Drought avoidance in
PP2C double knock-out mutants

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Journal research area: signal transduction and hormone action
Enhancement of abscisic acid sensitivity and reduction of water consumption in Arabidopsis by combined inactivation of the protein phosphatases type 2C ABI1 and HAB1

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This work was supported by grants BIO2002-03090 and BIO2005-01760 from Ministerio de Educación y Ciencia and Fondo Europeo de Desarrollo Regional (P.L.R.), and by National Institutes of Health grant R01GM060396 (J.I.S).

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Abstract
Abscisic acid 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) ABI1 and HAB1. Two new recessive loss-of-function alleles of ABI1, *abi1-*2 and *abi1-*3, were identified in an *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 *hab1-1abi1-*2 and *hab1-1abi1-*3 mutants strongly increased plant responsiveness to ABA. Thus, both *hab1-1abi1-*2 and *hab1-1abi1-*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.
Introduction

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 osmoregulation (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 wilty 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 1000 genes of the plant transcriptome (Hoth et al., 2002; Seki et al., 2002; Takashi 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 \textit{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). Furthermore, in guard cells several
studies have indicated the presence of intracellular ABA receptors (Schwartz et al., 1994; Allan 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 include Ca\(^{2+}\), cyclic ADP-ribose, reactive oxygen species, nitric oxide, phosphoinositides, phosphatidic acid, and sphingosine 1-phosphate (Schroeder and Hagiwara, 1989; McAinsh et al., 1990; Gilroy et al., 1990; Wu et al., 1997; Leckie et al., 1998; Jacob et al., 1999; Pei et al., 2000; Lemtiri-Chlieh et al., 2000; Ng et al., 2001; Allen 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 involve a complex network of protein kinases and phosphatases as well as other signal transducers (reviewed by Finkelstein et al., 2002). Finally, many transcriptional factors (TF) of ABA-inducible genes are known. The TFs comprise ABA-responsive element (ABRE)-binding proteins (ABI5/ABF/AREB/AtbZIP family), ABI3/VP1/B3, ABI4/AP2, MYC, MYB and HD-ZIP domain proteins (Giraudat et al., 1992; Suzuki et al., 1997; Finkelstein et al., 1998; Finkelstein and Lynch, 2000; Uno et al., 2000; Choi 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; Song et al., 2005; Pandey 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., 2001a), *hab1* (Saez et al., 2004; Leonhardt et al., 2004), *sad1* (Xiong et al., 2001b) and *gcr1* (Pandey
and Assmann, 2004) mutants. The intragenic revertants of abi1-1, 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 (ScaBP5) and its interacting protein kinase 3 (PKS3) 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), ostl (Mustilli et al., 2002), rcn1 (Kwak et al., 2002), rpk1 (Osakabe et al., 2005) and the rbohD/F double mutants (Kwak et al., 2003). Therefore, these loci point out to positive regulators of ABA signal transduction.

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; Meyer et al., 1994; Leung et al., 1997; Rodriguez, et al., 1998). Currently, at least four Arabidopsis 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; Saez et al., 2004; Leonhardt et al., 2004; Yoshida et al., 2006; Kuhn, 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 (Saez et al., 2004; Leonhardt et al., 2004). HAB1 is broadly expressed in the plant and strongly induced by ABA (Saez et al., 2004; Leonhardt 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 Gly180Asp 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 can not 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 knock-out alleles of ABI1, namely abi1-2 and abi1-3, in order to conclusively clarify its role in ABA signaling. Furthermore, double knock-out 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 knock-out alleles of ABI1

Two T-DNA insertion mutants of ABI1 were identified in the Salk collection (Columbia 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 2 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 impair ABI1 expression, based on RT-PCR (Fig. 1B) and quantitative real-time 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. Firstly, 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 μM ABA. In the next generation, F2 seeds showed an ABA-hypersensitive phenotype in approximately a 1:3 proportion (112 hypersensitive: 313 wt, χ²=0.42, P>0.5 for abi1-2; 121 hypersensitive: 319 wt, χ²=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 abil-2 and abil-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 two-fold lower for abil-2 and abil-3 than for the wild type (0.35, 0.37 and 0.67 µM 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 abil-2 and abil-3 (Fig. 2B and 2C). Both abil-2 and abil-3 mutants showed higher inhibition of germination and early growth by osmotic stress than wild type seeds.

