Molecular Mechanism for Inhibition of a Critical Component in the Arabidopsis thaliana Abscisic Acid Signal Transduction Pathways, SnRK2.6, by Protein Phosphatase ABI1*

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Background: The antagonistic complex of SnRK2.6 and ABI1 regulates abscisic acid (ABA) signaling in plants.

Results: Presented here are the structure of SnRK2.6 kinase domain, and biochemical and computational characterizations of the ABI1-SnRK2.6 complex.

Conclusion: Our studies revealed the molecular basis for ABI1-mediated inhibition of SnRK2.6.

Significance: The studies advanced our understanding of the downstream signal transduction of ABA through SnRK2s and protein phosphatase type 2Cs.

Subclass III SnRK2s (SnRK2.6/2.3/2.2) are the key positive regulators of abscisic acid (ABA) signal transduction in Arabidopsis thaliana. The kinases, activated by ABA or osmotic stress, phosphorylate stress-related transcription factors and ion channels, which ultimately leads to the protection of plants from dehydration or high salinity. In the absence of stressors, SnRK2s are subject to negative regulation by group A protein phosphatase type 2Cs (PP2Cs), whereas the underlying molecular mechanism remains to be elucidated. Here we report the crystal structure of the kinase domain of SnRK2.6 at 2.6-Å resolution. Structure-guided biochemical analyses identified two distinct interfaces between SnRK2.6 and ABI1, a member of group A PP2Cs. Structural modeling suggested that the two interfaces lock SnRK2.6 and ABI1 in an orientation such that the activation loop of SnRK2.6 is postis to the catalytic site of ABI1 for dephosphorylation. These studies revealed the molecular basis for PP2Cs-mediated inhibition of SnRK2s and provided important insights into the downstream signal transduction of ABA.

The phytohormone abscisic acid (ABA) protects plants in inclement environments, particularly during drought or high salinity. ABA also regulates seed dormancy, seedling development, stomatal aperture etc. (1–3). The molecular mechanism of ABA perception and signal transduction in Arabidopsis has been intensively studied, and a complex signaling network is being revealed (4, 5). Sitting in the central hub of the ABA signaling network are the antagonistic counterparts of subclass III SnRK2s (SNF1-related protein kinase 2) and group A PP2Cs (protein phosphatase type 2C), in addition to the ABA receptor PYL/PYR1/RACR family of proteins (PYLs for short) (4). SnRK2s are positive regulators of ABA signaling, whereas PP2Cs negatively affect the pathway by directly inhibiting SnRK2s.

Ten SnRK2s, SnRK2.1 through SnRK2.10, were identified in Arabidopsis and grouped to three subclasses (4, 6). Subclass I SnRK2s can be activated rapidly by osmotic stress, whereas II and III are activated by both osmotic stress and ABA, with subclass III proteins playing a more prominent role in ABA response (7–11). Subclass III consists of SnRK2.6 (SnRK2E), SnRK2.3 (SnRK2I), and SnRK2.2 (SnRK2D), which play a redundant role as positive regulators of ABA signaling. SnRK2-dei, the triple knock-out of subclass III SnRK2s gave rise to an ABA-insensitive phenotype (10–12). The substrates for SnRK2.6/3/2 include transcription factors responsible for the expression of the ABA-responsive genes (13–15), as well as ion channels that control the osmotic homeostasis, such as the anion channel SLAC1 (16) and potassium channel KAT1 (17). Activation of SnRK2s requires autophosphorylation (18), a process that is inhibited by group A PP2Cs, whose members include ABI1, ABI2, HAB1, HAB2, etc. (19). Genetic and biochemical characterizations revealed that group A PP2Cs are physically associated with and dephosphorylate SnRK2.6/3/2, hence impeding the kinase activities (20, 21).

The molecular mechanism of ABA-induced removal of the negative regulation by PP2Cs has been revealed (22–27). Upon ABA binding, PYLs bind to the active site of PP2Cs and inhibit the phosphatase activity of PP2Cs by blocking substrate entrance (25–27). A subfamily of PYLs was recently found to bind and inhibit PP2Cs even in the absence of ABA, although the physiological relevance remains elusive (28). Despite
advancement in the understanding of ABA perception and ABA-regulated inhibition of PP2Cs, the molecular basis by which PP2Cs inhibit SnRK2s remains poorly understood. To address this important question, we focused on SnRK2.6 for structural and biochemical characterizations. Here we report the crystal structure of a carboxyl terminus-truncated SnRK2.6. On the basis of structural analysis, we performed extensive mutagenesis analyses, biochemical characterizations, and kinetic studies, which collectively revealed the molecular basis for the recognition and inhibition of SnRK2.6 by ABI1. Our studies provide important insight into the understanding of the downstream signal transduction of ABA.

