Modulation of Abscisic Acid Signaling in Vivo by an Engineered Receptor-Insensitive Protein Phosphatase Type 2C Allele\textsuperscript{1[C][W]}

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The plant hormone abscisic acid (ABA) plays a crucial role in the control of the stress response and the regulation of plant growth and development. ABA binding to PYRABACTIN RESISTANCE1 (PYR1)/PYR1-LIKE (PYL)/REGULATORY COMPONENTS OF ABA RECEPTORS intracellular receptors leads to inhibition of key negative regulators of ABA signaling, i.e. clade A protein phosphatases type 2C (PP2Cs) such as ABA-INSSENSITIVE1 and HYPERSENSITIVE TO ABA1 (HAB1), causing the activation of the ABA signaling pathway. To gain further understanding on the mechanism of hormone perception, PP2C inhibition, and its implications for ABA signaling, we have performed a structural and functional analysis of the PYR1-ABA-HAB1 complex. Based on structural data, we generated a gain-of-function mutation in a critical residue of the phosphatase, hab1\textsuperscript{W385A}, which abolished ABA-dependent receptor-mediated PP2C inhibition without impairing basal PP2C activity. As a result, hab1\textsuperscript{W385A} caused constitutive inactivation of the protein kinase OST1 even in the presence of ABA and PYR/PYL proteins, in contrast to the receptor-sensitive HAB1, and therefore hab1\textsuperscript{W385A} qualifies as a hypermorphic mutation. Expression of hab1\textsuperscript{W385A} in Arabidopsis (Arabidopsis thaliana) plants leads to a strong, dominant ABA insensitivity, which demonstrates that this conserved tryptophan residue can be targeted for the generation of dominant clade A PP2C alleles. Moreover, our data highlight the critical role of molecular interactions mediated by tryptophan-385 equivalent residues for clade A PP2C function in vivo and the mechanism of ABA perception and signaling.

Abscisic acid (ABA) is required for biotic and abiotic stress responses as well as the control of plant growth and development. Plant growth can be severely im-

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beyond the structural data, no in planta evidence has been provided for its direct role in ABA signaling. Moreover, the ternary complexes analyzed at a structural level have not included PYR1, which plays a predominant role in germination (Park et al., 2009).

Plants harboring ab1G180D, ab2G180D, and hab1G246D dominant mutations have represented valuable tools to dissect ABA signaling (Leung et al., 1994, 1997; Meyer et al., 1994; Rodríguez et al., 1998; Robert et al., 2006). Their ABA-insensitive phenotypes are in agreement with a reduced capacity of the mutant PP2Cs to interact with PYR/PYL/RCAR receptors (Park et al., 2009; Santiago et al., 2009b; Umezawa et al., 2009). In spite of their utility, these alleles bear mutations close to the phosphatase catalytic site and have reduced basal PP2C activity (Bertauche et al., 1996; Leung et al., 1997; Leube et al., 1998; Rodríguez et al., 1998; Robert et al., 2006), which has complicated the interpretation of their in vivo phenotypes. Mutations in the conserved Trp residue described above have not been isolated by forward genetic screens, or engineered in Arabidopsis (Arabidopsis thaliana) plants, and the functional relevance of this residue has been documented uniquely on in vitro studies for the case of ABI1 (Miyazono et al., 2009). Since mutations in the Trp residue are expected to affect the stability of the ternary complex without compromising the phosphatase catalytic activity, they represent an ideal tool for studying in planta the effect of decoupling the receptor and phosphatase interaction.

Here we present a combined structural and functional analysis of the ternary complex formed by PYR1-ABA-HAB1. We analyzed the effect of PYR1-HAB1 mutations on OST1 kinase activity in vitro, since this SnRK2 is a key target of HAB1 (Vlad et al., 2009). We also performed in planta analysis of a hab1W385A mutation that decouples receptor and phosphatase interaction without impairing PP2C activity. These transgenic plants show an acute ABA insensitivity, demonstrating the importance of ABA-mediated PYR/PYL/RCAR-PP2C contacts for receptor function in vivo, and enabling a new method for probing PP2C function with dominant receptor-insensitive mutations.

