 trans-homodimer might exist in special tissues at particular stages of development, such as in seed maturation. Of course, the physiological conditions in vivo are so complicated and changeable that PYL3 may exist as an equilibrium mixture of trans-, cis-dimer, and monomer.

In addition, at a low proportion of PYL to HAB1 in the presence of (+)-ABA, PYL3 had stronger inhibitory ability to HAB1, whereas PYL2 lost nearly all inhibitory ability when the concentration of PYL2 was less than half of HAB1 (Figure S7B). Therefore, inhibition of HAB1 by PYL3 is kinetically favored at low protein levels, whereas inhibition by PYR1, PYL1 or PYL2 is thermodynamically favored at high protein levels. These results imply that the trans-homodimer may have important role at low expression level.

Recently, two independent groups proposed that PYR/PYL/RCAR proteins could be separated into two distinct subclasses (Dupeux et al., 2011; Hao et al., 2011) according to the oligomeric state of their apo forms, including monomeric PYLs and dimeric PYLs. Here, our structures support that PYL3 trans-dimers occur under ligands such as (+)-ABA and pyrabactin, which implies that the formation of trans-homodimer is a common and true mechanism for PYL3. Therefore, our findings extend the present classification of PYR/PYL/RCAR proteins. According to the conformations of ligand-bound PYLs, there are three PYR/PYL/RCAR subclasses, homodimeric PYLs such as PYL1, PYL1, and PYL2, transdimeric PYL3, and monomeric PYLs including PYL4–PYL6 and PYL8–PYL10. PYL7, PYL11, and PYL12 are not identified thus far. Our classification can deepen the understanding of the core question of PYLs receptors: how do the 13 ABA-responsive PYLs, which share a high degree of sequence identity, execute some distinct functions in ABA signaling in vivo? The three PYR/PYL/RCAR subclasses are corresponding to three different mechanisms of PYLs response to ABA, which display different behaviors on perception of ABA concentration, binding ability to ABA, and efficiency of inhibiting PP2C, etc. In different tissues and different physiological conditions, plants may employ different ABA receptors by different mechanisms to regulate different physiological processes and response to different abiotic stresses.

In the study of ABA signaling, a central question is how this small molecule is sensed. Productive and nonproductive modes in ligand binding to PYL family members have been proposed (Peterson et al., 2010) in which the gate closure responding to ligand exclusively underlies the capacity of PYL inhibiting PP2C (Melcher et al., 2009; Miyazono et al., 2009; Yin et al., 2009). In the PYL3 pyrabactin complex, we discovered an unexpected, nonproductive binding mode. In this mode, the gate
closes too tightly to bind to PP2C. The PYL3-pyrabactin structure with an excessive closure of L4 is incompatible for interaction with HAB1, and thus inhibits PP2C very weakly. Therefore, only the appropriate gate closure can induce PP2C binding. It was found that pyrabactin was an agonist for PYR1 and PYL1 by gate closure, an antagonist for PYL2 by gate open (Hao et al., 2010; Melcher et al., 2010; Peterson et al., 2010; Yuan et al., 2010). However, our structural analysis and biochemical data show that pyrabactin works as an antagonist for PYL3 using different mechanism. These data will provide unique evidence for designing small chemical compounds for improving plant performance in the future.

Our biochemical, mutational, and structural analyses also reveal how PYL3 differs from other PYLs upon ABA binding. A distinct molecular mechanism linking PYL3 to transcriptional regulation (Figure 8) was proposed here. Taken together, these results reveal a distinct insight for substrate selectivity and provide an approach for the design of special agonists and antagonists. These data and structural analyses also suggest that ligand-bound PYL3 differs greatly from apo-PYL3 and other PYLs. To determine whether this intermediate exists in other PYL proteins, more structural and functional data in vivo are needed.

EXPERIMENTAL PROCEDURES

Crystallization

Crystallizations were performed at room temperature by hanging-drop vapor diffusion method. Apo-PYL3 crystal was grown at room temperature in the hanging drop containing 1.0 µl purified PYL3 (residues 1–209) protein at 20 mg/ml and 1.0 µl of reservoir solution that included 2.3 M (NH4)2SO4, 0.1 M BTP pH 8.25. To get the PYL3-S(+)-ABA complex crystals, a homogeneous and stable PYL3-S(+)-ABA complex in solution should be first formed. S-(+) -ABA (hereafter referred to as (+)-ABA) was mixed with PYL3 (residues 25–209) at 5:1 ratios and stayed on ice for ~3 hr. The mixtures were applied to gel exclusion chromatography and the fractions corresponding to the homogeneous complexes were concentrated for crystallization. The complex PYL3-(+)-ABA crystal appeared in the hanging drop containing 1 µl of purified protein at 18 mg/ml mixed with 1 µl of well solution that contained 1.6 M (NH4)2SO4, 0.1 M HEPS pH 7.6. To obtain PYL3-pyrabactin complex crystal, the fragment of PYL3 (residues 21–209) was found to be better for complex crystallization. Pyrabactin was mixed with PYL3 at 10:1 ratios and stayed on ice overnight. PYL3-pyrabactin complex crystals were grown in the well buffer containing 1.7 M (NH4)2SO4, 0.1 M NaCaCodylate pH 6.3, 0.2 M NaCl. The crystals were transferred into the well solution containing 30% glycerol as cryo-protectant solution and flash cooled in liquid nitrogen before collecting data.

To form a homogeneous and stable HAB1-PYL3-(+)-ABA ternary complex, PYL3 (full length) and HAB1 (residues 169–511) was overexpressed and purified by Ni-charged resin (BIO-RAD) and followed by size exclusion chromatography, purified His tagged PYL3 mutants were subjected to disulphide formation. Sixty micrograms of His-tagged PYL3 was mixed with or without (+)-ABA for ~1 hr, then redox condition was applied by different ratio of oxidized glutathione (GSSH) and reduced glutathione (GSH) overnight in 4°C. For the subunit exchange experiment, an equal molar of N180C and T209C mutants were mixed. The protein samples were visualized by SDS-PAGE with or without 1% IEF followed by Coomassie blue staining.

Details of protein preparation, phosphatase activity assay, GST-mediated pulldown assay, crosslinking gel assay, gel exclusion chromatography, small angle X-ray scattering experiments, isothermal titration calorimetry (ITC) assays, analytical ultracentrifugation, experimental phasing, and yeast two-hybrid analysis are described in Supplemental Experimental Procedures.

ACCESSION NUMBERS

The atomic coordinates have been deposited in the Protein Data Bank (PDB) as 3OJJ, 3KLX, 3K1L, 4D8S, 4DSC, and 4DS8.

SUPPLEMENTAL INFORMATION

Supplemental Information includes one table, eight figures, and Supplemental Experimental Procedures and can be found with this article online at doi:10.1016/j.str.2012.02.019.

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