The Protein Phosphatase *AtPP2CA* Negatively Regulates Abscisic Acid Signal Transduction in Arabidopsis, and Effects of *abh1* on *AtPP2CA* mRNA[1][W]

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To identify new loci in abscisic acid (ABA) signaling, we screened a library of 35S::cDNA Arabidopsis (*Arabidopsis thaliana*)-expressing lines for ABA-insensitive mutants in seed germination assays. One of the identified mutants germinated on 2.5 μM ABA, a concentration that completely inhibits wild-type seed germination. Backcrosses and F_2 analyses indicated that the mutant exhibits a dominant phenotype and that the ABA insensitivity was linked to a single T-DNA insertion containing a 35S::cDNA fusion. The inserted cDNA corresponds to a full-length cDNA of the *AtPP2CA* gene, encoding a protein phosphatase type 2C (PP2C). Northern-blot analyses demonstrated that the *AtPP2CA* transcript is indeed overexpressed in the mutant (named PP2CAox). Two independent homozygous T-DNA insertion lines, *pp2ca-1* and *pp2ca-2*, were recovered from the Arabidopsis Biological Resource Center and shown to lack full-length *AtPP2CA* expression. A detailed characterization of PP2CAox and the T-DNA disruption mutants demonstrated that, whereas ectopic expression of a 35S::*AtPP2CA* fusion caused ABA insensitivity in seed germination and ABA-induced stomatal closure responses, disruption mutants displayed the opposite phenotype, namely, strong ABA hypersensitivity. Thus our data demonstrate that the PP2CA protein phosphatase is a strong negative regulator of ABA signal transduction. Furthermore, it has been previously shown that the *AtPP2CA* transcript is down-regulated in the ABA-hypersensitive nuclear mRNA cap-binding protein mutant *abh1*. We show here that down-regulation of *AtPP2CA* in *abh1* is not due to impaired RNA splicing of *AtPP2CA* pre-mRNA. Moreover, expression of a 35S::*AtPP2CA* cDNA fusion in *abh1* partially suppresses *abh1* hypersensitivity, and the data further suggest that additional mechanisms contribute to ABA hypersensitivity of *abh1*.

The phytohormone abscisic acid (ABA), which regulates many agronomically important aspects of plant life, including seed development and dormancy, plays a critical role in plant stress responses such as drought, salinity, cold shock, wounding, and pathogen attack (Schroeder et al., 2001; Finkelstein et al., 2002; Hetherington and Woodward, 2003; Fan et al., 2004). These physiological responses to ABA are in large part due to changes in gene expression and a complex signal transduction network (Hoth et al., 2002; Seki et al., 2002; Leonhardt et al., 2004; Takahashi et al., 2004). Several transcription factors mediating ABA responses have been isolated (Giraudat et al., 1992; Finkelstein et al., 1998; Finkelstein and Lynch, 2000; Uno et al., 2000; Himmelbach et al., 2002; Abe et al., 2003).

Whereas the biosynthesis of ABA is well understood (Seo and Koshiba, 2002), the mechanisms by which ABA regulates multiple plant responses is beginning to be revealed through genetic and physiological analyses in Arabidopsis (*Arabidopsis thaliana*). To date, genetic screens for ABA-hypersensitive mutants have indicated that processes including farnesylation (*era1*; Cutler et al., 1996; Pei et al., 1998), inositol 1,4,5-triphosphate (IP_3) dephosphorylation (*fry1*; Xiong et al., 2001b), and RNA metabolism (*hyl1*; Lu and Fedoroff, 2000; *abh1*, Hugouvieux et al., 2001; *sad1*, Xiong et al., 2001a) are required to attenuate the ABA signal. However, surprisingly few non-transcription factor-encoding genes have been identified as recessive ABA-insensitive disruption mutants, namely, the G-protein α-subunit *GPA1* (Wang et al., 2001), the *RCN1* protein phosphatase type 2A subunit (Kwak et al., 2002), the *OST1/SnRK2E* protein kinase (Mustilli et al., 2002; Yoshida et al., 2002), the *AtRBOHD/F* NADPH oxidases (Kwak et al., 2003), *ABI8* (Brocard-Gifford et al., 2004), *RPK1* (Osakabe et al., 2005), and *GCA2* (Himmelbach et al., 2003).

Many gene families in the Arabidopsis genome have large numbers of homologs relative to other sequenced genomes (Arabidopsis Genome Initiative, 2000). Therefore, the relatively low number of recessive

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ABA-insensitive mutants is most likely due to redundancy in genes encoding ABA transducers, requiring analyses of double or multiple mutations in (partially) redundant genes (Kwak et al., 2003). In addition, the ABA signaling pathway is mediated by a network of events and interacts with many other signaling pathways including drought, salinity, cold, sugar, GA_3, and ethylene (Finkelstein et al., 2002; Zhu, 2002; Himmelbach et al., 2003; Yamaguchi-Shinozaki and Shinozaki, 2005). Consequently, functional redundancy could also be explained by the fact that such complex networks have the ability to buffer a mutation's effects in a neighboring pathway (Cutler and McCourt, 2005).

To further enhance the chance of success in identifying new mutants in ABA signaling, we screened a library of 35S::cDNA Arabidopsis-expressing lines (LeClere and Bartel, 2001) for ABA-insensitive mutants in seed germination assays. In theory, the 35S::cDNA fusion can generate mutants due to (1) random insertional gene disruptions; (2) overexpression of the inserted full-length or truncated cDNA; or (3) silencing of the cDNA-corresponding endogenous gene (LeClere and Bartel, 2001). Consequently, this screen can identify both positive and negative regulators of a given signaling pathway. In this study, we report isolation of two strong ABA-insensitive mutants: a new insertional mutant of *ABI5*, coding for a basic Leu zipper transcription factor, a well-known positive regulator of ABA responses (Finkelstein and Lynch, 2000), as well as a constitutive overexpressor of *AtPP2CA* (named PP2CAox) encoding a protein phosphatase type 2C (PP2C).

Sixty-nine PP2Cs are encoded in the Arabidopsis genome (Kerk et al., 2002; Schweighofer et al., 2004), and a gene disruption phenotype has only been reported for one of these PP2Cs (Leonhardt et al., 2004; Saez et al., 2004). The protein phosphatase PP2CA belongs to group A of the Arabidopsis PP2C family, together with ABI1, ABI2, and AtP2C-HA (hereafter named AtP2C-HAB1; Schweighofer et al., 2004). Recently, reverse-genetics studies have demonstrated that AtP2C-HAB1 is a negative regulator of ABA signaling (Leonhardt et al., 2004; Saez et al., 2004). Isolation and characterization of the dominant negative *abi1-1* and *abi2-1* PP2C mutants and their intragenic revertants also support a negative role for these two PP2Cs (Leung et al., 1994, 1997; Meyer et al., 1994; Rodriguez et al., 1998; Gosti et al., 1999; Merlot et al., 2001). However, in transgenic Arabidopsis plants overexpressing the *ABI1* gene, no altered ABA sensitivity was found in seed germination or in suppression of ABA-mediated gene induction (Wu et al., 2003). To date, no allele of *ABI1* and *ABI2* in which the corresponding protein is not produced has been reported.

AtPP2CA has been shown to block ABA-induced gene induction when transiently overexpressed in protoplasts (Sheen, 1998). However, stable *AtPP2CA* overexpression in planta and corresponding knockout mutants have not been reported. In this study, the isolation and detailed phenotypic characterization of PP2CAox and two insertional mutants, *pp2ca-1* and *pp2ca-2*, unequivocally demonstrate that PP2CA acts as a strong negative regulator of ABA signaling not only at the seed germination level but also in vegetative tissues. It has been previously shown that *AtPP2CA* transcripts are reduced in the ABA-hypersensitive mutant *abh1*, which encodes a nuclear mRNA cap-binding protein (Hugouvieux et al., 2001). We therefore also analyzed *AtPP2CA* mRNA splicing and the effects of the introduction of a 35S::*AtPP2CA* fusion in the *abh1* background to determine the role of *AtPP2CA* in mediating the ABA hypersensitivity of *abh1*.

RESULTS

Screening of a 35S::cDNA-Expressing Line Library for ABA-Insensitive Mutants Identifies an Overexpressor of *AtPP2CA*

Because studies suggest genetic and network redundancy in ABA signal transduction, a screen was pursued that can include dominant ABA-insensitive mutants. Roughly one million seeds were screened from approximately 60,000 activation-tagged lines for ABA-insensitive seed germination at 5 μM ABA. Remarkably, after retesting putative mutants, no robust ABA-insensitive mutant line was isolated from the two activation-tagged populations that were tested twice independently. These findings may be attributed to the robustness of ABA signaling and the inherent limitations in the mutation rate of activation-tagged lines (Weigel et al., 2000).