EXPERIMENTAL PROCEDURES

Protein Preparation and Crystallization—SnRK2.6 (AT4G33950), ABI1 (AT4G26080), and ABF2 (AT1G45249) were subcloned from the Arabidopsis thaliana cDNA library using standard PCR-based protocol. All mutants of SnRK2.6 and ABI1 were generated with two-step PCR and verified by plasmid sequencing. The proteins were expressed and purified following the protocols reported in the supplementary data. Prior to crystallization, the His6 tag was removed by thrombin digestion. Crystals of SnRK2.6 (residues 1–317, C131A/C137A/C159A/S7A/S29A/S43A/S166A/T176A) were grown at 18 °C using the hanging drop vapor diffusion method. Typically, crystals appeared after 1 to 2 days in the buffer containing 50 mM MOPS, pH 7.0, 100 mM NaCl, and 4 mM MgCl2. To test the interaction of SnRK2.6 with ABI1, 0.5 mg of SnRK2.6 and 0.6 mg of ABI1 proteins were mixed in 200 μl of reaction mixture containing 50 mM MOPS, pH 7.0, 150 mM NaCl, and ABI1, 0.5 μg of GST fusion SnRK2.6 protein was added to 50 μl of glutathione S-Sepharose 4B (GE Healthcare). The reactions were initiated by the addition of purine nucleoside phosphorylase (PNPase) and its chromogenic substrate 7-methyl-6-thioguanosine (M 7-methyl-6-thioguanosine, 0.1 M) and lactate dehydrogenase (LDH) (33). The standard assay was carried out at 25 °C in 1.8 ml of reaction mixture containing 50 mM MOPS, pH 7.0, 100 mM NaCl, 0.1 mM EDTA, 10 mM MgCl2, 0.2 mM NADH, 1 mM ATP, 1.8 μM SnRK2.6, with varying amounts of ABI1. Progress of the reaction was monitored continuously by following the generation of NADH at 340 nm, on a PerkinElmer λ45 spectrophotometer equipped with a magnetic stirrer in the cuvette holder. The concentrations of ADP formed in the SnRK2.6-catalyzed reaction were determined using an extinction coefficient for NADH of 6220 cm⁻¹ M⁻¹ at 340 nm.

Phosphatase Activity Assay—Kinetic parameters for the dephosphorylation of SnRK2.6 by ABI1 were determined using a continuous spectrophotometric assay (34, 35). This assay incorporates a coupled enzyme system, which uses purine nucleoside phosphorylase (PNPase) and its chromogenic substrate 7-methyl-6-thioguanosine for the quantification of inorganic phosphate produced in the phosphatase reaction (36). All experiments were carried out at 25 °C in a 1.8-ml reaction mixture containing 50 mM MOPS, pH 7.0, 100 mM NaCl, 0.1 mM EDTA, 10 mM MgCl2, 100 μM 7-methyl-6-thioguanosine, 0.1 μg/ml of PNPase. The reactions were initiated by the addition of phosphatase unless indicated otherwise. The time courses of absorbance change at 360 nm were recorded on a PerkinElmer λ45 spectrophotometer equipped with a magnetic stirrer in the cuvette holder. Initial rates were determined from the linear slope of progress curves obtained and the experimental data were analyzed using a nonlinear regression analysis program.
Quantitation of phosphate release was determined using the extinction coefficient of 11,200 M\(^{-1}\) cm\(^{-1}\) for the phosphate-dependent reaction at 360 nm at pH 7.0 (37). The concentration of 7-methyl-6-thioguanosine was determined at 331 nm, using a molar extinction coefficient of 32,000 M\(^{-1}\) cm\(^{-1}\). With \(p\)-nitrophenyl phosphate (pNPP) as a substrate, the reaction was initiated by the addition of the enzyme in a reaction mixture. The initial rate for hydrolysis of pNPP by phosphatase was measured at 25 °C in 1.8 ml of reaction mixture containing 50 mM MOPS, pH 7.0, 100 mM NaCl, 0.1 mM EDTA, and 10 mM MnCl\(_2\). Nonenzymatic hydrolysis of the substrate was corrected by measuring the control reaction in the absence of enzyme. The amount of product \(p\)-nitrophenol was determined with the absorbance at 405 nm using a molar extinction coefficient of 18,000 M\(^{-1}\) cm\(^{-1}\) (38).