RESULTS

Architecture of the PYR1-ABA-ΔNHAB1 Ternary Complex

The PYR1 receptor and the catalytic domain of the HAB1 phosphatase (residues 179–511, ΔHAB1) were separately overexpressed in Escherichia coli, purified, and mixed in equimolar amounts in the presence of 1 mM (+)-ABA. The resulting complex was assayed for crystallization at the high-throughput crystallization facility of the EMBL Grenoble Outstation (https://embl.fr/htxlab; Dimasi et al., 2007). X-ray diffraction data were collected from orthorhombic crystals at the ID14-4 beam line of the European Synchrotron Radi-
ation Facility (ESRF) to 1.8 Å resolution. Initial phases were obtained by the molecular replacement method using the two central β-sheets of the catalytic domain of the human PP2Cα protein (1A6Q; Das et al., 1996) as a search model. The initial phases provided an easily interpretable electron density map extending outside the search model region. Successive rounds of automatic refinement and manual building resulted in a refined model with a Rwork and Rfree of 17.4% and 21.8%, respectively. In the refined model, the crystallographic asymmetric unit contains one molecule of PYR1, one molecule of ΔNHAB1, one molecule of ABA, and three manganese ions (Fig. 1; Table I).

The structure of PYR1 in the complex is very similar to that of the ABA-bound subunit in dimeric PYR1 (Nishimura et al., 2009; Santiago et al., 2009a). The ABA molecule is located in the receptor cavity stabilized by both polar and hydrophobic interactions and the gating loops are in the closed conformation, as described previously (Nishimura et al., 2009; Santiago et al., 2009a; Fig. 1). Subtle differences between the two PYR1 structures likely induced by interaction with HAB1 are found around Ser-85 in one of the gating loops, and the loop β7/α5, adjacent to the gating loops (Fig. 1, B and C). The structure of the HAB1 catalytic domain is similar to those of Arabidopsis ABI1 (Melcher et al., 2009; Miyazono et al., 2009; Yin et al., 2009) and the human PP2Cα protein phosphatase (Das et al., 1996). It is formed by a central 10-strand antiparallel β-sandwich flanked by two long α-helices at each side. A 55-amino acid α/β domain, which has been named the flap subdomain in some bacterial PP2Cs (Schlicker et al., 2008) is inserted between strands β8 and β12 of HAB1. This subdomain contains the HAB1 Trp-385 (Fig. 1A), which is highly conserved in plant clade A PP2Cs. Small conformational differences between the three phosphatases are found at the β2-β3 and α1-α2 loop regions of HAB1. In addition to this, HAB1 displays a 16-amino acid insertion at the α3/β4 loop not found in ABI1 and the human PP2Cα (Supplemental Fig. S1).

The catalytic site of HAB1 is located inside a deep channel formed at the top of the β-sandwich and flanked by the flap subdomain (Fig. 2; Supplemental Fig. S2). In our structure, the catalytic site of HAB1 contains three metal ions designated here as M1, M2, and M3 according to Alzari and coworkers (Wehenkel et al., 2007; Fig. 2). While some protein phosphatases contain two metal ions at the catalytic site, a few bacterial phosphatases have been shown to display a third conserved metal ion site, M3 (Pullen et al., 2004;
with HAB1 Trp-385 but also with the receptor gating loops (with the backbone carbonyl and amine groups of Pro-88 and Arg-116, respectively) and with the hormone itself, through its ketone group. Comparison of the present structure with the previously reported structures of isolated PYR1 reveals a conformational rearrangement in the β7/α5 loop of PYR1 upon binding to HAB1. This loop moves forward toward the flap domain of HAB1 (Fig. 1B), establishing new interactions that stabilize both the closed conformation of the gating loops and the receptor-phosphatase complex. These interactions involve Asn-151 of PYR1, which is hydrogen bonded to both the carbonyl group of HAB1 Gln-384 in the flap domain and PYR1 Arg-116, located in one of the gating loops. At the same time, in the present structure the side chain of PYR1 Ser-152 is involved in a helix-capping interaction (Presta and Rose, 1988) that stabilizes the forward movement of the β7/α5 loop.

