The PYL4 A194T Mutant Uncovers a Key Role of PYR1-LIKE4/PROTEIN PHOSPHATASE 2CA Interaction for Abscisic Acid Signaling and Plant Drought Resistance

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Because abscisic acid (ABA) is recognized as the critical hormonal regulator of plant stress physiology, elucidating its signaling pathway has raised promise for application in agriculture, for instance through genetic engineering of ABA receptors. PYRABACTIN RESISTANCE1/PYR1-LIKE (PYL)/REGULATORY COMPONENTS OF ABA RECEPTORS ABA receptors interact with high affinity and inhibit clade A phosphatases type-2C (PP2Cs) in an ABA-dependent manner. We generated an allele library composed of 10,000 mutant clones of Arabidopsis (Arabidopsis thaliana) PYL4 and selected mutations that promoted ABA-independent interaction with PP2CA/ABA-HYPERSENSITIVE3. In vitro protein-protein interaction assays and size exclusion chromatography confirmed that PYL4A194T was able to form stable complexes with PP2CA in the absence of ABA, in contrast to PYL4. This interaction did not lead to significant inhibition of PP2CA in the absence of ABA; however, it improved ABA-dependent inhibition of PP2CA. As a result, 35S:PYL4A194T plants showed enhanced sensitivity to ABA-mediated inhibition of germination and seedling establishment compared with nontransformed or 35S:PYL4 plants. Additionally, at basal endogenous ABA levels, whole-rosette gas exchange measurements revealed reduced stomatal conductance and enhanced water use efficiency compared with nontransformed or 35S:PYL4 plants and partial up-regulation of two ABA-responsive genes. Finally, 35S:PYL4A194T plants showed enhanced drought and dehydration resistance compared with nontransformed or 35S:PYL4 plants. Thus, we describe a novel approach to enhance plant drought resistance through allelic generation and engineering of a PYL4 mutation that enhances interaction with PP2CA.

Abscisic acid (ABA) plays a critical role both for plant biotic and abiotic stress response (Cutler et al., 2010). Because ABA is recognized as the critical hormonal regulator of plant response to water stress, both the ABA biosynthetic and signaling pathways can be considered as potential targets to improve plant performance under drought. Thus, it has been demonstrated that transgenic plants producing high levels of ABA display improved growth under drought stress compared with the wild type (Iuchi et al., 2001; Qin and Zeevaart, 2002). Priming of ABA biosynthesis can be obtained by direct over-expression of 9-cis-epoxyxarotenoid dioxygenase, a key enzyme in the biosynthetic pathway (Iuchi et al., 2001; Qin and Zeevaart, 2002), or through the use of chemicals that accelerate ABA accumulation (Jakab et al., 2005). On the other hand, some examples are also known of Arabidopsis (Arabidopsis thaliana) mutants affected in ABA signaling that show both an enhanced ABA response and drought-resistant phenotypes (Pei et al., 1998; Hugouvieux et al., 2001; Saez et al., 2006). For instance, enhancement of ABA sensitivity and reduction of water consumption has been achieved in Arabidopsis by combined inactivation of the protein phosphatases type 2C (PP2Cs) ABA-INSENSITIVE1 (ABI1) and HYPERSENSITIVE TO ABA1 (HAB1), leading to drought-resistant plants (Saez et al., 2006). Enhancing ABA signaling through the recently discovered PYRABACTIN RESISTANCE (PYR)/PYR1-LIKE (PYL)/REGULATORY COMPONENTS OF ABA RECEPTORS (RCAR) ABA receptors is another promising approach to improve plant drought resistance,

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for instance through overexpression or generation of constitutively active (CA) receptors (Santiago et al., 2009b; Saavedra et al., 2010; Mosquuna et al., 2011). However, pleiotropic effects due to sustained effects of high ABA levels or active ABA signaling might negatively affect plant growth, because abiotic stress responses divert resources required for normal growth. For instance, it was shown that vegetative expression of CA receptors was posttranscriptionally abolished in the case of PYL2 (Mosquuna et al., 2011).

Recent studies reveal at least two subclasses of PYR/PYL receptors, including monomeric and dimeric PYLs (Dupeux et al., 2011a; Hao et al., 2011). The dimeric receptors (PYR1, PYL1, and PYL2) show a higher dissociation constant for ABA (>50 μM, lower affinity) than monomeric ones (approximately 1 μM); however, in the presence of certain clade A PP2Cs, both groups of receptors form ternary complexes with high affinity for ABA (dissociation constant, 30–60 nM; Ma et al., 2009; Santiago et al., 2009a, 2009b). A third subclass appears when we consider the transdimeric PYL3 receptor, which suffers a cis-to-trans-dimer transition upon ligand binding to facilitate the subsequent dissociation to monomer (Zhang et al., 2012). Dimeric receptors occlude their surface of interaction with the PP2C in the dimer, so they are strongly ABA dependent for dissociation and adoption of a PP2C-binding conformation (Dupeux et al., 2011a). In vitro, monomeric ABA receptors are able to interact in the absence of ABA to some extent with the catalytic core of ABI1/ABI2/HAB1/PP2CA phosphatases, although less stable complexes are formed compared with ternary complexes with ABA (Dupeux et al., 2011a; Hao et al., 2011; Antoni et al., 2013). Boosting of such interaction might lead to faster association kinetics with PP2Cs or faster response to low-intermediate ABA levels. A subbranch of clade A PP2Cs comprises Highly ABA-Induced (HAI) phosphatases that show a more restrictive pattern of interaction with PYR/PYLs compared with ABI1/ABI2/HAB1/PP2CA (Bhaskara et al., 2012).

Yeast (Saccharomyces cerevisiae) two-hybrid (Y2H) assays reveal both ABA-independent and -dependent interactions among PYR/PYLs and PP2Cs (Ma et al., 2009; Park et al., 2009; Santiago et al., 2009b). However, tandem affinity purification of PYL8-interacting partners in planta was largely dependent on ABA to recover PYL8-PP2C complexes (Antoni et al., 2013). Y2H interactions of PYR/PYLs and PP2Cs that are dependent on exogenous ABA offer the possibility to set up screenings involving the generation of allele libraries and growth tests aimed to identify mutations that render ABA-independent interactions. Such mutations might lead in the plant cell to receptors that (1) show enhanced association kinetics with PP2Cs by generating additional contact points and (2) interfere with PP2C function by steric hindrance or, when several mutations are combined, to CA receptors that inhibit PP2Cs in the absence of ABA. The interaction in Y2H assays of PYL4 and PP2CA, two representative members of the PYR/PYL and clade A PP2C families, respectively, was shown to be ABA dependent (Lackman et al., 2011). PYL4 shows high expression levels in different tissues, and its inactivation is required to generate strongly ABA-insensitive combined pyr/pyl mutants (Gonzalez-Guzman et al., 2012). PP2CA plays a critical role to regulate both seed and vegetative responses to ABA and regulates stomatal aperture through interaction with the anion channel SLOW ANION CHANNEL1 (SLAC1) and the kinase OPEN STOMATA1 (OST1; Kuhn et al., 2006; Yoshida et al., 2006; Lee et al., 2009). Therefore, PP2CA is a physiologically relevant target to design PYR/PYL receptors that show enhanced interaction with the phosphatase, affecting ABA signaling and plant stress response.

