Unique Drought Resistance Functions of the *Highly ABA-Induced* Clade A Protein Phosphatase 2Cs[1][W][OA]

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Six Arabidopsis (*Arabidopsis thaliana*) clade A protein phosphatase 2Cs (PP2Cs) have established abscisic acid (ABA) signaling roles; however, phenotypic roles of the remaining three "*HAI*" PP2Cs, *Highly ABA-Induced1* (*HAI1*), *AKT1-Interacting PP2C1/HAI2*, and *HAI3*, have remained unclear. *HAI* PP2C mutants had enhanced proline and osmoregulatory solute accumulation at low water potential, while mutants of other clade A PP2Cs had no or lesser effect on these drought resistance traits. *hai1-2* also had increased expression of abiotic stress-associated genes, including dehydrins and late embryogenesis abundant proteins, but decreased expression of several defense-related genes. Conversely, the *HAI* PP2Cs had relatively less impact on several ABA sensitivity phenotypes. *HAI* PP2C single mutants were unaffected in ABA sensitivity, while double and triple mutants were moderately hypersensitive in postgermination ABA response but ABA insensitive in germination. The HAI PP2Cs interacted most strongly with PYL5 and PYL7 to -10 of the PYL/RCAR ABA receptor family, with PYL7 to -10 interactions being relatively little affected by ABA in yeast two-hybrid assays. HAI1 had especially limited PYL interaction. Reduced expression of the main HAI1-interacting *PYL*s at low water potential when *HAI1* expression was strongly induced also suggests limited PYL regulation and a role of HAI1 activity in negatively regulating specific drought resistance phenotypes. Overall, the *HAI* PP2Cs had greatest effect on ABA-independent low water potential phenotypes and lesser effect on classical ABA sensitivity phenotypes. Both this and their distinct PYL interaction demonstrate a new level of functional differentiation among the clade A PP2Cs and a point of cross talk between ABA-dependent and ABA-independent drought-associated signaling.

Plants have well-developed sensing and signal transduction systems to respond to water limitation during drought when soil water potential (Ψ_w) decreases. Accumulation of the stress hormone abscisic acid (ABA) is both one of the outputs of upstream stress sensing and signaling as well as a key regulator of downstream responses (Finkelstein and Rock, 2002; Cutler et al., 2010). ABA signaling regulates many, but not all, responses to drought and other abiotic stresses (Verslues and Zhu, 2005). There are nine clade A protein phosphatase 2Cs (PP2Cs) in Arabidopsis (*Arabidopsis thaliana*; Schweighofer et al., 2004; Xue et al., 2008), of which six have established functions as negative regulators of ABA signaling. These ABA signaling PP2Cs included ABA Insensitive1 (ABI1), ABI2, ABA Hypersensitive Germination1 (AHG1), AHG3/AtPP2CA, Hypersensitive to ABA1 (HAB1), and HAB2 (Koornneef et al., 1989; Leung et al., 1994, 1997; Meyer et al., 1994; Rodriguez et al., 1998; Sheen, 1998; Gosti et al., 1999; Merlot et al., 2001; Leonhardt et al., 2004; Saez et al., 2004; Kuhn et al., 2006; Yoshida et al., 2006; Nishimura et al., 2007). These PP2Cs have redundant functions in ABA signaling, as double and triple mutants have extreme ABA hypersensitivity of seed germination, stomatal regulation, and gene expression (Rubio et al., 2009).

The molecular role of ABA signaling clade A PP2Cs has been affirmed by several laboratories that have shown that ABI1, ABI2, HAB1, and AHG3 all interact with the RCAR/PYR/PYL family of ABA receptors (hereafter referred to as PYLs for convenience). PYL-ABA-PP2C interaction blocks PP2C activity and allows downstream targets, such as the Suc nonfermenting-related kinases group 2 (SnRK2s) and other PP2C substrates, to remain phosphorylated (Ma et al., 2009; Park et al., 2009; Umezawa et al., 2009; Vlad et al., 2009). This, in turn, leads to the phosphorylation of ABF/AREB/ABI5 bZIP family transcription factors and the activation of ABA-induced gene expression (Fujii et al., 2009) or the phosphorylation of Slow Anion Channel1 in guard cells (Geiger et al., 2009, 2010; Lee et al., 2009). Substantial data support this model of ABA-stimulated PYL-PP2C interaction in ABA signaling, including reconstitution of the pathway in Arabidopsis protoplasts

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and *Xenopus laevis* oocytes (Fujii et al., 2009; Brandt et al., 2012). However, at least one of the clade A PP2Cs (AHG1) is not inhibited by the PYLs, despite having ABA-hypersensitive seed germination (Nishimura et al., 2007; Antoni et al., 2012). Since there are 14 PYL family members and nine clade A PP2Cs, the number of possible interactions is large. Many of the possible PP2C-PYL interactions remain to be experimentally tested.

The remaining three clade A PP2Cs, *Highly ABA-Induced1* (*HAI1*), *AKT1-Interacting Phosphatase1* (*AIP1*; also known as *HAI2*), and *HAI3*, are of uncertain physiological function and unclear roles in PYL-PP2C signaling. We will refer to these collectively as the *HAI* PP2Cs. Mutants of the *HAI* PP2Cs do not exhibit ABA hypersensitivity (Yoshida et al., 2006); however, their expression is highly induced by exogenous ABA (Fujita et al., 2009). Interestingly, a number of PP2Cs, including *HAI1* and *AIP1*, were found to be misexpressed in the *eskimo1* freezing-tolerant mutant, which has constitutively high Pro and solute content (Xin and Browse, 1998; Xin et al., 2007). *HAI1* was identified as one of several stress-related genes controlled by the key developmental regulator SCARECROW (Iyer-Pascuzzi et al., 2011). These observations suggest a role of the *HAI* PP2Cs in abiotic stress; yet, no stress-related phenotype has been associated with the *HAI* PP2Cs. At the molecular level, the limited information we have about the *HAI* PP2Cs comes largely from testing their interaction with signaling proteins identified in studies of other clade A PP2Cs (Fujita et al., 2009). Thus, whether the *HAI* PP2Cs may have distinct targets and distinct functions in stress physiology remains uncertain. An exception to this is the role of AIP1 and HAI3 in regulating Arabidopsis K^+-Transporter1 (AKT1) as part of a calcineurin B-like-SnRK3-PP2C complex (Lee et al., 2007; Lan et al., 2011); however, the significance of this interaction in drought response is unclear.

Conversely, there are many drought responses for which the role of PYL-PP2C regulation is unclear. Two such phenotypes of long-standing interest are osmoregulatory solute accumulation and low-Ψ_w-induced Pro accumulation. Pro is essential for growth and redox buffering under low-Ψ_w and salt stress (Székely et al., 2008; Szabados and Savouré, 2010; Verslues and Sharma, 2010; Sharma et al., 2011). Pro accumulation is reduced in both ABA-deficient mutants (*aba2-1*) and the ABA-insensitive mutants *abi1-1* and *abi2-1*, demonstrating a role of ABA in promoting Pro accumulation (Verslues and Bray, 2006). However, ABA applied to unstressed plants elicits only a small fraction of the Pro accumulation seen during low Ψ_w (Sharma and Verslues, 2010). This shows that Pro accumulation also requires low Ψ_w-dependent signaling mechanisms in addition to ABA signaling.

Pro accumulation is one part of osmoregulatory solute accumulation whereby cellular solute content is adjusted to maintain appropriate volume (or turgor when the volume is restricted by a cell wall) and water content. A decrease in external Ψ_w caused by soil drying is compensated by an accumulation of solutes inside the plant cell. This is often referred to as osmotic adjustment (Kramer and Boyer, 1995; Zhang et al., 1999). Osmotic adjustment has a role in drought resistance (Blum, 2005) by allowing plant cells to maintain turgor under mild to moderate stress levels and resist excessive dehydration under more severe reductions in Ψ_w. Osmotic adjustment includes the accumulation of cytoplasmic compatible solutes, such as Pro, as well as K^+ and organic acids largely compartmentalized in the vacuole (Morgan, 1984; Sharp et al., 1990; Voetberg and Sharp, 1991; Zhang et al., 1999). The molecular mechanisms controlling osmoregulatory solute accumulation are largely unknown.

We found that *hai1*, *aip1*, and *hai3* mutants had increased Pro and osmoregulatory solute accumulation at low Ψ_w, while other clade A *pp2c* mutants had lesser or no effect. As osmoregulatory solute accumulation was not affected by ABA, these data suggest a low-Ψ_w signaling function of the *HAI* PP2Cs distinct from the ABA signaling clade A PP2Cs. The *HAI* PP2Cs had moderate ABA hypersensitivity, which could only be uncovered in double or triple mutants. *HAI* PP2C double and triple mutants had ABA-insensitive seed germination, which contrasted with the phenotypes of other clade A PP2C mutants. The HAI PP2Cs also interacted preferentially with certain members of the monomer-type PYLs, with HAI1 having especially limited PYL interaction. These combined results indicate that the *HAI* PP2Cs are functionally differentiated from other clade A PP2Cs both in their roles as negative regulators of a distinct set of low-Ψ_w responses and in the specificity and greater ABA independence of their PYL interactions.

RESULTS

HAI1, *AIP1*, and *HAI3* Are Negative Regulators of Low Ψ_w-Induced Pro Accumulation

We collected transferred DNA (T-DNA) mutants of all the clade A PP2Cs (Supplemental Fig. S1), including the *HAI* PP2Cs (shaded gray in Fig. 1A). Low Ψ_w-induced Pro accumulation of these lines was tested by transferring 7-d-old seedlings from control medium to polyethylene glycol (PEG)-infused plates of a range of Ψ_w representing mild to more severe stress. *HAI* PP2C mutants had Pro levels twice that of the ecotype Columbia (Col) wild type at mild low-Ψ_w severity (-0.5 to -0.7 MPa; Fig. 1B) and approximately 40% higher Pro at more severe low Ψ_w (-1.2 MPa). Of the other clade A PP2Cs, *abi1td*, *abi2td*, and *ahg1-3* had elevated Pro, but not to the extent seen in the *HAI* PP2C mutants. Pro accumulation of *ahg3-3*, *hab1-1*, and *hab2-1* did not differ from that of the wild type (Fig. 1C). The increased Pro of *abi1td* and *abit2td* was consistent with many observations that these PP2Cs regulate a broad spectrum of stress responses and with previous data showing that the *abi1-1* and *abi2-1* dominant