Another important interaction region involves the PYR1 β3/β4 loop containing Ser-85 and the catalytic site of the phosphatase (Fig. 1C). PYR1 Ser-85 takes part in a hydrogen bond network with the backbone amine of Gly-246 and the carboxylic group of Glu-203 at the catalytic site of HAB1. This interaction is likely to be responsible for the inhibition of the phosphatase activity, as the β3/β4 loop containing Ser-85 seems to block access to the phosphatase catalytic site (Fig. 2). The structure of the human PP2Ca contains a phos-

### Table 1. Crystallographic data collection and refinement statistics

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<td><strong>Bond length</strong></td>
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Wehenkel et al., 2007; Schlicker et al., 2008). The M3 site, located at the exit of the catalytic channel and is typically coordinated by one conserved aspartic residue also involved in coordination of the metal at M1 (Asp-432 for HAB1), and one residue from the flap domain. In some bacterial PP2Cs coordination of the third metal ion at M3 has been correlated with a change in position of the flap subdomain (Wehenkel et al., 2007), however, this site displays low metal-binding affinity and has been shown to be dispensable for catalysis (Wehenkel et al., 2007). To our knowledge, HAB1 is the first eukaryotic PP2C with three metal sites.

### Molecular Interactions Stabilizing the PYR1-ABA-HAB1 Complex

The PYR1-HAB1 interface comprises a total protein buried surface area of 1,691 Å². As in the case of the PYL2-HAB1 and PYL1-ABI1 structures (Melcher et al., 2009; Miyazono et al., 2009; Yin et al., 2009), HAB1 docks into the ABA-bound receptor establishing interactions with the gating loops (loops β3/β4 and β5/β6), the N-terminal part of the α5 helix, and the α4/β2 loop of PYR1 (Fig. 1, A–C). The HAB1 residues involved in those interactions are located both in the flap subdomain including Trp-385 and the phosphatase active site including the β1/β2, β3/α1, and α2/β4 loops (Figs. 1, A–C and 2; Supplemental Fig. S3). The HAB1 Trp-385 residue is inserted between the PYR1 gating loops with the nitrogen in the indole group establishing a hydrogen bond with the water located at the channel between the gating loops (Fig. 1B). This water molecule represents a critical point in the tertiary complex, establishing hydrogen bonds not only
phosphate ion at the catalytic site, which is likely mimicking the position of the phosphorylated amino acid substrate (Das et al., 1996). Interestingly, when PP2Ca and HAB1 catalytic cores are superimposed the phosphate ion of human PP2Ca is 2.9 Å away from the Cβ carbon of Ser 85 of PYR1 (Fig. 2; Supplemental Fig. S4), which suggests that a phospho-Ser substrate might enter the catalytic site in a similar manner.

**Mutational Analysis of the PYR1-HAB1 Interaction and Effect on the HAB1-Dependent Inhibition of OST1 Activity**

To test the biological relevance of the interactions observed in the PYR1-HAB1 complex, we analyzed the effect of a number of single-point mutations on both proteins. In the case of PYR1, we mutated key amino acid residues involved in either direct ABA binding (Glu-94Lys, Glu-141Lys, and Tyr-120Ala) or both ABA binding and PP2C interaction, particularly residues located in the gating loops (Ser-85Ala, Leu-87Ala, Pro-88Ser, Arg-116Ala) and the loop β7-α5 (Ser-152Leu). For HAB1 we chose the Gly-246Asp mutation, equivalent to abi1-1D and abi2-1D mutations, since expression of hab1^{G246D} in planta leads to a dominant ABA-insensitive phenotype (Robert et al., 2006) and Trp-385Ala, due to its critical interactions with the PYR1 gating loops and ABA. For each PYR1 mutant we first tested both its capacity to interact with HAB1 and inhibit its activity through yeast (Saccharomyces cerevisiae) two-hybrid (Y2H) interaction and in vitro phosphatase activity assays, respectively (Fig. 3, A and B; Supplemental Fig. S5). In general, the PYR1 mutations abolished or severely reduced the ABA-mediated interaction and the inhibition of HAB1 phosphatase activity as compared to the wild type. An exception is the PYR1 R157H variant. Although this mutation confers resistance to pyrabactin, a seed ABA agonist (Park et al., 2009), it shows very limited effect in both the Y2H and phosphatase activity assays.