Through the generation of a PYL4 allele library and Y2H assays, we selected several PYL4 mutations enabling ABA-independent interaction with PP2CA in yeast. We focused our work on the PYL4A194T protein, which interacted with PP2CA in the absence of ABA. This interaction led to a very modest inhibition of PP2CA in the absence of ABA (using p-nitrophenyl phosphate [pNPP] as a phosphatase substrate); however, it improved ABA-dependent inhibition of PP2CA. As a result, upon overexpression of PYL4A194T, we observed enhanced sensitivity to ABA compared with nontransformed or 35S:PYL4 plants both in seed and vegetative tissues. Moreover, 35S:PYL4A194T transgenic plants showed enhanced drought resistance compared with nontransformed or 35S:PYL4 plants. By contrast, a previous attempt to express a mutagenized version of an ABA receptor failed to detect protein expression in vegetative tissue, and therefore phenotype analysis could only be performed in seeds (Mosquuna et al., 2011).

## RESULTS

### Identification of PYL4 Mutations That Promote ABA-Independent Interaction with PP2CA in Yeast

PYL4 interacts in an ABA-dependent manner with PP2CA in Y2H assays (Lackman et al., 2011; Fig. 1A). We conducted error-prone PCR mutagenesis of the PYL4 receptor and generated an allele library of approximately 10,000 clones in the pGBK7T vector. The library was shuttled to yeast AH109 by cotransformation with pGAD7-PP2CA. Yeast transformants were pooled, and clones able to grow in the absence of exogenous ABA in medium lacking HIS and adenine were selected. Yeast plasmids were extracted, sequenced, and retransformed in yeast cells to recapitalize the phenotype. Thus, different mutations in the encoded PYL4 protein were identified that enabled constitutive interaction with PP2CA (Fig. 1, A and B). Through site saturation mutagenesis of PYR1, Mosquuna et al. (2011) identified mutations located in 10 different residues of the receptor that promoted PYR1-HAB1 interactions in the absence of ABA. These mutations were clustered in the receptor-phosphatase interaction surface, specifically in the gating loop of PYR1, its C-terminal α-helix, and H60. The H60 of PYR1 is a hotspot for activating mutations, and for instance, the H60P substitution destabilizes the PYR1

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dimer and increases its apparent ABA affinity and both PYR1<sup>H60R</sup> and PYR1<sup>H60R</sup> bound HAB1 in the absence of ABA (Dupeux et al., 2011a; Mosquna et al., 2011). The H60 equivalent residue in PYL4 is H82, and interestingly we found in our screening a PYL4<sup>H82R</sup> mutation that resulted in ABA-independent interaction with PP2CA (Fig. 1A). The H82R mutation was found combined with V97A but the individual V97A mutation did not affect the interaction in the absence of ABA, although it increased yeast growth in the presence of ABA (Fig. 1A). Other mutations that enhanced the interaction of PYL4 and PP2CA in the absence of ABA were A194T and the double mutation F130Y C176R (Fig. 1, A and B). Both A194T and C176R mutations are located in the C-terminal helix of PYL4, which represented another hotspot for activating mutations in PYR1 because this α-helix forms part of the receptor-phosphatase binding interface (Mosquina et al., 2011). The interaction of the PP2C HAB1 with PYL4 was also found to be ABA dependent in yeast (Lackman et al., 2011), so we decided to test whether these mutations affected the activity of two clade A PP2Cs, i.e. PP2CA and HAB1. Using pNPP as a substrate, we could detect a small inhibitory effect (20%) of PYL4<sup>A194T</sup> on the activity of PP2CA in the absence of ABA with respect to PYL4 (Fig. 2A). However, although the H82RV97A and F130Y C176R mutations promoted ABA-independent interactions in Y2H assay, they did not affect PP2CA activity in the absence of ABA. In the presence of 1 μM ABA, PYL4<sup>A194T</sup> also showed a higher inhibition of PP2CA than PYL4 (Fig. 2A). The other mutations behaved similarly to PYL4 except F130Y C176R, which showed lower capacity to inhibit PP2CA in the presence of ABA. PYL4 inhibited more efficiently HAB1 than PP2CA (inhibitory concentration to obtain 50% inhibition of 0.25 and 1 μM, respectively), and all PYL4 mutants inhibited HAB1 similarly to PYL4 (Fig. 2B).

**Effect of PYL4 Mutations on PP2CA Activity in Vitro**

Y2H assays reveal both ABA-independent and ABA-dependent interactions among PYR/PYLs and PP2Cs; however, PYR/PYL receptors inhibit the activity of clade A PP2Cs mostly in an ABA-dependent manner (Fujii et al., 2009; Ma et al., 2009; Park et al., 2009; Santiago et al., 2009b). Thus, an ABA-independent interaction in Y2H assay does not necessarily imply capacity to inhibit phosphatase activity in the absence of ABA. Although most of the monomeric PYR/PYL receptors show ABA-independent interaction with different PP2Cs in Y2H assay, effective phosphatase inhibition requires ABA, and for instance, the in vivo binding of PYL8 to five clade A PP2Cs was largely dependent on ABA (Ma et al., 2009; Park et al., 2009; Santiago et al., 2009b; Antoni et al., 2013). Therefore, we tested whether these mutations affected actually the activity of two clade A PP2Cs, i.e. PP2CA and HAB1. Using pNPP as a substrate, we could detect a small inhibitory effect (20%) of PYL4<sup>A194T</sup> on the activity of PP2CA in the absence of ABA with respect to PYL4 (Fig. 2A). However, although the H82RV97A and F130Y C176R mutations promoted ABA-independent interactions in Y2H assay, they did not affect PP2CA activity in the absence of ABA. In the presence of 1 μM ABA, PYL4<sup>A194T</sup> also showed a higher inhibition of PP2CA than PYL4 (Fig. 2A). The other mutations behaved similarly to PYL4 except F130Y C176R, which showed lower capacity to inhibit PP2CA in the presence of ABA. PYL4 inhibited more efficiently HAB1 than PP2CA (inhibitory concentration to obtain 50% inhibition of 0.25 and 1 μM, respectively), and all PYL4 mutants inhibited HAB1 similarly to PYL4 (Fig. 2B).