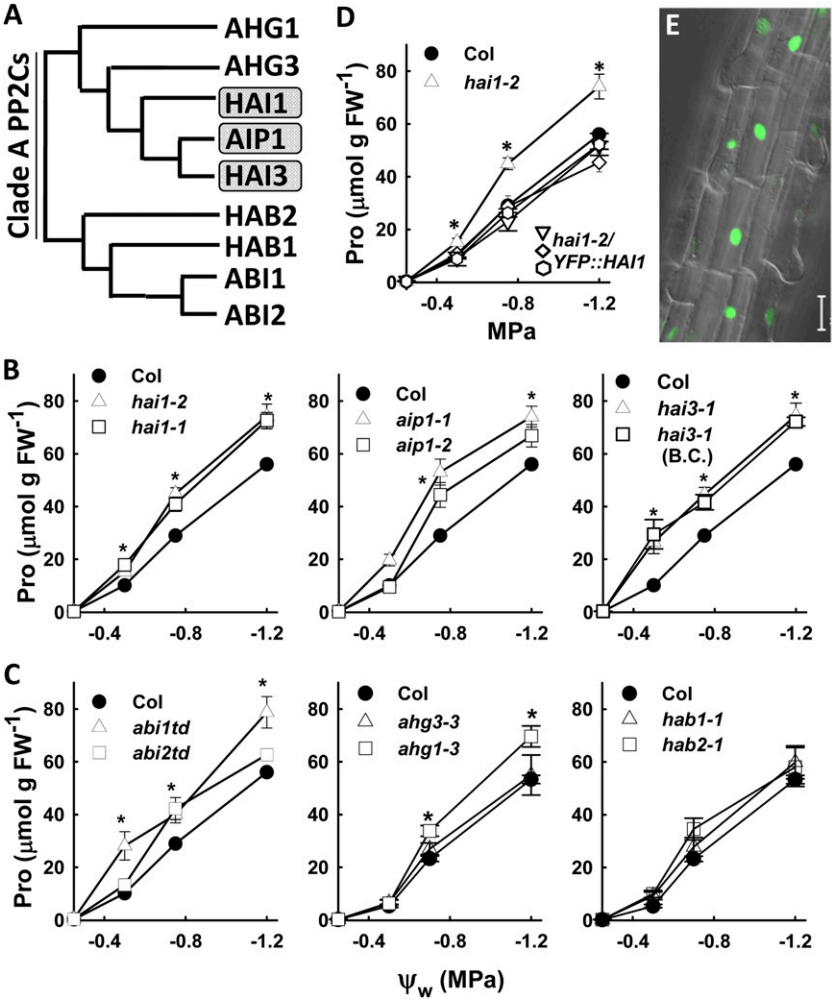

Figure 1. *HAI* PP2Cs are negative regulators of low-Ψ_w-induced Pro accumulation. A, Diagram showing the positions of the *HAI* PP2Cs (shaded) relative to other clade A PP2Cs. The diagram is adapted from the phylogenetic analysis of Schweighofer et al. (2004). T-DNA mutants used for our analyses are described in Supplemental Figure S1. B, Pro contents of the wild type (Col) and *HAI* PP2C mutants. Seven-day-old seedlings were transferred to PEG-infused agar plates of the indicated Ψ_w and Pro contents measured 96 h after transfer. Data are means ± SE (n = 9–12) combined from three independent experiments. Significant differences of mutant versus the wild type ($P \leq 0.05$) at a particular Ψ_w are marked by asterisks. Data from twice-backcrossed lines of *hai3-1* are also presented and represent means of three homozygous lines selected after backcrossing. FW, Fresh weight. C, Pro contents of other clade A PP2C mutants. The T-DNA mutants of *ABI1* and *ABI2* are referred to as *abi1td* and *abi2td* to clearly distinguish them from the dominant negative alleles of these genes. Data presentation is the same as in B. D, Pro content of the *hai1-2* mutant complemented with *35S-YFP:HAI1*. Data from three independent T3 homozygous lines are shown along with the Col wild type and the *hai1-2* mutant. Data are means ± SE (n = 6–9) combined from two independent experiments. E, Subcellular localization of YFP:HAI1. The image is from the root of a representative *35S-YFP:HAI1* transgenic line. Bar = 20 μm.

negative mutants had reduced Pro accumulation (Verslues and Bray, 2006). These data established the first low-Ψ_w phenotype of *hai1*, *aip1*, or *hai3*.

For both *HAI1* and *AIP1*, two independent T-DNA lines had the same phenotype. We also transformed *hai1-2* with *35S-YFP:HAI1* and found that the transgene could complement the Pro (Fig. 1D) and solute accumulation phenotypes (see below). The N-terminal YFP:HAI1 fusion protein was predominantly localized in the nucleus (Fig. 1E), in agreement with Antoni et al. (2012) and Fujita et al. (2009) and the presence of nuclear localization signals in the HAI1 sequence (Antoni et al., 2012). This contrasted with the Golgi localization of HAI1 reported by Zhang et al. (2012) using stable expression of a C-terminal fusion protein. We also generated transgenic plants using a C-terminal HAI1:YFP fusion construct but could not observe expression of the fusion protein. As Zhang et al. (2012) did not report whether their construct complemented the *hai1* mutant, it is possible that they observed a low level of mistargeted protein. The C-terminal fusion may have interfered with a C-terminal nuclear localization signal present in several clade A PP2Cs (Himmelbach et al., 2002). Of the two *aip1* mutants, *aip1-1* has a 5′ untranslated region T-DNA insertion and still produces a reduced level of *AIP1* mRNA (Supplemental Fig. S1B). However, its phenotype is identical to that of *aip1-2* (Fig. 1B; see below), indicating that *aip1-1* lacks AIP1 function, possibly through blocked or reduced translation of the mutant *AIP1* mRNA. For *hai3-1*, the high-Pro phenotype was still observed after the mutant was twice backcrossed to the wild type and plants homozygous for the T-DNA insertion were reselected (Fig. 1B). These results verified that it was disruption of the *HAI* PP2Cs that caused increased Pro accumulation at low Ψ_w.

Double and triple *HAI* PP2C mutants also had elevated Pro accumulation (Supplemental Fig. S2); however, they did not show additional effects above those seen in the single mutants, and the *hai1-2aip1-1hai3-1* triple mutant had less effect. This indicated that additional positive regulatory mechanisms may need to be activated to allow even higher Pro accumulation at low Ψ_w and that the double and triple mutants may have pleiotropic effects that prevent higher Pro accumulation.

None of the *HAI* PP2C mutants had increased Pro in response to salt stress (Supplemental Fig. S3). The *hai1-2aip1-1hai3-1* triple mutant had slightly reduced salt stress-induced Pro; thus, for this phenotype, there was an additive effect of knocking out multiple *HAI* PP2Cs. The lack of effect on salt stress-induced Pro accumulation suggested that the *HAI* PP2Cs have a more prominent role at low Ψ_w when there is a much higher accumulation of Pro as part of osmoregulatory solute accumulation.

HAI1, AIP1, and HAI3 Are Negative Regulators of Osmoregulatory Solute Accumulation

Compared with the Col wild type, *HAI* PP2C mutants all had lower osmotic potential (Ψ_s), indicating more solute accumulation, across several low-Ψ_w severities (Fig. 2A). *hai1* mutants had the largest effect: in the most severe low-Ψ_w treatment, the Ψ_s of *hai1-1* or *hai1-2* was 0.5 to 0.6 MPa lower than that of the wild type. This corresponds to more than a 200 mM difference in solute content and was larger than could be explained by the 20 to 30 mM difference in Pro content between the wild type and *hai1* (Fig. 1B). As the difference between cellular Ψ_s and Ψ_w (which in this case should be in near equilibrium with the medium Ψ_w indicated by the dashed lines in Fig. 2A) is a measure of turgor, these data suggest that *hai1* may maintain a higher turgor pressure at low Ψ_w. Complementation of *hai1-2* with *YFP:HAI1* returned Ψ_s to the wild-type level (Fig. 2D). For the other clade A PP2Cs, *abi1td* and *abi2td* had decreased Ψ_s only at the most severe low-Ψ_w treatment, while *ahg1-3*, *ahg3-3*, *hab1-1*, and