To further enhance the frequency of obtaining dominant mutants in ABA signaling, we screened Arabidopsis lines expressing a library of random 35S::cDNAs (LeClere and Bartel, 2001) for ABA-insensitive mutants in seed germination assays. This library is composed of approximately 400,000 T2 seeds coming from 33,000 different T1 lines in the ecotype Columbia (Col-0) background, each expressing a random 35S::cDNA together with a Basta resistance gene inserted in the T-DNA of the 35SpBARN vector. From the original screen of 400,000 T2 seeds, 902 putative individual T2 seeds were able to germinate (radicle emergence plus expanded green cotyledons) 5 d after stratification on 2.5 μM ABA, a concentration that completely inhibits wild-type seed germination. These young ABA-resistant seedlings were then transferred to soil and allowed to self pollinate. The progeny of each of the T2 plants were then tested again for ABA insensitivity in germination assays. From this secondary screen, the progeny of two T2 plants, named 54.7 and 393.1, exhibiting strong ABA resistance were selected and further characterized.

Southern-blot analyses revealed that both mutants contained tandem T-DNA insertions at one locus each (data not shown). Segregation analyses of both ABA and Basta resistance in the next generations as well as in F_1 and F_2 populations from backcrosses indicated

that both ABA insensitivities were linked to the corresponding T-DNAs. However, whereas 393.1 was heterozygous for the mutation and its ABA insensitivity was dominant, 54.7 was homozygous and its phenotype was due to a single recessive nuclear mutation.

Because the strong ABA insensitivity of the recessive mutant 54.7 was reminiscent of the loss-of-function phenotype of the classic positive regulators of ABA responses *ABI3*, *ABI4*, and *ABI5*, a PCR-based diagnosis was performed on 54.7 genomic DNA (see "Materials and Methods"). This analysis and sequencing showed that 54.7 has a tandem T-DNA insertion located in the first intron of *ABI5* approximately 500 bp before the start codon (data not shown). Thus, considering its strong ABA insensitivity and the location of the T-DNA insertion, 54.7 is most likely a new allele of *abi5*.

The cDNA contained within the T-DNA of the dominant 393.1 mutant was identified by PCR and perfectly matches the full-length cDNA of *AtPP2CA*, encoding a PP2C (Fig. 1A). The *AtPP2CA* gene belongs to group A of the Arabidopsis *PP2C* gene family (Schweighofer et al., 2004). A homozygous T3 line, PP2CAox (see below), was recovered from the progeny of the heterozygous 393.1 plant, and northern-blot analysis revealed that *AtPP2CA* was indeed overexpressed in PP2CAox compared to wild type (Fig. 1B). PP2CAox clearly exhibited ABA insensitivity in seed germination assays as it germinated almost completely on 2.5 μM ABA, whereas wild-type seeds do not germinate at all (Fig. 2A).

To further test whether the 35S::*AtPP2CA* fusion caused the ABA-insensitive phenotype in PP2CAox, the full-length *AtPP2CA* cDNA was cloned back in the 35SpBARN binary vector (LeClere and Bartel, 2001; see "Materials and Methods") and transformed into wild-type Arabidopsis plants. Fourteen independent T1 35S::*AtPP2CA* lines were recovered as well as 10 T1 35SpBARN lines (empty-vector controls) and the germination rate of their T2 seeds was analyzed in the presence of 2.5 μM ABA. Whereas none of the 10 T1 35SpBARN lines exhibited ABA insensitivity in their progeny compared to untransformed wild-type Arabidopsis (data not shown), all 14 35S::*AtPP2CA* lines displayed ABA resistance similar to the original heterozygous 393.1 mutant seeds (Fig. 2C). Thus, we conclude that ectopic expression of the 35S::*AtPP2CA* fusion is responsible for the ABA insensitivity observed for PP2CAox seeds.

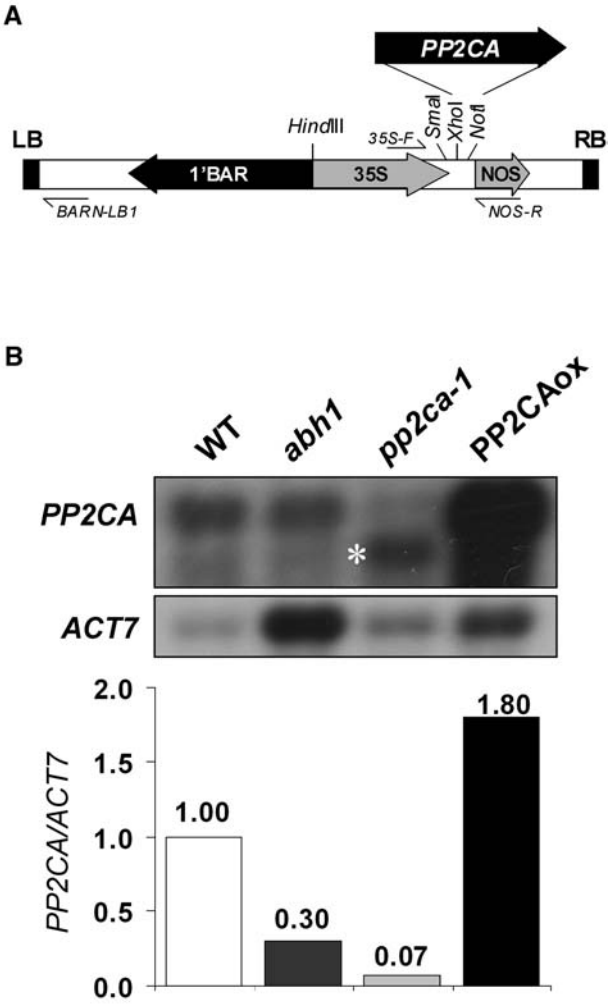

Figure 1. The ABA-insensitive 393.1 mutant is an overexpressor of *AtPP2CA* encoding a PP2C. A, Schematic representation of the 393.1 mutant T-DNA containing the full-length cDNA of *AtPP2CA*. The *AtPP2CA* cDNA is inserted at the original *Xho*I and *Not*I restriction sites of the 35SpBARN vector. Arrows indicate the direction of transcription. 35S-F and NOS-R are the primers used for amplifying the cDNA. BARN-LB1 is the primer used for T-DNA left-border sequencing. Note that the *Xho*I restriction site no longer exists because it has been filled with T4 polymerase during the original cloning procedure (LeClere and Bartel, 2001). B, Northern-blot analysis of *AtPP2CA* in wild-type, *abh1*, *pp2ca-1*, and PP2CAox leaves. Hybridization signals with *ACTIN7* cDNA (*ACT7*) were used for standardization of RNA and the value obtained from wild-type leaves was set to 1.

Seeds from Two Independent T-DNA Insertion Mutants for the *AtPP2CA* Gene Are Strongly ABA Hypersensitive

To further elucidate the role of *AtPP2CA* in ABA signaling, we isolated an insertion mutant from the Salk Institute Genomic Analysis Laboratory (SIGnAL; Alonso et al., 2003) database corresponding to donor stock number SALK_028132. Sequencing of the T-DNA flanking region indicated that the T-DNA is located at the end of exon II (position +864 [numbering refers to the ATG start codon]; Fig. 3A). Plants homozygous for the T-DNA insertion recovered by PCR genotyping were renamed *pp2ca-1*. Northern-blot analysis failed to detect a full-length *AtPP2CA* transcript in the *pp2ca-1* mutant (Fig. 1B), although a shorter transcript could be detected (Fig. 1B, asterisk). In addition, reverse transcription (RT)-PCR analysis did not result in any product corresponding to a full-length cDNA in the T-DNA disruption allele *pp2ca-1* (Fig. 3B).

