Pyrabactin is a synthetic abscisic acid (ABA) agonist that selectively inhibits seed germination. The use of pyrabactin was pivotal in the identification of the PYR1/PYL/RCAR family (PYL) of proteins as the ABA receptor. Although they both act through PYL proteins, pyrabactin and ABA share no apparent chemical or structural similarity. It remains unclear how pyrabactin functions as an ABA agonist. Here, we report the crystal structure of pyrabactin in complex with PYL1 at 2.4 Å resolution. Structural and biochemical analyses revealed that recognition of pyrabactin by the pocket residues precedes the closure of switch loop CL2. Structural comparison between pyrabactin- and ABA-bound PYL1 reveals a general principle in the arrangements of function groups of the two distinct ligands. The study provides a framework for the development of novel ABA agonists that may have applicable potentials in agriculture.

Abscisic acid (ABA) is ubiquitous in higher plants, regulates a variety of processes during plant development, and protects plant against inclement environments such as cold and drought. ABA, the conserved loop CL2 sits above the active site of PP2C and blocks substrate entry to PP2C, hence relieving PP2C-mediated inhibition of SnRK2 (9, 10, 12). Chemical genetic analysis played an essential role in the identification of ABA receptors. Pyrabactin, an ABA-selective agonist and a synthetic inhibitor of seed germination (5, 13), was exploited for the isolation of pyrabactin resistance 1 (Pyr1) mutant alleles (5). Pyr1, a representative member of PYL proteins, was shown to interact with PP2C in response to pyrabactin (5). Interestingly, although pyrabactin acts through PYL proteins, it shares no apparent chemical similarity with ABA (Fig. 1A). Thus, the available structural information fails to explain how pyrabactin agonizes ABA function.

Understanding the functional mechanism of pyrabactin will shed light on the development of novel ABA agonists that may have applicable potentials in agriculture. ABA is not a stable compound, and its costly synthesis further restricts its potential applications. Pyrabactin thus provides an alternative to the development of novel ABA-related compounds. In this paper, we performed biochemical and structural studies and report here the crystal structure of pyrabactin in complex with PYL1. Our study nicely illustrates how pyrabactin serves as an ABA agonist. Structural comparison with ABA-bound PYL1 revealed a general principle in the arrangement of function groups of the ligands.

**EXPERIMENTAL PROCEDURES**

**Protein Preparation and Crystallization—PYL1**: AT5G46790 and AB11 (AT4G26080) were subcloned from the Arabidopsis thaliana cDNA library using standard PCR-based protocol. All mutants of PYL1 were generated with two-step PCR, verified by plasmid sequencing. All proteins were purified according the protocol described previously (9). Wild type and all mutants of PYL1 were expressed in Escherichia coli strain BL21(DE3) using vector pET-15b induced at 22 °C for 12 h. The individual proteins were purified with nickel-nitrilotriacetic acid resin (Qiagen), followed by anion-exchange chromatography (Source-15Q; GE Healthcare) and size-exclusion chromatography.
Prior to crystallization, PYL1 (residues 22–210) protein was incubated with pyrabactin with a molecular ratio of 1:2. Crystals were grown at 18 °C using the hanging-drop vapor diffusion method. Crystals appeared after 2 days in the well buffer containing 1.45 M Na/K tartrate, 100 mM Tris, pH 8.0, 1% octyl-β-D-glucopyranoside (Anatrace), and 0.015% spermidine. Addition of 5.2 mM C8E5 (Hampton Research) into the crystallization solution further improved the diffraction of the crystals.

**Data Collection, Structure Determination, and Refinement**—PYL1/PYB data were collected at the Shanghai Synchrotron Radiation Facility (SSRF) beamline BL17U and integrated with MOSFLM (14). Further processing was carried out using programs from the CCP4 suite (15). Data collection statistics are summarized in Table 1. PYL1 model (Protein Data Bank (PDB) code 3KDJ) was translated into the PYL1/PYB cell with the program PHASER (16) by the molecular replacement method. Manual model iterative rebuilding and refinement were performed with COOT (17) and PHENIX (18). The pyrabactin molecules were built into the cavity of the host protein molecules. Their position and orientation were validated by the anomalous signal of bromide in the pyrabactin molecule.