In vitro reconstitution of an ABA signaling cascade can be achieved by combining PYR1, PP2C, SnRK2.6/OST1, and ABF2 in a test tube (Fujii et al., 2009). In this system, OST1 activity is measured as autophosphorylation as well as transphosphorylation of its natural substrate ABF2. We used this assay to determine how the different mutations affect the control of the OST1 activity. Figure 3 shows that HAB1 dephosphorylates OST1 and inhibits its kinase activity (lanes 1 and 2, Fig. 3, C and D). However, if ABA and PYR1 are added, HAB1 is inactivated, and consequently a significant recovery of OST1 activity is observed (lane 5, Fig. 3, C and D). All the PYR1 mutants assayed, except R157H, showed a strongly decreased capacity to antagonize the HAB1-mediated dephosphorylation of OST1 and were unable to promote ABA-dependent recovery of the OST1 protein kinase activity.

Both HAB1 Trp-385Ala and Gly-246Asp mutations abolished the ABA-dependent interaction between HAB1 and PYR1, as revealed by the Y2H and in vitro phosphatase activity assays (Fig. 4, A and B). In agreement with these results and in contrast to wild-type HAB1, both mutant PP2Cs were able to dephosphorylate OST1 in the presence of ABA and PYR1 (Fig. 4C). Thus, both mutant PP2Cs were refractory to inhibition by PYR1 under these experimental conditions. This result indicates that both hab1^{W385A} and hab1^{G246D} qualify as hypermorphic mutants compared to wild-type HAB1 in the presence of ABA and PYR1 (Wilkie, 1994). However, the basal dephosphorylation of OST1 by hab1^{G246D} was less effective than wild type in the absence of ABA and PYR1 (Vlad et al., 2009; this work), which can be explained because this mutation is located close to the PP2C active site. Indeed, using p-nitrophenol as substrate, hab1^{G246D} showed 4-times-lower specific activity as compared to wild-type HAB1 (4.86 ± 0.43 and 18.76 ± 2.13 nmol inorganic phosphate/ min mg, respectively). Instead, the activity of hab1^{W385A} was similar to wild type both in the p-nitrophenyl phosphate (pNP; 20.52 ± 2.53 nmol inorganic phosphate/ min mg) and the OST1 dephosphorylation assays (Fig. 4C).

In summary, the mutational analysis of both PYR1 and HAB1 confirms that the interactions revealed by the structural analysis of the ternary complex are crucial for the inhibition of HAB1 activity. Additionally, these results illustrate that certain mutations in the PP2C lead to escape of the inhibitory ABA-mediated PYR/PYL mechanism. The results obtained for hab1^{G246D} provide additional support to the model proposed by Merlot and coworkers (Vlad et al., 2009) to explain the negative regulation of OST1 activity by HAB1 and the strong ABA-insensitive phenotype of 35S:hab1^{G246D} plants (Robert et al., 2006), assuming that a general escape from PYR/PYL receptors occurs in these plants. Indeed, we have demonstrated in vitro that hab1^{G246D} phosphatase, as well as hab1^{W385A}, are refractory to inhibition by different PYR/PYL proteins (Fig. 4D).

**Expression of hab1^{W385A} in Arabidopsis Plants Leads to Reduced ABA Sensitivity**

To test the biological relevance of the PYR1-ABA-HAB1 interaction mediated by the residue Trp-385 of HAB1, we generated 35S:hab1^{W385A} transgenic lines and examined their ABA response compared to 35S:HAB1 plants (Fig. 5). For this analysis, we selected three 35S:hab1^{W385A} transgenic lines that showed expression levels of the recombinant protein similar to those of the previously described 35S:HAB1 plants (Saez et al., 2004), as determined by immunoblot analysis against the hemagglutinin (HA) epitope added to each protein (Fig. 5C). Germination and early seedling establishment of 35S:HAB1 and 35S:hab1^{W385A} seeds were less sensitive to ABA-mediated inhibition than wild-type seeds (Fig. 5, A and B). Moreover, 35S:hab1^{W385A} seeds were able to germinate and establish seedlings at 10 μM ABA, which is an inhibitory concentration for establishment of 35S:HAB1 seeds (Fig. 5, A and B).
Stomatal closing is a key ABA-controlled process that preserves water under drought conditions. We mimicked drought by exposing plants to the drying atmosphere of a flow laminar hood and under these conditions we measured water loss in 2-week-old seedlings (Fig. 5, D and E). Both 35S:HAB1 and 35S:hab1W385A plants showed a higher transpiration rate than wild type, and water loss in plants overexpressing the mutated phosphatase was higher than in the wild-type PP2C. The increased insensitivity to ABA of the 35S:hab1W385A plants as compared to 35S:HAB1, is consistent with the inability of the PYRL/PYL/RCAR receptors to inhibit in vitro the activity of hab1W385A (Fig. 4D). Finally, the expression of ABA-inducible genes was severely reduced in 35S:hab1W385A plants as compared to the wild type (Fig. 5F). The accumulation of these transcripts was also impaired in 35S:HAB1 plants; in some cases, RAB18 and RD29B, the effect was similar to 35S:hab1W385A plants, however, ABA induction of other transcripts, KIN1, RD29A, P5CS, and RD22, was less affected (Fig. 5F).

