The Protein Phosphatase AtPP2CA Negatively Regulates Abscisic Acid Signal Transduction in Arabidopsis, and Effects of abh1 on AtPP2CA mRNA

Josef M. Kuhn, Aurélien Boisson-Dernier, Marie B. Dizon, Mohammad H. Maktabi, and Julian I. Schroeder

Division of Biological Sciences, Cell and Developmental Biology Section, and Center for Molecular Genetics, University of California, La Jolla, California 92093–0116

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 F1 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 (erva; Cutler et al., 1996; Pei et al., 1998), inositol 1,4,5-triphosphate (IP3) 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), ABI18 (Brocard-Gifford et al., 2004), RPK1 (Osakabe et al., 2005), and GCA2 (Himmelbach et al., 1998).

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

1 This work was supported by National Institutes of Health (R01GM060396) and National Science Foundation (MCB0417118) grants (to J.I.S.), and by a European Molecular Biology Organization fellowship (to J.M.K.).

2 These authors contributed equally to the paper.

* Corresponding author; email julian@biomail.ucsd.edu; fax 858–534–7108.

The author responsible for distribution of materials integral to the findings presented in this article in accordance with the policy described in the Instructions for Authors (www.plantphysiol.org) is: Julian I. Schroeder (julian@biomail.ucsd.edu).

[1W] The online version of this article contains Web-only data.

Article, publication date, and citation information can be found at www.plantphysiol.org/cgi/doi/10.1104/pp.105.070318.
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 abh1-1, 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 abh1-1.

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, 920 putative individual T2 seeds were able to germinate (radicle emergence plus expanded 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.

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
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. 2A 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. 2A 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...
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 ACT7 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; Kmiecik 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).

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
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). Despite the large gene family of PP2Cs and similar expression patterns 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). Despite the large gene family of PP2Cs and similar expression patterns of AtP2C-HAB1 and AtPP2CA, the...
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 AtPP2C-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, ABI2 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; Forster 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 overexpressers. 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 CSS4450 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 S4.7 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 S4.7 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 S4.7 genomic DNA (data not shown). PCR genotyping reactions from S4.7 genomic DNA were then carried out with ABI5-F3/ABI5-R2 and with BARN-LB1 (Table I; Fig. 1A), a specific primer of the 530bpBARN 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 Wisc-DeLox341D03 lines, respectively. Genotyping PCR reactions for pp2ca-1 and pp2ca-2 were performed with PP2CAEx1-F/PP2CAEx-R primers and with PP2CAEx1-F/SALK-LBai primers (SALK_028132) or with PP2CAEx1-F/ P745 primers (Wisc-DeLox341D03) and amplified products were sequenced (Table I).

Molecular Characterization of the PP2Cox 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 vector with SmaI and Nof restriction enzymes, subcloned into SmaI/Nof linearized 35S::BARN vector (Fig. 1A; LeClerc 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 abh1-1 plants. After amplification from total wild-type (Col-0) cDNA (first-strand cDNA synthesis kit; Amersham BioSciences) and cloning the AtPP2CA and cDNA into the pGEM-T Easy vector (Promega), they sequenced cDNA was excised with Sall and Sph restriction enzymes and subcloned into the PS173 vector previously digested with Xhol and XbaI 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-primer 32P-labeled probes (Megaprimr DNA labeling system; Amersham-Pharmacia). AtPP2CA and ACTIN7 probes were amplified by PCR from cDNA using PP2CAEx1-F/PP2CAEx-R and ACTIN7-S/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 Mira 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 nutrient medium, no Suc) supplemented with the indicated ABA concentrations on

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 CaCl2, and 10 mM MES (pH 6.15). After incubation in ABA for 2.5 h, leaves were blanched and the stomatal aperture was measured. Control experiments were performed in parallel with no ABA added.

Sequence data from this article can be found in the GenBank/EMBL data libraries under accession number NM_115974.

ACKNOWLEDGMENTS

We thank David Waner and Nasef Nasi for screening the activation-tagging populations, Bonnie Bartel for providing the 35S::BARN vector as well as helpful discussions, Nadia Robert, Jeanel Combet, and Dongyul Sung for discussions, and Tae-Hoon Kim and Jared Young for comments on the manuscript.

Received August 24, 2005; revised October 13, 2005; accepted October 23, 2005; published December 16, 2005.

LITERATURE CITED