Although phosphatase activity is usually measured using small phosphorylated molecules such as pNPP or phosphopeptides, in vivo phosphatase activity is addressed against phosphorylated proteins and therefore could involve substrate-dependent effects. Therefore, we also performed in vitro reconstitution of the
ABA signaling cascade and measured the capacity of PYL4A194T or PYL4 to inhibit the dephosphorylation of several PP2CA targets, i.e. OST1/SnRK2.6, C-terminal deletion (ΔC)-ABRE binding factor2 (ABF2; residues 1–173), and ΔC-ABA-INSENSITIVE5 (ABI5; residues 1–200) transcription factors or N-terminal fragment (residues 1–186) of the anion channel SLAC1 (Fig. 2C). First, a phosphorylation reaction was performed where OST1 was autophosphorylated in vitro, and, in turn, it phosphorylated ΔC-ABI5, and SLAC11–186 proteins. Next, these proteins were used as substrates of PP2CA that was preincubated (or not) for 10 min with PYL4 or PYL4A194T either in the absence or presence of 30 μM ABA. In the absence of ABA, we did not find significant differences among PYL4 and PYL4A194T. In the presence of 30 μM ABA, PYL4A194T inhibited better than PYL4 the dephosphorylation by PP2CA of ΔC-ABI5 and SLAC11–186, although it was not more effective than PYL4 to inhibit the dephosphorylation of ΔC-ABF2 (Fig. 2C). PYL4A194T shows enhanced capacity to interact with PP2CA in the absence of ABA, probably because novel contact points are generated by the mutation. Therefore, we reasoned that this mutation might also lead to enhanced association kinetics in the presence of ABA, particularly at low ABA levels or low phosphatase:receptor ratios. We performed dephosphorylation assays at low ABA concentrations (0.1, 0.5, and 1 μM) and 1:1 phosphatase:receptor ratio (Fig. 2D). In the presence of 0.5 to 1 μM ABA, PYL4A194T inhibited better (2–3-fold) than PYL4 the dephosphorylation of ΔC-ABF2, ΔC-ABI5, and SLAC11–186. Protection of OST1 phosphorylation

Figure 2. PYL4A194T prevents better than PYL4 the PP2CA-mediated dephosphorylation of protein substrates in the presence of ABA. A and B, Phosphatase activity of either PP2CA or HAB1, respectively, was measured in vitro using pNPP as a substrate in the absence or presence of PYL4 or different PYL4 mutant versions at the indicated ABA concentrations. Assays were performed in a 100-μ reaction volume that contained 2 μM phosphatase and 4 μM receptor. Data are averages ± se from three independent experiments. Asterisk indicates P < 0.05 (Student’s t test) when comparing data of mutant and wild-type PYL4 in the same assay conditions. C and D, Effect of PYL4A194T or PYL4 on PP2CA-mediated dephosphorylation of OST1, ABF2 (1–173), ABI5 (1–200), and SLAC1 (1–186) phosphorylated proteins. C, The quantification of the autoradiography (numbers below) shows the percentage of protected phosphorylated substrate in each experiment relative to 100% in the absence of PP2CA. A 1:10 phosphatase: receptor stoichiometry was used in this assay. D, PYL4A194T prevents better than PYL4 the PP2CA-mediated dephosphorylation of OST1, ABF2 (1–173), ABI5 (1–200), and SLAC1 (1–186). Value 1 expresses protection of each substrate in the absence of ABA, and the normalized ratio expresses the fold number that either PYL4A194T or PYL4 enhanced protection of the substrate at the indicated concentration of ABA. A 1:1 phosphatase:receptor stoichiometry was used in this assay.
was also improved by PYL4<sup>A194T</sup> compared with PYL4 (Fig. 2D).

**In Vitro and in Vivo Interaction of PYL4<sup>A194T</sup> and PP2CA**

The phenotype described below for 35S: PYL4<sup>A194T</sup> plants prompted us to further analyze the interaction between PYL4<sup>A194T</sup> and PP2CA using in vivo and in vitro protein-protein interaction tests. First, bimolecular fluorescent complementation (BiFC) assays were used to analyze the interaction of PYL4 or PYL4<sup>A194T</sup> and either PP2CA or HAB1 in tobacco (*Nicotiana tabacum*) cells (Fig. 3A). To this end, we performed transient expression of PP2CA-Yellow fluorescent protein N-terminal fragment (YFP<sup>N</sup>) and Yellow fluorescent protein C-terminal fragment (YFP<sup>C</sup>)-PYL4 in epidermal cells of *Nicotiana benthamiana* using agroinfiltration. The nuclear interaction between PP2CA and PYL4 did not require the addition of exogenous ABA; endogenous ABA levels in tobacco cells after agroinfiltration appear to be enough to promote such interaction. The subcellular localization of PP2CA was previously investigated by Antoni et al. (2012) using both transient expression of PP2CA-GFP in tobacco cells and biochemical fractionation of Arabidopsis transgenic lines that express hemmaglutinin (HA)-tagged PP2CA. We found that PP2CA was localized both to nucleus and cytosol; however, PP2CA-GFP appears to be predominantly localized in the nucleus, and presumably a higher concentration of the protein is found in this compartment (Fig. 3A). Both red fluorescent protein (RFP)-PYL4 and RFP-A194T show a similar localization pattern to each other (Fig. 3A). Injection of ABA enhanced the relative fluorescence of the PP2CA-PYL4 complex and made evident additional interaction at the cytosol (Supplemental Fig. S1). PYL4<sup>A194T</sup> was able to interact with PP2CA in the cytosol at endogenous ABA levels, and the relative fluorescence emission was higher in the PYL4<sup>A194T</sup>-PP2CA interaction compared with PYL4 (Fig. 3B). Therefore, our results suggest that A194T is able to interact in the cytosol with PP2CA at low ABA levels, whereas PYL4 is not. In the nucleus, the higher concentration of PP2CA-GFP probably facilitates the BiFC interaction. By contrast, the interaction of HAB1 and either PYL4 or PYL4<sup>A194T</sup> did not differ significantly (Fig. 3, A and B).

Finally, we performed in vitro protein-protein interaction assays. Non-His-tagged PYL4<sup>A194T</sup> could be copurified with 6His-ΔNPP2CA using Ni affinity chromatography in the absence of ABA, in contrast to PYL4 (Fig. 3C). Size exclusion chromatography and SDS-PAGE analysis of the eluted fractions confirmed that both proteins formed a 1:1 complex in the absence of ABA (Fig. 3D). Finally, a pull-down assay showed that whereas the interaction of PYL4 and PP2CA was dependent on the addition of ABA, ABA-independent binding could be observed for PYL4<sup>A194T</sup> and PP2CA (Fig. 3E). Therefore, both in vivo and in vitro assays show a differential interaction of PYL4<sup>A194T</sup> and PP2CA with respect to PYL4.