**RESULTS**

**In Vitro Reconstitution of ABI1-mediated Dephosphorylation of SnRK2.6**—Subclass III SnRK2s play a redundant role in ABA signaling. To understand the inhibitory mechanism of SnRK2s by PP2Cs, we selected SnRK2.6 for in vitro structural and biochemical characterizations. SnRK2.6 (also known as OST1, open stomata 1) is functionally divided to three domains, the catalytic kinase domain (residues 21–282), the activation motif (residues 283–318, domain I), and the C-terminal ABI1 binding domain (residues 319–347, domain II) (Fig. 1A) (21). We expressed recombinant SnRK2.6 and ABI1 in *Escherichia coli* and sought to purify the proteins to homogeneity. During the purification of SnRK2.6, however, smearable bands with molecular masses close to 40 kDa were observed on SDS-PAGE (supplemental Fig. S1A, left lane). Incubation with the catalytic domain of ABI1 (residues 125–429) reduced the multiple bands to a single one corresponding to an apparent molecular masses of ~41 kDa (supplemental Fig. S1A, right lane). These observations suggested that SnRK2.6 may be phosphorylated during recombinant expression and purification. Mass spectrometry (MS) analysis confirmed that multiple Ser/Thr residues were phosphorylated in the recombinantly expressed SnRK2.6, an observation similar to the protein obtained from plant cells (18, 20). Interestingly, when the dephosphorylated SnRK2.6 was further purified and then incubated with ATP and Mg\(^{2+}\), the dominant phosphorylation site was Ser\(^{175}\) (Fig. 1B, supplemental Figs. S1B and S2), although the reaction was not 100% complete even after incubation overnight.

To examine the kinase activity of the recombinant pSnRK2.6, we reconstituted a coupled spectrophotomeric assay using purified ABF2 (residues 1–199) as substrate (supplemental Fig. S3) (33). The \(k_{cat}\) and \(K_m\) values for the ABF2 phosphorylation by pSnRK2.6 were determined to be 0.04 ± 0.003 s\(^{-1}\) and 19.3 ± 2.52 \(\mu\)M, respectively. Substitution of Ser\(^{175}\) with Ala completely abolished the kinase activity of SnRK2.6 (Fig. 1C); by contrast, T176A only exhibited a moderate effect on the kinase activity. This observation corroborates the in vivo characterization that Ser\(^{175}\) plays a key role in the activity of SnRK2.6 (18, 42). Glutamate is generally used to mimic the phosphorylated serine. In SnRK2.6, however, the S175E mutant displayed significantly impaired activity (Fig. 1C).

Addition of ABI1 led to dephosphorylation of pSnRK2.6 (supplemental Fig. S1). We determined the kinetic parameters for the ABI1-catalyzed dephosphorylation of SnRK2.6 using the continuous spectrophotometric assay for protein phosphatases (34, 35). ABI1 exhibited a highly specific and efficient phosphatase activity for pSnRK2.6 with \(K_m\) 0.097 ± 0.013 \(\mu\)M and \(k_{cat}\) 0.924 ± 0.020 s\(^{-1}\) (Fig. 1D). MS analysis revealed that phospho-Ser\(^{175}\) was the dominant substrate for ABI1 within the first 5 min of the reaction (supplemental Fig. S1C).

Phosphorylation of Ser\(^{175}\), which is located on the activation loop (also known as T-loop) of SnRK2.6, was reported to be essential to the kinase activity of SnRK2.6 (18, 20). Experiments with purified recombinant proteins here demonstrated that activation of SnRK2.6 can be achieved by autophosphorylation, whereas ABI1 inhibits SnRK2.6 by the effective dephosphorylation of SnRK2.6-Ser(P)\(^{175}\).

**Crystal Structure of the C terminus-truncated SnRK2.6**—To better understand the recognition and inhibition of SnRK2.6 by ABI1, we sought to obtain the crystal structures of SnRK2.6 or the complex of SnRK2.6 and ABI1. After many trials, the structure of the C terminus-truncated SnRK2.6 (SnRK2.6ΔC) was determined and refined to 2.6-Å resolution (Fig. 2 and supplemental Table S1). To obtain better diffracting crystals, three cysteine residues were replaced by Ala to avoid unwanted oxidation. The Ser and Thr residues that may be heterogeneously phosphorylated during expression and purification, including Ser\(^{7}\), Ser\(^{29}\), Ser\(^{43}\), Ser\(^{156}\), and Thr\(^{176}\), were also mutated to Ala.

