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Full Length Research Paper

Overexpression of Arabidopsis *PP2CA2* gene shows phenotypes related to abscisic acid responses

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In this study we overexpressed *PP2CA2* gene in Arabidopsis to provide genetic evidence on this gene function in seed germination and other plant responses. The average seed germination percentage of constitutive expression of *AtPP2CA2* under a cauliflower mosaic virus 35S promoter (*35S::AtPP2CA2*) was lower than that of wild type under 0.3 and 1 uM exogenous abscisic acid (ABA), indicating an increased sensitivity of *35S::AtPP2CA2* seeds to ABA with respect to wild type. The *35S::AtPP2CA2* seedlings showed an increased inhibition of root growth compared with wild-type plants. In the absence of exogenous ABA, the water loss in *35S::AtPP2CA2* plants was lower than that in wild type. There is no obvious difference of water loss between *35S::AtPP2CA2* and wild type plants under 10 uM ABA treatment. The *35S::AtPP2CA2* plants had smaller stomatal apertures than did wild-type plants under ABA treatment. Furthermore, the *35S::AtPP2CA2* seedlings showed a reduced inhibition of hypocotyl growth compared with wild-type plants under the 0, 10, 20, 30 and 40 uM exogenous ABA. The study on overexpression of *AtPP2CA2* gene could provide an approach for improving crop performance under stress conditions.

Key words: Arabidopsis, *AtPP2CA2* gene, phenotype, abscisic acid signal transduction.

INTRODUCTION

The phosphorylation and dephosphorylation of proteins are the fundamental mechanism by which living organisms modulate cellular processes including cell cycle events, growth factor responses, hormone and environmental stimuli responses, metabolic control, and developmental processes. The protein phosphatases can be divided into two major classes based on substrate specificity: protein tyrosine phosphatases (PTPs) and protein serine/threonine phosphatases. Protein tyrosine phosphatases include PTPs and dual specificity phosphatases (DSPTPs). According to distinct amino acid sequences and crystal structures, the protein serine/threonine phosphatases are classified into the PPP and PPM gene families. The PPP family includes so-called signature phosphatases [types 1 (PP1), 2A (PP2A) and 2B (PP2B)], whereas the PPM family includes type 2C (PP2C) and pyruvate dehydrogenase phosphatase (Cohen, 1997). Regulated dephosphorylation by protein phosphatases (PPs) has emerged as a universal control mechanism in physiology and development. Important roles have been identified for a variety of PP species in plants. For example, PP2As in plants have been demonstrated to play important roles in hormone homeostasis and signaling, defense responses, cell division, morphogenesis, and reproduction (DeLong, 2006). Analysis of Arabidopsis mutants showed PP2As are important regulators of some proteins to control micro-tubule dynamics (Camilleri et al., 2002) and components of the auxin transport apparatus (Shin et al., 2005; Michniewicz et al., 2007). Similarly, the DSPTPs have been shown to regulate carbohydrate metabolism (Kerk et al., 2006; Sokolov et al., 2006) and oxidative, saline, and genotoxic stress tolerance (Ulm et al., 2002; Lee and Ellis, 2007). PP2C are monomeric enzymes present in both prokaryotes and eukaryotes. Plant PP2Cs have been demonstrated to play important roles in abscisic acid (ABA)-activated multiple signal transductions, such as ABA-induced seed dormancy/germination, guard cell closure, ion tunnel regulation and stress acclimation (Yoshida et al., 2006; Ma et al., 2009; Rubio et al., 2009; Umezawa et al., 2009; Vlad et al., 2009; Brock et al.,

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2010). Protein phosphatases type 2C from group A, which includes the ABI1/HAB1 and PP2CA branches, are some key negative regulators of ABA signaling. Specifically, HAB1, ABI1, ABI2, and PP2CA have been shown to affect both seed and vegetative responses to ABA (Rubio et al., 2009). The plant hormone ABA regulates many key processes in plants, including seed germination and development. It has critical functions in stress resistance and in growth and development. Understanding ABA signal transduction events has been a major goal of plant research (Lee et al., 2009; Hubbard et al., 2010; Cutler et al., 2010; Fujii et al., 2010). In the present study, to understand the function of *AtPP2CA2* (At5g59220) gene in Arabidopsis, we isolated a *35S::AtPP2CA2* transgenic plant and investigate its possible phenotypes related to abscisic acid responses.