**Analysis of Transgenic Lines Overexpressing PYL4 Mutants**

To study the putative effect of PYL4 mutations on ABA signaling in vivo, we generated transgenic plants that overexpressed HA-tagged versions of PYL4 or the mutant versions PYL4<sup>V97A</sup>, PYL4<sup>A194T</sup>, PYL4<sup>C176R F130Y</sup>, and PYL4<sup>H82R V97A</sup>. Expression of the proteins in vegetative tissue was detected by immunoblot analysis and transgenic lines that expressed similar levels of PYL4, and mutant PYL4 proteins were selected for further analysis; however, PYL4<sup>H82R V97A</sup> lines consistently showed lower expression of the transgene compared with PYL4 or other mutant proteins (Fig. 4A). Overexpression of PYL4 or PYL4<sup>V97A</sup> enhanced ABA-mediated inhibition of seedling establishment compared with nontransformed plants, whereas ABA sensitivity of PYL4<sup>C176R F130Y</sup> overexpressing (OE) plants was similar to nontransformed plants. Interestingly, both PYL4<sup>A194T</sup> and PYL4<sup>H82R V97A</sup> OE plants showed higher sensitivity to ABA-mediated inhibition of seedling establishment than PYL4 OE plants (Fig. 4B). Low concentrations of ABA (0.25–0.5 μM) delay seedling establishment of the nontransformed ecotype Columbia (Col-0) wild type and have a limited inhibitory effect on further growth of the seedlings (Fig. 4C). This effect was enhanced in PYL4 or PYL4<sup>V97A</sup> OE plants, particularly evident at 0.5 μM ABA (Fig. 4C). In the case of PYL4<sup>A194T</sup> and PYL4<sup>H82R V97A</sup> OE plants, the effect was even visible at 0.25 μM ABA, indicating that these lines show higher sensitivity to ABA-mediated inhibition of shoot growth than PYL4 OE plants (Fig. 4, C and D).

Subsequent generations of PYL4<sup>H82R V97A</sup> transgenic plants showed reduced levels of the protein compared with homozygous T3, so we concentrated further analysis on PYL4<sup>A194T</sup> transgenic lines, where expression of the transgene remained stable. Seed germination and seedling establishment analyses of PYL4<sup>A194T</sup> OE lines confirmed the enhanced sensitivity to ABA observed in T3 seeds (Supplemental Fig. S2). Moreover, root and shoot growth analyses also revealed enhanced sensitivity to ABA in vegetative tissues (Fig. 5). We transferred 4-d-old seedlings to Murashige and Skoog (MS) medium plates lacking or supplemented with 10 μM ABA, and root growth was measured 10 d after transfer. Both PYL4 and PYL4<sup>A194T</sup> OE plants showed enhanced ABA-mediated inhibition of root growth compared with nontransformed plants (Fig. 5, A and B). Shoot growth was evaluated by measuring the maximum rosette radius of plants grown for 11 d in MS medium lacking or supplemented with 10 μM ABA (Fig. 5C). Finally, we measured expression of two ABA-responsive genes, *ABA-responsive18* (RAB18) and *desiccation-responsive29B* (RD29B), in mock- or 10 μM ABA-treated plants (Fig. 5D). In the absence of exogenous ABA treatment, expression of RAB18 and RD29B was 6- and 23-fold, respectively, up-regulated in PYL4<sup>A194T</sup> OE plants compared with nontransformed plants. These results indicate a partial derepression of ABA-responsive genes in this line compared with nontransformed plants.
However, after ABA treatment, the induction of these genes was not higher than in nontransformed plants.

**PYL4<sup>Δ194T</sup> OE Plants Show Enhanced Water Use Efficiency and Drought Resistance**

Regulation of stomatal aperture by ABA is a key adaptive response to cope with drought stress. To probe stomatal function in nontransformed Col-0, PYL4, and PYL4<sup>Δ194T</sup> OE plants, we performed diurnal analysis of stomatal conductance (G<sub>st</sub>) and transpiration in whole plants under basal conditions (Fig. 6, A and B). Interestingly, both PYL4 and PYL4<sup>Δ194T</sup> OE lines showed lower G<sub>st</sub> and transpiration values than nontransformed Col-0 plants during the day and night. Moreover, PYL4<sup>Δ194T</sup> OE plants showed lower G<sub>st</sub> values than PYL4 OE plants. Diurnal course of G<sub>st</sub> was generally not affected in transformed plants; both OE lines closed their stomata alike the nontransformed Col-0 wild type during the night and showed maximum G<sub>st</sub> values around midday, followed by predark stomatal closure. Still, predawn stomatal opening was more pronounced.

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**Figure 3.** BiFC assay shows a different interaction of PYL4 or PYL4<sup>Δ194T</sup> and PP2CA in tobacco leaves. PYL4<sup>Δ194T</sup> binds ΔNPP2CA in the absence of ABA in vitro. A, Laser scanning confocal imaging of epidermal leaf cells infiltrated with a mixture of *A. tumefaciens* suspensions harboring the indicated BiFC constructs and the silencing suppressor p19. Right panels show the location of GFP and RFP fusions of PP2Cs and receptors, respectively. B, Quantification of the fluorescent protein signal. Images of A were analyzed using ImageJ software, and signal intensity was calculated after subtracting the mean background density. C, SDS-PAGE showing the Ni<sup>2+</sup> affinity chromatography purification from *E. coli* lysates containing recombinant 6His-ΔNPP2CA and either PYL4 (top) or PYL4<sup>Δ194T</sup> (bottom). A lane showing PYL4 and PYL4<sup>Δ194T</sup> is also displayed at the right of each gel. In the absence of ABA, PYL4<sup>Δ194T</sup> copurifies with 6His-ΔNPP2CA, while PYL4 does not. D, Elution profiles after size exclusion chromatography in absence of ABA of pure PYL4<sup>Δ194T</sup> (green) and 6His-ΔNPP2CA (orange) and the eluted fractions described above containing the copurified PYL4<sup>Δ194T</sup>/6His-ΔNPP2CA (blue) proteins. Insets from each peak show SDS-PAGE analysis. The figure shows the formation of 1:1 PYL4<sup>Δ194T</sup>/6His-ΔNPP2CA complex and the monomeric nature of PYL4<sup>Δ194T</sup>. E, SDS-PAGE shows a pull-down assay, where 6His-ΔNPP2CA is incubated with PYL4 or PYL4<sup>Δ194T</sup> in absence or presence of 100 μM ABA. M, Molecular mass makers; SF, soluble fraction; FT, flow through the column; E1 to E4, eluted fractions at 500 mM imidazole. [See online article for color version of this figure.]
in the nontransformed Col-0 wild type and PYL4 OE compared with PYL4A194T OE plants. The latter result could be directly related to an enhanced ABA sensitivity of PYL4A194T OE plants, because diurnal stomatal movements are linked to ABA concentration via its effect on ion and sugar fluxes (Tallman, 2004).