The catalytic core of SnRK2.6 exhibits a canonical kinase fold with distinct N- and C-lobes. We included ATP or AMP_PPNP in the crystallization trials; nevertheless, the structure is in an apo-form. As in the case of a number of apo-kinase structures, the activation loop (residues 164–177) is invisible, probably due to the intrinsic flexibility in the absence of a co-factor (Fig. 2A). Residues 287–296 of the activation motif are invisible, whereas residues 301–313 form a helix that lies against the N-lobe. The activation helix is positioned approximately in parallel with helix αC through extensive van der Waals contacts (Fig. 2B).

It is known that the mobility of helix αC is an important regulating factor to the kinase activity (43). Placement of the activation helix in contact with helix αC suggests that the activation motif may regulate the catalytic activity of SnRK2.6 through modula-
tion of the spatial position of helix αC, a caveat requiring further investigations.

Biochemical Identification of the Interface between SnRK2.6 and ABI1—Cell-based co-immunoprecipitation, BiFC, and Y2H screenings showed that SnRK2.6 and ABI1 are physically associated, and domain II of SnRK2.6 is required for the interaction (20, 21). With the purified recombinant proteins, we attempted to identify the interface between the kinase and phosphatase. Glutathione S-transferase (GST)-fused SnRK2.6 variants, including the full-length protein (GST-FL), the N-terminal fragment (residues 1–317, GST-N), and domain II (residue 318–362, GST-C) were immobilized on GS4B resin and used as bait to pull down ABI1 (Fig. 3A). Consistent with the in vivo observations (21), GST-FL and GST-C of SnRK2.6 could successfully pull down the catalytic domain of ABI1, whereas GST-N of SnRK2.6 could only pull down a barely visible band of ABI1 (Fig. 3A). We then sought to identify the motif on ABI1 that recognizes SnRK2.6. Notably, domain II of all the subclass III SnRK2s is highly enriched of acidic residues (Fig. 3B and supplemental Fig. S4). We reasoned that the counterpart of ABI1 may involve a cluster of positively charged residues. Therefore, we carefully examined the crystal structure of ABI1 (PDB accession code 3KDJ) and identified four positively charged surface patches (Fig. 3C).

To examine whether any of them are involved in SnRK2 recognition, we generated four ABI1 variants, each containing two to four point mutations and named mut1 through mut4 (mut1, R137D/K412D; mut2, K270D/R363A/K364A/R365A; mut3, R386A/R387A/K388A; and mut4, K334A/K337A). We then tested the binding of these variants to both SnRK2.6 FL and
domain II. Although mut1, mut3, and mut4 of ABI1 retained interaction with FL or domain II of SnRK2.6, mut2 failed to do so. Therefore ABI1 residues Lys270, Arg363, Lys364, and Arg365 are involved in association to the Glu/Asp-rich domain II of SnRK2.6. The polar interface is likely to be conserved for subclass III SnRK2s and most of group A PP2Cs, as the positively charged residues are conserved among four PP2Cs, ABI1, ABI2, HAB1, and HAB2 (supplemental Fig. S5).

We also examined the effect of the interface mutations on the dephosphorylation of pSnRK2.6 by ABI1. The $K_m$ values were both increased by 2–3-fold for ABI1-mut2 or domain II-truncated SnRK2.6 (Table 1). The activity assay supported the identification of the interface. On the other hand, the moderate effect on $K_m$ by the interface mutations may suggest the existence of an additional interface other than the identified one. This notion was supported by an extra line of evidence. When SnRK2.6 and ABI1 were subjected to SEC for the binding test, they were co-eluted, indicating formation of a stable SnRK2-ABI1 complex (Fig. 4A, top three panels on the right). By contrast, the interaction between SnRK2.6 domain II and ABI1, which were detected by the GST-mediated pulldown assay, could not sustain SEC (Fig. 4A, bottom two panels on the right). These observations demonstrated that SnRK2.6 FL exhibits a higher binding affinity with ABI1 than domain II, and that the N-terminal fragment of SnRK2.6 (residues 1–317) may possess a second recognition site for ABI1.

