

Enhancement of Abscisic Acid Sensitivity and Reduction of Water Consumption in Arabidopsis by Combined Inactivation of the Protein Phosphatases Type 2C ABI1 and HAB1^{1[W]}

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Abscisic acid (ABA) plays a key role in plant responses to abiotic stress, particularly drought stress. A wide number of ABA-hypersensitive mutants is known, however, only a few of them resist/avoid drought stress. In this work we have generated ABA-hypersensitive drought-avoidant mutants by simultaneous inactivation of two negative regulators of ABA signaling, i.e. the protein phosphatases type 2C (PP2Cs) ABA-INSENSITIVE1 (ABI1) and HYPERSENSITIVE TO ABA1 (HAB1). Two new recessive loss-of-function alleles of ABI1, *abi1-2* and *abi1-3*, were identified in an Arabidopsis (*Arabidopsis thaliana*) T-DNA collection. These mutants showed enhanced responses to ABA both in seed and vegetative tissues, but only a limited effect on plant drought avoidance. In contrast, generation of double *hab1-1 abi1-2* and *hab1-1 abi1-3* mutants strongly increased plant responsiveness to ABA. Thus, both *hab1-1 abi1-2* and *hab1-1 abi1-3* were particularly sensitive to ABA-mediated inhibition of seed germination. Additionally, vegetative responses to ABA were reinforced in the double mutants, which showed a strong hypersensitivity to ABA in growth assays, stomatal closure, and induction of ABA-responsive genes. Transpirational water loss under drought conditions was noticeably reduced in the double mutants as compared to single parental mutants, which resulted in reduced water consumption of whole plants. Taken together, these results reveal cooperative negative regulation of ABA signaling by ABI1 and HAB1 and suggest that fine tuning of ABA signaling can be attained through combined action of PP2Cs. Finally, these results suggest that combined inactivation of specific PP2Cs involved in ABA signaling could provide an approach for improving crop performance under drought stress conditions.

The plant hormone abscisic acid (ABA) plays a crucial role in plant responses to several abiotic stresses such as drought, salt, and cold, as well as plant growth and development. In vegetative tissues, water stress produced by drought or high osmoticum treatment boosts ABA biosynthesis, leading to a variety of adaptive ABA-mediated responses such as stomatal closure and differential gene expression (Finkelstein et al., 2002; Nambara and Marion-Poll, 2005). ABA signaling in guard cells leads to stomatal closure, which occurs through rapid changes of ion fluxes and osmoreg-

ulation (Schroeder et al., 2001; Hetherington and Woodward, 2003). ABA regulation of the transpiration flow through stomatal pores is a crucial response of the plant to water deficit, as exemplified by the wilted phenotype of both ABA-deficient and ABA-insensitive mutants (Zeevaart and Creelman, 1988). Additionally, the ABA-dependent signaling pathway regulates stress-inducible gene expression, leading to a coordinated remodeling of gene expression that affects more than 1,000 genes of the plant transcriptome (Hoth et al., 2002; Seki et al., 2002; Takahashi et al., 2004).

Biochemical and genetic analyses have resulted in the identification of many elements of the ABA signal transduction pathway, although important pieces are still lacking. Recently, the RNA-binding protein FCA has been identified as an ABA-binding receptor with a singular role in flowering control, however, key responses to ABA such as inhibition of seed germination or stomatal response were not affected in the *fca-1* mutant (Razem et al., 2006). Accordingly, FCA appears to be an ABA receptor involved in controlling flowering time but additional ABA receptors must perform ABA perception. Putative candidates might be some plasma membrane receptors, such as RPK1, which is known to be involved in ABA signaling (Osakabe et al., 2005).

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Furthermore, in guard cells several studies have indicated the presence of intracellular ABA receptors (Allan et al., 1994; Schwartz et al., 1994; Schwarz and Schroeder, 1998; Levchenko et al., 2005).

It is well known that a variety of second messengers contribute to the transmission of the ABA signal, which includes Ca^{2+} , cADP-Rib, reactive oxygen species, nitric oxide, phosphoinositides, phosphatidic acid, and sphingosine 1-P (Schroeder and Hagiwara, 1989; Gilroy et al., 1990; McAinsh et al., 1990; Wu et al., 1997; Leckie et al., 1998; Jacob et al., 1999; Lemtiri-Chlieh et al., 2000; Pei et al., 2000; Allen et al., 2001; Ng et al., 2001; Neill et al., 2002; Guo et al., 2003). It is also known that phosphorylation/dephosphorylation events play a crucial role in ABA signaling, which involves a complex network of protein kinases and phosphatases as well as other signal transducers (for review, see Finkelstein et al., 2002). Finally, many transcriptional factors (TFs) of ABA-inducible genes are known. The TFs comprise ABA-responsive element (ABRE)-binding proteins (ABA-INSENSITIVE5 [ABI5]/ABF/AREB/AtbZIP family), ABI3/VP1/B3, ABI4/APETALA2, MYC, MYB, and HD-ZIP domain proteins (Giraudat et al., 1992; Suzuki et al., 1997; Finkelstein et al., 1998; Choi et al., 2000; Finkelstein and Lynch, 2000; Uno et al., 2000; Bensmihen et al., 2002; Himmelbach et al., 2002; Abe et al., 2003). Most of these TFs play a positive role in ABA signaling, but some of them function as repressors of ABA response (Himmelbach et al., 2002; Pandey et al., 2005; Song et al., 2005).

Genetic analyses of ABA signal transduction have identified both negative and positive regulators of ABA signaling (McCourt, 1999; Finkelstein et al., 2002). For instance, recessive mutations leading to ABA hypersensitivity were found in the *era1* (Cutler et al., 1996), *abh1* (Hugouvieux et al., 2001), *fry1* (Xiong et al., 2001b), *hypersensitive to ABA1* (*hab1*; Leonhardt et al., 2004; Saez et al., 2004), *sad1* (Xiong et al., 2001a), and *gcr1* (Pandey and Assmann, 2004) mutants. The intragenic revertants of *abi1-1* and *abi1-1R1* to *R7* also carry recessive mutations that lead to enhanced responsiveness to ABA (Gosti et al., 1999). Loss-of-function mutants generated by RNA interference for the *SOS3-like calcium-binding protein 5* and its interacting *protein kinase 3* were also hypersensitive to ABA (Guo et al., 2002). As loss of function of the above-mentioned genes leads to enhanced ABA responsiveness, their corresponding gene products must represent negative regulators of ABA signaling. On the other hand, recessive mutations leading to reduced ABA sensitivity have been identified in the *abi3* (Giraudat et al., 1992), *abi4* (Finkelstein et al., 1998), *abi5* (Finkelstein and Lynch, 2000), *ost1* (Mustilli et al., 2002), *rcn1* (Kwak et al., 2002), *rpkl1* (Osakabe et al., 2005), and the *rbhd/F* double mutants (Kwak et al., 2003). Therefore, these loci point out to positive regulators of ABA signal transduction.