DISCUSSION

The structure of the PYR1-ABA-HAB1 complex presented here and those of the ternary complexes studied previously (Melcher et al., 2009; Miyazono et al., 2009; Yin et al., 2009) contribute to explain how ABA binding induces the interaction between receptor and phosphatase and its inhibitory nature on phosphatase activity. Interestingly, these complexes show a 1:1 receptor:phosphatase stoichiometry. Since it has been shown that PYR1 forms a dimer in vivo (Nishimura et al., 2009), evidence that is not yet available for PYL1 and PYL2, our data confirm that PYR1...
dimer dissociation is required for the formation of the ternary complex, as Yan and coworkers have suggested (Yin et al., 2009). However, a detailed understanding of the dimer dissociation process is not available yet.

Once the hormone enters the receptor cavity, the cyclic moiety of the ABA molecule establishes interactions with the receptor gating loops, which favors their closed conformation. This closed conformation offers an optimal surface for the docking of the phosphatase, which contributes in turn to the stability of the ternary complex by locking the gating loops in their closed conformation and trapping the hormone inside the binding cavity. For instance, PYL9 and PYL5 bind to ABA with a Kd of 0.70 and 1.1 μM, respectively, whereas inclusion of ABI2 and HAB1 in the binding assay leads to a Kd of 64 and 38 nM, respectively (Ma et al., 2009; Santiago et al., 2009b). The HAB1 Trp-385 residue plays a major role in this stabilization process by inserting between the gating loops, and additionally via an indirect contact with the ABA's ketone group through a hydrogen bond network mediated by a critical water molecule. This water molecule establishes hydrogen bonds not only with HAB1 Trp-385 and the hormone, but also with key residues (Pro-88 and Arg-116) of the receptor gating loops. This complex network of interactions provides a mechanism through which the phosphatase is able to monitor hormone occupancy of the ABA binding cavity, and