**Ile$^{298}$ and Trp$^{300}$ of ABI1 Are Involved in SnRK2.6 Recognition**—During an attempt to identify the second interface between SnRK2.6 and ABI1, when an ABI1 variant containing the Trp$^{300}$ to Glu mutation (ABI1-W300E) was incubated with SnRK2.6 and subjected to SEC analysis, their interaction was completely abolished (Fig. 4B). In addition, mutation of an adjacent hydrophobic residue, Ile$^{298}$, of ABI1 to Glu also led to complete loss of interaction between SnRK2.6 and ABI1 on SEC. Supporting this observation, in the phosphatase assay, ABI1 variants containing single point mutations of I298E or W300E both displayed increased $K_m$ values by about 8-fold with respect to the WT protein (Table 1). Moreover, the $K_m$ value of double mutations (I298E/W300E) was increased by ~60-fold. These observations suggested that Ile$^{298}$ and Trp$^{300}$ are involved in the association of ABI1 with SnRK2.6.

Interestingly, these ABI1 mutants retained interaction with FL or domain II of SnRK2.6 in the GST-mediated pulldown assay (supplemental Fig. S6), indicating that their interaction counterpart is not Glu/Asp-rich domain II. Supporting this analysis, Ile$^{298}$ and Trp$^{300}$ are located at a loop region about 40 Å away from the positive patch of ABI1 recognized by the SnRK2.6 domain II (Fig. 4B, left panel). It is likely that the hydrophobic region comprised of Ile$^{298}$ and Trp$^{300}$ represents a separate binding site for SnRK2.6.

**C-lobe of Kinase Domain of SnRK2.6 Is Recognized by ABI1**—We examined the structure of SnRK2.6 to look for hydrophobic
clusters that may be recognized by Ile<sup>298</sup> and Trp<sup>300</sup> of ABI1. Notably, Ile<sup>298</sup> and Trp<sup>300</sup> are located in the vicinity of the catalytic site of ABI1. We reasoned that their recognition site on SnRK2.6 was likely to be on the same side of the structure as the activation loop, because phospho-Ser<sup>175</sup> was readily dephosphorylated by ABI1. Based on structural analysis, we generated four variants of SnRK2.6, each containing double point mutations surrounding the potential position for the activation loop (mutI, F103D/Y215D; mutII, C159A/F161A; mutIII, V187D/Y193D; and mutIV, F226D/I230D) (Fig. 4). SEC was exploited to test the interactions between the SnRK2.6 variants and ABI1 (Fig. 4D).

Although mutI and mutII retained a stable interaction with ABI1 as WT SnRK2.6 does (Fig. 4D, top two panels), mutIII showed a decreased binding affinity (Fig. 4D, panel 3). In particular, mutIV was completely dissociated from ABI1 (Fig. 4D, bottom panel).

Val<sup>187</sup>/Tyr<sup>193</sup> and Phe<sup>226</sup>/Ile<sup>230</sup> are located at the adjacent structural motifs on the C-lobe of SnRK2.6. Therefore, this region may represent a second surface patch that is recognized by ABI1, most likely through van der Waals contacts with Ile<sup>298</sup> and Trp<sup>300</sup>. Notably, Val<sup>187</sup>, Tyr<sup>193</sup>, Phe<sup>226</sup>, and Ile<sup>230</sup> are highly conserved among the subclass III SnRK2s, and Ile<sup>298</sup> and Trp<sup>300</sup> are invariant among group A PP2Cs (supplemental Fig. S5).

**TABLE 1**

| Phosphatase | Substrate | \(k_{cat}\) | \(K_m\) | \(k_{cat}/K_m\) |
|-------------|-----------|-------------|--------|-----------------|
| ABI1 WT     | SnRK2.6   | 0.924 ± 0.02 | 0.097 ± 0.013 | (9.53 ± 1.44) × 10<sup>6</sup> |
| ABI1 (RKR363A,K270D) | SnRK2.6   | 0.962 ± 0.009 | 0.260 ± 0.012 | (3.70 ± 0.21) × 10<sup>6</sup> |
| ABI1 (I298E,W300E) | SnRK2.6   | 1.03 ± 0.03 | 0.358 ± 0.035 | (2.88 ± 0.37) × 10<sup>6</sup> |
| ABI1 (I298E,W300E, RKR363A,K270D) | SnRK2.6   | 1.11 ± 0.03 | 0.632 ± 0.048 | (1.76 ± 0.18) × 10<sup>6</sup> |
| ABI1 (W300E) | SnRK2.6   | 0.939 ± 0.013 | 5.59 ± 0.23 | (1.68 ± 0.08) × 10<sup>6</sup> |
| ABI1 (I298E) | SnRK2.6   | 1.17 ± 0.02 | 0.751 ± 0.057 | (1.57 ± 0.15) × 10<sup>6</sup> |
| ABI1 (I298E,W300E, RKR363A,K270D) | SnRK2.6   | NA<sup>a</sup> | NA | NA |