Protein phosphatases type 2C (PP2Cs) were identified as components of ABA signaling pathway from pioneer work with the ABA-insensitive *abi1-1* and

abi2-1 mutants (Koornneef et al., 1984; Leung et al., 1994, 1997; Meyer et al., 1994; Rodriguez, et al., 1998). Currently, at least four Arabidopsis (*Arabidopsis thaliana*) PP2Cs, ABI1, ABI2, PP2CA, and HAB1 (formerly named AtP2C-HA), are known to regulate ABA signaling. Evidence on their role as negative regulators of ABA signaling has been provided by genetic approaches (Gosti et al., 1999; Merlot et al., 2001; Tahtiharju and Palva, 2001; Gonzalez-Garcia et al., 2003; Leonhardt et al., 2004; Saez et al., 2004; Kuhn, et al., 2006; Yoshida et al., 2006). For instance, the recessive T-DNA insertion mutant *hab1-1* shows ABA-hypersensitive inhibition of seed germination and enhanced ABA-mediated stomatal closure (Leonhardt et al., 2004; Saez et al., 2004). *HAB1* is broadly expressed in the plant and strongly induced by ABA (Leonhardt et al., 2004; Saez et al., 2004). Constitutive expression of *HAB1* under a 35S promoter led to reduced ABA sensitivity both in seeds and vegetative tissues, compared to wild-type plants (Saez et al., 2004).

In the case of *ABI1*, recessive alleles were isolated as intragenic revertants of the originally dominant *abi1-1* mutation, and named *abi1-1R1* to *R7* (Gosti et al., 1999). Therefore, these recessive alleles, in addition to the original Gly-180 Asp mutation, carry a second mutation that abolishes the dominant character of the *abi1-1* mutation. The same approach was applied to the dominant mutant *abi2-1*, leading to the identification of the recessive *abi2-1R1* allele (Merlot et al., 2001). It cannot be excluded that intragenic revertants of *abi1-1* still retain some activity (not necessarily an enzymatic one) in the corresponding gene products, even though their in vitro protein phosphatase activity was shown to be negligible (Gosti et al., 1999). Thus, we were interested in the isolation of direct knockout alleles of *ABI1*, namely *abi1-2* and *abi1-3*, to conclusively clarify its role in ABA signaling. Furthermore, double knockout mutants in PP2Cs have not yet been generated and we have analyzed *hab1 abi1* double loss-of-function mutants here to determine whether these PP2Cs are strictly redundant or additive in their functions. Phenotypic analysis of *abi1-2* and *abi1-3* provided new data regarding the role of *ABI1* in ABA-induced stomatal closure, transpiration, and ABA-mediated regulation of gene expression. The phenotypic effect on ABA signaling observed in single *hab1-1*, *abi1-2*, and *abi1-3* mutants was notably reinforced in double mutants, which showed both enhanced responsiveness to ABA and drought avoidance. Thus, these results show a new biotechnological approach to increase plant drought avoidance, i.e. the combined inactivation of PP2Cs involved in ABA signaling.

RESULTS

Identification and Characterization of Knockout Alleles of *ABI1*

Two T-DNA insertion mutants of *ABI1* were identified in the Salk collection (Columbia [Col] background),

corresponding to donor stock numbers SALK_72009 and SALK_76309, and they were named *abi1-2* and *abi1-3*, respectively. Homozygous individuals were identified by PCR and Southern-blot analyses (data not shown). Sequencing of the T-DNA flanking region in *abi1-2* showed that the insertion was localized two nucleotides upstream of the ATG start codon (Fig. 1A). In the case of *abi1-3*, the T-DNA insert was localized 546 nucleotides downstream from the ATG start codon (Fig. 1A). Both T-DNA insertions severely impaired *ABI1* expression, based on reverse transcription (RT)-PCR (Fig. 1B) and quantitative RT-PCR (qRT-PCR) analyses (Fig. 1C). Expression of *HAB1* and *ABI1* in wild type was quite similar to that in *abi1-2/abi1-3* and *hab1-1* mutant backgrounds, respectively (Fig. 1C).

Progeny of both *abi1-2* and *abi1-3* homozygous individuals was harvested and different analyses to test their sensitivity to ABA were performed. First, the sensitivity of the mutants to inhibition of seed germination by ABA was analyzed (Fig. 2A). In the absence of exogenous ABA, *abi1-2* and *abi1-3* mutant seeds showed a germination ratio similar to wild type. However, in the presence of exogenous ABA, both the *abi1-2* and *abi1-3* mutants showed ABA-hypersensitive inhibition of seed germination (Fig. 2A; Supplemental Fig. 1). F₁ seeds that were hemizygous for the T-DNA insertion present either in *abi1-2* or *abi1-3* showed wild-type germination on 0.5 μ M ABA. In the next generation, F₂ seeds showed an ABA-hypersensitive phenotype in approximately a 1:3 proportion (112 hypersensitive:313 wild type, $\chi^2 = 0.42$, $P > 0.5$ for *abi1-2*; 121 hypersensitive:319 wild type, $\chi^2 = 1.4$, $P > 0.1$ for *abi1-3*). Finally, F₂ ABA-hypersensitive seedlings showed linkage between the ABA-hypersensitive phenotype and the presence of a homozygous T-DNA insertion in *ABI1* as determined by PCR analysis ($n = 40$). Taken together, these data indicate that both the *abi1-2* and *abi1-3* mutations are recessive and segregate as a single nuclear locus linked to the T-DNA insertion present in the *ABI1* gene. The ABA inhibitory concentration to achieve 50% inhibition (IC₅₀) of seed germination was approximately 2-fold lower for *abi1-2* and *abi1-3* than for the wild type (0.35, 0.37, and 0.67 μ M ABA, respectively; Supplemental Fig. 1).

ABA plays a critical role promoting inhibition of both seed germination and early seedling growth under high osmoticum (Gonzalez-Guzman et al., 2002). Thus, whereas ABA-hypersensitive mutants are generally more sensitive than wild type to the inhibition of seed germination promoted by osmotic stress (Saez et al., 2004), both ABA-deficient and ABA-insensitive mutants are more tolerant to osmotic stress at this stage (Leon-Kloosterziel et al., 1996; Gonzalez-Guzman et al., 2002). Dose-response analyses of germination and early growth in media supplemented with increasing concentrations of NaCl or mannitol were performed for *abi1-2* and *abi1-3* (Fig. 2, B and C). Both *abi1-2* and *abi1-3* mutants showed higher inhibition of germination and early growth by osmotic stress than wild-type seeds.