Figure 4. The hab1W385A and hab1G246D PP2Cs are refractory to inhibition by PYR1 and dephosphorylate OST1 in the presence of ABA and PYR1. A, The HAB1 mutations Trp-385Ala and Gly-246Asp abolish the interaction of the PP2C and PYR1 in a Y2H assay. Immunoblot analysis using antibody against the Gal4 activation domain (GAD) is shown to verify the expression of the different fusion proteins. Ponceau staining from a representative yeast protein is shown as loading control. GBD, Gal4 binding domain. B, Phosphatase activity of HAB1, hab1W385A, and hab1G246D proteins was measured in vitro using pNPP as substrate in the absence or presence of PYR1 and ABA, as indicated. Assays were performed in a 100 μL reaction volume containing 2 μM phosphatase and, when indicated, 4 μM HIS6-PYR1 and 1 μM (+)-ABA. Data are averages ± SD from three independent experiments. C, In vitro OST1 kinase activity in the presence of wild-type and mutated versions of HAB1, PYR1, and ABA, as indicated. The autoradiography shows the level of autophosphorylation of OST1 in each reaction. The graphs show the quantitative analysis of the autoradiogram. D, hab1W385A and hab1G246D proteins are resistant to ABA-mediated inhibition by different PYR/PYLs. The assay was performed as described in B. [See online article for color version of this figure.]
therefore ensuring that the conserved Trp residue will only contribute to the stabilization of the receptor-phosphatase complex if the hormone is present. The in vitro data presented here for hab1W385A and by Miyazono et al. (2009) for abi1W300A support this conclusion. Moreover, our results show that this hormonesensing mechanism is critical for ABA response in planta. Thus, expression of hab1W385A in Arabidopsis plants leads to a strong ABA-insensitive phenotype, which can’t be explained solely by enhanced PP2C gene dosage, since 35S:HAB1 plants, although less sensitive to ABA than wild type, show milder phenotypes. The reduced sensitivity to ABA-mediated inhibition of seed germination and seedling establishment, enhanced water loss, and reduced expression of ABA-responsive genes in 35S:hab1W385A plants support the relevance of this locking interaction, postulated by structural studies. Additionally, these plants represent a valuable tool to dissect the ABA pathway by using dominant receptor-insensitive PP2C mutants that do not compromise the intrinsic phosphatase activity. Taking into account the large number of screenings performed to identify ABA-insensitive plants, the failure to isolate mutants harboring missense mutations in
This Trp residue is somehow surprising. However, since ethylmethanesulfonate mutagenesis usually leads to G → A transitions, such mutation in the Trp codon (UGG) would lead to stop codons and presumably loss-of-function alleles. The locking mechanism provided by the Trp residue appears to be a particular evolution of the plant clade A PP2Cs, since with the exception of AHG1, they are the unique plant PP2Cs that present this residue in the appropriated position of the flap PP2C subdomain. Interestingly, AHG1 was less sensitive to ABA-dependent PYL8-mediated inhibition than other clade A PP2Cs, such as PP2CA and At5g59220 (Supplemental Fig. S6).

This work and previous structural analyses indicate that the insertion of the PYRI Ser-85 containing β3-β4 loop (Ser-112 of PYL1 and Ser-89 of PYL2) into the phosphatase catalytic site could account for the inhibition of PP2C catalytic activity by blocking access of potential substrates to the phosphatase catalytic site in a competitive manner. However, although this mechanism looks plausible, the phosphatase catalytic channel remains open in its lower part in the ternary complexes formed by both HAB1 and ABI1 (Supplemental Fig. S2). This lower part of the phosphatase catalytic groove might represent an alternative entry site for substrates and indeed initial studies based on biochemical assays with a nonpeptidic substrate, suggested that inhibition of the PP2C activity by PYR/PYL/RCAR proteins occurs by a noncompetitive, rather than competitive mechanism (Ma et al., 2009). In contrast, in other studies the inhibition of HAB1 by ABA-bound PYL2 was overcome by increasing concentrations of an OST1 phosphopeptide containing residues of the kinase activation loop (Melcher et al., 2009). Unfortunately the structure of a PP2C in complex with a natural peptide substrate is lacking, which could contribute to resolve this issue. However, one striking observation arising from this structural analysis is the proximity of Ser-85 in the gating loop of the PYRI receptor to the position expected to be occupied by the phosphoryl group of the substrate of the phosphatase reaction. Superposition of this structure and the catalytic domain of human PP2Ca shows that the β-carbon of PYRI Ser-85 is next to the phosphate ion oxygen atom that Barford and coworkers have proposed as the seryl/threonyl oxygen in their analysis of the PP2Ca catalytic site (Das et al., 1996). This would suggest that the PYRI Ser-85, and its equivalent in other PYR/PYL proteins, might act as a product mimic and occupy a similar position as the phosphorylated Ser residues in SnRK2s and other PP2C targets. In our view, this important observation lends weight to the interpretation that the formation of the receptor-phosphatase complex prevents access of natural PP2C substrates to the catalytic site, supporting the competitive nature of the inhibition mechanisms. At the same time it would support the catalytic mechanism proposed by Barford (Das et al., 1996), where the water molecule linked to the metal at the M2 site and Glu-37 of human PP2Ca (Glu-203 in HAB1) would contribute to catalysis by facilitating the protonation of the oxygen atom in the P-O scissile bond.