ABA germination assays were carried out with seeds from *pp2ca-1* in parallel with seeds from the PP2CAox

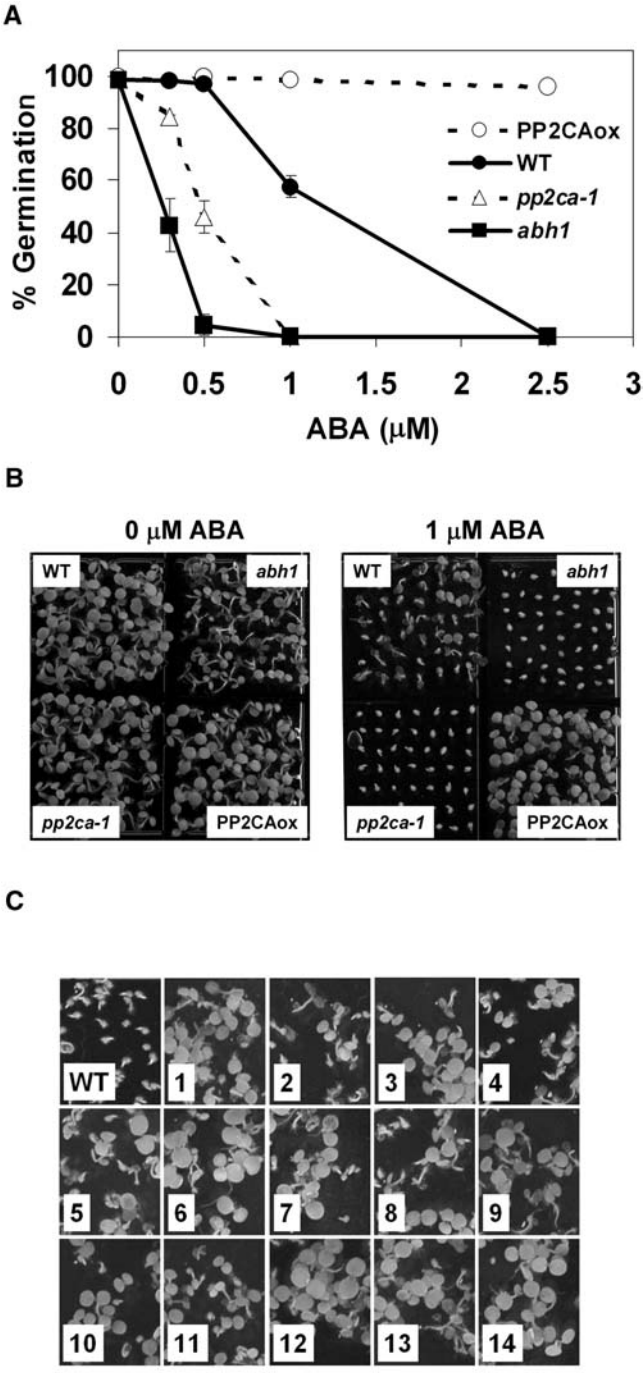

Figure 2. Constitutive expression of *AtPP2CA* results in ABA insensitivity, while disruption of *AtPP2CA* causes ABA hypersensitivity during seed germination. A, Comparison of germination rates of wild-type (Col-0; black circles), *pp2ca-1* (white triangles), *abh1* (black squares), and PP2CAox (white circles) seeds exposed to 0, 0.3, 0.5, 1, and 2.5 μM ABA at 5 d. Data represent the mean ± SEM of three independent experiments with 36 seeds per genotype and experiment. See Supplemental Figure 1A for similar data with *pp2ca-2*. Error bars are smaller than symbols, if not visible. B, Comparison of germination for wild-type, *pp2ca-1*, *abh1*, and PP2CAox seeds at 1 μM ABA or in the absence of ABA after 5 d. C, Germination of seeds from wild-type and 14 individual T1 lines with constitutive 35S::*AtPP2CA* expression at 2.5 μM ABA 5 d after germination.

line, wild-type plants, and the ABA-hypersensitive mutant, *abh1* (Fig. 2A). In the absence of exogenous ABA, *pp2ca-1* mutant seeds germinated as well as wild-type seeds (Fig. 2, A and B). And in the presence of 1 μM ABA, a clear ABA-hypersensitive inhibition of seed germination was observed. Indeed, the ABA hypersensitivity of *pp2ca-1* was almost as strong as that of *abh1* (Fig. 2, A and B). Later, a second T-DNA insertion mutant line WiscDsLox341D03 was released by the Arabidopsis Biological Resource Center (ABRC; stock no. CS851888; Sussman et al., 2000). Plants homozygous for the T-DNA insertion recovered by PCR genotyping were renamed *pp2ca-2* and sequencing of the T-DNA flanking region showed that the insertion lay at the beginning of exon III (position +966; Fig. 3A). As for *pp2ca-1*, RT-PCR failed to amplify a product corresponding to a full-length cDNA in the *pp2ca-2* mutant (Fig. 3B). Seeds from *pp2ca-2* were also tested in germination assays and displayed a strong ABA hypersensitivity very similar to that of *pp2ca-1* (Supplemental Fig. 1A). Together, our data obtained from PP2CAox, as well as the *pp2ca-1* and *pp2ca-2* T-DNA disruption mutants, demonstrate an important role of *AtPP2CA* as a negative regulator of ABA signaling during seed germination.

AtPP2CA Affects ABA-Promoted Inhibition of Root Growth

To test whether *AtPP2CA* gene disruption or constitutive expression of *AtPP2CA* in plants could affect other ABA responses, and because *AtPP2CA* was shown to be expressed in the stele of the Arabidopsis root system (Cherel et al., 2002), we investigated ABA inhibition of root growth by transferring 6-d-old seedlings on 0.25× Murashige and Skoog plates with 0, 2.5, 5, 10, 25, and 50 μM ABA. Elongation of the primary root was measured 6 d after the transfer in three independent experiments (Fig. 3C). Disruption of *AtPP2CA* in *pp2ca-1* and *pp2ca-2* plants exhibits a moderate, but significant, increase in ABA sensitivity compared to wild type during root growth on 0.25× Murashige and Skoog media supplemented with 2.5, 5, 10, 25, and 50 μM ABA (Fig. 3C). Interestingly, ABA inhibition of root elongation in the originally isolated PP2CAox plants was the same as in wild type at all ABA concentrations measured ($P >$ 0.04 to 0.36 in all conditions tested; Fig. 3C), suggesting that elevated *AtPP2CA* transcript levels have no dramatic effect on ABA regulation of root elongation. A possible explanation could be that during the ABA inhibition of root elongation, the expression of PP2CA interacting partners is the rate-limiting step rather than *PP2CA* levels. Moreover, due to the moderate ABA hypersensitivity of *pp2ca-1* and *pp2ca-2* in root assays compared to the strong seed germination phenotype, it is conceivable that partial redundancy with other PP2Cs is more pronounced in roots. When plants were exposed to 50 μM ABA for extended time periods (18 d), *pp2ca-1* plants show