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Generation and Analysis of *hab1-1 abi1-2* and *hab1-1 abi1-3* Double Mutants

Sequence similarity analysis of the Arabidopsis PP2C gene family reveals a branch composed by four members: *ABI1*, *ABI2*, *HAB1*, and *HAB2* (Saez et al., 2004). *ABI1* and *HAB1* appear to play a predominant role over *ABI2* and *HAB2*, respectively, according to their mRNA expression levels and mutant phenotype (Merlot et al., 2001; Leonhardt et al., 2004; Saez et al., 2004; Kuhn, et al., 2006; A. Saez, N. Robert, J. I. Schroeder, and P. L. Rodriguez, unpublished data). Double loss-of-function phenotypes in plant PP2Cs have not yet been analyzed in knockout mutants. To unravel a possible functional redundancy between *ABI1* and *HAB1*, we decided to generate double mutant lines that contained knockout alleles of both genes. To this end we crossed the previously described

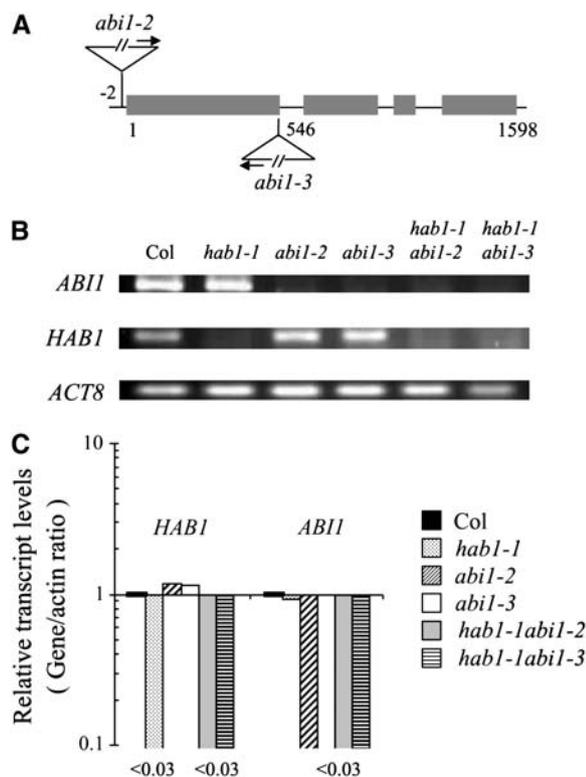


Figure 1. Map of *abi1-2* and *abi1-3* mutants. *ABI1* and *HAB1* transcript levels in wild type, *hab1-1*, *abi1-2*, *abi1-3*, and double *hab1-1 abi1-2/abi1-3* mutants. A, Scheme of the *ABI1* gene and localization of the T-DNA insertions in *abi1-2* and *abi1-3* mutants. The numbering begins at the ATG translation start codon. The T-DNA left border primer (LBpROK2) that was used to localize the T-DNA insertion is indicated by an arrow. B, RT-PCR analysis shows absence of full-length transcripts of *ABI1* or *HAB1* in genotypes containing either the *abi1-2/abi1-3* or *hab1-1* alleles, respectively. PCR reactions were performed as indicated in "Materials and Methods" and amplification of β -actin-8 was used as control. Samples were taken for analysis after 25 PCR cycles. C, Expression of *HAB1* and *ABI1* in wild type was similar to that in *abi1-2/abi1-3* and *hab1-1* mutants, respectively.

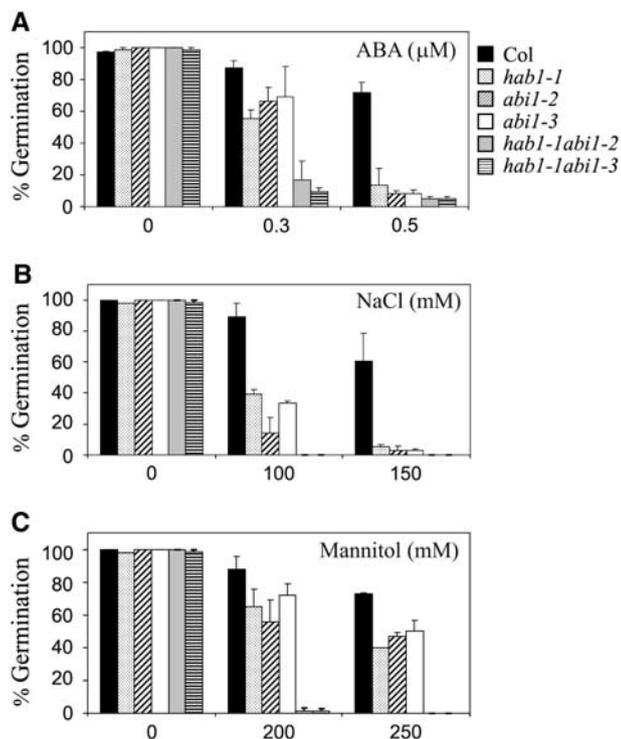


Figure 2. ABA-hypersensitive germination inhibition of *hab1-1*, *abi1-2*, *abi1-3*, and double *hab1-1 abi1-2/hab1-1 abi1-3* mutants as compared to wild-type seeds. A to C, Percentage of seeds that germinated and developed green cotyledons in the presence of the indicated concentrations of ABA, NaCl, and mannitol. Approximately 200 seeds of each genotype were sowed on each plate and scored for germination and early growth 10 d later. Values are averages \pm SD for three independent experiments.

hab1-1 mutant with either *abi1-2* or *abi1-3*. PCR (data not shown) and RT-PCR analyses (Fig. 1B) of the resulting F₂ population allowed the identification of *hab1-1 abi1-2* and *hab1-1 abi1-3* double mutants, and their response to ABA was analyzed in germination, growth, and transpiration assays.