<sup>a</sup> NA, not applicable.
On the basis of the biochemical analyses as well as the available structures of SnRK2.6 and ABI1, we were able to generate a structural model of the SnRK2-ABI1 complex through computational approaches. First, we generated a structural model with the program Modeler 9.9 (39) for domain II of SnRK2.6 (Fig. 5A). Residues 339–347 were calculated as a helix, whereas the other residues were loops. Then we exploited the docking method Hodock (44) to predict the structure of the complex. The two biochemically identified interfaces were set as restraints for conformational searching and model selecting. In total, 11,000 models were generated, and the one with the highest score was picked up (Fig. 5B). We are fully aware of the speculative nature of this model, which mainly serves to examine the spatial compatibility of the two biochemically identified interfaces. Interestingly, this model showed that the two interfaces between ABI1 and SnRK2.6 anchor the proteins in an orientation that the activation loop of SnRK2.6 is positioned into the active site of ABI1 (Fig. 5B), and thus provided a plausible molecular basis for the efficient dephosphorylation of phospho-Ser175 of SnRK2.6 by ABI1.

Molecular Basis of PYLs-mediated Activation of SnRK2.6—SnRK2.6 can be activated by both ABA and osmotic stress in planta. Despite the poorly understood ABA-independent activation mechanism, the biochemical and structural investigations presented here, together with previous reports, provide the molecular basis for the efficient dephosphorylation of phospho-Ser175 of SnRK2.6 by ABI1.
important role in the recognition between ABI1 and PYLs (25–27). Here we further identified that ABI1-Trp300 represented an essential binding site for SnRK2.6 (Fig. 3B). Therefore, in addition to directly blocking the catalytic site of PP2Cs, PYLs also compete with SnRK2.6 for binding to Trp300 of ABI1 (Fig. 5C), thus disrupting the hydrophobic interface between ABI1 and SnRK2.6.

It is noteworthy that disruption of the hydrophobic interface between ABI1 and SnRK2.6 resulted in a nearly 60-fold increase of $K_m$ value for the dephosphorylation of SnRK2.6 by ABI1. By contrast, loss of the polar interface only led to a slight augment of the $K_m$ value (Table 1). The hydrophobic interface is closer to the active site of ABI1 and phospho-Ser$^{175}$ of SnRK2.6, and may directly facilitate a stable association between the enzyme and the substrate. On the other hand, domain II of SnRK2.6 may be rather flexible as suggested by structural prediction (Fig. 5A). Domain II may serve as a tethering factor for the initial recognition by PP2Cs, which is indispensable for complex formation (Fig. 2A). Once the proteins were pulled close enough, the hydrophobic interface may help anchor the enzyme and the substrate with the appropriate orientation so that phospho-Ser$^{175}$ of SnRK2.6 is subject to dephosphorylation by ABI1. When ABA-bound or ABA-independent PYLs arrive, the hydrophobic interface is disrupted and the catalytic site of ABI1 is blocked. Our preliminary analyses showed that PYLs, ABI1, and SnRK2.6 may be able to form a ternary complex (supplemental Fig. S7), as the polar interface between SnRK2.6 and ABI1 is not significantly affected by PYLs. Nonetheless, due to the long and flexible linker between the catalytic domain and domain II, the kinase domain of SnRK2.6 is relieved and activated in response to ABA (Fig. 5C).

**DISCUSSION**

In summary, the biochemical and structural characterizations reported here revealed the molecular basis for the recognition and inhibition of subclass III SnRK2s by group A PP2Cs, and served as an important platform to the understanding of the downstream signal transduction of ABA. Nevertheless, an important question awaits further investigations. Subclass III SnRK2s can be activated in both ABA-dependent and -independent manners. Our current studies revealed the molecular mechanism for ABA-dependent activation of SnRK2s. It remains elusive how osmotic stress activates SnRK2s. As reported previously, a subclass of PYLs inhibit PP2Cs independent of ABA (28). Further studies are required to elucidate whether osmotic stress directly regulates SnRK2s, or through inhibition of PP2Cs by ABA-independent PYLs. It is also plausible that unidentified factors or mechanisms are involved in the activation of SnRK2s by osmotic stress.

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