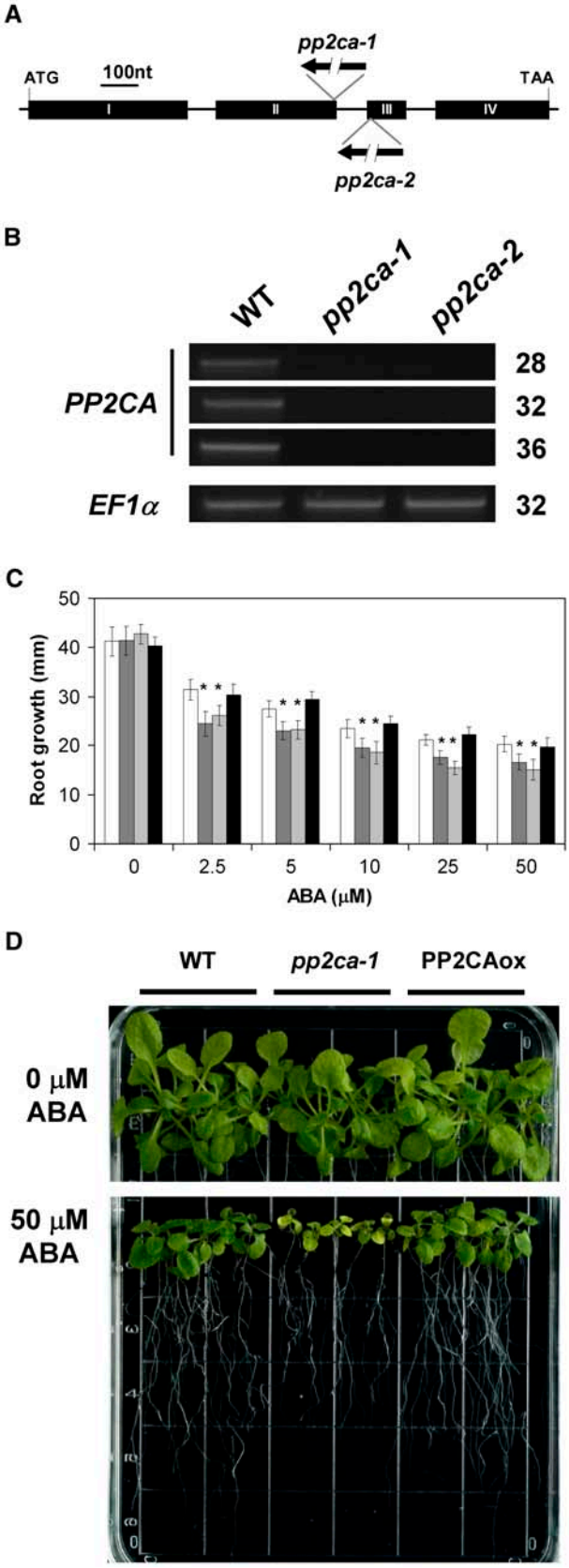

Figure 3. Disruption of *AtPP2CA* causes reduced root elongation in response to ABA. A, Schematic representation of the genomic organization of the *AtPP2CA* gene with four exons (black boxes). Positions of the *pp2ca-1* and *pp2ca-2* T-DNA insertions are indicated and orientation of the left-border sequence of the respective T-DNAs is represented by broken arrows. B, RT-PCR analysis shows no full-length transcript in *AtPP2CA* T-DNA disruption lines *pp2ca-1* and *pp2ca-2*. PCR reactions were performed with oligonucleotides PP2CAEx1-F and PP2CAEx4-R (Table I) and samples were withdrawn from the reaction after 28, 32, and 36 cycles. Amplification of *EF1α* cDNA with primers EF1α-F and EF1α-R (Table I) was used for controls. C, Comparison of root elongation of wild-type (white bars), *pp2ca-1* (dark gray bars), *pp2ca-2* (light gray bars), and PP2CAox (black bars) seedlings; 6-d-old seedlings were transferred to plates supplemented with 0, 2.5, 5, 10, 25, and 50 μM ABA, and root elongation was monitored after 6 d. Each data point represents the mean of three independent experiments with eight seedlings each. Asterisks (*) indicate a significant change between wild-type and *pp2ca-1* or *pp2ca-2* plants ($P < 0.001$). D, Growth of wild type, *pp2ca-1*, and PP2CAox on 0.25× Murashige and Skoog medium supplemented with 50 μM ABA. The photographs show plants on plates with 0 (top) and 50 μM ABA (bottom) 18 d after transfer of 6-d-old seedlings from 0.25× Murashige and Skoog medium.

ABA-hypersensitive inhibition of root elongation as well as retarded growth and chlorosis in aerial parts of plants (Fig. 3D), further exhibiting an enhanced sensitivity to ABA.

The *AtPP2CA* Transcript Level Is Up-Regulated by Both ABA and Drought Treatments

AtPP2CA is expressed ubiquitously in plant organs with the highest transcript levels in leaves and its expression is up-regulated by stresses, including ABA, cold, drought, and salt treatment (Tahtiharju and Palva, 2001; Cherel et al., 2002). However, the time courses of *AtPP2CA* gene induction in ABA or drought experiments have not yet been reported, to our knowledge. Thus, to assess the stress-related regulation of this gene, we studied the accumulation of *AtPP2CA* transcripts from wild-type plant leaves in response to ABA and drought treatments. The results clearly show that the *AtPP2CA* mRNA is highly and rapidly up-regulated by both treatments (Fig. 4). Significant transcript increases were detected within 30 min of exposure to ABA or drought (Fig. 4). However, whereas the peak of ABA induction occurred at 1 h, induction by drought increased rather progressively before reaching a peak at approximately 6 h.

AtPP2CA Disruption Causes ABA Hypersensitivity in Stomatal Guard Cells

The control of water loss by ABA is a crucial survival mechanism for plants during drought periods. To investigate the role of *AtPP2CA* in regulating water homeostasis, we measured the loss of fresh weight of detached rosette leaves (Fig. 5A). Overexpression of the *AtPP2CA* cDNA in planta led to an approximately 1.5-fold increase in the water-loss rate (Fig. 5A). In contrast, the gene disruption line *pp2ca-1* did not exhibit significant differences to transpiration

rates of wild-type leaves (Fig. 5A). Similarly, some stomatal ABA response mutants show no detached-leaf water-loss phenotype, including earlier findings on mutant alleles of the *PP2Cs ABI1*, *ABI2*, and *AtP2C-HAB1* (Gosti et al., 1999; Merlot et al., 2001; Saez et al., 2004). This lack of a phenotype in detached-leaf wilting assays may be attributable to the limited resolution of this method.

Therefore, we more directly analyzed stomatal movement responses to ABA in loss- and gain-of-function *AtPP2CA* plants (Fig. 5, B and C). Compared to wild type, guard cells from PP2CAox plants exhibit a clear insensitivity in ABA-induced stomatal closure analyses. Guard cells in these plants show a significantly reduced response to 10 μM ABA, which clearly results in stomatal closure in wild-type plants (Fig. 5B). In contrast, *AtPP2CA* gene disruption results in an ABA-hypersensitive stomatal closure response at 1 μM ABA (Fig. 5B for *pp2ca-1*, and 5C for *pp2ca-2*). These data show that *AtPP2CA* plays an important role in ABA signal transduction events and the regulation of stomatal aperture. Analyses of stomatal aperture responses (Fig. 5, B and C) and ratios of stomatal apertures to stomatal heights illustrate the same findings in *AtPP2CA* disruption and gain-of-function lines (Supplemental Fig. 1, B and C).

Analysis of *AtPP2CA* mRNA Splicing in the *abh1* Mutant

Interestingly, the *AtPP2CA* mRNA was previously shown to exhibit a reduced mRNA level in the ABA-hypersensitive *abh1* mutant (Hugouvieux et al., 2001).

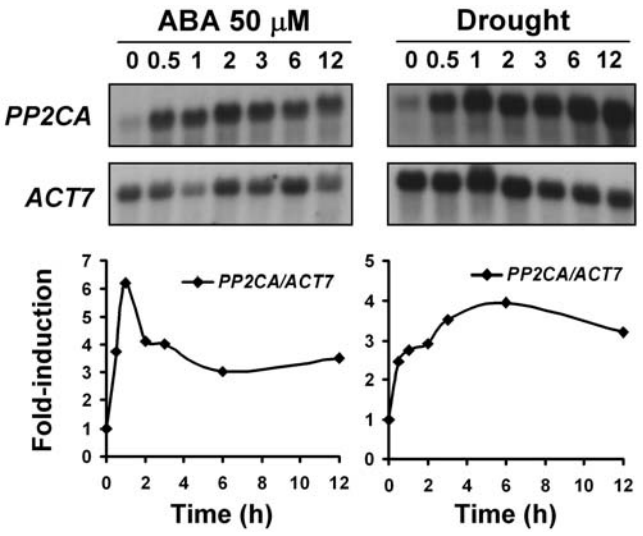

Figure 4. *AtPP2CA* transcripts are rapidly and highly up-regulated by both ABA and drought treatments. Northern-blot analyses of *AtPP2CA* in wild-type leaves either treated with ABA (50 μM; left) or excised and subjected to desiccation (right). Total RNA was extracted from leaves at times specified by the number above each lane. Hybridization signals with *ACTIN7* cDNA (*ACT7*) were used for standardization of equal amounts of RNA. Values obtained prior to the indicated treatments were set to 1.