The lower Gs values of PYL4 and PYL4A194T OE plants suggest that under steady-state conditions, the stomata of PYL4 and PYL4A194T OE plants have reduced aperture compared with nontransformed Col-0 plants. Direct measurements of stomatal aperture using whole-leaf imaging revealed that stomata of both PYL4 and particularly PYL4A194T OE plants were more closed than those of nontransformed Col-0 plants (Fig. 6C). Finally, we also performed water loss assays of nontransformed Col-0, PYL4, and PYL4A194T OE lines (Fig. 6D). Water loss experiments were done using 15-d-old seedlings grown in a growth chamber, which were excised from petri dishes and submitted to the drying atmosphere of a laminar flow hood (Fig. 6D). Water loss kinetics indicated that PYL4A194T OE lines lost less water than nontransformed or PYL4 OE lines (Fig. 6D). Finally, when scoring stomatal aperture, we noticed that stomatal density of PYL4 and PYL4A194T OE lines was
circa 10% lower than that of nontransformed plants (Fig. 6E). Therefore, a partial explanation of the reduced water loss observed in these lines can be attributed to 10% reduced stomatal density. However, because stomatal density was similar in PYL4 and PYL4A194T OE lines, but lower transpiration was found in PYL4A194T compared with PYL4 OE lines, we conclude that A194T has a higher effect compared with PYL4.

Reduced net photosynthesis (Anet) could be a drawback caused by the reduced stomatal opening and Gs found in PYL4 and PYL4A194T OE lines. Therefore, to evaluate the performance of these plants compared with nontransformed plants, we determined water use efficiency (WUE), i.e. the amount of carbon gained per unit water lost, Anet/transpiration. Both PYL4 and PYL4A194T OE lines showed a reduced Anet compared with nontransformed plants; however, PYL4A194T OE lines showed enhanced WUE compared with nontransformed and PYL4 OE plants because the strongly reduced transpiration compensated the effect on Anet (Fig. 6F).

Finally, we performed drought resistance experiments under greenhouse conditions (Fig. 7). Plants were grown in a greenhouse under normal watering conditions for 15 d, and then irrigation was stopped. This day was taken as 0 d, when average rosette radius did not differ significantly among nontransformed Col-0, PYL4 OE, and PYL4A194T OE plants (Fig. 7, A and B). However, we found that during the subsequent 5-d period, plant growth was reduced in nontransformed Col-0 and PYL4 OE plants compared with PYL4A194T OE plants (Fig. 7B). Severe wilting and yellowing of leaves were observed at 16 d in the wild type, in contrast to PYL4A194T OE lines. Finally, at 19 d, watering was resumed and survival of the plants was scored at 23 d. Col-0 wild-type plants did not survive after drought stress, whereas around 30% and 60% to 70% of PYL4 and PYL4A194T OE lines survived, respectively (Fig. 7C). Finally, because PYL4A194T OE lines were hypersensitive to ABA and showed derepression of ABA/drought-responsive genes, we tested whether they showed enhanced survival after suffering severe dehydration in petri dishes. These experiments were done using 15-d-old seedlings by submitting them to dehydration for 12 h in a laminar flow hood, followed by rehydration and scoring survival rate 3 d afterward. Dehydration experiments revealed enhanced resistance of PYL4A194T OE compared with PYL4 OE and nontransformed plants. Thus, approximately 40% of PYL4A194T plants survived after 12 h of dehydration followed by rehydration (Fig. 7D).

**DISCUSSION**

Under nonstress conditions, endogenous ABA levels play a critical role to regulate stomatal aperture, as
revealed by the open stomata phenotype of multiple pyr/pyl mutants, and basal ABA signaling is also required for proper plant growth and root development (Barrero et al., 2005; Gonzalez-Guzman et al., 2012; Antoni et al., 2013; Merilo et al., 2013). On the other hand, plant response to drought is largely dependent on enhanced ABA biosynthesis and signaling to regulate both stomatal aperture and gene expression under water stress conditions (Cutler et al., 2010). Thus, some mutants or transgenic plants showing enhanced response to ABA also display enhanced drought resistance and reduced water consumption (Pei et al., 1998; Hugouvieux et al., 2001; Saez et al., 2006). In this work, we describe a novel approach to confer drought resistance through genetic engineering of a mutated PYL4 receptor. The PYL4A194T mutation here described cannot be considered a CA receptor, because we did not observe strong inhibition of PP2C activity in the absence of ABA. Triple and quadruple combinations of mutations were required to obtain CA PYR1, PYL2, and

Figure 6. Leaf gas exchange measurements reveal reduced Gs and enhanced WUE in PYL4A194T OE plants compared with nontransformed Col-0 and PYL4 OE plants. A and B, Gs and transpiration values of nontransformed Col-0, PYL4, and PYL4A194T OE plants. Plants were kept in custom-made whole-rosette gas exchange measurement device (Kollist et al., 2007), and Gs and transpiration were followed during a diurnal light/dark cycle for 27 h. Values are mean ± se (n = 5). White and black bars above represent light and dark periods, respectively. C. Reduced stomatal aperture of both PYL4 and PYL4A194T OE lines compared with nontransformed Col-0 plants. Asterisk indicates P < 0.05 (Student's t test) when comparing data of OE lines and nontransformed Col-0 plants in the same assay conditions. D. Loss of fresh weight of 15-d-old plants submitted to the drying atmosphere of a laminar flow hood. E. Stomatal density is reduced in PYL4 and PYL4A194T OE lines compared with nontransformed Col-0 plants. F. WUE is enhanced in PYL4A194T OE lines compared with PYL4 OE and nontransformed plants. The values of Anet and WUE are averages for the whole day/light period. The different letters denote significant differences (P < 0.05; n = 5; ANOVA and Fisher's LSD test).
PYL9 receptors, which showed full ABA-independent inhibition of HAB1, ABI1, and ABI2 (Mosquina et al., 2011). As a result, expression of a 35S:GFP-PYL2CA transgene in Arabidopsis seeds activated ABA signaling even in the ABA-deficient aba2-1 mutant (Mosquina et al., 2011). However, the existence of a posttranscriptional mechanism that abolished expression of PYL2CA in vegetative tissue precluded further analysis and testing of drought resistance in adult plants (Mosquina et al., 2011). The PYL4A194T mutation improved ABA-dependent inhibition of PP2CA, and expression of the receptor could be detected in vegetative tissues of 35S:PYL4A194T plants, which showed ABA hypersensitivity both in seed and vegetative responses. Moreover, 35S:PYL4A194T exhibited enhanced drought resistance compared with nontransformed or 35S:PYL4 OE plants. Particularly interesting features were the partial derepression of ABA-responsive genes, reduced stomatal aperture, and transpiration of these lines at basal endogenous ABA levels. Additionally, because PYL4A194T showed enhanced capacity to inhibit PP2CA at low ABA levels, it is likely that 35S:PYL4A194T plants are primed for an accelerated response to stress conditions, which likely contributes to the enhanced drought resistance observed in these plants.