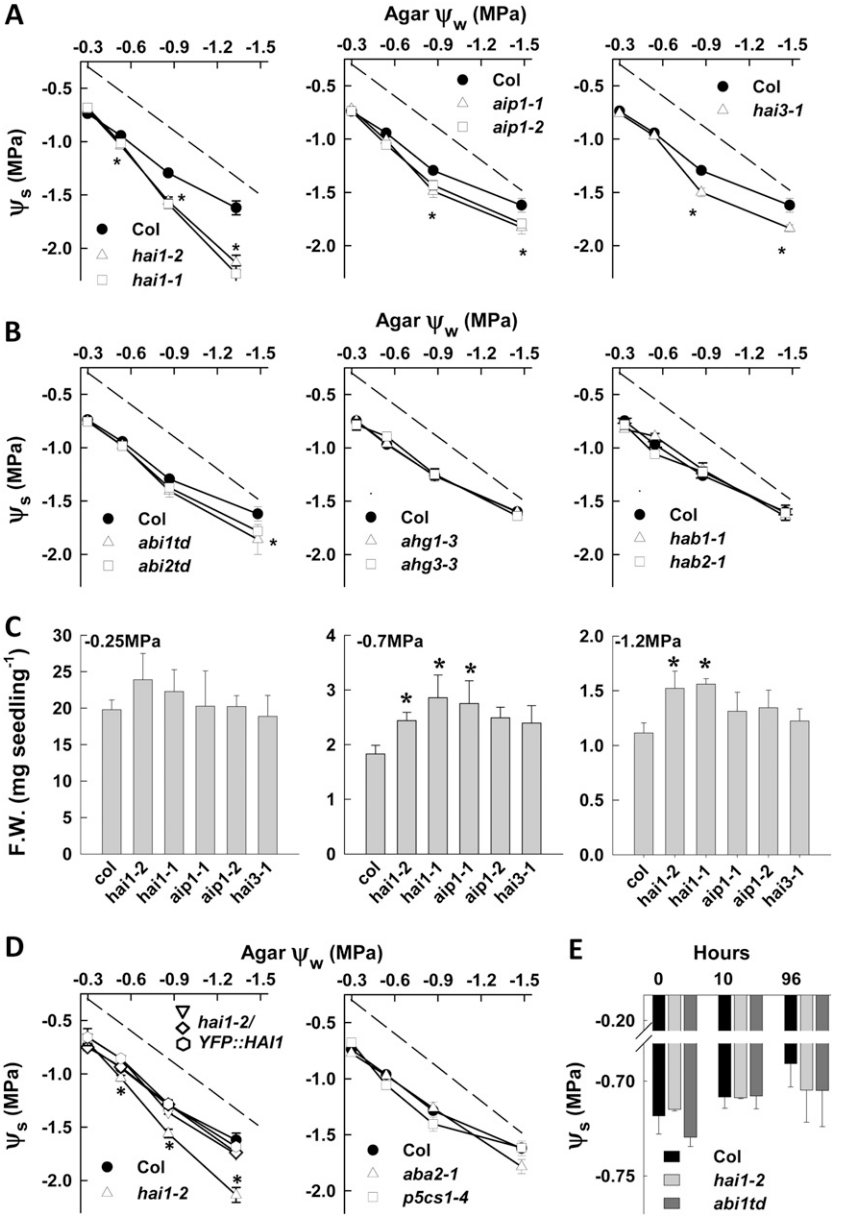

Figure 2. Mutants of the *HAI* PP2Cs have increased osmoregulatory solute accumulation and fresh weight at low Ψ_w. A, Ψ_s of *HAI* PP2C mutants measured 96 h after transfer of 7-d-old seedlings to PEG-infused plates of a range of Ψ_w. Data are plotted as the osmotic potential versus the agar water potential measured at the time of sample collection. The dashed line in each panel plots where agar Ψ_w = seedling Ψ_s. Points below the dashed line are consistent with a positive turgor pressure, as more reduced Ψ_s indicates greater accumulation of osmotically active solutes. Data are means ± SE (n = 3–5) of measurements combined from two to three independent experiments. Significant differences between the mutant and the wild type ($P \leq 0.05$) are indicated by asterisks. B, Ψ_s of ABA signaling clade A PP2Cs. Data presentation is as described for A. C, Seedling fresh weight (F.W.) measured 96 h after transfer of wild-type and mutant seedlings to a high-Ψ_w control (−0.25 MPa), an intermediate Ψ_w (−0.7 MPa), or more severe low Ψ_w (−1.2 MPa) treatment. Data are means ± SE (n = 3–5) combined from two to three independent experiments. Significant differences between the mutant and the wild type ($P \leq 0.05$) are indicated by asterisks. D, Seedling Ψ_s of *hai1-2* complemented with *35S-YFP:HAI1* (left panel; data of three independent T3 homozygous lines are shown) as well as *p5cs1-4*, which has reduced Pro accumulation, and *aba2-1*, which is ABA deficient (right panel). Experimental procedures and data presentation are as described for A. E, Effect of ABA treatment (5 μM) on Ψ_s. Seven-day-old seedlings were transferred to ABA-containing plates, and Ψ_s was measured 10 and 96 h later. Data are means ± SE (n = 3–5) combined from two independent experiments.

hab2-1 did not differ from wild-type Ψ_s at any severity of low-Ψ_w stress (Fig. 2B). The fresh weight of *hai1-1* and *hai1-2* seedlings was greater than that of the wild type at −0.7 and −1.2 MPa (Fig. 2C), demonstrating that increased solute deposition in *hai1* was able to drive increased water retention. *aip1* and *hai3-1* had slightly increased or unchanged fresh weight compared with the wild type. These data demonstrated that the higher solute concentrations in the *HAI* PP2C mutants were a result of increased solute uptake and synthesis rather than an indirect effect of decreased growth or increased water loss, which could concentrate the same amount of solutes in a smaller volume.

These observations suggested several intriguing aspects of *HAI* PP2C physiological function. First, the greatly increased osmoregulatory solute accumulation and increased water content indicated a general effect on osmoregulation, potentially involving many solutes, rather than a specific effect on Pro. Consistent with this, we found that *p5cs1-4*, which lacks Δ^1-Pyrroline Carboxylate Synthetase1 activity and is deficient in Pro accumulation (Székely et al., 2008; Sharma et al., 2011), did not differ from the wild type in Ψ_s (Fig. 2D). Thus, loss of Pro accumulation in *p5cs1-4* may be compensated by additional accumulation of other compounds, and the *HAI* PP2Cs affect this overall osmoregulatory control mechanism. Also, osmoregulatory solute accumulation may be independent of ABA sensitivity, as *HAB* and *AHG* mutants did not differ from the wild type in Ψ_s. Consistent with this, the ABA-deficient mutant *aba2-1* did not differ from the wild type in Ψ_s (Fig. 2D) either in these experiments or in a previous study (Verslues and Bray, 2006). In addition, application of 5 μM ABA did not affect Ψ_s (Fig. 2E), even though it was sufficient to induce ABA-regulated gene expression (see below). These observations support a role of the *HAI* PP2Cs in osmoregulatory solute accumulation that is distinct from the ABA signaling roles of other clade A PP2Cs.

We also assayed Ψ_s and fresh weight of double and triple mutants of *hai1-2*, *aip1-1*, and *hai3-1* and found that they had decreased Ψ_s while maintaining fresh weight (Supplemental Fig. S4). The double and triple mutants had similar or less effect than single mutants, indicating that pleiotropic effect of the combined *hai* mutations may prevent further increases in solute accumulation.

HAI1 Is a Negative Regulator of Osmotic Adjustment during Soil Drying

We planted mutant and wild-type plants together in the same pot to ensure that they interrooted and were exposed to the same soil moisture conditions and subjected 30-d-old plants to a 12-d water-withholding period. Soil Ψ_w decreased from −0.15 MPa to approximately −1.3 MPa during this period (Fig. 3A, inset). Ψ_s of *hai1-2* was lower than that of the wild type on days 8 and 10 of water withholding

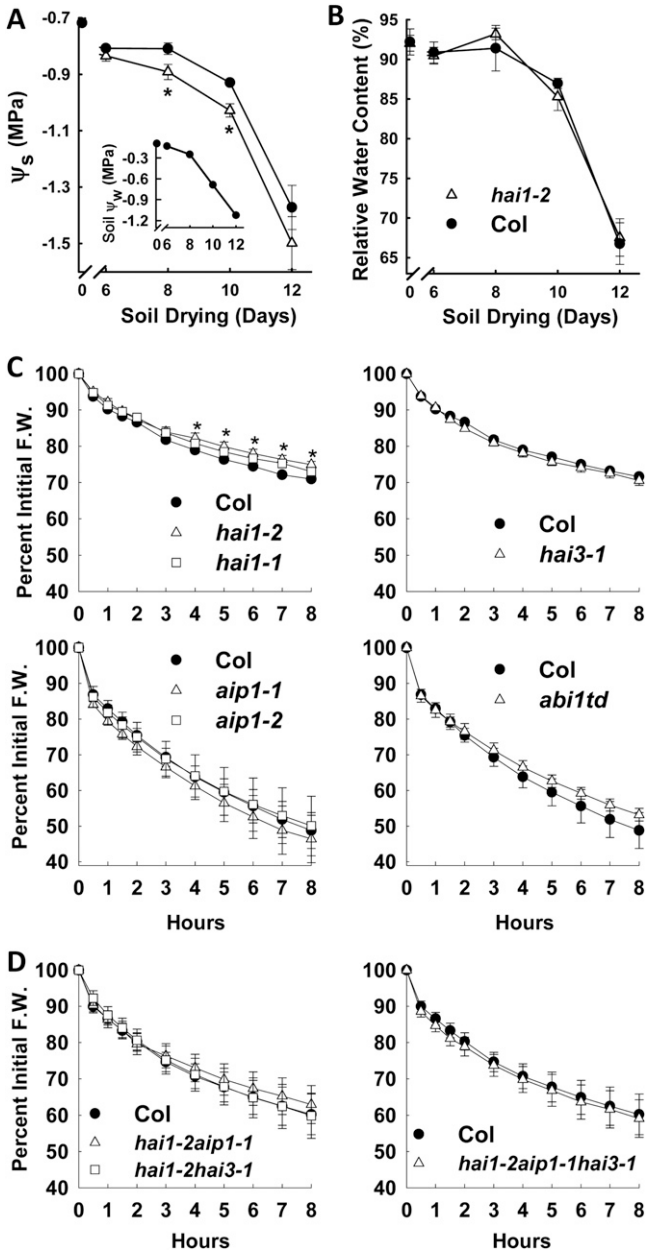

Figure 3. *hai1-2* has increased osmotic adjustment during soil drying, while neither it nor other *HAI* PP2Cs substantially affect leaf water loss. A and B, Ψ_s and RWC of the wild type (Col) and *hai1-2* before water with holding (day 0) and 6 to 12 d after the start of water withholding. The two genotypes were grown together in the same pots to ensure that they were exposed to the same Ψ_w and the inset shows the mean soil Ψ_w at various times after water withholding. Data are means ± SE (n = 3–9) from two to three independent experiments. Significant differences between the mutant and the wild type ($P \leq 0.05$) are indicated by asterisks. Photographs of representative plants during soil drying can be seen in Supplemental Figure S5. C and D, Water loss from detached leaves of the wild type (Col), *HAI* PP2C mutants, and an *ABI1* T-DNA mutant (*abi1td*). Data are means ± SE (n = 3–6) from two to three independent experiments. Significant differences between the mutant and the wild type ($P \leq 0.05$) are indicated by asterisks. F.W., Fresh weight.

(Fig. 3A). Leaf relative water content (RWC) declined during soil drying but did not differ between the wild type and the mutant (Fig. 3B). Thus, *hai1-2* had greater osmotic adjustment, since a given decrease in RWC was accompanied by greater solute accumulation. Plants of *hai1-2* were of similar size and appearance as the wild type (Supplemental Fig. S5), again indicating that increased solute accumulation of *hai1-2* was not caused by decreased growth or altered development.

HAI PP2C Mutants Have Little Effect on Leaf Water Loss

HAI PP2C mutants did not differ from the Col wild type in leaf water loss over the first 3 h after leaf detachment; however, *hai1* mutants had decreased water loss over 4 to 8 h (Fig. 3C). This late decrease in leaf water loss was not seen in *abi1td*, which was assayed for comparison (Fig. 3C), and may be related to the increased osmoregulatory solute accumulation of *hai1*. The lack of difference in leaf water loss between the *abi1td* single mutant and the wild type was consistent with previous reports (Saez et al., 2004; Rubio et al., 2009). Double and triple mutants of the *HAI* PP2Cs were unaffected in leaf water loss (Fig. 3D). This contrasted with double and triple mutants of the other clade A PP2Cs, which have greatly decreased leaf water loss as part of their severe ABA hypersensitivity (Rubio et al., 2009).

hai1-2 Has Enhanced Expression of Dehydration-Protective Genes But Decreased Expression of Defense Genes

We conducted microarray analysis of *hai1-2*, as it had the most unique and prominent phenotypes. A 96 h, low-Ψ_w (−1.2 MPa) treatment was used to identify differentially expressed genes associated with longer term low-Ψ_w response, similar to Pro and osmoregulatory solute accumulation. In the Col wild type, we found 1,474 genes up-regulated (Supplemental Table S1) and 1,530 genes down-regulated (Supplemental Table S2) at least 1.5-fold by this longer term low-Ψ_w treatment. Comparison of *hai1-2* with the wild type at low Ψ_w found 61 genes up-regulated and 76 genes down-regulated in *hai1-2* relative to the wild type (Supplemental Tables S5 and S6). A smaller number of genes were altered in *hai1-2* relative to the wild type in the high-Ψ_w control: 17 genes up-regulated and 26 genes down-regulated (Supplemental Tables S3 and S4). These gene expression changes were further analyzed for enrichment of specific Gene Ontology (GO) terms and coexpression-based clustering to identify groups of similarly expressed genes affected by *hai1-2*.