*Generation and analysis of hab1-1abi1-2 and hab1-1abi1-3 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; Saez et al., 2004; Leonhardt et al.,
Double loss-of-function phenotypes in plant PP2Cs have not yet been analyzed in knock-out mutants. In order to unravel a possible functional redundancy between *ABI1* and *HAB1*, we decided to generate double mutant lines that contained knock-out alleles of both genes. To this end we crossed the previously described *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 *hab1-1abi1-2* and *hab1-1abi1-3* 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. 2B and 2C). Thus, a concentration of 100 mM NaCl practically abolished germination of the double mutants, whereas 15-40 % 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*, *abi2-1* and *35S:HAB1* plants, show ABA-resistant growth compared to wild type plants (Leung et al., 1994; Meyer et al., 1994; Leung et al., 1997; 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 La-er wild type (Gosti et al., 1999). Fig. 3 shows that both abi1-2 and abi1-3 displayed enhanced sensitivity to ABA-mediated growth inhibition than wild-type plants. After ten days 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 hab1-1abi1-2 and hab1-1abi1-3 double mutants

ABA signaling, by regulating stomatal aperture, plays a crucial role to reduce water loss under water shortage. Different analyses were performed in order 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 hab1-1abi1-2 (Fig. 4B). Stomatal aperture
measurements indicated that \textit{abi1-2}, \textit{habl-1} and double mutant \textit{hab1-abi1-2} were hypersensitive to ABA-induced stomatal closing in the range of 10-100 nM ABA. Moreover, the response of the double mutant \textit{hab1-abi1-2} to 10 nM ABA was more sensitive as compared to the single parental mutants (Fig. 4B). Similar results to those obtained for \textit{abi1-2} and double mutant \textit{hab1-abi1-2} were obtained for \textit{abi1-3} and double mutant \textit{hab1-abi1-3}, respectively (Supplemental Fig. 2).

The \textit{era1}, \textit{abh1} and \textit{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). Therefore, we examined water-loss of the different genetic backgrounds described here. Water-loss data were obtained, under greenhouse conditions, after exposing 21-days-old plants to drought stress by completely terminating irrigation and minimizing soil evaporation. Fig. 4D shows that after 14 days without watering, wild-type plants wilted and many rosette leaves yellowed. In contrast, \textit{hab1-abi1-2} and \textit{hab1-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 days without watering (Fig. 4C). Under these experimental conditions, where the plants were submitted to a long period of drought, the single \textit{habl-1}, \textit{abi1-2} and \textit{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, particularly in the case of the \textit{hab1-1abi1-2} and \textit{hab1-1abi1-3} double mutants.

\textit{Enhanced expression of ABA-inducible genes in PP2C mutants as compared to wild type}