Y2H, in vitro protein-protein interaction, and BiFC assays revealed that PYL4A194T showed a distinct pattern of interaction with PP2CA with respect to PYL4 (Figs. 1 and 3). Thus, both Y2H and pull-down assays indicated that PYL4A194T interacted with PP2CA in the absence of ABA. BiFC assays showed enhanced interaction of PYL4A194T and PP2CA compared with PYL4 OE plants. Particularly interesting features were the partial derepression of ABA-responsive genes, reduced stomatal aperture, and transpiration of these lines at basal endogenous ABA levels. Additionally, because PYL4A194T showed enhanced capacity to inhibit PP2CA at low ABA levels, it is likely that 35S:PYL4A194T plants are primed for an accelerated response to stress conditions, which likely contributes to the enhanced drought resistance observed in these plants.
at the endogenous ABA levels present in agroinfiltrated tobacco cells. ABA-independent interaction in Y2H assay does not necessarily imply capacity to inhibit phosphatase activity in the absence of ABA (Fujii et al., 2009; Ma et al., 2009; Park et al., 2009; Santiago et al., 2009b). In the absence of ABA, we only found a modest inhibitory effect of PYL4A194T on PP2CA activity using pNPP but no significant effect when phosphorylated proteins were used as phosphatase substrate. By contrast, these substrates were better protected from PP2CA-mediated dephosphorylation by PYL4A194T compared with PYL4 when ABA was present. It is likely that PYL4A194T displays enhanced association kinetics with PP2CA than PYL4, particularly at low-intermediate levels of ABA, leading to enhanced formation of ternary complexes. Thus, PYL4A194T might act at endogenous ABA levels or prime ABA-dependent interaction with PP2CA to speed initial response to stress. Additionally, the PYL4A194T-PP2CA interaction might lead to steric hindrance of phosphatase activity in vivo by restricting access to the substrates. Steric inhibition of kinase activity by catalytically inactive phosphatase is well known in the field of ABA signaling (Lee et al., 2009; Soon et al., 2012). Thus, it was reported that an inactive form of PP2CA was able to inhibit SnRK2.6 kinase activity (Lee et al., 2009) and catalytically inactive HAB1 was still able to inhibit SnRK2.6 (Soon et al., 2012).

The effect of PYL4A194T appeared to be specific for PP2CA with respect to HAB1, because it did not show a differential effect on HAB1 compared with PYL4. However, at this stage, we cannot exclude that other clade A phosphatases (for instance, other members of the PP2CA branch) might also be differentially affected by PYL4A194T. Alignment of clade A PP2Cs reveals two subgroups (the ABI1 and PP2CA branches) and subtle differences in some regions of the proteins that could affect the interaction with PYR/PYLs (Bhaskara et al., 2012; Santiago et al., 2012). For instance, Bhaskara et al. (2012) noticed that HAI PP2Cs showed a differential interaction with PYR/PYLs and marked preference for monomeric receptors. Previous results revealed a certain specificity in the multiple interactions of the nine clade A PP2Cs and 14 PYR/PYLs (Santiago et al., 2009b; Szostkiewicz et al., 2010), and a differential inhibition of PP2CA by PYR/PYLs was recently reported (Antoni et al., 2012). Structural evidence for the PYL4A194T-PP2CA complex is currently not available; however, taking as a model other complexes can be observed a clear difference in the length of the α2β4 loop of clade A PP2Cs, which is close to the receptor-phosphatase binding interface (Fig. 8, A

Figure 8. Modeling of PYL4A194T and PYL4H82R mutations based on the PYR1-ABA-HAB1 structure. A, Location on PYR1 of equivalent PYL4 A194T and H82R residues using structure of the PYR1 (green)-ABA-HAB1 (orange) complex (protein data bank code 3QN1). The variable α2β4 loop of clade A PP2Cs is displayed as a yellow ribbon for HAB1. The equivalent α2β4 loop from ABI1 (as deduced from the PYL1-ABA-ABI1 complex, protein data bank code 3KDJ) is displayed as a blue ribbon. B, The length of the α2β4 loop (black box) notably differs among clade A PP2Cs. PP2CA displays the longer α2β4 loop, and residues of the loop that show high identity with HAI PP2Cs (HAI1, At5g59220; HAI2, At1g07430; and HAI3, At2g29380) are highlighted in green. Amino acid residues of HAB1/ABI1 involved in the interaction with PYR/PYL receptors are marked as green dots. C, The location of the PYL4 A194 residue at the C-terminal α-helix and equivalent Thr residue of monomeric receptors PYL5, PYL10, and PYL13 is highlighted. [See online article for color version of this figure.]
and C). This αβ4 loop is clearly different in PP2CA/HAI2P2Cs compared with the ABI1 branch, and it represents a potential point of interaction with PYL4A194T. Additionally, the A194 residue is located at the C-terminal helix of PYL4, close to the receptor-phosphatase binding interface. Therefore, the A194T mutation might also indirectly influence the interaction of the C-terminal helix of PYL4 with PP2CA or establish new contact points with the αβ4 loop of certain clade A P2Cs. Interestingly, PYL10, which shows ABA-independent inhibition of PP2CA (Hao et al., 2011), contains a Thr residue at the equivalent position of PYL4A194T (Fig. 8B).

In summary, genetic engineering of ABA receptors might serve as a new tool to ameliorate drought stress through (1) CA receptors (Mosqueta et al., 2011), (2) mutations that enhance ABA-dependent inhibition of P2Cs (this work), or (3) natural receptor versions that enhance ABA-independent inhibition of P2Cs (Hao et al., 2011). Expression driven by a strong constitutive promoter might lead to some pleiotropic effects that negatively affect growth or yield of crop plants. Such a drawback could be bypassed by introducing stress-inducible or tissue-specific promoters that would drive the expression of the receptor only under stress conditions or in certain tissues.

MATERIALS AND METHODS

Plant Material and Growth Conditions

Arabidopsis (Arabidopsis thaliana) plants were routinely grown under greenhouse conditions (40%–50% relative humidity) in pots containing a vermiculite:soil (1:3) mixture. For plants grown under growth chamber conditions, seeds were surface sterilized by treatment with 70% (v/v) ethanol for 20 min, followed by commercial bleach (2.5% (v/v) sodium hypochlorite) containing 0.05% (v/v) Triton X-100 for 10 min, and, finally, four washes with sterile distilled water. Stratification of the seeds was conducted in the dark at 4°C for 3 d. Seeds were sowed on MS plates composed of MS basal salts, 0.1% (v/v) MES, 1% (v/v) Suc, and 1% (w/v) agar. The pH was adjusted to 5.7 with KOH before autoclaving. Plates were sealed and incubated in a controlled environment growth chamber at 25°C under a 16-h-light/8-h-dark photoperiod at 80 to 100 μE m−2 sec−1.