"Response to water deprivation" was the most significantly enriched GO term in the genes up-regulated in *hai1-2* under control or low-Ψ_w conditions (Supplemental Table S7). Several other terms related to seed dormancy and germination, cold stress, and GA and ABA signaling were also significantly enriched. Clustering analysis identified a group of highly coexpressed genes (Fig. 4A) that included many, but not all, of the genes up-regulated in *hai1-2*. This cluster included the dehydrin *XERO1*, several late embryogenesis abundant (LEA) proteins including *EM1*, and the seed storage protein *CRUCIFERIN3*. Other *hai1-2* up-regulated genes included stress-induced NAC domain transcription factors (NAC019 and NAC040), dehydrin XERO2, seed germination regulator *SOMNUS*, and *GAST homolog protein2* (*GASA2*) and *GASA3*. Quantitative reverse transcription (RT)-PCR analysis confirmed the increased expression of several of these genes in *hai1-2* (Fig. 4B). Overall, these gene expression changes were consistent with HAI1 negative regulation of dehydration resistance.

In contrast, "systemic acquired resistance" and several other GO categories associated with defense responses, defense-related metabolism, or cell wall modification were significantly enriched among the genes down-regulated in *hai1-2* (Supplemental Table S8). Clustering analysis found several clusters of coexpressed genes (Supplemental Fig. S6), and we used quantitative RT-PCR to verify the expression of genes in one of the main clusters (Fig. 4, C and D). In addition to the pathogen response genes differentially regulated in *hai1-2*, *TAT3*, *PBS3*, *GLIP1*, and *GDSL* also have defense roles (Lopukhina et al., 2001; Oh et al., 2005; Nobuta et al., 2007; Kwon et al., 2009; Okrent et al., 2009). The contrasting sets of up- and down-regulated genes suggest a role for HAI1 in balancing abiotic stress versus defense responses.

Despite the increased Pro accumulation of *hai1-2*, we did not see any evidence in our microarray data for altered expression of genes related to Pro metabolism. Quantitative RT-PCR detected a small increase in expression of the Pro synthesis gene *P5CS1* in *hai1-2* after 96 h at low Ψ_w but no other differential expression of the core Pro metabolism genes (Supplemental Fig. S7). Thus, HAI1 may affect Pro accumulation by unknown mechanisms other than transcriptional regulation of Pro metabolism genes.

HAI PP2C Double and Triple Mutants Have Differential Effects on Germination and Postgermination ABA Sensitivity

To further define unique and overlapping functions among clade A PP2Cs, we examined the ABA sensitivity of seed germination as well as postgermination (vegetative) ABA sensitivity. In seed germination, *HAI* PP2C single mutants did not differ from the wild type except for a small but significant ABA-insensitive phenotype observed in *hai1-2* (Fig. 5). These results were in agreement with previous tests of ABA sensitivity (Yoshida et al., 2006; Guo et al., 2010; Antoni et al., 2012). Double and triple *HAI* PP2C mutants had more pronounced ABA-insensitive germination (Fig. 5), which contrasted with the strong ABA hypersensitivity of

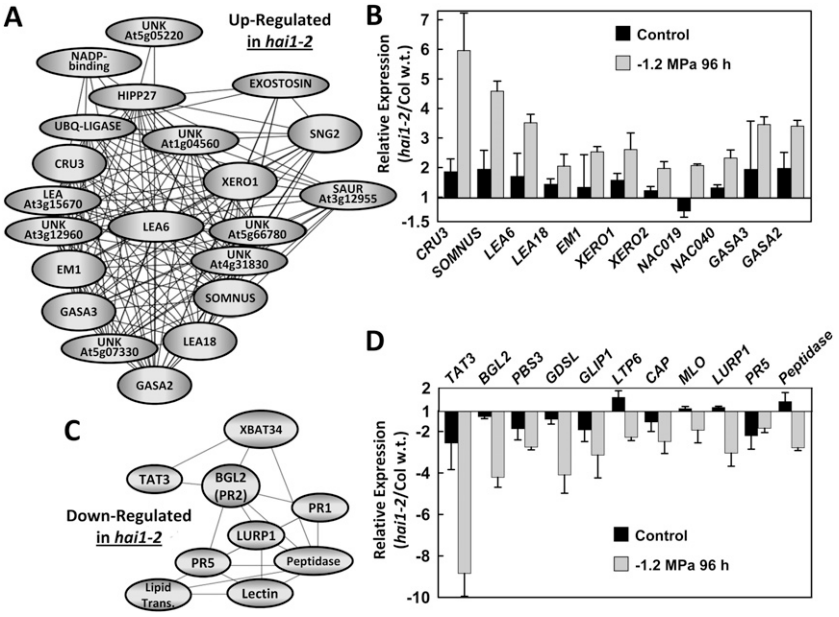

Figure 4. Abiotic stress-associated genes up-regulated and defense genes down-regulated in *hai1-2*. A, Cluster of coexpressed genes up-regulated in *hai1-2*. Coexpression-based clustering was performed using a database of 3,800 arrays (see "Materials and Methods"). Each edge connecting two genes represents a coexpression relationship having a Pearson correlation coefficient ≥ 0.75. See Supplemental Tables S3 and S5 for full lists of genes up-regulated in *hai1-2* relative to the Col wild type under either control or low-Ψ_w conditions. UNK, Gene of unknown function. B, Quantitative RT-PCR verification of genes found to be more highly expressed in *hai1-2* at low Ψ_w by microarray analysis. The genes analyzed along with gene descriptions are given in Supplemental Table S5. Gene expression analysis was performed for Col wild-type (w.t.) and *hai1-2* seedlings under control conditions or 96 h after transfer to low Ψ_w (−1.2 MPa). Data are means ± SE ($n = 3$) using samples collected from three independent experiments. All gene expression differences at −1.2 MPa were found to be significant ($P \leq 0.05$) by one-sided *t* test. Note that *XERO2*, *NAC19*, and *NAC40* are not part of the coexpression cluster shown in A. C, Cluster of coexpressed genes down-regulated in *hai1-2*. Analysis was performed as described for A. The full set of coexpressed clusters of genes down-regulated in *hai1-2* can be seen in Supplemental Figure S6, and the full list of genes down-regulated in *hai1-2* relative to the Col wild type is shown in Supplemental Tables S4 and S6. D, Quantitative RT-PCR verification of genes found to have reduced expression in *hai1-2* at low Ψ_w by microarray analysis. Procedures and data presentation are as described for B. The genes analyzed along with gene descriptions are given in Supplemental Table S6.

double and triple mutants of other clade A PP2Cs (Rubio et al., 2009). We also measured the germination of ABI, HAB, and AHG mutants and found the expected ABA-hypersensitive phenotypes (Supplemental Fig. S8).

We examined postgermination ABA sensitivity first by measuring the portion of seedlings forming green cotyledons on 0.5 μM ABA, a concentration that had minimal effect on germination. *HAI* PP2C single mutants again showed no difference compared with the Col wild type (Fig. 6, A and B). Double and triple *HAI* PP2C mutants showed greater inhibition of cotyledon growth than the wild type; however, the response of the *hai1-2aip1-1hai3-1* triple mutant was equivalent to that of the *ahg1-3* single mutant (Fig. 6, A and B). Similar results were obtained in experiments where root elongation was quantified after transfer of seedlings from control medium to medium containing a range of ABA concentrations (Fig. 6, C and D). *HAI* PP2C single mutants did not show ABA-hypersensitive root growth inhibition. In fact, root elongation of *aip1-1* and *hai3-1* was greater than in the wild type at low ABA concentrations (Fig. 6C). ABA hypersensitivity was seen in double and triple *HAI* PP2C mutants (Fig. 6, C and D), but the effect was similar to *abi1td* and *abi2td* single mutants (Fig. 6E) rather than *hab1-1abi1-2ahg3* or *hab1-1abi1-2abi2-2*, which had constitutively reduced root elongation because of their extreme ABA hypersensitivity (Rubio et al., 2009). *HAI* PP2C double and triple mutants also showed enhanced ABA induction of *NCED3* and *COR15A* expression, which was again similar to the *abi1td* single mutant (Fig. 6F). Of the *HAI* PP2C single mutants, only *hai1-2* showed an increase in *COR15A* expression, and none of the single mutants affected *NCED3* expression. ABA-induced Pro was also assayed as an additional measure of ABA sensitivity. Exogenous ABA applied to unstressed plants elicits a low level of Pro accumulation (Sharma and Verslues, 2010). Only the *HAI* PP2C triple mutant had a small increase in ABA-responsive Pro accumulation (Supplemental Fig. S9). This contrasted with the robust increase in low-Ψ_w-responsive Pro accumulation seen in single mutants of the *HAI* PP2Cs (Fig. 1B). These assays collectively showed that loss of all three

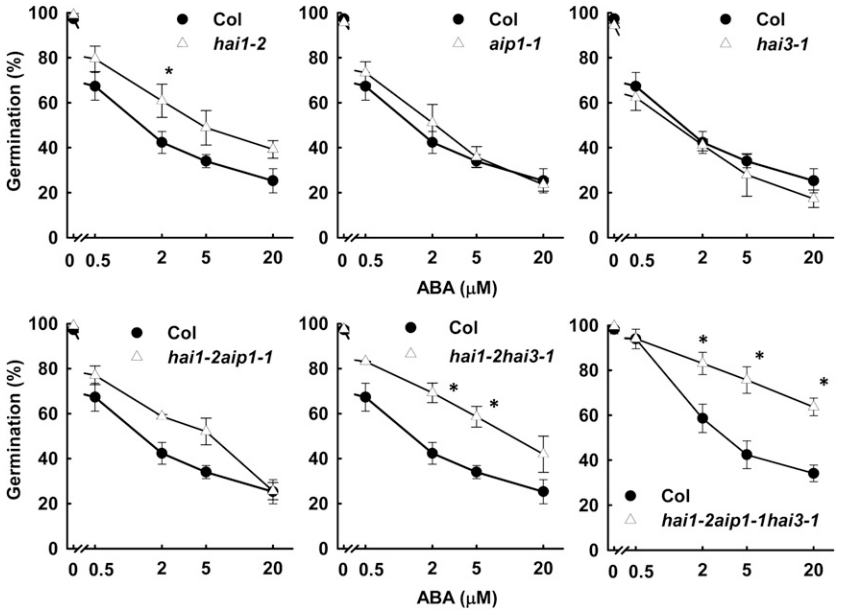

Figure 5. *HAI* PP2C double and triple mutants have ABA-insensitive seed germination. Germination was scored based on radicle emergence 4 d after the end of stratification. Data are means ± SE (n = 3–4) from independent experiments. Significant differences between the mutant and the wild type ($P \leq 0.05$) are indicated by asterisks. For comparison, germination assays of the ABA signaling clade A PP2Cs conducted under the same conditions can be seen in Supplemental Figure S8.