The effect of the isolated single and double \textit{hab1} and \textit{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 \textit{RAB18}, \textit{P5CS1}, \textit{RD29B}, \textit{KIN1}, \textit{RD29A} and \textit{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 Frank, 1990; Yamaguchi-Shinozaki and Shinozaki, 1994; Lang and Palva, 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 \textit{hab1-1abi1-2} and \textit{hab1-1abi1-3} mutants showed approximately 2-fold higher mRNA levels of some gene markers (\textit{RAB18}, \textit{RD29A}, \textit{RD29B}) as compared to Columbia wild type (Table I). In the case of single mutants and under control conditions, only the \textit{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 \textit{RAB18}, \textit{RD29B} and \textit{P5CS1} (between 4- and 8-fold higher expression level than wild type). Gene markers that contain both DRE and ABRE elements, \textit{KIN1} and \textit{RD29A}, were also hyper-induced by ABA in the double mutants, although to a
lower level (2-3 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 *hab1-1abi1-2* and *hab1-1abi1-3* double mutants. The knock-out *abi1-2* and *abi1-3* mutants (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* (La-er background). ABA-induced stomatal closing was also ABA-hypersensitive in *abi1-2* and *abi1-3* (Supplemental Fig. 2) in the range of 10-100 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 knock-out 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 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 knock-out 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 hab1-1abi1-2 and hab1-1abi1-3 double mutants led to an additive ABA-hypersensitivity compared to the single parental mutants. Thus, the IC50 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 (Yoshida et al., 2006; Kuhn, et al., 2006). The ABA-mediated seed germination phenotype of pp2ca or hab1-1abi1-2/ hab1-1abi1-3 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 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 super-induced in the double mutant compared to single parental mutants. For instance, inhibition of growth upon prolonged cultured in medium supplemented with ABA was particularly dramatic in hab1-1abi1-2 and hab1-1abi1-3 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 & Zeevaart 2002). Priming of ABA biosynthesis can be obtained by direct over-expression of 9-cis-epoxycarotenoid dioxygenase, a key enzyme in the biosynthetic pathway (Iuchi et al. 2001; Qin & 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 ger1) 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 super-induction of ABA-responsive genes, have compromised tolerance to drought stress (Xiong et al., 2001a, b). Likewise, the calcineurin B-like 9 (cbl9), the calcineurin B-like-interacting protein kinase (cipk3) and the AP2-like ABA repressor 1 (abr1) 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, super-induction 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-labi1-2*/*hab1-labi1-3* 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* knock-out line (*abi1-ko*, corresponding to SALK_076309, here named *abi1-3*) was used to show a genetic interaction with the phospholipase Dα1 mutant (*pldα1*). Thus, whereas the single mutant *pldα1* abolished both ABA promotion of stomatal closure and ABA inhibition of stomatal closure, the double mutant *pldα1abi1-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 PLDα1 is not needed for ABA-induced stomatal closing when ABI1 is deleted. These findings are interesting in light of the present and other recent findings that several PP2Cs function as negative regulators of ABA signaling (Saez et
al., 2004; Leonhardt et al., 2004; Kuhn et al., 2006; Yoshida 2006), but deletion of the ABI1 PP2C is sufficient to restore PLDα1-independent ABA-induced stomatal closing in pldα1 (Mishra et al., 2006). Finally, we show here that the abi1-2 and abi1-3 knock-out 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 μM) of ABA was assayed.
Experimental procedures

Plant material and growth conditions

*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 4ºC for 3 days. Then, seeds were sowed on Murashige-Skoog (1962) (MS) plates composed of MS basal salts, 0.1% 2-[N-morpholino]ethanesulfonic acid, 1% agar and 1% sucrose. The pH was adjusted to 5.7 with KOH before autoclaving. Plates were sealed and incubated in a controlled environment growth chamber at 22ºC under a 16 h light, 8 h dark photoperiod at 80-100 µE m⁻² sec⁻¹.

Mutant identification by PCR screening

Two lines containing a single T-DNA insertion in *ABI1* were identified in the SALK T-DNA collection (Alonso et al., 2003), SALK_72009 and SALK_76309, and obtained from the Nottingham Arabidopsis Stock Center (http://nasc.nott.ac.uk). In order 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´- CTCTGTTCCTGCTGATCATCT. Line SALK_76309, 5´- CCGGCCCTCGAGATGATCAGCAGAAGAGTG and 5´-
CCGCCCTCGAGTCAGTTCAAGGGTTTGCT. As T-DNA left border primer of the pROK2 vector, we used LBpROK2: 5’-GCCGATTTCCGGAACCACCACATC.

In order to generate the hab1-1abi1-2 and hab1-1abi1-3 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 MS basal salts, 1% sucrose 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. In order 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 days of the transfer of 5-day-old seedlings onto MS plates supplemented with 10 μM ABA. Data were obtained for 3 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 30 min in stomatal opening buffer containing 10 mM KCl, 7.5 mM iminodiacetic acid and 10 mM MES/Tris, pH 6.2, at 20ºC. Then, they were incubated for 2 h 30 min in the same buffer supplemented or not with 10 and 100 nM ABA. Data
were expressed as the average of 4 experiments where 30-40 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-days-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 (ten individuals per experiment, three independent experiments) were grown under normal watering conditions for 21 days 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 de-mineralized water for 3 h and weighed again. The difference in weight was considered as water loss.