**Phosphatase Activity Assay**—The phosphatase activity was measured by the Ser/Thr phosphatase assay system (Promega). Each reaction was performed in a 100-μl reaction volume containing 1.8 μg of ABI1, 1 μg of wild type or mutant PYL1 proteins. 10 μM ABA (Sigma-Aldrich) or pyrabactin was added when required. After incubation with peptide substrate (supplied with the Promega kit) in the buffer containing 50 mM imidazole, pH 7.2, 5 mM MgCl2, 0.2 mM EGTA, and 0.1 mg/ml bovine serum albumin at 30 °C for 15 min, the reaction was stopped by the addition of 100 μl of molybdate dye and incubated for another 15 min at room temperature. Absorbance at 630 nm was measured. For the IC50 assay, the ligands were applied at the concentrations indicated in Fig. 1. The represented data are means ± S.D. from three independent experiments.

**RESULTS**

**Overall Structure of Pyrabactin-bound PYL1**—PYL1 inhibits PP2Cs phosphatase activity in an ABA-dependent manner. To examine whether PYL1 responds to pyrabactin similarly, we reconstituted a PP2C phosphatase activity assay in vitro. Consistent with the *in planta* observations (5), pyrabactin cannot directly inhibit the phosphatase activity of ABI1, but achieves the inhibition through PYL proteins (supplemental Fig. 1). Further characterization revealed that pyrabactin exhibits an IC50 (1.14 ± 0.07 μM) ~10-fold higher than that of ABA (93.8 ± 8.4 nM) (Fig. 1B). We sought to understand the functional mechanism of pyrabactin by determining the crystal structure of pyrabactin-bound PYL1.
Crystal Structure of PYL1 in Complex with Pyrabactin

In the presence of pyrabactin, PYL1 was crystallized in the space group P3121. There are two PYL1 molecules, named Mol A and Mol B, in each asymmetric unit (Fig. 2, A and B). The crystals diffracted anisotropically between 2.5 and 3 Å. The structure of PYL1 was determined by molecular replacement, and the final atomic model was refined to 2.8 Å resolution (Table 1 and supplemental Fig. 2). After all protein atoms were in place, a headset-shaped electron density appeared in the conserved pocket of each PYL1 molecule (Fig. 2A). This electron density allowed modeling of the pyrabactin molecule, and the position of the bromide atom in pyrabactin was confirmed by the anomalous signal (Fig. 2A and supplemental Fig. 3). Within the protein-ligand complex, pyrabactin adopts a U-shaped conformation. The pyridine and the naphthalene rings face each other with an angle of ~45 degrees (supplemental Fig. 3B).

We next examined the two PYL1 molecules within each asymmetric unit. When Mol A and Mol B are superimposed, a prominent difference was observed in CL2 (supplemental Fig. 4A). Although CL2 in Mol A adopts a closed conformation, it remains open in Mol B even with pyrabactin binding. Pyrabactin binds to Mol A and Mol B in a similar manner, except for the lack of coordination by CL2 in Mol B (supplemental Fig. 4, A and B). This observation suggests that ligand binding into the pocket may precede the conformational change of CL2. Further examination of crystal packing revealed that Mol A exists as a monomer, whereas Mol B forms a homodimer with the adjacent symmetry-related molecule (supplemental Fig. 4C). The structure of the dimeric pyrabactin-bound PYL1 (Mol B) is almost identical to that of apo-PYL1 (supplemental Fig. 4B), corroborating the hypothesis that dimer formation of PYLs may prevent conformational change of CL2 (9).

During the revision of this manuscript, we obtained the crystals of pyrabactin-bound PYL1 diffracting x-ray to 2.1 Å, when an additive, the detergent C8E5, was included in the crystallization solution. Due to the anisotropic diffraction (2.1 Å × 2.5 Å), the structure was refined to 2.4 Å resolution (Table 1). The high resolution structure is identical to the previous one, except that the anomalous signal of bromide was not detected in Mol B. The missing of the pyrabactin molecule in Mol B was probably due to the slightly modified crystallization condition. The electron density of pyrabactin and its surrounding waters in Mol A is of excellent quality (Fig. 2C and supplemental Fig. 5C), so we therefore focus on Mol A for the analysis of the interaction between pyrabactin and PYL1.