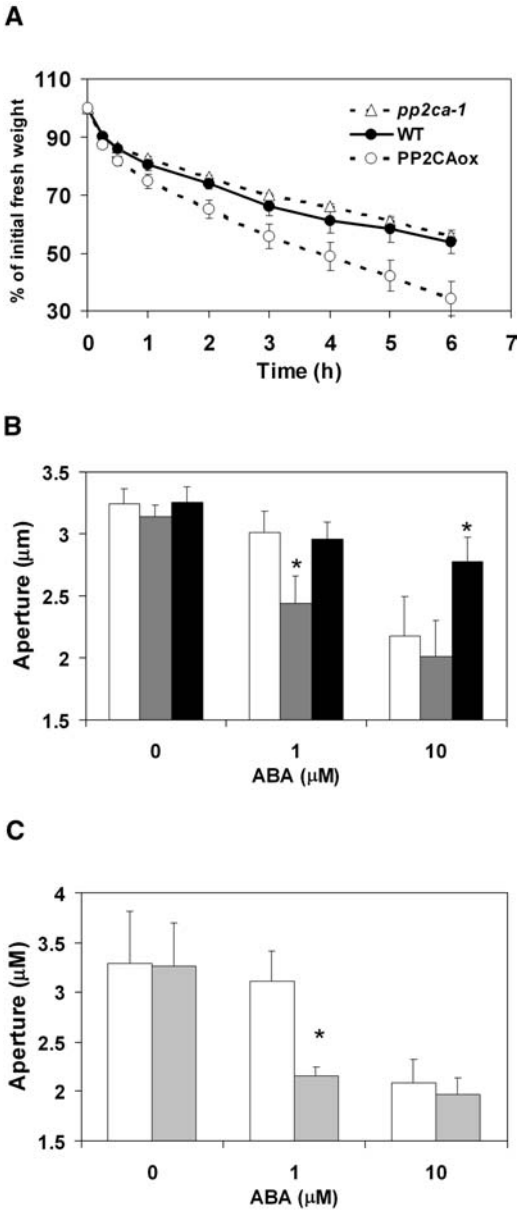

Figure 5. *AtPP2CA* modulates the stomatal response to ABA. A, Ectopic expression of *AtPP2CA* in plants causes enhanced leaf evaporation rate compared to wild type. Loss of fresh weight of detached rosette leaves at the same developmental stages was measured for wild-type (black circles), *pp2ca-1* (white triangles), and PP2CAox plants (white circles) at the indicated time points. Data represent the mean of three independent experiments ± SEM. B, Stomatal closing is ABA hypersensitive in *pp2ca-1* and ABA insensitive in PP2CAox plants. Stomatal aperture measurements of wild type (white bars), *pp2ca-1* (shaded bars), and PP2CAox (black bars) in response to 0, 1, and 10 μM ABA. Data represent the mean of $n = 4$ independent experiments ± SEM with 4×50 stomata per data point. Asterisks (*) indicate significant changes between the indicated genotype and wild type ($P < 0.001$). C, Stomatal closing is ABA hypersensitive in *pp2ca-2*. Stomatal aperture measurements of wild type (white bars) and *pp2ca-2* (shaded bars) in response to 0, 1, and 10 μM ABA. Data represent the mean of two independent experiments ± SEM with 2×50 stomata per data point. Asterisks (*) indicate significant changes between the indicated genotype and wild type ($P < 0.001$). See Supplemental Figure 1, B and C, for stomatal aperture ratios from experiments in Figure 5, B and C.

Transcript levels were analyzed in northern-blot analyses in *abh1* and compared to those in wild-type controls. *AtPP2CA* transcripts normalized to *Actin7* mRNA were 3.3-fold lower in *abh1* compared to wild-type controls (Fig. 1B).

ABH1 is the Arabidopsis homolog of an 80-kD subunit of the dimeric mRNA cap-binding complex, which additionally consists of a 20-kD subunit, AtCBP20 (Hugouvieux et al., 2001; Kmieciak et al., 2002; Papp et al., 2004). In yeast (*Saccharomyces cerevisiae*) and human HeLa cells, the cap-binding complex was shown to participate in pre-mRNA splicing (Izaurralde et al., 1994; Lewis and Izaurralde, 1997; Fortes et al., 1999). We investigated whether the *AtPP2CA* transcript undergoes differential splicing in *abh1* compared to wild type. Differential splicing could contribute to the down-regulation of the *AtPP2CA* mRNA in *abh1* due to a reduced turnover of *AtPP2CA* transcript maturation (Clark et al., 2002). We designed an RT-PCR approach to analyze qualitative and quantitative differences in *AtPP2CA* transcript maturation (Fig. 6A). Forward primers were selected for amplification of the full reading frame or for amplification of intron sequences with the corresponding reverse primer being located at the 3′ part of the most downstream exon IV (Fig. 6A).

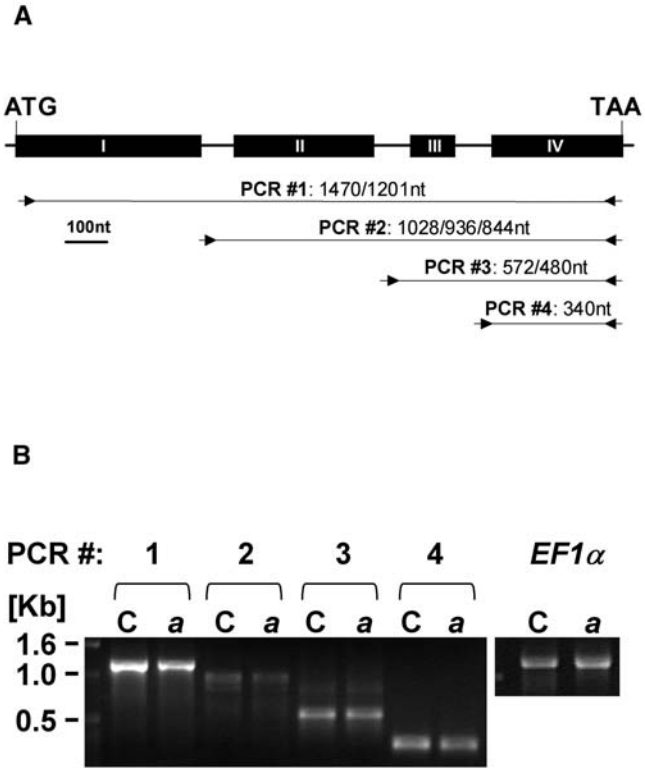

Figure 6. Analysis of *AtPP2CA* intron splicing in wild type and *abh1*. A, Schematic representation of *AtPP2CA* genomic organization with exons (black boxes) and introns (lines between exons). Positions of primers (arrows) used for the RT-PCR analysis are indicated and resulting amplification products (dotted lines) are shown for each PCR reaction performed, numbered from 1 to 4. Product sizes for full-length amplification, corresponding to completely unspliced transcripts, or for smaller fragments, corresponding to fully or partially processed mRNAs, are given for each PCR reaction. B, DNA fragments of fully and partially spliced *AtPP2CA* mRNA amplification products (36 cycles) generated in PCR reactions 1 to 4 (Fig. 6A) for wild-type controls (C) and *abh1* (a) are size fractionated on a 1.8% agarose gel. Amplification (28 cycles) of the *EF1α* was used as internal control. Sizes of coelectrophoresed DNA standard fragments are given in kilobase pairs.

RT-PCR on DNase-treated total RNA from leaves yielded similar amplification product qualities and quantities for all four reactions (Fig. 6B). PCR product 1 resulted in a single band corresponding to the full-length reading frame. PCR product 2, with the forward primer location in the first intron, showed faint bands with sizes corresponding to a fully unspliced pre-mRNA (1,028 nucleotides). PCR product 2 also resulted in splice intermediates emerging from intron I independent of removal of intron II or intron III (936 nucleotides) and even removal of both introns II and III (844 nucleotides) from the pre-mRNA without remarkable differences between wild type and *abh1* (Fig. 6B). This holds equally true for PCR products 3 and 4 (Fig. 6B). Identical results were obtained for an analysis on RNA isolated from independently grown plants (data not shown). We conclude from these results that splicing of the *AtPP2CA* pre-mRNA is not affected in the *abh1* background and down-regulation of *AtPP2CA* transcripts is more likely caused by other mechanisms.

To elucidate whether elevated transcript levels of *AtPP2CA* can restore normal ABA sensitivity or even cause ABA insensitivity in *abh1*, we transformed wild-type (Col-0) and *abh1* plants with a *AtPP2CA* cDNA under the control of the cauliflower mosaic virus 35S promoter in a binary vector different from 35SpBARN (see ''Materials and Methods''). Forty independent T1 plants were isolated each for wild type and for *abh1* and tested for ABA responses. Individual lines with single-insertion segregation patterns and the strongest ABA insensitivity in seed germination were selected to obtain homozygous lines. ABA germination assays were performed in triplicate and confirmed earlier findings (Fig. 2C) that introduction of a 35S::*AtPP2CA* fusion in the wild type always confers a strong ABA insensitivity in seed germination independent of the binary vector used (data not shown).