Construction of a PYL4 Mutant Library and Analysis of Y2H Interaction with PP2CA

We conducted error-prone PCR mutagenesis by amplification of the PYL4 open reading frame using the primers FPYL4NcoI, 5′-CGACCAAGCATGTTCCGCCTTCACCCTCTCTC-3′ and RPYL4EcoRIstop, CGCACGAATTCACAGAGACATC25l oopof 25 mM Tris-HCl, pH 8.0, 50 mM NaCl, 50 mM imidazole, and 5 mM Mg2+, mixed with 8 mg of either pure nontagged (through tobacco etch virus cleavage) PYL4 or PYL4A194T, and disrupted by sonication. After centrifugation (40 min, 40,000g) at 27 K, the clear supernatant was filtered (pore diameter, 045 mm; Millipore Corporation). The 6His-tagged proteins were purified using nickel-nitrilotriacetic acid agarose (Qiagen) according to the manufacturer’s instructions. The filtered supernatant was mixed with the previously equilibrated beads. After incubation, a washing step with 10 volumes of 25 mM Tris-HCl, pH 8.0, 50 mM NaCl, 20 mM imidazole, and 5 mM β-mercaptoethanol buffer was performed, followed by the elution from the Ni2+ resin in a buffer with 300 mM imidazole. Imidazole was removed using a PD-10 column (GE Healthcare), and the His tag was cleaved using tobacco etch virus protease.

BiFC Assay in Nicotiana benthamiana

Experiments were performed basically as described by Voit et al. (2003). The different binary vectors described above were introduced into Agrobacterium tumefaciens C58C1 (pGV2260; Deblaere et al., 1985) by electroporation, and transformed cells were selected in Luria-Bertani (LB) plates supplemented with kanamycin (50 μg mL−1). Then they were grown in liquid LB medium to late exponential phase, and cells were harvested by centrifugation and resuspended in 10 mM morpholinoethanesulfonic acid-KOH (pH 5.6) containing 10 mM MgCl2 and 150 mM acetylsorcinol to an optical density at 600 nm of 1. These cells were mixed with an equal volume of A. tumefaciens C58C1 (pCh32355p19) expressing the silenced suppressor p19 (Horsch et al., 1985). The mix was vortexed on a shaking platform (300 rpm) for 3 min, and 500 μL of the final density of A. tumefaciens solution was added to 1. Bacteria were incubated for 3 h at room temperature and then injected into young fully expanded leaves of 4-week-old N. benthamiana plants. Leaves were examined after 3 to 4 d under a Leica TCS-CL confocal microscope and laser scanning confocal imaging system. Quantification of fluorescent protein signal was done as described (Gampala et al., 2007) using the National Institutes of Health (NIH) Image software ImageJ v1.37.

Constructs were done in pSPYNE-35S (Walter et al., 2004) as well as gateway vector pYFP6-43 (a derivative of pmD43 where GFP is replaced by YFP) (Belda-Palazón et al., 2012). The coding sequence of AtG353810 (PYL4) was cloned into the pENTR223.1-S entry vector, kindly provided by the Arabidopsis Biological Resource Center (clone G12606). The coding sequence of PYL4A194T was PCR amplified, cloned into the pCR8/GW/TOPO vector and verified by sequencing. Next, constructs containing PYL4 and PYL4A194T were recombined by LR reaction into pYFP6-43 destination vector or into pETPWRG2 to generate GFP fusion proteins. The coding sequence of HAB1 and PP2CA was excised from a pCR8/GW/TOPO construct using a double digestion BamHI-SalI and subcloned into BamHI-SalI doubly digested pSPYNE-35S.

Protein Expression and Purification

For small-scale protein purifications, E. coli BL21 (DE3) cells transformed with the corresponding constructs were grown in 100 mL of LB medium to an optical density at 600 nm of 0.6 to 0.8. At this point, 1 mM isopropyl-β-D-thiogalactoside was added, and the cells were harvested after overnight incubation at 20°C. Pellets were resuspended in lysis buffer (50 mM Tris, pH 7.5, 250 mM KCl, 10% (v/v) glycerol, and 1 mM β-mercaptoethanol) and lysed by sonication with a Branson Sonifier 250. The clear lysate obtained after centrifugation was purified by Ni affinity. A washing step was performed using 50 mM Tris, 250 mM KCl, 20% (v/v) glycerol, 30 mM imidazole, and 1 mM β-mercaptoethanol washing buffer, and finally the protein was eluted using 50 mM Tris, 250 mM KCl, 20% (v/v) glycerol, 250 mM imidazole, and 1 mM β-mercaptoethanol elution buffer.

For protein–protein interaction experiments, the pET28aΔNPP2CA, pETM11_PYL4wt, and pETM11_PYL4A194T plasmids were transformed into E. coli BL21 (DE3). A total of 8 mL of an overnight culture was subcultured into 800 mL fresh 2TY broth (16 g bacto-tryptone, 10 g yeast extract, and 5 g NaCl per liter of solution) plus kanamycin (50 μg mL−1). Protein expression was induced with 0.3 mM isopropyl-β-D-thiogalactoside and the cells were harvested after overnight incubation at 20°C. Pellets were resuspended in 25 mL Tris-HCl, pH 8.0, 50 mM NaCl, 50 mM imidazole, and 5 mM β-mercaptoethanol and disrupted by sonication. After centrifugation (40 min, 40,000g) at 27 K, the clear supernatant was filtered (pore diameter, 0.45 mm; Millipore Corporation). The 6His-tagged proteins were purified using nickel-nitrilotriacetic acid agarose (Qiagen) according to the manufacturer’s instructions. The filtered supernatant was mixed with the previously equilibrated beads. After incubation, a washing step with 10 volumes of 25 mM Tris-HCl, pH 8.0, 50 mM NaCl, 20 mM imidazole, and 5 mM β-mercaptoethanol buffer was performed, followed by the elution from the Ni2+ resin in a buffer with 300 mM imidazole. Imidazole was removed using a PD-10 column (GE Healthcare), and the His tag was cleaved using tobacco etch virus protease.

Blocking Assay of 6His-ΔNPP2CA and PYL4

6His-ΔNPP2CA pellets were resuspended in 25 mL Tris-HCl, pH 8.0, 150 mM NaCl, 50 mM imidazole, 5 mM β-mercaptoethanol, and 5 mM Mg2+, mixed with 8 mg of either pure nontagged (through tobacco etch virus cleavage) PYL4 or PYL4A194T, and disrupted by sonication. The crude extracts were treated as described above using His-Trap HP columns from GE Healthcare to the capture step according to the manufacturer’s instructions. In all cases, the purified proteins were subjected to a size exclusion chromatography using a Superdex200 10/300 (Amersham Biosciences Limited) to analyze the behavior in a gel filtration of each protein and to isolate the complex. To perform pull-down assays, 6His-ΔNPP2CA was purified, next immobilized on nickel-nitrilotriacetic acid agarose beads (Qiagen), and incubated with either pure nontagged PYL4 or PYL4A194T. The mix was swirled 30 min at 4°C and incubated in the absence or presence of
PP2C and OST1 in Vitro Activity Assays