MATERIALS AND METHODS

Plant material and growth conditions

We used the Col-0 accession of Arabidopsis (Arabidopsis thaliana) and *35S::AtPP2CA2* expressing lines for germination, gene expression, hypocotyl and root growth, leaf water loss and stomatal closure measurements. The plants were routinely grown in a growth chamber under 40% humidity, a temperature of 22°C, and with a 16 h light/8 h dark photoperiod at 100 to 150 µmol m^{-2} s^{-1} of light in pots containing a 1: 3 vermiculite-soil mixture. For in vitro culture, seeds were surface-sterilized in 70% ethanol for 10 min, soaked for 10 min in 7% NaClO, and finally, washed four times in sterile distilled water. Stratification of the seeds was conducted during 3 d at 4°C. Afterward, seeds were sowed on Murashige and Skoog (1962) plates containing solid medium composed of Murashige and Skoog basal salts and 1% (w/v) sucrose, solidified with 1% (w/v) agar and the pH was adjusted to 5.7 with KOH before autoclaving. Plates were sealed and incubated in a controlled environment growth chamber.

Reconstruction of Arabidopsis *35S::AtPP2CA2* lines

The *AtPP2CA2* was amplified by Polymerase Chain Reaction (PCR) from genomic DNA with the following primers: F(5'-CCGGAATTCGCCAGACCGTCCGGACGA-3'), R(5'-CGCCTCGAGCTACGTGTCTCGTCGTAGATCA-3'). The purified DNA was then cloned into pGEM-T Easy vector (Promega) and sequenced. The PCR fragment was excised from pGEM-T Easy with EcoRI and XhoI restriction enzymes, subcloned into EcoRI/XhoI linearized pEGAD vector with the 35S promoter, and then used to transform Arabidopsis wild-type plants. The reconstructed vector was transferred to Agrobacterium tumefaciens strain GV3101 by electroporation, which was then used to transform wild-type (Col-0) plants by floral dipping (Clough and Bent, 1998). Transgenic lines were selected by spraying a 1:1,000 dilution of Basta on 2-week-old plants. Homozygous T3 lines containing a single insertion were used.

Quantitative real-time RT-PCR analyses

Total RNA was isolated using Puprep RNAeasy mini kit (Ambiogen Life Tech Ltd). DNA-free RNA was obtained by RQ1 DNase I treatment according to the manufacturer's instructions (Promega). The first strand cDNA was synthesized by M-MLV reverse transcriptase (RT, Promega), and then used for real-time qRT-PCR. Primers were designed using the program Primer Express™ (Applied Biosystems, Forster City, CA, USA). The β-actin gene was used as a control to normalize the level of total RNA. The PCR primers are as follows: Act2F(5'-CACTGTGCCAATCTACGAGGGT-3'), Act2R(5'-CACAAACGAGGGCTGGAACAAG-3'); PP2CA2F (5'-CAGCGGGTGGTCGTGTTA-3'), PP2CA2R (5'-CGCAAGCCTCGTCAGCAA-3'). Real-time RT-PCR reactions were prepared according to the following protocol: 12.5 µl 2×SYBR™ Green PCR Mix (Applied Biosystems), 500 nM forward primer, 500 nM reverse primer, cDNA template from wild type and *35S::AtPP2CA2* plants and 25 µl H2O. A master mix of sufficient cDNA and 2 SYBR® Green reagent was prepared prior and was dispensed into individual wells reducing pipetting errors and to ensure that each reaction contained an equal amount of cDNA. Stratagene Mx3000P QPCR System was used for the quantification of mRNA transcripts using RT-PCR. The PCR protocol starts with a denaturing step for 10 min at 95°C followed by 40 cycles of 15 s at 95°C and a primer extension reaction at 60°C for 1 min. After the run, the entire mix was denatured for 5 min at 95°C before it was slowly cooled down to 60°C while the kinetics of the reassociation of the complementary DNA strands of the PCR products were monitored. Reassociation kinetics provided information about additional unspecific product accumulation in a dissociation curve. All PCR reactions and the negative controls were run in duplicates with three biological replicates each.