Analysis of germination and early seedling growth in media supplemented with 0.3 μM ABA indicated an enhanced responsiveness to ABA of the double mutants as compared to the single parental mutants (Fig. 2A; Supplemental Fig. 1). Thus, the IC₅₀ of ABA in seed germination was 0.18 μM for the double mutants versus 0.35 and 0.37 μM for *abi1-2* and *abi1-3*, respectively. In agreement with this result, the double mutants were particularly sensitive to inhibition of germination and early growth promoted by both NaCl and mannitol (Fig. 2, B and C). Thus, a concentration of 100 mM NaCl practically abolished germination of the double mutants, whereas 15% to 40% germination was still observed in the single parental mutants (Fig. 2B). Likewise, 200 mM mannitol leads to almost complete inhibition of germination for the double mutants, whereas more than 50% germination is still observed in the single parental mutants (Fig. 2C).

ABA has an inhibitory effect on plant growth when the medium is supplemented with micromolar concentrations of the hormone. For instance, the ABA-insensitive mutants *abi1-1* and *abi2-1* and 35S:*HAB1* plants show ABA-resistant growth compared to wild-type plants (Leung et al., 1994, 1997; Meyer et al., 1994; Rodriguez, et al., 1998; Saez et al., 2004). In contrast, the recessive *abi1-1R1* to *R7* alleles were more sensitive to ABA inhibition of root growth than Landsberg *erecta* wild type (Gosti et al., 1999). Figure 3 shows that both *abi1-2* and *abi1-3* displayed enhanced sensitivity to ABA-mediated growth inhibition than wild-type plants. After 10 d in 10 μM ABA, both *abi1-2* and *abi1-3* plants showed yellowing and impaired growth of both leaves and roots. Under these conditions, the *hab1-1* mutant also showed reduced growth as compared to wild-type plants, although growth was inhibited less in *hab1-1* than in *abi1-2* and *abi1-3* mutants (Fig. 3). Finally, both double mutants showed a dramatic growth inhibition in medium supplemented with 10 μM ABA, and they were markedly more sensitive to ABA than the single parental mutants (Fig. 3).

Enhanced ABA-Induced Stomatal Closing and Reduced Water Loss of the *hab1-1 abi1-2* and *hab1-1 abi1-3* Double Mutants

ABA signaling, by regulating stomatal aperture, plays a crucial role to reduce water loss under water shortage. Different analyses were performed to evaluate responses in wild type and the different mutant backgrounds (Fig. 4). Thus, short-term water-loss assays were performed by evaluating the decline in fresh weight of detached leaves (Verslues et al., 2006). The single loss-of-function *abi1-2* and *abi1-3* mutants, as well as *hab1-1*, did not exhibit significant differences in the transpiration rate of detached leaves compared to wild type (Fig. 4A). In contrast, combined inactivation of *HAB1* and *ABI1* resulted in a phenotype of reduced water loss in both double mutants (Fig. 4A).

To further analyze stomatal responses to ABA in the mutants, direct measurements of stomatal closing were performed (Fig. 4B). ABA-induced stomatal closing was assayed in the single *abi1-2* and *hab1-1* mutants, as well as in the double mutant *hab1-1 abi1-2* (Fig. 4B). Stomatal aperture measurements indicated that *abi1-2*, *hab1-1*, and double mutant *hab1-1 abi1-2* were hypersensitive to ABA-induced stomatal closing in the range of 10 to 100 nM ABA. Moreover, the response of the double mutant *hab1-1 abi1-2* to 10 nM ABA was more sensitive as compared to the single parental mutants (Fig. 4B). Similar results to those obtained for *abi1-2* and double mutant *hab1-1 abi1-2* were obtained for *abi1-3* and double mutant *hab1-1 abi1-3*, respectively (Supplemental Fig. 2).

The *era1*, *abh1*, and *gcr1* mutants display enhanced ABA-induced stomatal closing and reduced water loss as compared to wild-type plants (Pei et al., 1998; Hugouvieux et al., 2001; Pandey and Assmann, 2004).

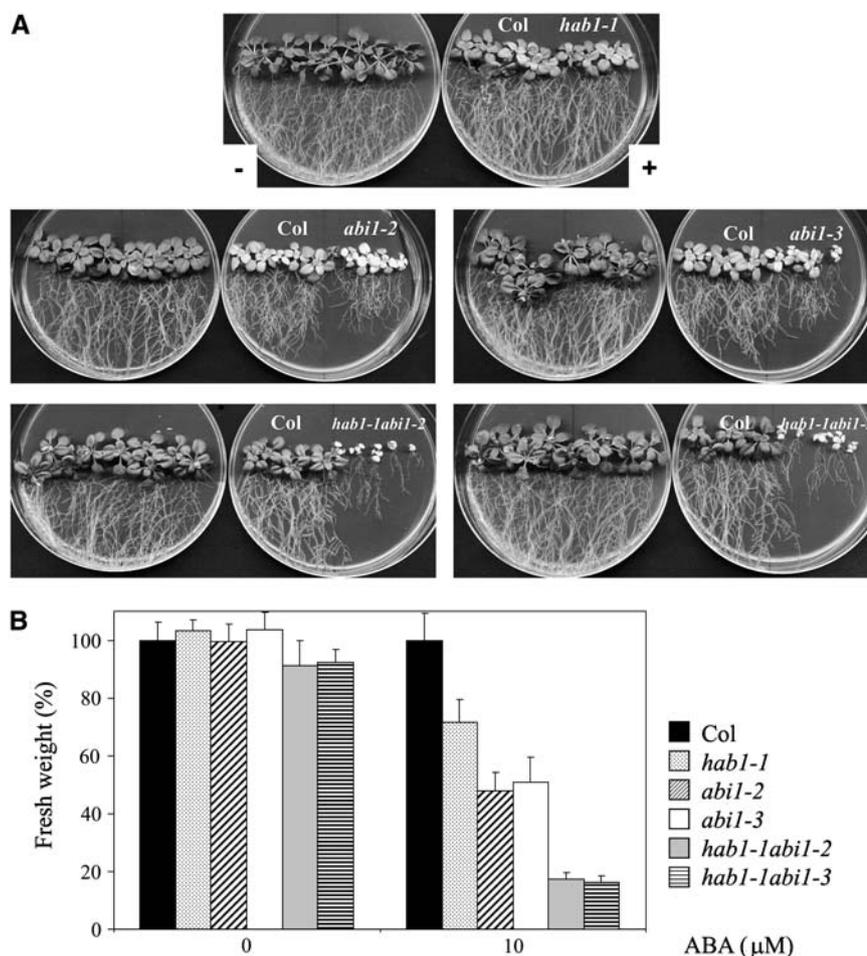


Figure 3. ABA-hypersensitive growth inhibition of *hab1-1*, *abi1-2*, *abi1-3*, and double *hab1-1 abi1-2/hab1-1 abi1-3* mutants as compared to wild-type plants. A, Growth of the different mutants and wild type in medium supplemented (+) or not (–) with 10 μM ABA. The photographs were taken after 12 d of the transfer of 5-d-old seedlings from Murashige and Skoog medium to plates lacking or containing 10 μM ABA. B, Percentage of fresh weight from the different mutants as compared to wild type. The percentage was calculated with respect to the fresh weight of wild type in Murashige and Skoog medium either lacking or containing 10 μM ABA. Fresh weight of wild type was reduced by 35% in plates supplemented with ABA as compared to medium lacking ABA. Values are averages \pm SD ($n = 30$).