Phosphatase activity was measured using as a substrate either pNPP or phosphorylated ΔC-ABF2, ΔC-AB5, and SLAC1c proteins. For the pNPP substrate, assays were performed in a 100-µL solution containing 25 mM Tris-HCl pH 7.5, 2 mM MnCl2, and 5mM pNPP. Assays contained 2 µM phosphatase (PP2CA or HA1), 4 µM receptor, and the indicated concentrations of ABA. Phosphatase activity was recorded with a Väktér XS reader at 405 nm every 60 s over 30 min, and the activity obtained after 30 min is indicated in the graphics. To obtain phosphorylated ΔC-ABF2, ΔC-AB5, and SLAC1c proteins, phosphorylation reactions were done using the OST1 kinase basically as described previously (Dupieux et al., 2011b). ΔC-ABF2 and SLAC1c N-terminal fragments were prepared as described (Vahisalu et al., 2010; Antoni et al., 2012). ΔC-AB5 recombinant protein (amino acid residues 1–200) containing the C1, C2, and C3 target sites of ABA-activated SnRK2s was expressed in the pETM1 vector as described above. The reaction mixture containing the OST1 kinase and either ΔC-ABF2, ΔC-AB5, or SLAC1c recombinant proteins was incubated for 50 min at room temperature in 30 µL of kinase buffer: 20 mM Tris-HCl, pH 7.8, 2 mM MgCl2, 2 mM MnCl2, and 3.5 µCi of γ-32P-ATP (3,000 Ci mmol−1). Thus, OST1 was autophosphorylated, and in turn, it phosphorylated ΔC-ABF2, ΔC-AB5, and SLAC1c proteins. Next, they were used as substrates of PP2CA that was preincubated [or not] for 10 min with PYL4 or PYL4V194D either in the absence or presence of the indicated ABA concentration. The reaction was stopped by adding Laemmli buffer, and the proteins were separated by SDS-PAGE using an 8% (v/v) acrylamide gel and transferred to an Immobilon-P membrane (Millipore). Radioactivity was detected and quantified using a Phosphorimage system (FLA5100, Fuji). After scanning, the same membrane was used for Forcex staining. The data presented are averages of at least three independent experiments.

Generation of Transgenic Lines

PYL4 or PYL4 mutants were cloned into pCR8/GW/TOPO entry vector (Invitrogen) and recombined by LR reaction into the Gateway-compatible ALLIGATOR2 vector (Bensmihen et al., 2004). This construct drives expression of PYL4 under control of the Cauliflower mosaic virus 35S 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 At2S3. The ALLIGATOR2-35S:3HA-PYL4 or mutant constructs were transferred to A. thaliana C380C (pgC2260; Deblaere et al., 1985) by electroporation and used to transform Col-0 wild-type plants by the floral dip method. Ti transgenic seeds were selected based on GFP visualization and sowed in soil to obtain the T2 generation. At least three independent transgenic lines were generated for each construct. Homozygous T3 progeny was used for further studies, and expression of HA-tagged protein in 21-d-old seedlings was verified by immunoblot analysis using anti-HA-peroxidase (Roche).

Seed Germination and Seedling Establishment Assays

After surface sterilization of the seeds, stratification was conducted in the dark at 4°C for 3 d. Approximately 100 seeds of each genotype were sowed on MS plates supplemented with different ABA concentrations per experiment. To score seed germination, radial emergence was analyzed at 72 h after sowing. Seedling establishment was scored as the percentage of seeds that developed green expanded cotyledons and the first pair of true leaves.

Root and Shoot Growth Assays

Seedlings were grown on vertically oriented MS plates for 4 to 5 d. Afterward, 20 plants were transferred to new MS plates lacking or supplemented with the indicated concentrations of ABA. The plates were scanned on a flatbed scanner after 10 d to produce image files suitable for quantitative analysis of root growth using the NIH software ImageJ v1.37. As an indicator of shoot growth, the maximum rosette radius was measured.

RNA Analyses

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

Drought Resistance through Abscisic Acid Receptor Mutagenesis

Whole-Rosette Stomatal Conductance and Transpiration Measurements

The Arabidopsis whole-rosette gas exchange measurement device, plant growth practice, and custom written program to calculate transpiration and Gs for water vapor have been described previously (Kollist et al., 2007; Vahisalu et al., 2008). For gas exchange experiments, 25- to 28-d-old plants (rosette area, 6–18 cm2) were used. Until measurements, plants were grown in growth chambers (AR-66LX and AR-22L, Percival Scientific) at 12/12 photoperiod, 23°C/18°C temperature, air relative humidity of 70% to 75% and 150 µmol m−2 s−1 light. During gas exchange measurements, temperature, air relatively humidity, photoperiod, and light in the cuvettes were kept as similar as possible to the values in growth chambers. Photographs of plants were taken before and after the experiment, and rosette leaf area was calculated using the NIH software ImageJ 1.37v. Leaf area values for the intermediary experimental period were calculated using linear regression between starting and final leaf area.

Water Loss and Stomatal Aperture Assays

Two- to three-week-old seedlings grown in MS plates were used for water loss assays. Four seedlings per genotype with similar growth (three independent experiments) were submitted to the drying atmosphere of a laminar flow hood. Kinetic analysis of water loss was performed and represented as the percentage of initial fresh weight loss at each scored time point. Stomatal aperture measurements were done in leaves of 5-week-old plants grown under greenhouse conditions using whole-leaf imaging (Chitrakar and Melotto, 2010). Staining of whole leaves with propidium iodide was conducted, and the aperture of 30 to 40 stomata (ratio, width/length; two independent experiments) was measured using a Leica TCS-SP2 confocal microscope.

Drought Stress

Plants grown under greenhouse conditions (10 individuals per experiment, three independent experiments) were grown under normal watering conditions for 15 d and then subjected to drought stress by stopping irrigation during 20 d. Next, watering was resumed, and survival rate was calculated after 3 d by counting the percentage of plants that had more than four green leaves. Photographs were taken at the start of the experiment (day 0), after 16 and 19 d of drought, and 3 d after rewatering. Quantification of shoot growth was performed at 2, 5, 7 and 9 d after stopping irrigation (day 0) by measuring the maximum rosette radius of the plants.

Dehydration Treatment

Two-week-old seedlings grown in MS plates were used for these experiments. Twenty seedlings per genotype (two independent experiments) were submitted to the drying atmosphere of a laminar flow hood for 12 h (25°C ± 1°C, 25% ± 2% relative humidity) and then rehydrated with 25 mL of water. Survival percentage was scored 3 d after rehydration by counting the percentage of plants that had at least four green leaves. The Arabidopsis Genome Initiative locus identifiers for PYL4 and PP2CA are Atlg38310 and Atlg11410, respectively.

Supplemental Data

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

Supplemental Figure S1. ABA infiltration reveals BiFC interaction of PYL4 and PP2CA, both at nucleus and cytosol.

Supplemental Figure S2. Enhanced sensitivity to ABA-mediated inhibition of germination and early seedling growth in T4 PYL4V194D OE plants compared with nontransformed Col-0 and PYL4 OE plants.

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