In the *abh1* background, ectopic expression of the *AtPP2CA* cDNA had a much weaker effect than in the wild-type background, with only two homozygous single-insertion lines being able to restore wild-type-like ABA responses during germination (Fig. 7A, white diamonds). Most of the 40 *abh1* mutant lines expressing the 35S::*AtPP2CA* construct exhibited a range of ABA sensitivities between *abh1* and wild type in T2 generation germination experiments (data not shown). Based on the hypothesis that *abh1* might affect *AtPP2CA* transcripts in these constitutively cDNA-expressing lines, we tested *AtPP2CA* transcript integrity in RT-PCR experiments (Fig. 7B). Amplification of the full-length reading frame resulted in a single band in all

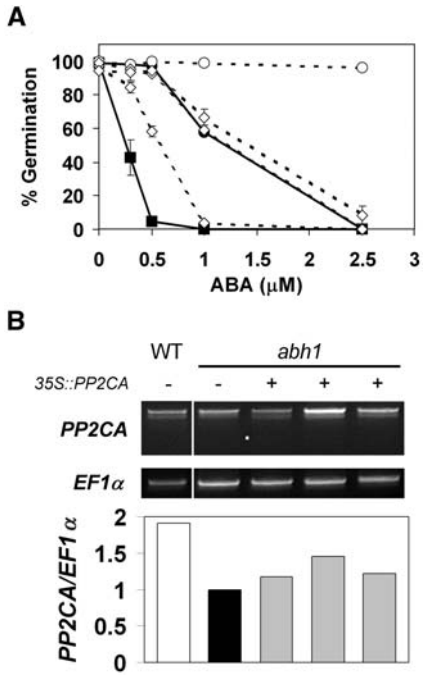

Figure 7. Ectopic expression of *AtPP2CA* partially suppresses the ABA hypersensitivity of *abh1*. A, Comparison of germination rates of wild type (black circles), PP2CAox in wild type (white circles), *abh1* (black squares), and seeds from three independent *abh1* 35S::*AtPP2CA* (white diamonds) lines germinated on Murashige and Skoog plates containing 0, 0.3, 0.5, 1, and 2.5 μM ABA after 5 d. Data represent the mean ± SEM of three independent experiments. Error bars are smaller than symbols, if not visible. Data for wild type, *abh1*, and PP2CAox in A are the same as in Figure 2A for reference purposes. B, RT-PCR analysis of *AtPP2CA* transcript levels in wild type, *abh1*, and the three independent *abh1* 35S::*AtPP2CA* lines displayed in A from the more sensitive to the more ABA resistant. DNA fragments from RT-PCR analyses of *EF1α* were used for standardization of equal amplification rates and the value obtained for *AtPP2CA* quantity in *abh1* was set to 1. RT-PCR reactions were performed for 20, 24, 28, and 32 cycles to quantify relative mRNA levels and representative images after 28 cycles are shown.

lines investigated, indicating no detectable qualitative impact of *abh1* on the full-length transcripts (Fig. 7B). Ratiometric analysis of the *AtPP2CA* transcript levels, with *elongation factor 1α* (*EF1α*) as a reference, indicated that *AtPP2CA* mRNA abundance in 35S::*AtPP2CA*/*abh1* lines was indeed increased up to 1.5-fold compared to *abh1* (Fig. 7B). However, the transcript abundance was persistently lower than in wild-type plants (Fig. 7B). Northern analyses of *AtPP2CA* transcript levels confirmed these results (data not shown). Thus, elevated *AtPP2CA* transcript levels can partially suppress the ABA hypersensitivity of *abh1* and the overall lower levels of *AtPP2CA* transcripts in *abh1* compared to wild type indicate a continued down-regulation of *AtPP2CA* in *abh1* (Fig. 7).

DISCUSSION

Here we report isolation of a strong dominant ABA response mutant overexpressing the *AtPP2CA* cDNA during an ABA-insensitive screen of a library of 33,000 35S::cDNA-expressing Arabidopsis lines. We characterize *AtPP2CA* gene disruption and overexpression phenotypes in Arabidopsis. We show that T-DNA insertions in the *AtPP2CA* gene result in a strongly increased sensitivity to the phytohormone ABA during seed germination (Fig. 2, A and B; Supplemental Fig. 1A) and also render guard cells more sensitive to ABA during stomatal closure at 1 μM ABA (Fig. 5, B and C) and affect root elongation in the presence of exogenous ABA (Fig. 3, C and D). On the other hand, overexpression of *AtPP2CA* impairs stomatal closure in response to 10 μM ABA (Fig. 5B). Seed germination of PP2CAox lines displays a greatly decreased sensitivity to ABA (Fig. 2, A and C). Moreover, constitutive expression of *AtPP2CA* in the ABA-hypersensitive mutant *abh1* is shown to partially restore ABA sensitivity in *abh1* (Fig. 7A). Together, these results point to an important function of *AtPP2CA* as a negative regulator of ABA signal transduction events. The identification of a negative regulator in ABA signaling based on a cDNA overexpression screen shows that this approach can be used to isolate mutants in genes that modulate complex signaling networks in plants (Schroeder et al., 2001; Fedoroff, 2002; Finkelstein et al., 2002; Hetherington and Woodward, 2003; Himmelbach et al., 2003; Fan et al., 2004; Cutler and McCourt, 2005).

In an earlier study, *AtPP2CA* was shown to be linked to cold acclimation in Arabidopsis (Tahtiharju and Palva, 2001). It was shown that *AtPP2CA* is highly induced during cold acclimation. Plants with suppression of *AtPP2CA* transcripts by an antisense approach were shown to exhibit an ABA-dependent accelerated development of freezing tolerance (Tahtiharju and Palva, 2001) and an increased ABA sensitivity in seed germination was mentioned. As the antisense construct used shows nucleotide homology to five of the nine group A PP2Cs, and to avoid the possible effects of cosuppression of these related group A *PP2C* genes, we pursued analyses of T-DNA disruption lines of the *AtPP2CA* gene (Fig. 3A). Accordingly, northern-blot and RT-PCR analyses of the *pp2ca-1* and the *pp2ca-2* lines show absence of mature *AtPP2CA* transcripts (Figs. 1B and 3B).

AtPP2CA, a Negative Regulator of Physiological ABA Responses

We report that *AtPP2CA* gene disruption lines show a strongly increased sensitivity to ABA during seed germination, which appears to be more pronounced than in a *AtP2C-HAB1* disruption line (Leonhardt et al., 2004; Saez et al., 2004). Thus, we investigated the effect of *AtPP2CA* gene disruption and ectopic expression at the whole-plant level.

Application of exogenous ABA is well established to affect root growth as an antagonist of auxin, impairing cell elongation and causing an arrest in mitotic cell cycle activity (Himmelbach et al., 1998). The ABA-insensitive dominant *abi1-1* mutant and sustained ex-

pression of *AtP2C-HAB1* in 35S::*AtP2C-HAB1* plants have been shown to exhibit less sensitivity to ABA inhibition of root growth (Beaudoin et al., 2000; Ghassemian et al., 2000; Saez et al., 2004). However, previous studies have not analyzed *PP2C* gene disruption lines for altered ABA-dependent root elongation. In our study, we show that *AtPP2CA* gene disruption results in a moderate hypersensitive response to ABA in root elongation compared to wild type under the conditions tested (Fig. 3, C and D).

Furthermore, we show that ectopic expression of *AtPP2CA* results in increased transpiration rates of detached rosette leaves (Fig. 5A). It has been shown previously that the dominant ABA-insensitive mutants *abi1-1* and *abi2-1* are sensitive to water stress conditions and impair early ABA signal transduction (Koornneef et al., 1984; Finkelstein, 1994; Leung et al., 1994, 1997; Meyer et al., 1994; Pei et al., 1997; Allen et al., 1999). More recently, plants overexpressing the *AtP2C-HAB1* protein phosphatase were also shown to exhibit increased transpiration rates (Saez et al., 2004). However, we could not observe a difference in the transpiration rates of detached leaves from the *pp2ca-1* plants (Fig. 5A), resembling findings on the *abi1-1R1* to *abi1-1R7* intragenic revertant lines and the T-DNA insertion line of *AtP2C-HAB1* (Saez et al., 2004), even though these mutants show ABA-hypersensitive responses in stomatal movements (Gosti et al., 1999; Leonhardt et al., 2004). Previous leaf water loss analyses from detached leaves have shown that these assays can show phenotypic differences in mutations in which steady-state stomatal apertures already differ from wild-type controls prior to excising leaves (Kwak et al., 2001; Cominelli et al., 2005; Liang et al., 2005). More direct (double-blinded) analyses of ABA-induced stomatal closing show that *AtPP2CA* disruption causes ABA-hypersensitive stomatal closing (Fig. 5, B and C), which also appears to be more pronounced than in the *AtP2C-HAB1* gene disruption line (Leonhardt et al., 2004). Together, these results emphasize the importance of *AtPP2CA* as a strong negative regulator of ABA signal transduction during seed germination and stomatal closure.