HAI PP2Cs did uncover ABA hypersensitivity but also indicated a lesser role of the *HAI* PP2Cs in ABA sensitivity compared with other clade A PP2Cs.

To determine whether low-Ψ_w-induced ABA accumulation was affected by the *HAI* PP2Cs, we measured ABA content at 0, 10, and 96 h after transfer to −1.2 MPa for mutants of all of the clade A PP2Cs (Supplemental Fig. S10; the 10-h time point represents the peak ABA accumulation, while 96 h is the steady-state ABA level [Verslues and Bray, 2006]). Decreased ABA accumulation was observed in *hab1-1* and *ahg3-3*. However, we saw no difference in ABA content of *HAI* PP2C single mutants. Only in *hai1-2aip1-1hai3-1* at 96 h did we see an increase in ABA (Supplemental Fig. S10), the opposite effect to that seen in triple mutants of other clade A PP2Cs (Rubio et al., 2009). The ABA content data further illustrated functional differentiation among the clade A PP2Cs and also indicated that differences in ABA content were not responsible for the gene expression, Pro, or osmotic potential phenotypes of the *HAI* PP2C mutants.

HAI PP2Cs Have a Distinct Pattern of PYL Interaction

One basis for the unique physiological function of the HAI PP2Cs could be differences in PYL interaction and regulation compared with other clade A PP2Cs. Sequence comparison found that several amino acids shown by structural and mutational analysis (Miyazono et al., 2009; Nishimura et al., 2009; Santiago et al., 2009a; Yin et al., 2009; Dupeux et al., 2011) to be important for PYL interaction of ABI1, ABI2, and HAB1 were not conserved in the HAI PP2Cs (Supplemental Fig. S11). To test the importance of these differences, we first performed a set of qualitative yeast two-hybrid assays to compare the PYL interactions of HAI1 with those of HAB1, whose PYL interactions have been characterized previously (Melcher et al., 2009; Park et al., 2009; Santiago et al., 2009b). We used full-length HAI1 but an N-terminal deletion of HAB1 (ΔNHAB1), as full-length HAB1 autoactivated both in our experiments and in previous work (Saez et al., 2008).

HAB1 interacted with all of the PYLs except PYL13 (Fig. 7A). Interaction with the dimeric PYLs (PYR1, PYL1, and PYL2; Dupeux et al., 2011; Hao et al., 2011) was ABA dependent except for a low level of PYL1 interaction detected without added ABA. PYL4 to -10 have been shown to be monomers (except PYL7, which is predicted to be monomeric but has not been experimentally tested; Dupeux et al., 2011; Hao et al., 2011). HAB1 interaction with these PYLs was not dependent on the addition of ABA (with the exception of PYL4; consistent with the results of Park et al. [2009]). In contrast, HAI1 had detectable interaction only with PYL5 and PYL8 to -10 (Fig. 7A). Also, β-galactosidase staining could only be seen after much longer incubation times for HAI1 than for HAB1, suggesting that the HAI1 interactions were weaker than those of HAB1. There was a weak HAI1-PYL9 interaction without added ABA that could be reproducibly detected in quantitative assays (see below) but was not seen in β-galactosidase staining.

For more detailed analysis of HAI PP2C-PYL interactions, we performed quantitative yeast two-hybrid assays (Fig. 7B) and found that all three HAI PP2Cs interacted with PYL5 and PYL8 to -10 but had no detectable interaction with the dimeric PYLs (PYR1, PYL1, and PYL2). HAI1 differed from AIP1 and HAI3 in that it had the most limited range of PYL interaction, and its PYL interactions were consistently weaker (note the difference in scale between the top panel of Fig. 7B and the two other panels). Perhaps most striking

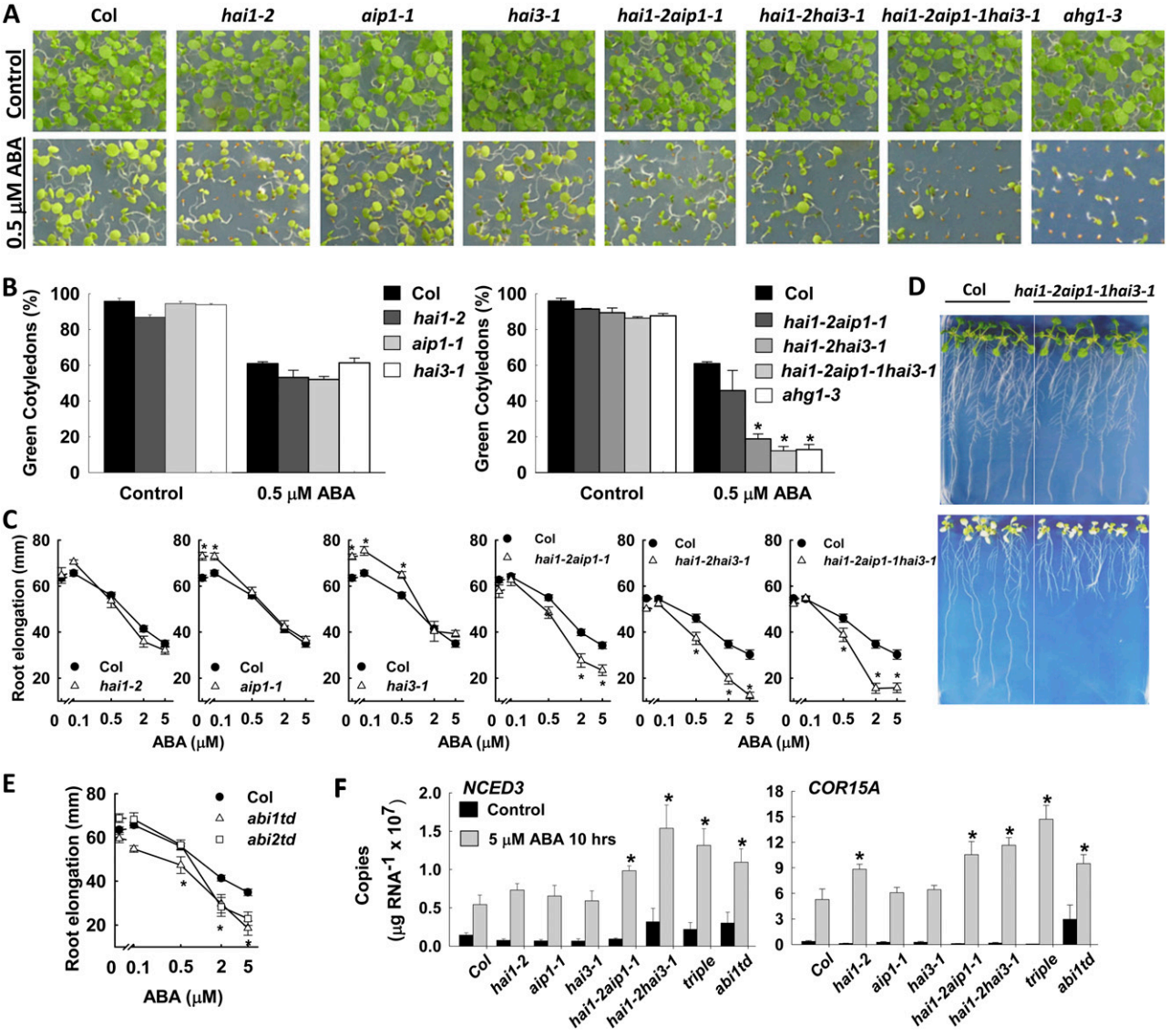

Figure 6. *HAI* PP2C double and triple mutants have postgermination ABA hypersensitivity similar to that of single mutants of other clade A PP2Cs. A, Green cotyledon emergence of the wild type, *HAI* PP2C mutants, and *ahg1-3*, which is shown for comparison. Seeds of each genotype were plated on control medium or medium containing 0.5 μM ABA, and photographs were taken after 8 d of growth. B, Quantification of green cotyledon emergence after 5 d of growth for all genotypes shown in A. Data are means ± SE ($n = 3$) combined from three independent experiments. Significant differences of the mutant versus the wild type are marked by asterisks. C, Four-day-old seedlings were transferred to the indicated ABA concentration, and root elongation of Col wild-type and mutant seedlings was measured over the subsequent 7 d. Data are means ± SE ($n = 10$–15) combined from three independent experiments. Significant differences of the mutant versus the wild type at a particular ABA concentration are marked by asterisks. D, Photographs of representative mutant and wild-type seedlings 7 d after transfer to control (no ABA) plates (top panel) or plates containing 2 μM ABA (bottom panel). E, Root elongation of *abi1td* and *abi2td* assayed for comparison with *HAI* PP2C data in C. F, Expression of the ABA-responsive genes *NCED3* and *COR15A* in the Col wild type, *HAI* PP2C mutants, and *abi1td*. Seven-day-old seedlings were transferred to control medium or medium containing 5 μM ABA for 10 h. Data are means ± SE ($n = 3$) combined from two independent experiments. Significant differences of the mutant versus the wild type are marked by asterisks.

was the lack of HAI1 interaction with PYL7, which strongly interacted with AIP1 and HAI3 (Fig. 7, B and C). HAI3 had a somewhat broader interaction range than HAI1 or AIP1 in that it also had detectable interaction with PYL3, PYL6, PYL11, and PYL12 (Fig. 7B). These experiments used an N-terminal AIP1 truncation (ΔNAIP1, lacking amino acids 1–118; equivalent to ΔNHAB1), as full-length AIP1 caused autoactivation. Full-length HAI3 did not cause autoactivation.