Recognition of Pyrabactin by PYL1—The U-shaped pyrabactin sits in the conserved ligand-binding pocket of PYL1 with the bromonaphthalene ring positioned in proximity to CL2, while the pyridine group is away from the loop region and buried deep into the pocket (Fig. 3A). The coordination of pyrabactin is mediated by both polar and van der Waals contacts. Charged residues from CL1, β3, β4, and β7 play an important role in coordinating pyrabactin via hydrogen bonds (Fig. 3B). The carboxylate of Glu121 in strand β4 interacts with the sulfonamide of pyrabactin with both direct and water-mediated hydrogen bonds. Notably, a water molecule plays an important role in organizing the polar contacts between pyrabactin and PYL1. It accepts hydrogen bonds from the side chains of Lys86 in CL1 and Arg106 in strand 7, and it donates hydrogen bonds to the carboxylate of Glu121 and the sulfone group of pyrabactin. The existence and the position of the pyridyl nitrogen were found essential for pyrabactin agonistic activity (5). The current structure showed that the pyridyl nitrogen is coordinated by the amine group of Lys86 in CL1 and the carboxylate group of Glu121 in β7 through water-mediated hydrogen bonds, supporting its essential role in mediating the ligand-protein interaction (Fig. 3B).

In contrast to the central portion of the U-shaped pyrabactin, the two arms of the ligand are relatively hydrophobic. They are buried in a hydrophobic environment surrounded by amino
acids mainly from CL1, CL2, CL3, and α2, which include Phe
88 in CL1; Val
110, Leu
114, and Ala
116 in CL2; His
142, Leu
144, and 
Tyr
147 in CL3; and Phe
189, Val
193, and Ile
194 in α2 (Fig. 3C). Note that the geometric plate of CL2 is nearly in parallel with the hydrophobic naphthalene ring of pyrabactin. In particular, the bromide of pyrabactin is in close proximity to Val
110 and Leu
114 of CL2 (supplemental Fig. 5), indicating an important role of the bromide in rendering the closure of CL2.

In ABA, the two polar modules, the carboxylate and the hydroxyl groups, point into the conserved pocket and are coordinated mainly through water-mediated hydrogen bonds with charged or polar residues of PYL proteins (8–12). The two polar portions of pyrabactin, the pyridyl nitrogen and the sulfonamide group, adopt the same orientation. A closer examination revealed that the distance between the corresponding polar module 1 in ABA and pyrabactin, namely the carboxylate oxygen of ABA and the pyridyl nitrogen of pyrabactin, is 2.2 Å, whereas those between the corresponding polar module 2, the hydroxyl group of ABA and the amine-sulfone groups of pyrabactin, are 2.5 Å, 2.7 Å, and 2.9 Å, respectively. These distances are close to the length of hydro-
The analyses also explained why the position of the pyridyl nitrogen is essential for pyrabactin function (5). If the nitrogen is located at any other sites on the pyridyl ring, it will be out of reach of the polar residues in PYL1 even in the presence of water molecules. In addition, an amine group on any other positions of the pyridyl ring would interfere with the hydrophobic module 2’, which might be unfavorable to the hydrophobic environment within the receptor (Figs. 3C and 4B). To corroborate the analyses, we synthesized a few unreported variants of pyrabactin and examined their function. Removal of the pyridyl nitrogen (PYB1) or the bromide (PYB2) severely crippled the effect of the compounds on PYL1, whereas replacement of the bromide with a methyl group (PYB3) retains its function (Fig. 4E). PYL1 responds to PYB3 with an IC₅₀ of 2.6 ± 0.17 µM, comparable with that of pyrabactin. This observation indicates that a bulkier hydrophobic plate is required at hydrophobic module 1’ so as to attract the CL2 switch to bend over (Fig. 4D).

It is noteworthy that there is no salt bridge mediating the interaction between pyrabactin and PYL1, whereas the salt bridge between the carboxylate of ABA and the amine group of the conserved Lys⁸⁶ in PYL1 is essential for ABA binding (9). This variation may partially account for the lower efficacy of pyrabactin in the PYL1-mediated PP2C inhibition compared with that of ABA. Thus, modification of the function groups may result in varied efficacies of the ligands. In addition, water molecules play an important role in mediating the interaction between the ligands and the receptor. If a compound contains extended polar groups that occupy where the waters are localized, it may exhibit an increased binding affinity with the receptor because of the direct polar contacts.