Therefore, we examined water loss of the different genetic backgrounds described here. Water-loss data were obtained, under greenhouse conditions, after exposing 21-d-old plants to drought stress by completely terminating irrigation and minimizing soil evaporation. Figure 4D shows that after 14 d without watering, wild-type plants wilted and many rosette leaves yellowed. In contrast, *hab1-1 abi1-2* and *hab1-1 abi1-3* double mutant plants did not show symptoms of wilting and they had turgid green rosette leaves. A limited improvement was observed under these conditions in single mutants (Fig. 4D), although far from the phenotype observed in the double mutants. Water loss was estimated by comparing fresh and turgid weight of rosette leaves after 12 d without watering (Fig. 4C). Under these experimental conditions, where the plants were submitted to a long period of drought, the single *hab1-1*, *abi1-2*, and *abi1-3* mutants showed a reduced water loss as compared to wild type (Fig. 4C). Detached-leaf water-loss assays are likely not sensitive enough as to detect such variations (Kuhn et al., 2006), which are apparent after long periods of drought. Thus, whereas wild-type plants exhibited a marked water loss under these conditions, the ABA-hypersensitive mutants exhibited a reduced water loss, partic-

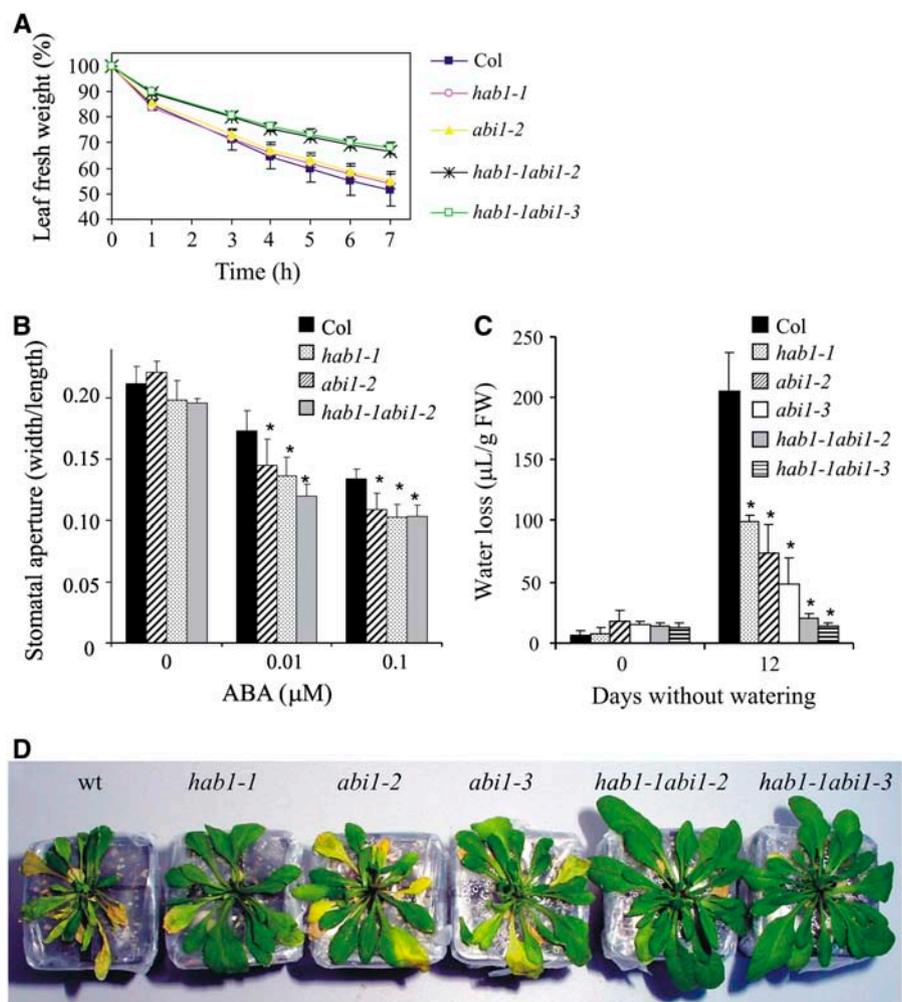
ularly in the case of the *hab1-1 abi1-2* and *hab1-1 abi1-3* double mutants.

Enhanced Expression of ABA-Inducible Genes in PP2C Mutants as Compared to Wild Type

The effect of the isolated single and double *hab1* and *abi1* loss-of-function mutations was analyzed on ABA-regulated gene expression. To this end, we used qRT-PCR to analyze the expression of the ABA- and drought-responsive *RAB18*, *P5CS1*, *RD29B*, *KIN1*, *RD29A*, and *RD22* genes, in wild type, single, and double mutants. These gene markers have been widely used to monitor the ABA and stress response pathways in plants (Kurkela and Franck, 1990; Lang and Palva, 1992; Yamaguchi-Shinozaki and Shinozaki, 1994; Strizhov et al., 1997; Abe et al., 2003). In general, in the absence of ABA or stress treatments, these gene markers show a low expression, which is strongly up-regulated in response to the inductive signal.