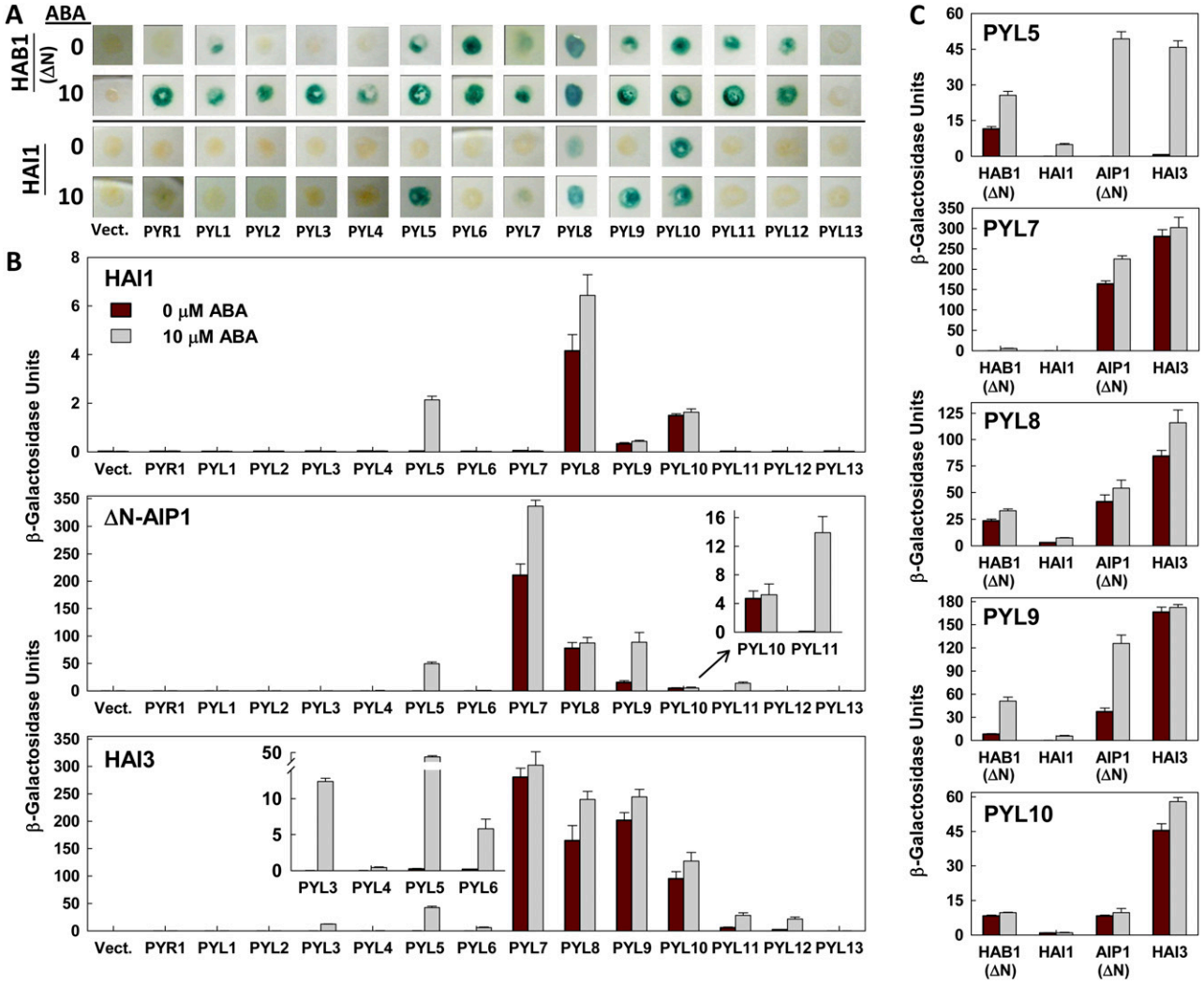

Figure 7. Differing PYL interactions of the HAI PP2Cs and the ABA signaling PP2C HAB1. A, Colony-lift β-galactosidase staining assay comparing the PYL interaction of HAI1 and ΔNHAB1 (N-terminal deletion construct) without ABA or with 10 μM ABA added to the yeast culture. Note that the colony lifts for HAB1 were incubated for 2 to 3 h while those of HAI1 were incubated longer (12 h or overnight) to allow the weaker HAI1 interactions to be seen with similar staining intensity. B, Quantitative yeast two-hybrid assay of PYL interactions of HAI1 (full length), ΔN-AIP1, and HAI3 (full length) either without ABA or with 10 μM ABA added to the yeast culture. Note the difference in scale between the top panel (HAI1) and the other two panels. Insets show selected data replotted using an expanded *y* axis scale for clarity. Data are means ± SD of β-galactosidase activity from three to four independent yeast colonies. C, Repeated quantitative yeast two-hybrid assays testing PYL5, PYL7, PYL8, or PYL10 interaction with all four PP2Cs in the same experiment. Data are means ± SD from three to four independent yeast colonies. Note the difference in scale between the different panels.

We performed several additional experiments to verify these results, particularly the limited PYL interaction of HAI1. First, we selected several PYLs having differing PP2C interactions (PYL5, -7, -8, -9, and -10) and again assayed their interactions with HAB1, HAI1, AIP1, and HAI3 to ensure that the patterns found in Figure 7, A and B, could be confirmed when all PP2Cs were tested in the same experiment. These experiments again found that the PYL interactions of HAI1 were consistently weaker than those of the other PP2Cs (Fig. 7C). Interestingly, PYL5 interacted with HAB1 without added ABA but had no or very weak interaction with HAI1, AIP1, or HAI3 unless ABA was added. PYL7 had weak or nondetectable interaction with HAB1 and HAI1 but interacted strongly with AIP1 and HAI3. Interaction of PYL7, -8, and -10 was little affected by added ABA. PYL9 had a slightly different pattern, with HAI3 having strong interaction without ABA but HAB1 and AIP1 having ABA-stimulated interaction (Fig. 7C). PYL9 interaction with HAI1 was consistently detected but was much weaker; thus, it was difficult to determine the effect of ABA on this interaction. For PYL5, -8, -9, and -10, a range of ABA concentrations were tested, and HAI1 interaction was always less than that of

HAB1 (Supplemental Fig. S12A). We also constructed ΔNHAI1 and found that it had weak PYL interaction, essentially identical to full-length HAI1 (Supplemental Fig. S12B). Western blotting demonstrated that the weak PYL interactions of ΔNHAI1 were not caused by differences in protein expression (Supplemental Fig. S12C). Overall, these data demonstrated a distinctive pattern of PYL interaction for the HAI PP2Cs, with varying effects of ABA on these interactions and an especially limited PYL interaction of HAI1 either with or without added ABA.

Differential Effects of Low Ψ_w on *HAI* PP2C and *PYL* Expression Further Suggest Minimal PYL Regulation of HAI1 at Low Ψ_w

Our microarray analysis indicated that the three *HAI* PP2Cs were more highly induced by low Ψ_w than any of the other clade A PP2Cs (Fig. 8A), and this was confirmed by quantitative RT-PCR (Fig. 8C; note the differing scales for each graph). Conversely, expression of many *PYL*s was down-regulated by low Ψ_w in the microarray analysis (Fig. 8B). Quantitative RT-PCR confirmed the strong low-Ψ_w down-regulation of *PYL5* and *PYL8* and the moderate down-regulation of *PYL9* in the Col wild type (Fig. 8C). In contrast, expression of *PYL7* and *PYL10* was little affected by low Ψ_w. While gene expression differences are at best imperfect indicators of protein abundance, the down-regulation of the HAI1-interacting PYLs further suggests a minimal PYL regulation of HAI1 at low Ψ_w. The steady expression of PYL7 and PYL10 indicates that they may be more important in regulating AIP1 and HAI3 at low Ψ_w.

We also assayed *PP2C* and *PYL* expression during low Ψ_w in the ABA-deficient mutant *aba2-1*. Low Ψ_w induction of the *HAI* PP2C expression was greatly inhibited in *aba2-1*, indicating that their expression was at least partially dependent on ABA (Fig. 8C). In contrast, repression of *PYL5*, *PYL8*, and *PYL9* by low Ψ_w was largely independent of ABA. *PYL10*, and to a lesser extent *PYL7*, were low Ψ_w induced rather than repressed in *aba2-1*. As overexpression of *PYL5* (Santiago et al., 2009b) or *PYL9* (Ma et al., 2009) leads to ABA hypersensitivity, the regulation of *PYL* expression may be a point of cross talk between ABA-dependent and ABA-independent signaling factors.

DISCUSSION

Our analysis found both some similarity as well as prominent differences between the physiological function and PYL interaction of the HAI PP2Cs compared with other clade A PP2Cs. Single mutants of the *HAI* PP2Cs had increased levels of low-Ψ_w-induced Pro and osmoregulatory solute accumulation. These phenotypes were seen to a lesser extent in *abi1td* and *abi2td* but not in mutants of other clade A PP2Cs. Mutants of *hai1* also maintained higher fresh weight at low Ψ_w, indicating that greater osmotic adjustment in *hai1* led to increased water uptake. *hai1-2* also had increased expression of a number of drought-protective

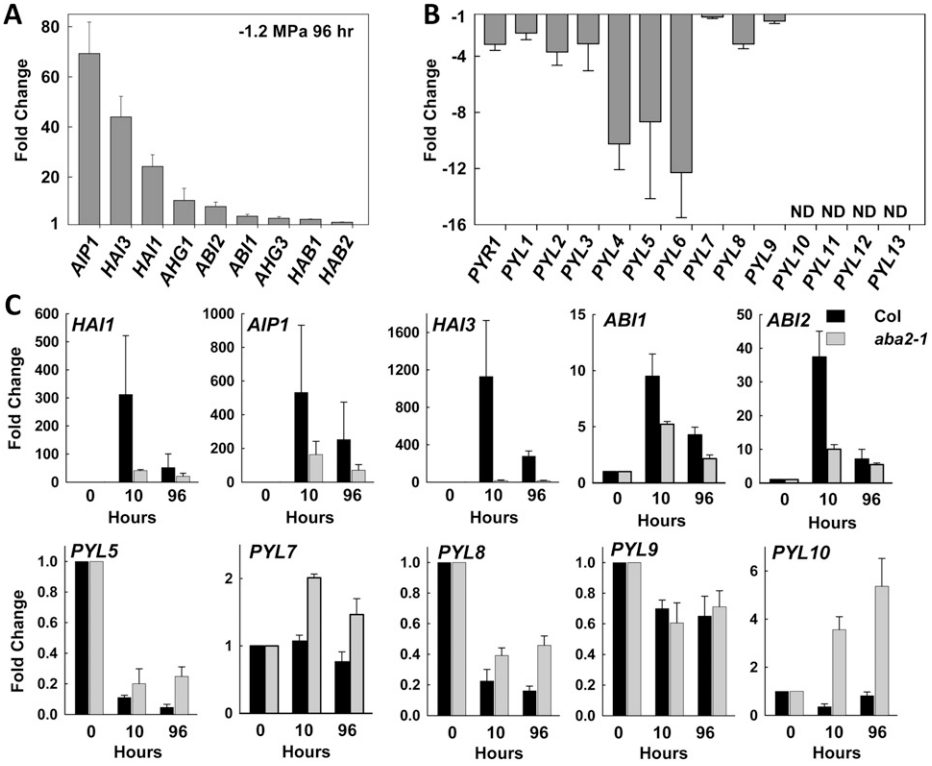

Figure 8. Differing patterns of *PP2C* and *PYL* expression at low Ψ_w. A, Effect of low Ψ_w on gene expression of clade A *PP2C*s in the Col wild type. Data are from the microarray analysis described in Figure 4 and are shown as means ± SD from three experiments. B, Effect of low Ψ_w on *PYL* expression in the Col wild type from the microarray analysis. ND, Not detected. C, Quantitative RT-PCR was conducted on samples 0, 10, or 96 h after transfer of 7-d-old seedlings to −1.2 MPa low-Ψ_w treatment. Data are normalized to the expression level at time 0 for both the Col wild type and *aba2-1* and are means ± SE (n = 3–4) of samples collected from three or four independent experiments.

genes, including dehydrins and LEAs. Overall, the data demonstrated that the *HAI* PP2Cs regulate a distinct set of drought responses related to dehydration resistance rather than avoidance of leaf water loss, which is a prominent phenotype of other clade A *pp2c* mutants.