“Electromagnet” Model for Ligand Perception of PYL Proteins—With the similar geometric arrangements of the function groups of pyrabactin and ABA, it is not surprising to see that the residues coordinating pyrabactin are almost identical to those involved in ABA recognition. These residues are mostly conserved in the PYL family of proteins (Fig. 5A and supplemental Fig. 5). We examined whether all of the four charged residues, Lys³⁶, Arg¹⁰⁶, Glu¹²¹, and Glu¹⁷¹, are essential in the ligand coordination pyrabactin and PYL1 are superimposed as shown in A. Only the ligands are shown here. The two polar modules are highlighted in pink and annotated by 1 and 2. Two hydrophobic modules are highlighted in cyan and annotated by 1’ and 2’. Two approximately perpendicular views are shown. Pyrabactin and ABA are shown in orange and yellow. The distances are measured in angstroms. The green line depicts the division of the polar and hydrophobic modules. C, water molecules exploited to compensate for the missing polar groups of either ligand for hydrophilic interaction with PYL1. A water molecule (yellow sphere) from the structure of ABA-bound PYL1 is located in the same position of the amine group of pyrabactin; likewise, a water molecule (orange sphere) from the structure of pyrabactin-bound PYL1 occupies the position of the carboxylate of ABA. D, geometric diagram of the ligands. The hydrophobic and the polar modules of the ligands are located in two perpendicular but not crossed plates. E, chemical characterization of the key function groups of pyrabactin. The red arrows beside the labels PYB1 and PYB2 indicate the compromised effects of the compounds on PYL1.
binding. Single missense mutation of Lys\(^ {86} \), Arg\(^ {106} \), Glu\(^ {121} \), and Glu\(^ {171} \) to Ala all rendered PYL1 insensitive to pyrabactin (Fig. 5B). These observations highlighted the essential roles of these highly conserved, charged residues of PYL proteins in ligand coordination. This result also suggests that neither of the polar modules of the ligands is dispensable for the ligand-receptor interaction. It thus further explains why the existence and the position of the pyridyl nitrogen are pivotal for the function of pyrabactin.

From the structural comparison we noticed that the four charged residues adopt identical conformations in ABA-bound and pyrabactin-bound PYL1. Are these conformations induced upon ligand binding or are they preexisting? To address this question, we superimposed all of the available structures of PYL proteins, including PYR1 and PYL2, in both apo- and ABA-bound forms, and PYL1 in apo-, ABA-bound, and pyrabactin-bound forms. In all seven structures, the four residues are arranged in an identical way (Fig. 5C), indicating a preexisting and rigid polar environment, in contrast to the induced hydrophobic portion exemplified by CL2. This facet should be taken into account in the future design of ABA agonists targeting PYL proteins.

The observations suggest an electromagnet model for the ligand perception of PYL proteins. The polar modules of the ligands function like plugs. Once the polar plug is correctly anchored to the preexisting polar socket, the hydrophobic module of the ligand subsequently attracts the switch loop of PYLs to achieve a closed conformation as well as creating a PP2C-binding surface (Fig. 5D).

**DISCUSSION**

Structural analyses of ABA-bound and pyrabactin-bound PYL1 revealed a general principle in designing ABA agonists that target PYL proteins (Fig. 4D). The ligand should be amphipathic with four modules: two polar modules positioned on one plate at one side of the compound; and at the other side a planar, bulky, hydrophobic module perpendicular to, but not crossed with, the polar modules. The polar modules are the anchor to be recognized by the hydrophilic pocket residues of
PYLs, whereas the hydrophobic module is responsible for the structural rearrangement of CL2.

In summary, our study reveals the molecular basis of pyrabactin agonistic activity on the ABA signaling pathway and provides a framework to develop novel ABA-related compounds for potential application in agriculture. However, it is known that pyrabactin is a selective agonist of ABA. It has a strong effect on PYR1, PYL1, etc., but has little or no effect on PYL2 and PYL4. Sequence alignment revealed that the residues involved in the coordination of pyrabactin are conserved among most of the 14 members of PYLs (supplemental Fig. 6). The current study and the available structural information cannot provide an explanation to the selectivity of pyrabactin by PYL proteins. Nonetheless, the existence of the "closed" monomer (Mol A) and the "open" dimer (Mol B) of PYL1 in the crystal structure provides a clue to address this question. It is possible that pyrabactin may be able to bind to other PYLs but unable to induce the closure of CL2 in PYL2 or PYL4 if these PYLs have a tighter dimer formation so as to effectively prevent the conformational change of CL2. The answer to this question may require additional structures of PYL proteins in complex with pyrabactin as well as associated computational, biochemical, and biophysical analyses.

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