Interestingly, in the absence of exogenous ABA treatment, the double *hab1-1 abi1-2* and *hab1-1 abi1-3* mutants showed approximately 2-fold higher mRNA levels of some gene markers (*RAB18*, *RD29A*, and *RD29B*) as compared to Col wild type (Table I). In the

Figure 4. Reduced water loss of double *hab1-1 abi1-2/hab1-1 abi1-3* mutants as compared to wild type or single parental mutants. A, Detached-leaves water-loss assays show reduced water loss in double *hab1-1 abi1-2/hab1-1 abi1-3* mutants. Five leaves per individual at the same developmental stage and size from 21-d-old plants were excised and fresh weight was determined after submitting the leaves to the drying atmosphere of a flow laminar hood ($n = 4$). Results for *abi1-2* and *abi1-3* were almost identical. B, ABA-induced stomatal closing is ABA hypersensitive in *hab1-1*, *abi1-2*, and double mutant *hab1-1 abi1-2* as compared to wild-type plants. Stomatal apertures were measured 2 h and 30 min after addition of 0.01 or 0.1 μM ABA. Data represent the average of three independent experiments \pm SEM ($n = 30\text{--}40$ stomata per experiment). C, Quantification of water loss in 5-week-old plants after 12 d without watering. Data shown are the average amounts of water loss measured in 10 leaves ($\mu\text{L/g}$ fresh weight) collected from four different plants. Asterisks in B and C indicate $P < 0.01$ (Student's t test) when data was compared from mutant and wild type. D, Enhanced drought tolerance of double *hab1-1abi1-2/hab1-1 abi1-3* mutants with respect to wild type or single parental mutants. Photograph was taken 14 d after water was withheld. Shoot was cut to better show the effect of drought on rosette leaves.



case of single mutants and under control conditions, only the *RD29B* marker was 2-fold up-regulated in all the single mutants. Upon ABA treatment, as a general trend, induction by ABA was higher in the mutants than in wild type. This enhanced response to ABA was particularly apparent in the double mutants for gene markers that contain ABRE but no typical drought-responsive element (DRE) at the promoter, such as *RAB18*, *RD29B*, and *P5CS1* (between 4- and 8-fold higher expression level than wild type). Gene markers that contain both DRE and ABRE elements *KIN1* and *RD29A*, were also hyperinduced by ABA in the double mutants, although to a lower level (2- to 3-fold). Finally, ABA-mediated induction of *RD22*, which lacks both ABRE and DRE consensus sequences at its promoter, was also up-regulated.

DISCUSSION

In this work, we report the identification and characterization of two new *ABI1* recessive alleles, *abi1-2* and *abi1-3*, as well as *hab1-1 abi1-2* and *hab1-1 abi1-3* double mutants. The knockout *abi1-2* and *abi1-3* mu-

tants (Col background) showed enhanced ABA sensitivity in germination and growth assays, which is in agreement with previous results reported for intragenic revertants of *abi1-1* (Landsberg *erecta* background). ABA-induced stomatal closing was also ABA hypersensitive in *abi1-2* and *abi1-3* (Supplemental Fig. 2) in the range of 10 to 100 nM, in contrast to the recessive *abi1-1R4* allele, which showed a wild-type response at 100 nM ABA (Merlot et al., 2001). This discrepancy might be due to the different genetic background of each mutant or might reflect that *abi1-1R4* is not a knockout mutation. In spite of the enhanced response to ABA in stomatal closure assays, water-loss measurements in detached-leaf assays did not reveal significant differences with respect to wild type for single mutants. This may be due to the finding that detached-leaf water-loss assays to a degree reflect differences in stomatal apertures of wild type compared to a mutant at the beginning of drought experiments rather than later wilting-induced signaling events (Kuhn et al., 2006). In intact plants after a longer drought period, both *abi1-2* and *abi1-3* showed reduced water loss as compared to wild type (Fig. 4C). Finally, both *abi1-2* and *abi1-3* showed an enhanced up-regulation of some

Table 1. Enhanced expression of ABA-inducible genes in PP2C mutants with respect to wild type

Numbers indicate the induction level of the stress-responsive genes under mock or ABA treatment (10 μM for 3 h) in wild type and mutants. Values are the expression level reached in each mutant genotype with respect to the wild type (value 1). qRT-PCR analyses were made in triplicate on RNA samples obtained from mock-treated plants or plants treated once with 10 μM ABA.

	Genotype					
	<i>RAB18</i>	<i>KIN1</i>	<i>RD22</i>	<i>P5CS1</i>	<i>RD29a</i>	<i>RD29b</i>
Mock						
Col	1	1	1	1	1	1
<i>hab1-1</i>	0.9	0.8	1.5	0.9	2.7	2.0
<i>abi1-2</i>	1.1	1.0	1.4	1.1	1.3	2.6
<i>abi1-3</i>	0.8	0.7	1.1	0.8	1.0	2.6
<i>hab1-1 abi1-2</i>	2.1	1.6	2.1	1.7	2.0	2.5
<i>hab1-1 abi1-3</i>	2.3	1.2	1.6	1.9	2.0	2.7
ABA treatment						
Col	1	1	1	1	1	1
<i>hab1-1</i>	2.6	1.7	2.5	3.0	3.5	2.1
<i>abi1-2</i>	3.7	1.6	1.7	2.6	1.7	2.4
<i>abi1-3</i>	2.7	1.5	1.5	2.0	1.5	1.3
<i>hab1-1 abi1-2</i>	6.0	2.7	3.0	6.4	2.2	3.9
<i>hab1-1 abi1-3</i>	8.6	3.6	3.1	6.0	2.2	4.9

ABA- and drought-inducible genes compared to wild type, although to a modest level (1.5- to 3-fold). In general, a similarly enhanced response to ABA was observed in the *hab1-1* mutant, except that ABA-mediated inhibition of growth was stronger in both *abi1-2* and *abi1-3* than *hab1-1*, indicating that *ABI1* plays a predominant role in this particular response to ABA. Finally, these phenotypes conclusively indicate that *ABI1* is a global negative regulator of ABA signaling. We speculate that the reduced sensitivity to ABA observed in the dominant *abi1-1* allele might be due to the formation of an inactive complex between *abi1-1* and one of its substrates (Gosti et al., 1999), which might be a master positive regulator of ABA signaling. In both *abi1-2* and *abi1-3* recessive mutants the putative target of *ABI1* might be hyperactive in response to ABA; conversely, it would be inactivated by the effect of the *abi1-1* dominant allele.

Previous studies have not analyzed double knockout mutants in plant PP2Cs. An *abi1-1R4 abi2-1R1* double mutant was more responsive to ABA than the single parental mutants (Merlot et al., 2001). Combined inactivation of *HAB1* and *ABI1* in the *hab1-1 abi1-2* and *hab1-1 abi1-3* double mutants led to an additive ABA hypersensitivity compared to the single parental mutants. Thus, the IC_{50} for ABA-mediated inhibition of germination was 2-fold lower in the double mutants than in single parental mutants. The double mutants were also more sensitive than single parental mutants to inhibition of germination and early growth mediated by osmotic stress. Imposing osmotic stress at the seedling stage leads to increased ABA biosynthesis and consequently to early growth arrest (Lopez-Molina et al., 2001; Gonzalez-Guzman et al., 2004). Thus, whereas in adult plants ABA plays a

crucial role to coordinate the various aspects of the low water potential response to allow plant survival, in seeds and seedlings ABA action is mainly focused to prevent germination and to arrest seedling growth. Interestingly, lowering the osmotic potential of the media by using 200 mM mannitol (-0.5 MPa) had a limited effect on wild type or single mutants, but practically abolished early growth of the double mutants (Fig. 2C). According to the dramatic effect of the combined loss-of-function phenotype, *ABI1* and *HAB1* must cooperate to negatively regulate ABA signaling at the seed and seedling stage. Another PP2C, *PP2CA*, was recently shown to strongly and negatively regulate ABA signaling during germination (Kuhn, et al., 2006; Yoshida et al., 2006). The ABA-mediated seed germination phenotype of *pp2ca* or *hab1-1 abi1-2/hab1-1 abi1-3* mutants was apparent even though *HAB1* and *ABI1*, or *PP2CA*, respectively, were functional. Therefore, at least two branches of ABA signaling (or not completely redundant functions of these proteins) appear to exist during seed germination, and the impairing of any of them leads to strong ABA hypersensitivity.