ABA application to unstressed plants cannot duplicate the effect of low Ψ_w on Pro or osmoregulatory solute accumulation. Thus, these traits are controlled by low-Ψ_w-specific signaling, which is attenuated by the *HAI* PP2Cs. It is not without precedent to find differing effects of low Ψ_w versus exogenous ABA. For example, the use of ABA-deficient mutants has shown that during low Ψ_w, ABA is a promoter of root growth rather than an inhibitor (Sharp et al., 1994; Sharma et al., 2011). Interestingly, low-Ψ_w-induced *HAI* PP2C expression was largely dependent on ABA, suggesting that the *HAI* PP2Cs are a point of cross talk between ABA signaling and signaling directly responsive to low Ψ_w (Fig. 9).

Double and triple *hai* mutants did exhibit ABA hypersensitivity in postgermination responses to ABA, including inhibition of cotyledon emergence and root growth and induction of ABA-regulated genes. This is consistent with previous findings (Fujita et al., 2009; Antoni et al., 2012) that the *HAI* PP2Cs may participate in feedback regulation that reduces ABA sensitivity. However, these ABA sensitivity differences could only be seen in double and triple *HAI* PP2C mutants, and even then they were more similar to single mutants of the other clade A PP2Cs rather than the severe ABA hypersensitivity of *hab1-1abi1-2ahg3-2* or *hab1-1abi1-2abi2-2* (Rubio et al., 2009). An even more clear contrast between *HAI* PP2Cs versus other clade A PP2Cs is that double and triple *HAI* PP2C mutants were ABA insensitive in seed germination. Also, none of the *HAI* PP2C single, double, or triple mutants had the reduced ABA accumulation that we observed for *hab1-1* and *ahg3-3* and that was even more apparent in *abi1ahg3hab1* (Rubio et al., 2009). Thus, the combined data indicate that the *HAI* PP2Cs have a more prominent role in controlling Pro and osmoregulatory solute accumulation, which were clearly affected in *HAI* PP2C single mutants, rather than ABA sensitivity, which only differed in double or triple mutants.

The PYL interaction pattern of the HAI PP2Cs differed dramatically from that of the ABA signaling PP2C HAB1. The HAI PP2Cs had marked preference for interaction with monomer-type PYLs. We did not detect any interaction of the HAI PP2Cs with the dimeric ABA receptors PYR1, PYL1, and PYL2, although we do not exclude the possibility that these PYLs have limited ability to inhibit HAI PP2C activity, as suggested by in vitro assays (Antoni et al., 2012). The HAI PP2C interaction with PYL7 to -10 was unaffected or only moderately stimulated by added ABA. This is consistent with previous analysis of monomeric PYLs (Dupeux et al., 2011; Hao et al., 2011). The relatively ABA-independent PYL interaction of the HAI PP2Cs would seem consistent with their prominent roles in low-Ψ_w-specific signaling regulating Pro and osmoregulatory solute accumulation. The specific PYL interaction of the HAI PP2Cs and differences in their protein sequences compared with other clade A PP2Cs also suggest different specificity for dephosphorylation substrates (for further explanation, see below). HAI1 (as well as AIP1 and HAI3) may have relatively strong recognition of substrates in low-Ψ_w signaling but weaker recognition of substrates in ABA signaling (Fig. 9). The converse may be true for other clade A PP2Cs, which have strong effects on ABA sensitivity. Differing PP2C substrate specificities could be another basis for cross talk between low Ψ_w and ABA signaling (Fig. 9).

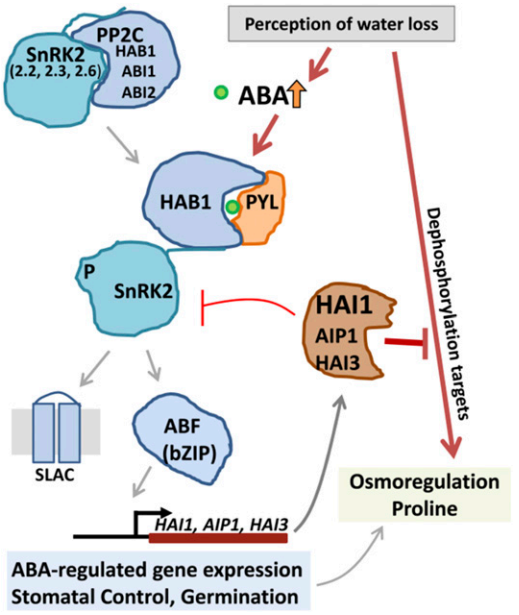

Figure 9. Model of HAI PP2C function during low-Ψ_w stress. Perception of water loss by the plant elicits ABA accumulation that causes the activity of clade A PP2Cs, such as HAB1, to be inhibited by ABA-stimulated PYL interaction. This, in turn, allows SnRK2 kinases to remain active and phosphorylate targets such as ABF transcription factors controlling ABA-dependent gene expression and guard cell slow anion channel (SLAC) ion channels influencing stomatal aperture. Expression of the *HAI* PP2Cs is induced in a partially ABA-dependent manner. Because of their highly induced expression and limited PYL interaction, the HAI PP2Cs can remain active and act in feedback regulation of ABA signaling, possibly through dephosphorylation of SnRK2s (Fujita et al., 2009; Antoni et al., 2012). However, the more prominent role of the HAI PP2Cs, particularly HAI1, is in attenuating the low-Ψ_w signaling controlling Pro and osmoregulatory solute accumulation. The specific PYL interaction pattern of HAI PP2Cs also suggests differences in their substrate recognition. Such differential recognition of substrates in low Ψ_w and ABA signaling may be a basis for cross talk between these two types of signaling mediated by the clade A PP2Cs.

Implications of the PYL Interaction Pattern of HAI PP2Cs

Recent studies have continued to add complexity and specificity to the core model of PP2C-ABA-PYL interaction. Examples include both the ABA-independent PP2C interactions of the monomeric PYLs (Dupeux

et al., 2011; Hao et al., 2011) as well as differences in PYL regulation of different clade A PP2Cs. For example, AHG1 lacks the critical Trp for interaction with PYL-bound ABA and thus is not PYL regulated (Dupeux et al., 2011; Antoni et al., 2012). Other clade A PP2Cs also differ in their PYL regulation both in the ABA concentration required for half-maximal inhibition of phosphatase activity (IC_{50}) and the effect of PYL-PP2C ratio on the ABA IC_{50} (Szostkiewicz et al., 2010). In general, higher PYL-PP2C ratios favored inhibition at low levels of ABA, while lower PYL-PP2C ratios allowed greater phosphatase activity. Antoni et al. (2012) observed that HAI1 activity could be inhibited to some extent by both dimeric and monomeric PYLs, at least when no other proteins were present to compete for interaction with the PP2C. However, the IC_{50} values for the dimeric PYLs were very high, indicating the HAI1 was resistant to inhibition by these PYLs. Although their study did not examine the full range of PYLs, the results were consistent with ours in that PYL5 and PYL8 were most effective in inhibiting HAI1 activity and gave the strongest interactions in our yeast two-hybrid experiments. The phosphatase activity assays of Antoni et al. (2012) were constructed with a 4:1 or 10:1 PYL:PP2C ratio, and it was pointed out that a 4:1 PYL:PP2C ratio gave a more realistic indication of PP2C regulation than a 100:1 PYL:PP2C ratio, which caused severalfold greater inhibition of phosphatase activity (Hao et al., 2011). Our data showing several hundred fold induction of *HAI1* expression at the same time that expression of its interacting PYLs was mostly down-regulated suggest that even a 4:1 PYL:HAI1 ratio may be unlikely to exist in vivo. It has also been demonstrated that HAB1 mutations that weakened its interaction with PYR1 allowed it to remain active and dephosphorylate the SnRK2 OST1 even in the presence of PYR1 and ABA concentrations that inhibited the activity of wild-type HAB1 (Dupeux et al., 2011). Wild-type HAI1 already has such weakened interaction. Thus, the combination of weak PYL interaction and high expression indicates that PYL regulation may not be a major determinant of HAI1 function at low Ψ_w. It was also suggested that the ABA-independent PP2C interactions of the monomeric PYLs were less effective in PP2C regulation than PP2C-ABA-PYL ternary complexes (Antoni et al., 2012). The strong interaction of the monomeric PYLs, particularly PYL7, with AIP1 and HAI3 observed in our experiments suggests that this may not always be the case. Phosphatase activity assays using the monomeric PYLs with AIP1 or HAI3 will be of interest for future studies.

This model of limited PYL regulation, which allows the HAI PP2Cs, particularly HAI1, to remain active during low Ψ_w, also suggests the importance of their dephosphorylation substrate proteins as regulators of the low-Ψ_w response. Structural studies have shown that PYLs and SnRK2 kinases mimic each other in their binding to PP2Cs (Soon et al., 2012). Thus, sequence differences in the HAI PP2Cs compared with other clade A PP2Cs (Supplemental Fig. S11) in and around the amino acids critical for PYL interaction may also indicate different specificity in their interactions with SnRK2s or other unidentified substrates. Other differences between the HAI PP2Cs compared with other clade A PP2Cs are found in the ABA box, which tethers some SnRK2s and PP2Cs (such as HAB1) to each other (Soon et al., 2012), and in the motif that determines SnRK3 interaction specificity of ABI1 and ABI2 (Ohta et al., 2003; Supplemental Fig. S11). These factors indicate that identifying dephosphorylation targets of the HAI PP2Cs will be key to understanding their unique function in low-Ψ_w signaling.

Unique Stress Physiology Function of the *HAI* PP2Cs

Osmoregulatory solute accumulation (osmotic adjustment) has been well studied by crop physiologists, who have found substantial genetic variability in this trait (Morgan, 1984, 1991). The increased solute accumulation of *hai1*, *aip1*, and *hai3* establish the *HAI* PP2Cs as one of the few molecular components known to regulate osmotic adjustment. Interestingly, one of the few other genes shown to regulate osmotic adjustment are group C mitogen-activated protein kinases of cotton (*Gossypium hirsutum*), whose overexpression could increase both Pro and osmoregulatory solute accumulation (Zhang et al., 2011). It is tempting to speculate that these phosphatases and kinases may directly antagonize each other at the molecular level.

Our data also raise the question of why the negative regulation mediated by the *HAI* PP2Cs is maintained when it may seem that maximizing osmotic adjustment and Pro would be a better drought-adaptive strategy that would emerge through natural selection. An explanation is suggested by the observation that *hai1-2* had opposing effects on a number of abiotic stress-associated genes (up-regulated in *hai1-2* relative to the wild type) and defense-related genes (down-regulated in *hai1-2*). Antagonism between ABA/abiotic stress signaling versus biotic stress/defense signaling is an emerging topic in plant-environment interaction (Yasuda et al., 2008; Huang et al., 2010; Kim et al., 2011). Fitness tradeoffs between pathogen defense and drought response would suggest that maximizing drought responses may not always be best for overall adaptation and that negative regulation, such as that mediated by HAI1 and other PP2Cs, is important to balance the two responses.