AtPP2CA Shows a Similar Expression Profile to Other Group A *PP2C* Genes

Sixty-nine *PP2C* genes are encoded in the Arabidopsis genome compared to 15 *PP2C* genes in humans (Kerk et al., 2002; Schweighofer et al., 2004). This implies genetic redundancy and a more specific role of plant PP2Cs during developmental stages and responses to environmental changes. With the exception of *AtP2C-HAB1*, no gene disruption phenotype for other plant *PP2Cs* has been reported (Leonhardt et al., 2004; Saez et al., 2004). *AtP2C-HAB1* is highly expressed in guard cells in response to ABA and gene disruption causes ABA-hypersensitive stomatal closure (Leonhardt et al., 2004) and increased sensitivity during seed germination (Leonhardt et al., 2004; Saez et al., 2004).

AtPP2CA transcript levels are the second highest expressed of all group A *PP2Cs* in dry seeds of Arabidopsis and are significantly down-regulated upon imbibition (Supplemental Fig. 2; Zimmermann et al., 2004; Nakabayashi et al., 2005). A similar, but overall lower, expression pattern can be seen for *AtP2C-HAB1* (Supplemental Fig. 2). Since other not-yet-characterized *PP2Cs* of group A also show high transcript levels in dry seeds and down-regulation during imbibition, an ABA-related function in seed germination can be anticipated for these genes. Moreover, *AtPP2CA* was found to be among the mRNAs with the highest levels of ABA-mediated induction in guard cells, showing an average 7-fold ABA induction (Leonhardt et al., 2004), again similar to the transcript abundance of *AtP2C-HAB1* (Supplemental Fig. 3). Guard cell expression of *AtPP2CA* and *AtP2C-HAB1* was also shown in independent studies, which investigated their promoter activities in planta with reporter gene fusions (Cherel et al., 2002; Saez et al., 2004).

Despite the large gene family of PP2Cs and similar expression patterns of *AtP2C-HAB1* and *AtPP2CA*, the

Table I. *Oligonucleotide primers used in this study*

Primer Name	Sequence (5′-3′)
PP2CAEx1-F	CAAATGGCTGGGATTTGTTGC
PP2CAInt1-F	CTCATGTACGTTAATTTCCTCTGTTTAG
PP2CAInt2-F	CGAATTGGTTAGTATGAATCAAGATGGC
PP2CAInt3-F	CTGATGATTATTGTTTTTGTTTGTATAGGT
PP2CAEx4-R	AAGACGACGCTTGATTATTCCTC
SALK-LBa1	TGGTTCACGTAGTGGGCCATCG
p745	AACGTCCGCAATGTGTTATTAAGTTGTC
PP2CAEx1Sal1	TACCGTCGACAAATGGCTGGGATTTGTTGC
PP2CAEx4Spe1	TCCACTAGTTTAAGACGACGCTTGATTATTCC
EF1a-F	GGCCACGTCGATTCTGGAAA
EF1a-R	GGCTTGGTTGGAGTCATCTT
35S-F	CGCACAATCCCACTATCCTTCGCAAG
NOS-R	GATAATCATCGCAAGACCGGCAACAGG
1′BAR-F	GCGCAAGACGTGACGTAAGTATCCG
1′BAR-R	CCCTCTAGAGTCGACCTGCAGGCATGC
BARN-LB1	GGGCCAGGCGGTGAAGGGCAATC
ACTIN7-F	GGCCGATGGTGAGGATATTCAGCCACTTG
ACTIN7-R	TCGATGGACCTGACTCATCGTACTCACTC
ABI3F1	CGGTTTTAGATTACTTATTAGC
ABI3R1	CCACCGCCTAGTCTTCTTGCC
ABI3F2	GGCGGTGGTAAAGAAGCGATGAAGC
ABI3R2	CCGAGGTTACCCACGTCGC
ABI3F3	GGGTAACCTCGGAAGGATCG
ABI3R3	CCCATGCATGCACGAGAAG
ABI4F1	GGGATGCTCATCGTATATAATATG
ABI4R1	GGACCCTTTAGCTTCCCAAC
ABI5F1	GGGAACACTAGTAAAGCAG
ABI5R1	CCGCTTTGTAGGAAGACTGTTG
ABI5F2	GCGGAGCTGGAAGTGTCAAAG
ABI5R2	CCGCCTCCTACCCATTTATC
ABI5F3	GCGCCATGGACGACTCTACTTCTCGC
ABI5R3	GGTAACGAAAACTTTATTGG

limited functional redundancy in single gene disruption lines during the process of ABA signal transduction in seeds and guard cells may imply a high degree of specificity toward downstream targets of these two PP2Cs. In plants, our knowledge about PP2C targets is still limited and no target has been identified for AtP2C-HAB1. However, the inward-rectifying potassium channel AKT2 was shown to interact with AtPP2CA in yeast and AKT2 channel activity is negatively modulated by *AtPP2CA* in heterologous expression systems (Cherel et al., 2002). Also, the ABI2 protein phosphatase has been found to interact in yeast with the protein kinase PKS3 (Guo et al., 2002). In addition, ABI1 can interact with the ABA-inducible homeodomain transcription factor AtHB6 (Himmelbach et al., 2002). Moreover, phospholipase Dα1 (*PLDα1*)-derived phosphatidic acid has been shown to bind and regulate ABI1 (Zhang et al., 2004).

Modulation of *AtPP2CA* in *abh1*

The *abh1* mutation causes ABA hypersensitivity in seed germination and stomatal movements and modulates ion channel activities in guard cells (Hugouvieux et al., 2001, 2002). Down-regulation of the *AtPP2CA* transcript level was previously reported in the *abh1* mutant (Hugouvieux et al., 2001). Because a protein homologous to ABH1, the 80-kD subunit of the dimeric nuclear cap-binding protein CBP80, has been shown to affect splicing during pre-mRNA maturation in yeast and mammalian cells (Izaurralde et al., 1994; Lewis and Izaurralde, 1997; Fortes et al., 1999; Clark et al., 2002), we investigated the hypothesis that the *AtPP2CA* transcript undergoes differential splicing in *abh1* compared to wild type, therefore causing down-regulation of the transcript. An RT-PCR analysis specifically designed to amplify splice intermediates based on differential intron splicing efficiencies in *abh1* and wild type did not reveal any differences in pre-mRNA splicing of *AtPP2CA* (Fig. 6). Therefore, pre-mRNA splicing seems unlikely to cause down-regulation of the *AtPP2CA* transcript, an effect that was observed for some transcripts in the yeast *gcr3* mutant that encodes the CBP80 protein (Clark et al., 2002).

In this study, we investigated the hypothesis that *AtPP2CA* down-regulation in *abh1* contributes to the ABA hypersensitivity in *abh1*. Constitutive expression of *AtPP2CA* in the *abh1* mutant suppressed the ABA hypersensitivity of *abh1* plants. Interestingly, however, overexpression did not render *abh1* plants as ABA-insensitive as 35S::*AtPP2CA* wild-type plants (Fig. 7A). Out of 40 *abh1* plants harboring the 35S::*AtPP2CA* construct, only two homozygous single-insertion lines resulted in ABA sensitivity similar to wild-type plants in seed germination experiments. The comparison of *AtPP2CA* transcript levels by RT-PCR in these gain-of-function lines revealed that the *AtPP2CA* transcript levels were up to 1.5-fold higher than the *AtPP2CA* transcript level in *abh1*, but still significantly lower than in wild-type plants (Fig. 7B). Because the identification of strong *AtPP2CA* gain-of-function phenotypes in the *abh1* background proved substantially more difficult than in wild type, a negative feedback mechanism may limit *AtPP2CA* expression in *abh1*. With *AtPP2CA* gene disruption lines being less ABA hypersensitive in seed germination than *abh1* (Fig. 2, A and B) and because *AtPP2CA* overexpression only partially restores wild-type-like ABA sensitivity in *abh1*, we conclude that additional mechanisms contribute to ABA hypersensitivity in *abh1*.

In conclusion, we demonstrate that the protein phosphatase *AtPP2CA* acts as a strong negative regulator of ABA signal transduction during seed germination (Fig. 2A) and the regulation of stomatal closure (Fig. 5B). Yoshida et al. (2006) have conducted an independent screen for ABA signaling components in Arabidopsis. They have characterized the same protein phosphatase *AtPP2CA* also showing ABA hypersensitivity in loss-of-function mutants and insensitivity in *AtPP2CA* overexpressors. Despite the large number of *PP2C* genes in the Arabidopsis genome, this study demonstrates that loss- and gain-of-function of *AtPP2CA* causes strong modulation of ABA responses. With the negative regulatory role of PP2CA in ABA signal transduction, a challenging question for future research will be to uncover the interacting proteins of PP2CA.