In addition to enhanced ABA-mediated inhibition of seed germination, vegetative responses to ABA were superinduced in the double mutant compared to single parental mutants. For instance, inhibition of growth upon prolonged culture in medium supplemented with ABA was particularly dramatic in *hab1-1 abi1-2* and *hab1-1 abi1-3* double mutants. Transpiration water loss was also noticeably reduced in the double mutants, either measured as detached-leaf assays or after a long period of drought. Finally, ABA-inducible gene expression was notably up-regulated in the double mutants compared to single parental mutants, particularly for those stress-responsive genes mostly regulated through an ABA-dependent pathway, such as *RAB18*, *RD29B*, and *P5CS1*. Taken together, these results indicate partially overlapping functions for *HAB1* and *ABI1* as negative regulators of ABA signaling, although a predominant role for *ABI1* in growth control can be deduced from the ABA-mediated growth-inhibition phenotype observed in *abi1-2* and *abi1-3*. Additionally, these results reveal fine modulation of ABA signaling through the combined action of *HAB1* and *ABI1* and suggest that different degrees of ABA sensitivity can be engineered in plants through PP2C modulation of the ABA signal transduction pathway.

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 than wild type (Iuchi et al., 2001; Qin and Zeevaart, 2002). Priming of ABA biosynthesis can be obtained by direct overexpression of 9-cis-epoxycarotenoid 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). Alternatively, mutants affected in ABA signal transduction might also show an enhanced ABA response leading to stress-tolerant phenotypes.

Many examples of ABA-hypersensitive mutants have been reported (Finkelstein et al., 2002); however, in spite of the critical role of ABA to coordinate plant response to drought, a general correlation between enhanced response to ABA and drought tolerance has not been well established. Thus, although some mutants (i.e. *era1*, *abh1*, and *gcr1*) with enhanced response to ABA have been shown to cause reduced water consumption (Pei et al., 1998; Hugouvieux et al., 2001; Pandey and Assmann, 2004), many examples of mutants that do not match this assertion are known. For instance, the *fry1* and *sad1* mutants, which show ABA-hypersensitive inhibition of seed germination and superinduction of ABA-responsive genes, have compromised tolerance to drought stress (Xiong et al., 2001a, 2001b). Likewise, the *calcineurin B-like 9*, the *calcineurin B-like-interacting protein kinase*, and the *APETALA2-like ABA repressor 1* mutants, which display ABA hypersensitivity and enhanced expression of ABA signaling genes, do not correlate with stress-tolerance phenotypes (Kim et al., 2003; Pandey et al., 2004, 2005). Therefore, superinduction of ABA- and stress-inducible genes in ABA-hypersensitive mutants does not appear to be sufficient to induce drought avoidance. A differential feature of the *era1*, *abh1*, and *gcr1*, as well as *hab1-1 abi1-2/hab1-1 abi1-3* double mutants is an enhanced response to ABA in stomata and reduced water loss. Thus, an important consideration for engineering drought avoidance by enhancing ABA responses may include amplifying the molecular mechanisms through which ABA closes stomata. Prospecting of fully or partially sequenced plant genomes from other plants than *Arabidopsis* reveals the presence of gene products that are likely orthologous to the PP2Cs involved in ABA signaling in *Arabidopsis*, such as ABI1 and HAB1. Therefore, based on the results presented here, we suggest that silencing in crop plants of genes encoding PP2Cs with similar roles to ABI1 and HAB1 may provide a new biotechnological approach to enhance drought avoidance mechanisms.

A major advance in the study of ABA effect on stomatal closure and opening has been recently reported by Mishra et al. (2006). This work shows that ABA signaling bifurcates at ABI1 and the heterotrimeric G-protein α -subunit GPA1 to regulate ABA-mediated stomatal closure and inhibition of stomatal opening. In this work, an *abi1* knockout line (*abi1-ko*, corresponding to SALK_076309, here named *abi1-3*) was used to show a genetic interaction with the *phospholipase D α 1* mutant (*pld α 1*). Thus, whereas the single mutant *pld α 1* abolished both ABA promotion of stomatal closure and ABA inhibition of stomatal closure, the double mutant *pld α 1 abi1-ko* remained insensitive to ABA in the ABA inhibition of stomatal closing response, but was sensitive to ABA for promotion of stomatal closure. This result suggests that inhibition of stomatal opening by ABA is not governed through

ABI1, whereas ABI1 inhibits ABA promotion of stomatal closure. The results further suggest that PLD α 1 is not needed for ABA-induced stomatal closing when ABI1 is deleted. These findings are interesting in light of these and other recent findings that several PP2Cs function as negative regulators of ABA signaling (Leonhardt et al., 2004; Saez et al., 2004; Kuhn et al., 2006; Yoshida et al., 2006), but deletion of the ABI1 PP2C is sufficient to restore PLD α 1-independent ABA-induced stomatal closing in *pld α 1* (Mishra et al., 2006). Finally, we show here that the *abi1-2* and *abi1-3* knockout lines show enhanced ABA-induced stomatal closing. The fact that the *abi1-3* line reported by Mishra et al. (2006) did not show an ABA-hypersensitive phenotype in the stomatal closure response can likely be explained because a high dose (50 μ M) of ABA was assayed.

MATERIALS AND METHODS

Plant Material and Growth Conditions

Arabidopsis (*Arabidopsis thaliana*) plants were routinely grown under greenhouse conditions in pots containing a 1:3 vermiculite-soil mixture. For in vitro culture, seeds were surface sterilized by treatment with 70% ethanol for 20 min, followed by commercial bleach (2.5% sodium hypochlorite) containing 0.05% 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. Then, seeds were sowed on Murashige and Skoog (1962) plates composed of Murashige and Skoog basal salts, 0.1% 2-[N-morpholino]ethanesulfonic acid, 1% agar, and 1% Suc. The pH was adjusted to 5.7 with potassium hydroxide before autoclaving. Plates were sealed and incubated in a controlled environment growth chamber at 22°C under a 16-h light, 8-h dark photoperiod at 80 to 100 μ E m⁻² s⁻¹.