MATERIALS AND METHODS

Plant Material and Stress Treatments

T-DNA insertion lines of Arabidopsis (*Arabidopsis thaliana*) were obtained from the Arabidopsis Biological Resource Center, and primers used for genotyping are given in Supplemental Table S9. Double and triple mutants were generated by crossing *hai1-2* to *aip1-1* and then crossing *hai1-2aip1-1* to *hai3-1*. *hai3-1* was further analyzed by twice backcrossing to the wild type and selecting homozygous plants for Pro analysis. *hai1-2* was also used for transgenic complementation. The open reading frame of *HAI1* was amplified

(primers are given in Supplemental Table S9), cloned into pDONR207, transferred to pGWB442 (Nakagawa et al., 2007) to generate *35S:YFP-HAI1*, and transformed into *hai1-2*.

For seedling growth and stress treatments, sterilized seeds were plated on one-half-strength Murashige and Skoog medium with MES buffer (pH 5.7) but without added sugar. Plates were stratified for 4 d at 4°C, and the seedlings were grown by placing the plates vertically in a growth chamber (25°C, continuous light at 80–100 μmol photons m^{-2} s^{-1}). Seven-day-old seedlings were transferred to PEG-infused plates (Verslues et al., 2006) to impose low-Ψ_w stress. Alternatively, seedlings were transferred to plates containing NaCl or S(+)-ABA added to the medium after sterilization. For germination or cotyledon emergence assays, approximately, 100 seeds per genotype were sown on plates with or without S(+)-ABA, radicle emergence was scored after 4 d, and green cotyledon emergence was examined after 5 or 8 d.

For leaf water loss experiments, plants were grown under long-day conditions, and fully expanded rosette leaves were collected from 4-week-old plants and weighed over the course of 8 h to monitor water loss. For soil drying experiments, plants were grown under short-day conditions in a growth chamber, and the normal potting mixture was supplemented with 40% fine sand to facilitate even soil drying. Watering was stopped after 30 d of growth, and measurements were conducted over the subsequent 6 to 12 d of water withholding.

Physiological Assays

For Pro measurement, seedlings were collected 96 h after transfer to various Ψ_w treatments and analyzed by ninhydrin assay adapted to a 96-well plate format (Bates et al., 1973; Verslues, 2010). For Ψ_s measurement, seedling or leaf samples were frozen, macerated with a microfuge tube pestle, and centrifuged to pellet insoluble material. Ψ_s of the cell sap was measured using a Wescor Psypro system with c-52 sample chambers. Ψ_w of agar or soil medium collected at the same time was also measured. Seedling fresh weight was measured by weighing groups of six to 10 seedlings and calculating the per seedling weight. RWC was measured by detaching fully expanded leaves, weighing, floating on water for 9 to 10 h, reweighing, and drying overnight in a 60°C oven. RWC was calculated as (fresh weight − dry weight)/hydrated weight − dry weight) × 100.

ABA analysis was performed by extracting freeze-dried seedlings (50–200 mg fresh weight) in 80% methanol with 25 pmol of [D_6]ABA (Plant Biotechnology Institute) as an internal standard. Extracts were passed through a C_{18} solid-phase extraction cartridge (Supelco), evaporated to dryness, resuspended in diethyl ether:methanol (9:1), and derivatized by the addition of trimethylsilydiazomethane (Sigma). After derivatization, remaining trimethyldiazomethane was destroyed by the addition of 0.5 M acetic acid in hexane (Schmelz et al., 2003). The samples were then evaporated, resuspended in a small volume of ethyl acetate, injected onto a VF-14MS (Varian/Agilent) column, and analyzed by tandem mass spectrometry. Methanol chemical ionization was used to generate precursor ions (261 mass-to-charge ratio [m/z] for ABA and 267 m/z for [D_6]ABA). Daughter ions of 229 m/z (ABA) and 233 + 234 m/z ([D_6]ABA) were used for quantification (Müller et al., 2002). The ABA content of samples was quantified by a standard curve over 2 to 60 pmol of ABA prepared using the ratio of the 229 and 233 + 234 peak areas.

Gene Expression

Total RNA was extracted from control or low-Ψ_w-treated seedlings using RNeasy plant mini kits (Qiagen). Microarray analysis was conducted at the Affymetrix Gene Expression Service Laboratory of Academia Sinica using Arabidopsis ATH1 chips (Affymetrix) and standard protocols. Three biological replicates were used for all microarray analysis. Data were analyzed using GeneSpring software. A Benjamini-Hochberg corrected value of $P \leq 0.05$ and change greater than 1.5-fold were used to define genes differentially expressed between the wild type and *hai1-2*. Coexpression clustering was done using the Maccu toolbox (Lin et al., 2011) based on data of 3,800 slides downloaded from the NASCarrays database (Craigon et al., 2004). GO enrichment was computed using the TopGO elim method (Alexa et al., 2006) using GOBU with its MultiView plugin (Lin et al., 2006).

For quantitative RT-PCR analysis, RNA (typically 1 μg) from at least three independently collected samples was reverse transcribed using SuperScript III (Invitrogen). Quantitative RT-PCR was performed using the KAPA SYBR FAST qPCR kit (Kapa Biosystems), and fold change in expression was calculated by the comparative cycle threshold method following normalization based on *Actin8* expression. All primers used are given in Supplemental Table S9. The expression of Pro metabolism genes, *NCED3* and *COR15A*, was quantified using TaqMan probes and standard curves for each gene as described previously (Sharma and Verslues, 2010).

Yeast Two-Hybrid Analysis

Constructs for yeast two-hybrid analysis were prepared by PYL and PP2C amplification from either Col wild-type DNA (for *PYL*s lacking introns) or from full-length complementary DNA clones obtained from the Arabidopsis Biological Resource Center (Supplemental Table S9), cloning into pDONOR207, and transfer to yeast two-hybrid destination vectors by Gateway reaction. Yeast two-hybrid screening was performed using the ProQuest two-hybrid system (Invitrogen). Yeast strain MaV203a was transformed with pEXP32-HAI1 or ΔN HAI1 (lacking amino acids 1–104), ΔN AIP1 (lacking amino acids 1–118), or HAI3 or ΔN HAB1 (lacking amino acids 1–178; Santiago et al., 2009b) bait plasmid and pEXP22-PYL prey plasmid using the lithium acetate method. Primary transformants were selected for growth on Trp/Leu dropout plates. Trp-Leu^+ colonies were analyzed for β-galactosidase activity by colony-lift filter assay. Quantitation of interaction strength was performed using chlorophenol red-β-D-galactopyranoside (CPRG) assay following the manufacturer's instruction (Invitrogen). At least three different colonies per two-hybrid pair were grown in selective minimal medium overnight at 30°C and then were inoculated in 5 mL of yeast extract-peptone-adenine-dextrose (YPAD) medium with or without ABA until the A_{600} reached 1.0 to 1.5. Cells were collected and washed with cold water. The cell pellet was resuspended in 100 μL of buffer 1 (100 mM HEPES,154 mM NaCl, 4.5 μM L-Asp, 0.1 g L^{-1} bovine serum albumin, and 500 μL L^{-1} Tween 20, pH 7.25–7.30). Cells were broken using a bead beater and acid-washed 0.5-mm glass beads. The CPRG reaction was started by mixing lysed cells with 900 μL of buffer 2 (27.1 mg of CPRG in 20 mL of buffer 1), and A_{574} was measured.

For western-blot detection of fusion proteins in yeast, an overnight culture in selective medium was subcultured in YPAD for 4 to 6 h, and equal amounts of cells were collected based on A_{600}. Proteins were extracted (Kushnirov, 2000), separated by SDS-PAGE, electroblotted onto polyvinylidene difluoride membranes, probed with antibody against the GAL4 DNA-binding domain (Abcam) and horseradish peroxidase-conjugated secondary antibody (Abcam), and developed using TMA-6 reagent (Lumigen).

Statistical Analysis

Data were analyzed by ANOVA (using Sigma Plot 11) or *t* test as indicated in the text or figure legends.

Complete data from the microarray analysis of the Col wild type and *hai1-2* under control conditions and after 96 h of low-Ψ_w treatment are available in the National Center for Biotechnology Information Gene Expression Omnibus under accession number GSE35258.

Supplemental Data

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

Supplemental Figure S1. Clade A *PP2C* T-DNA lines.

Supplemental Figure S2. Low water potential-induced Pro accumulation of *HAI* PP2C double and triple mutants.

Supplemental Figure S3. Salt stress-induced Pro accumulation of *HAI* PP2C double and triple mutants.

Supplemental Figure S4. Osmotic potential and seedling fresh weight of *HAI* PP2C double and triple mutants after low water potential treatment.

Supplemental Figure S5. Wild-type (Col) and *hai1-2* plants during soil drying.

Supplemental Figure S6. Coexpression clusters formed from genes down-regulated in *hai1-2* relative to the Col wild type.

Supplemental Figure S7. Quantitative RT-PCR analysis of Pro metabolism genes shows similar expression in the wild type and *hai1-2*.

Supplemental Figure S8. Confirmation of ABA-hypersensitive germination in mutants of the ABA-signaling Clade A PP2Cs.

Supplemental Figure S9. ABA-induced Pro accumulation does not differ in *HAI* PP2C single mutants and is only slightly increased in the triple mutant.

Supplemental Figure S10. Low water potential-induced ABA accumulation of Clade A PP2C mutants.

Supplemental Figure S11. Alignment of the C-terminal regions containing the active site and PYL-interaction sites of the HAI PP2Cs, ABI1, ABI2, and HAB1 shows sequence differences which may affect PYL interaction and substrate specificity of the HAI PP2Cs.

Supplemental Figure S12. Additional yeast two-hybrid assays and western-blot assays with HAB1 and HAI1.

Supplemental Table S1. Genes up-regulated in Col wild-type seedlings at low water potential.

Supplemental Table S2. Genes down-regulated in Col wild-type seedlings at low water potential.

Supplemental Table S3. Genes up-regulated in *hai1-2* relative to the wild type in control (high water potential).

Supplemental Table S4. Genes down-regulated in *hai1-2* relative to the wild type in control (high water potential).

Supplemental Table S5. Genes up-regulated in *hai1-2* relative to the wild type at low water potential.

Supplemental Table S6. Genes down-regulated in *hai1-2* relative to the wild type at low water potential.

Supplemental Table S7. GO enrichment analysis of genes up-regulated in *hai1-2* relative to the wild type under either unstressed control or low water potential treatments.

Supplemental Table S8. GO enrichment analysis of genes down-regulated in *hai1-2* relative to the wild type under either unstressed control or low water potential treatments.

Supplemental Table S9. Primer sequences used in this study.

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