**RNA analyses**

Plants were grown on MS plates supplemented with 1% sucrose. After 7 days, approximately 30-40 seedlings were either mock- or 10 µM 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 µg of the RNA solution obtained was reverse transcribed using 0.1 µg oligo(dT)\(_{15}\) primer and M-MLV reverse transcriptase (Roche), to finally obtain a 40 µl cDNA solution. RT-QPCR 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’-AACTGCTGTTGTTGCCTTG and reverse 5’-GGTTCTGGTCTTGAACCTTTCT; for \(ABI1\) (At4g26080), forward 5´-ATGATCAGCAGAACACAGAG and reverse 5´-TCAGTTCAAGGGTTGCT; for \(KIN1\) (At5g15960), forward 5´-GCTGGCAAAGCTGAGGAA and reverse 5´-TTCCCGCTGTGTCTGCT; for \(RD29A\) (At5g52310), forward 5´-GTCCAAAGTTAC TGATCCAC and reverse 5´-CTTCATATCAAATCAGACT; for \(P5CS1\) (At2g39800), forward 5´-TTTATGGTGCTATAGACCA and reverse 5´-GAATGTCTGATGCTGTAAAC; for \(RAB18\) (At5g66400), forward 5´-ATG GCG TCT TACCAGAACGT and reverse 5´-CCAGATCCAGGCGGTGAAGC; 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 CCG TTC CGG ATT CTG ATC and reverse 5´-GAC ATT CAT TTC TTT CCC GCG AAC and for \(\beta\)-actin-8 (At1g49420), forward 5´-AGTGGTCTGTCACAACCGGTATTGT and reverse 5´-GAGGATAGCATGTTGGAAAGTGAGAA.

RT-Q-PCR amplifications were monitored using the Eva-Green™ fluorescent stain (Biotium). Relative quantification of gene expression data was carried out using the \(2^{-\Delta\Delta CT}\) or comparative \(C_T\) method (Livak and Schmittgen 2001). Expression levels were normalized using the \(C_T\) values obtained for the \(\beta\)-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.
Acknowledgements

We thank Joseph Ecker and the Salk Institute Genomic Analysis Laboratory for providing the sequence-indexed *Arabidopsis* T-DNA insertion mutants, and ABRC/NASC for distributing these seeds.
Literature cited

Abe H, Urao T, Ito T, Seki M, Shinozaki K, Yamaguchi-Shinozaki K (2003) Arabidopsis AtMYC2 (bHLH) and AtMYB2 (MYB) function as transcriptional activators in abscisic acid signaling. Plant Cell 15: 63-78


Figure legends

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 hab1-1abi1-2/ hab1-1abi1-3 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 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 hab1-1abi1-2/ hab1-1abi1-3 mutants as compared to wild type seeds. A-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 days later. Values are averages ±SD for three independent experiments.

Figure 3. ABA-hypersensitive growth inhibition of hab1-1, abi1-2, abi1-3 and double hab1-1abi1-2/ hab1-1abi1-3 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 days of the transfer of 5-day-old seedlings from MS 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 MS 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 ±SD (n = 30).

**Figure 4.** Reduced water loss of double *hab1-1abi1-2/ hab1-1abi1-3* mutants as compared to wild type or single parental mutants. A, Detached-leaves water loss assays show reduced water loss in double *hab1-1abi1-2/ hab1-1abi1-3* mutants. Five leaves per individual at the same developmental stage and size from 21-days-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 *hab1-1abi1-2* as compared to wild type plants. Stomatal apertures were measured 2 h 30 min after addition of 0.01 or 0.1 µM ABA. Data represent the average of three independent experiments ±SEM (n = 30-40 stomata per experiment) C, Quantification of water loss in 5-week-old plants after 12 days without watering. Data shown are the average amounts of water loss measured in ten leaves (µL/g fresh weight) collected from four different plants. Asterisk in B and C indicates P<0.01 (Student’s t test) when compared data from mutant and wild type. D, Enhanced drought tolerance of double *hab1-1abi1-2/ hab1-1abi1-3* mutants with respect to wild type or single parental mutants. Photograph was taken 14 days after water was withheld. Shoot was cut to better show the effect of drought on rosette leaves.
Table I. 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 treated once with 10 µM ABA.

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