Mutant Identification by PCR Screening

Two lines containing a single T-DNA insertion in *ABI1* were identified in the SALK T-DNA collection (SALK_72009 and SALK_76309; Alonso et al., 2003) and obtained from the Nottingham Arabidopsis Stock Center (<http://nasc.nott.ac.uk>). To identify individuals homozygous for the T-DNA insertion, genomic DNA was obtained from kanamycin-resistant seedlings and submitted to PCR genotyping using the following *ABI1* primers: line SALK_72009, 5'-AGGAAACCCTTATTGAAATTC and 5'-CTCTGTCTGCTGATCATCT; line SALK_76309, 5'-CCGGCCCTCGAGATGATCAGCAGAACAGAGAGT and 5'-CCGGCCCTCGAGTCAGTCAAGGGTTTGTCT. As T-DNA left border primer of the pROK2 vector, we used LBpROK2 (5'-GCCGATTTCGGA-ACCACCATC).

To generate the *hab1-1 abi1-2* and *hab1-1 abi1-3* double mutants, we transferred pollen of either *abi1-2* or *abi1-3* to the stigmas of emasculated flowers of *hab1-1*. The resulting F₂ individuals were genotyped by PCR for the presence of homozygous *hab1-1* (Saez et al., 2004), *abi1-2*, and *abi1-3* alleles (see above).

Germination Assays

To measure ABA sensitivity, seeds were plated on solid medium composed of Murashige and Skoog basal salts, 1% Suc, and increasing concentrations of ABA. To determine sensitivity to inhibition of germination by high osmoticum the medium was supplemented with increasing concentrations of either sodium chloride or mannitol, respectively. To score seed germination, the percentage of seeds that had germinated and developed fully green expanded cotyledons was determined.

Growth and Stomatal Aperture Assays

The ABA-resistant growth was scored by weighting whole plants after 12 d of the transfer of 5-d-old seedlings onto Murashige and Skoog plates supplemented with 10 μ M ABA. Data were obtained for three independent

experiments, each done with 15 plants. For assays of ABA-induced stomatal closing, leaves of 5- to 6-week-old plants were used. Measurements were performed on epidermal peels, which were first incubated for 2 h and 30 min in stomatal opening buffer containing 10 mM KCl, 7.5 mM iminodiacetic acid, and 10 mM MES/Tris, pH 6.2, at 20°C. Then, they were incubated for 2 h and 30 min in the same buffer supplemented or not with 10 and 100 nM ABA. Data were expressed as the average of four experiments where 30 to 40 stomata were measured for each one.

Drought Stress and Water-Loss Assays

Two different water-loss assays were performed. Short-term assays were performed in detached leaves at the same developmental stage and size from 21-d-old plants. Five leaves per individual were excised and fresh weight was determined after submitting the leaves to the drying atmosphere of a flow laminar hood. Kinetics analysis of water loss was performed and represented as the percentage of initial fresh weight at each time point.

Long-term assays were performed after removing watering in plants maintained under greenhouse conditions. To this end, plants (10 individuals per experiment, three independent experiments) were grown under normal watering conditions for 21 d and then subjected to drought stress by completely terminating irrigation and minimizing soil evaporation by covering pots with plastic Saran Wrap film. Ten leaves from each plant were removed at the time points indicated. Subsequently, leaves were weighted, incubated in demineralized water for 3 h, and weighed again. The difference in weight was considered as water loss.

RNA Analyses

Plants were grown on Murashige and Skoog plates supplemented with 1% Suc. After 7 d, approximately 30 to 40 seedlings were either mock or 10 μ M ABA treated. After 3 h, plant material was collected and frozen in liquid nitrogen. Total RNA was extracted using a Qiagen RNeasy plant mini kit and 1 μ g of the RNA solution obtained was reverse transcribed using 0.1 μ g oligo(dT)₁₅ primer and Moloney murine leukemia virus reverse transcriptase (Roche) to finally obtain a 40 μ L cDNA solution. qRT-PCR amplifications and measurements were performed using an ABI PRISM 7000 sequence detection system (Perkin-Elmer Applied Biosystems). The sequences of the primers used for PCR amplifications were the following ones: for *HAB1* (At1g72770), forward 5'-AACTGCTGTTGCTTGCCTTG and reverse 5'-GGTTCGGTCT-TGAACTTCT; for *ABI1* (At4g26080), forward 5'-ATGATCAGCAGAAC-AGAGAGT and reverse 5'-TCAGTTCAAGGGTTTGCT; for *KIN1* (At5g15960), forward 5'-GCTGGCAAAGCTGAGGAGAA and reverse 5'-TTCCCGCTG-TTGTGCTC; for *RD29A* (At5g52310), forward 5'-GTCCAAAGTTAC-TGATCC-CAC and reverse 5'-CTTCATATCAAATCATGACT; for *P5CS1* (At2g39800), forward 5'-TTTATGGTCTATAGATCACA and reverse 5'-GAATGCTC-TGATGGGTGTAAC; for *RAB18* (At5g66400), forward 5'-ATG GCG TCT TACCAGAACCGT and reverse 5'-CCAGATCCGGAGCGGTGAAGC; for *RD29B* (At5g52300), forward 5'-ATG GAG TCA CAG TTG ACA CGT CC and reverse 5'-GAG ATA GTC ATC TTC ACC ACC AGG; for *RD22* (At5g25610), forward 5'-ATG GCG ATT CGG CTT CCT CTG ATC and reverse 5'-GAC ATT CAT TTT CCC GCG AAC; and for β -*actin-8* (At1g49420), forward 5'-AGTGGTCGTACAACCGGTATTGT and reverse 5'-GAGGATAGCATGTGGAAGTGAGAA.

qRT-PCR amplifications were monitored using the Eva-Green fluorescent stain (Biotium). Relative quantification of gene expression data was carried out using the $2^{-\Delta\Delta C_T}$ or comparative C_T method (Livak and Schmittgen, 2001). Expression levels were normalized using the C_T values obtained for the β -*actin-8* gene. The presence of a single PCR product was further verified by dissociation analysis in all amplifications. All quantifications were made in triplicate on RNA samples obtained from plants treated once with ABA.

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