MATERIALS AND METHODS

Plant Material, Growth Conditions, and Transformation

Arabidopsis (*Arabidopsis thaliana*) plants (Col-0) were grown in a Conviron growth chamber (Controlled Environments Limited) in plastic pots filled with ready-to-use soil (Professional Blend). After sowing, pots were kept at 4°C for 4 to 7 d. Growing conditions were 22°C, 75% humidity, with a 16-h-light/8-h-dark photoperiod regime at approximately 75 μmol m^{-2} s^{-1}. Seeds used for comparative studies were from plants grown and harvested in parallel.

Seeds of the activation-tagged lines for identification of ABA-insensitive mutants were kindly provided by D. Weigel (Max-Planck-Institute for Developmental Biology, Tuebingen, Germany; Weigel et al., 2000) and by W. Scheible (Carnegie Institution, Stanford, CA; Sedbrook et al., 2004). Seeds from the library of 35S::cDNA Arabidopsis-expressing lines (LeClere and Bartel, 2001) were obtained from ABRC and correspond to the CS84450 stock number. The binary vectors constructed and described below were introduced into *Agrobacterium tumefaciens* strain GV3101 by electroporation, which was then used to transform wild-type (Col-0) or *abh1* plants by floral dipping (Clough and Bent, 1998).

Molecular Characterization of a New *abi5* Allele and *pp2ca-1* and *pp2ca-2* Insertional Mutants

Given the recessive nature of the strong ABA insensitivity in the homozygous 54.7 mutant, we hypothesized that its phenotype could be mediated by disruption of positive regulators such as *ABI3*, *ABI4*, and *ABI5*. To test this hypothesis, PCR on genomic DNA from 54.7 and wild-type plants with a set of specific primers for *ABI3*, *ABI4*, and *ABI5* (Table I) was carried out. Only one PCR reaction with ABI5-F3/ABI5-R2 primers (Table I) did not lead to any products from 54.7 genomic DNA (data not shown). PCR genotyping reactions from 54.7 genomic DNA were then carried out with ABI5-F3/ABI5-R2 and with BARN-LB1 (Table I; Fig. 1A), a specific primer of the 35SpBARN T-DNA left border. This led to amplification of two PCR products whose sequencing showed that a tandem T-DNA insertion occurred in the first intron of *ABI5* approximately 500 bp before the start codon.

pp2ca-1 and *pp2ca-2* mutants of the *AtPP2CA* gene (At3g11410) were obtained from ABRC and correspond to the SALK_028132 and WiscDsLox341D03 lines, respectively. Genotyping PCR reactions for *pp2ca-1* and *pp2ca-2* were performed with PP2CAEx1-F/PP2CAEx4-R primers and with PP2CAEx1-F/SALK-LBa1 primers (SALK_028132) or with PP2CAEx1-F/p745 primers (WiscDsLox341D03) and amplified products were sequenced (Table I).

Molecular Characterization of the PP2CAox Mutant and Generation of the Reconstructed 35S::*AtPP2CA* Lines

The cDNA within the T-DNA of 393.1 plants was PCR amplified from genomic DNA with 35S-F/NOS-R primers (Table I; Fig. 1A) and purified with the QIAEX II kit (Qiagen). The amplified DNA was then cloned into pGEM-T Easy vector (Promega) and sequenced. Because the PCR fragment matched the full-length cDNA of *AtPP2CA* perfectly, it was then excised from pGEM-T Easy with *Sma*I and *Not*I restriction enzymes, subcloned into *Sma*I/*Not*I linearized 35SpBARN vector (Fig. 1A; LeClere and Bartel, 2001), and then used to transform Arabidopsis wild-type plants.

Vector PS173 (kindly provided by Professor Jeff F. Harper, University of Nevada, Reno) was used to constitutively express *AtPP2CA* cDNA in wild-type and *abh1* plants. After amplification from total wild-type (Col-0) cDNA (first-strand cDNA synthesis kit; Amersham Biosciences) and cloning the *AtPP2CA* cDNA into the pGEM-T Easy vector (Promega), the sequenced cDNA was excised with *Sal*I and *Spe*I restriction enzymes and subcloned into the PS173 vector previously digested with *Xho*I and *Xba*I restriction enzymes.

Northern-Blot and RT-PCR Analyses

Total RNA was extracted from leaves using TRIzol reagent (Life Technologies/Gibco-BRL) and quantified by absorption and migration of an aliquot on agarose gel. For ABA and drought treatments, rosette leaves of 3- to 4-week-old wild-type plants were either sprayed with 50 μM ABA or excised and subjected to desiccation for 0.5, 1, 2, 3, 6, or 12 h before extraction. Fifteen micrograms of total RNA were separated in a denaturing formaldehyde-agarose gel and blotted to a Hybond-N membrane (Amersham-Pharmacia). Blots were hybridized with random-priming ^{32}P-labeled probes (Megaprime DNA labeling system; Amersham-Pharmacia). *AtPP2CA* and *ACTIN7* probes were amplified by PCR from cDNA using PP2CAEx1-F/PP2CAEx4-R and ACTIN7-F/ACTIN7-R primers, respectively (Table I). PCR fragments were purified using the QIAEX II kit (Qiagen).

RT-PCR experiments were performed on total RNA isolated as described above after DNase I treatment (DNA-*free*; Ambion). Reverse transcription (first-strand cDNA synthesis kit, Amersham Biosciences) was performed on 2.5 μg of RNA and 2 μL were used for PCR reactions (*Ex Taq* DNA polymerase; TaKaRa Mirus Bio). Samples were withdrawn after 20, 24, 28, and 32 cycles (splicing) or 28, 32, and 36 cycles (T-DNA disruption lines) and products were analyzed by agarose gel electrophoresis. Hybridization/PCR signals were quantified using Adobe Photoshop 5.5 software (Adobe Systems) after subtraction of background levels. Expression levels for northern-blot and RT-PCR analyses were normalized against the corresponding *ACTIN7* and *EF1α* RNA levels, respectively.

Root Growth and Germination Assays

For ABA germination assays, sterilized seeds were plated on minimal medium (0.25× Murashige and Skoog medium, no Suc) supplemented with increasing ABA concentrations. After stratification of 4 d at 4°C, plates were transferred to a Conviron growth chamber (Controlled Environments Limited). To score seed germination, the percentage of seeds that had germinated and developed fully green expanded cotyledons was determined in three independent experiments (36 seeds per genotype and experiment).

Root growth assays to assess ABA sensitivity were carried out by transferring 6-d-old seedlings onto minimal medium (0.25 × Murashige and Skoog medium, no Suc) supplemented with the indicated ABA concentrations on 0.8% agar (Phytagel; Sigma) plates. Root growth was measured 6 d after the transfer in three independent experiments with eight individuals per genotype and experiment. *t*-Test (one-tailed, homoscedastic) *P*-values are as follows: *pp2ca-1*, P = 3.6E-07 (2.5 μM ABA); P = 3.3E-06 (5 μM ABA); P = 4.6E-04 (10 μM ABA); P = 1.6 E-07 (25 μM ABA); and P = 6.8E-06 (50 μM ABA). *pp2ca-2*, P = 9.7E-06 (2.5 μM ABA); P = 6.5E-06 (5 μM ABA); P = 7.2E-05 (10 μM ABA); P = 8.9E-13 (25 μM ABA); and P = 1.2E-07 (50 μM ABA).

Leaf Water Loss and Stomatal Closure Measurements

Time-dependent analyses of loss of fresh weight were performed with detached rosette leaves at the same developmental stage and size from single 3-week-old plants. Three leaves per genotype were excised, kept in the Conviron growth chamber (Controlled Environments Limited), and fresh weight was measured at the indicated periods of time in three independent experiments.

Double-blind stomatal movement assays were performed such that the genotype and applied ABA concentrations were unknown. Stomatal responses were analyzed in 3- to 4-week-old plants grown in a Conviron growth chamber. Leaves were floated for 2.5 h in stomatal opening solution (Pei et al., 1997) containing 50 mM KCl, 50 μM CaCl, and 10 mM MES (pH 6.15). After incubation in ABA for 2.5 h, leaves were blended and the stomatal aperture was measured. Control experiments were performed in parallel with no ABA added. *t*-Test (one-tailed, homoscedastic) *P*-values were calculated: P = 2.3E-08 for *pp2ca-1* and P = 5.7E-13 for *pp2ca-2* at 1 μM ABA, and P = 2.0E-09 for PP2CAox at 10 μM ABA.

Sequence data from this article can be found in the GenBank/EMBL data libraries under accession number NM_111974.

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