Since Ser-85 of PYRI, Ser-112 of PYL1, and Ser-89 of PYL2 insert into the PP2C active site and establish contacts with Gly-180 of ABI1 or Gly-246 of HAB1, the structural data provide a framework to explain the effect of ab1G180D and hab1G246D mutations. However, no direct biochemical evidence had been previously provided in the case of hab1G246D. This analysis shows that hab1G246D is insensitive to inhibition by various PYR/PYL proteins, which leads to the escape from the ABA-dependent PYR/PYL inhibitory mechanism and the subsequent constitutive inhibition of OST1 activity. Therefore, these data are in agreement with the notion that hab1G246D behaves as a hypermorphic mutation in the presence of ABA, as noted by Schroeder and coworkers (Robert et al., 2006). Paradoxically, in the absence of ABA, hab1G246D shows lower intrinsic phosphatase activity than wild-type HAB1, probably because this mutation perturbs the PP2C active site to some extent.

Even though other ABA receptors have been identified (Pandey et al., 2009; Shang et al., 2010) and therefore other input sources exist for ABA signaling, the phenotypes of both 35S:hab1G246D and 35S:hab1W385A plants indicate that constitutive activation of the PP2Cs (and the consequent inactivation of the SnRK2s) leads to a severe blockade of ABA signaling. Therefore, the action of the SnRK2s is likely localized downstream of the other putative inputs and could represent a core ABA signaling component shared by all ABA receptors. This would be in agreement with the extreme ABA insensitivity of triple snrk2.2/2.3/2.6 mutant plants (Fujii and Zhu, 2009).

**MATERIALS AND METHODS**

**Construction of Plasmids**

Plasmids pETM11 or pET28a were used to generate N-terminal His-tagged recombinant proteins. The cloning of 6×His-ΔHAB1 (lacking residues 1–178), PYRI, PYL4, PYL5, and PYL8 constructs was previously described (Santiago et al., 2009b). Using a similar approach, PYL1 and PYL6 were cloned in pETM11, whereas PYL9 was cloned in pET28a. HAB1(W385A), HAB1(G246D), PYR1(S85A), PYR1(R116A), PYR1(L87A), and PYR1(Y120A) mutants were produced using the overlap extension procedure (Ho et al., 1989) and cloned into pETM11. PYR1(S152L), PYR1(P88S), PYR1(R157H), PYR1(E141K), and PYR1(E94K) mutants were obtained from the pyr1-2, pyr1-3, pyr1-4, pyr1-5, and pyr1-6 alleles, respectively (Park et al., 2009), and cloned into pET28a. The coding sequences of OST1 and a C-terminal deletion of AB2 (ACABF2, amino acids 1–173) were cloned into pET28a.

**Protein Expression and Purification**

BL21(DE3) cells transformed with the corresponding constructs in pETM11 or pET28a vectors were grown in Luria-Bertani medium to an OD 600 of 0.6 to 0.8. At this point 1 mM isopropylthio-b-galactoside was added and the cells were harvested after overnight incubation at 20°C. Proteins used for crystallization were purified as described (Santiago et al., 2009a). For small-scale protein preparations, the following protocol was used. Pellets were suspended in lysis buffer (50 mM Tris pH 7.5, 250 mM KCl, 1% glycerol, 1 mM β-mercaptoethanol) and lysed by sonication with a Branson Sonifier 250. The clear lysate obtained after centrifugation was purified by nickel affinity. A washing step was performed using 50 mM Tris, 250 mM KCl, 20% glycerol, 30

Cry stallization and Structure Solution

The PYRI-ABA-HAB1 ternary complex was prepared by mixing PYRI, ΔNHA1B, and 1 μM ABA to a final concentration of 3 mg/mL, 5 mg/mL, and 1 μM, respectively, in 20 mM Tris pH 7.5, 150 mM NaCl, 1 mM MnCl2, and 1 mM β-mercaptoethanol. Crystallization conditions for the complex were identified at the High Throughput Crystallization Laboratory of EMBL Grenoble Outstation (https://htcrlab.emb-bl.fr) as described in Marquez et al. (2007). The crystals used for data collection were obtained by vapor diffusion method in 0.25 M NaCl, 19% Peg 3530 at 20°C. X-ray diffraction data were collected at the ID14-4 beam line of the ESRF to 1.8 Å resolution. Initial phases were obtained by the molecular replacement method using the two central sheets of the catalytic domain of the human PP2C protein (1A6Q; Das et al., 1996) as a search model and the program Phaser (McCoy et al., 2007). Successive rounds of automatic refinement and manual building were carried out with RefMac5 (Murshudov et al., 1997) and Coot (Emsley and Cowtan, 2004). Atomic coordinates from the final model have been deposited in the Protein Data Bank under accession code 3QN1.

PP2C and OST1 in Vitro Activity Assays

Phosphatase activity was measured using either the Ser/Thr phosphatase assay system (Promega) using the RRA(phosphoT)VA peptide as substrate or pNPP. In the first case assays were performed in a 100-μL reaction volume containing 25 mM Tris-HCl pH 7.8, 10 mM MgCl2, 1 mM dithiothreitol, 25 μM peptide substrate, and the PP2C. When indicated, PYR-PYL recombinant proteins and ABA were included in the PP2C activity assay. After incubation for 60 min at 30°C, the reaction was stopped by addition of 30 μL molybdate dye (Baykov et al., 1988) and the absorbance was read at 630 nm with a 96-well plate reader. For the pNPP phosphatase activity assays a 100 μL solution containing 25 mM Tris-HCl pH 7.5, 2 mM MnCl2, and 5 mM pNPP substrate and the indicated amount of the PP2Cs was used. Measurements were taken with a ViktorX5 reader at 405 nm every 60 s over 30 min.

Phosphorylation assays were done basically as described previously (Belin et al., 2006; Vlad et al., 2009). Assays to test recovery of OST1 activity were done by previous incubation for 10 min of the protein phosphatase HAB1 together with the PYR1 wild type or PYR1 mutant proteins in the presence of the indicated concentration of (+)-ABA. Next, the reaction mixture was incubated for 50 min at room temperature in 30 μL of kinase buffer: 20 mM Tris-HCl pH 7.8, 20 mM MgCl2, 2 mM MnCl2, and 3.5 μCi of γ-32ATP (3,000 Ci/ mmol). The reaction was stopped by adding Laemmli buffer. When indicated, ΔCABF2 recombinant protein (100 μg) was added as substrate of OST1. After the reaction proteins were separated by SDS-PAGE using an 8% acrylamide gel and transferred to an Immobilon-P membrane (Millipore). Radioactivity was detected using a phosphorimage system (FLA5100, Fujifilm). After scanning, the same membrane was used for Ponceau staining. The data presented are averages of at least three independent experiments.

Y2H Assays

Protocols were similar to those described previously (Saez et al., 2006).

Generation of 35S:habi<sup>W385A</sup> Transgenic Lines

The mutated habi<sup>W385A</sup> was cloned into pCR8/GW/TOPO entry vector (Invitrogen) and recombined by LR reaction into the gateway-compatible ALLIGATOR2 vector. This construct drives expression of habi<sup>W385A</sup> under control of the 35S cauliflower mosaic virus promoter and introduces a triple HA epitope at the N terminus of the protein. Selection of transgenic lines is based on the visualization of GFP in seeds, whose expression is driven by the specific seed promoter At2BS. The ALLIGATOR2-35S:3HA-habi<sup>W385A</sup> construct was transferred to <i>Agrobacterium tumefaciens</i> C58C1 (pGV2260; Deblaere et al., 1985) by electroporation and used to transform Columbia wild-type plants by the floral-dip method. T1 transgenic seeds were selected based on GFP visualization and sowed in soil to obtain the T2 generation. Homozygous plants by the floral-dip method. T1 transgenic seeds were selected based on the HA epitope at the N terminus of the protein. Selection of transgenic lines are presented are averages of at least three independent experiments.

RNA Analyses

ABA treatment, RNA extraction, and reverse transcription-quantitative PCR amplifications were performed as previously described (Saez et al., 2004).

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure S1. Structural superposition of ternary receptor complexes.

Supplemental Figure S2. Detail of the catalytic groove of HAB1.

Supplemental Figure S3. Amino acid sequence and secondary structure alignment of plant PP2Cs and the catalytic core of human PP2C.

Supplemental Figure S4. Detail of the HAB1 catalytic site around the PYR1 Ser-85.

Supplemental Figure S5. Comparison of the ABA-dependent inhibitory effect of PYR1 wild-type and mutant proteins on HAB1 activity.

Supplemental Figure S6. Amino acid sequence alignment of Arabidopsis clade A PP2Cs and representative PP2Cs from other groups.

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LITERATURE CITED


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