Germination assays, hypocotyl and root growth

To measure ABA sensitivity and score seed germination, seeds were plated on solid medium composed of Murashige and Skoog basal salts, 1% sucrose, and different concentrations of ABA (0, 0.1, 0.3, 1, 10, 20, 30 and 40 uM). For germination, three replicates of 150 seeds were used in each treatment. The hypocotyl and root length for scoring ABA sensitivity were done after 6 d of the transfer of 2 day old seedlings onto vertical Murashige and Skoog plates containing 10, 20, 30 and 40 uM ABA. Root and hypocotyl length were measured 12 day after the transfer in three independent experiments. The criterion for hypocotyl measurement was the distance from the collet of root hairs to the apical hook.

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. Five leaves per genotype were excised, and leaves were floated on incubation in 0 and 10 uM ABA for 2.5 h, respectively. We measured the fresh weight loss in detached leaves *35S::AtPP2CA2* and wild type with or without ABA in three independent experiments. Stomatal aperture responses were analyzed from 5-week-old plants of *35S::AtPP2CA2* and wild type grown in a growth chamber. Leaves were floated for 2.5 h in stomatal opening solution (Pei et al., 1997) containing 50 mM KCl, 50 mM 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. Data were expressed as the average of four experiments where 30 to 40 stomata were measured for each one.

RESULTS

Generation and characterization of transgenic Arabidopsis plants overexpressing *35S::AtPP2CA2*

To investigate the role of *35S::AtPP2CA2* in the ABA

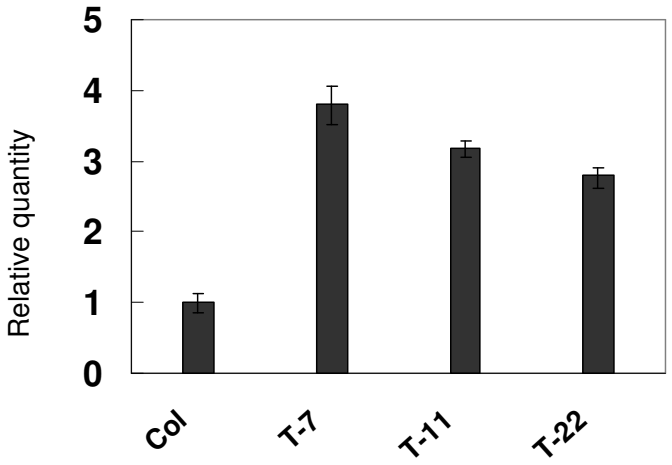

Figure 1. Relative expression levels of *PP2CA2* gene in 35S*::AtPP2CA2* plants (T-7, T-11 and T-22) compared to wild type. The expression level of *PP2CA2* gene in Col-0 was defined as "1."

response, we used an overexpression approach in Arabidopsis. Transgenic plants were selected in the presence of Basta. The approximate 3:1 segregation ratio was also confirmed when testing Basta-resistant T2 generation, possibly indicating a single insertion of the full-length *35S::AtPP2CA2* transgene. Then, transgenic lines were selected by spraying a 1:1,000 dilution of Basta. We selected homozygous lines 7, 11 and 22 of T3 progenies for the quantitative RT-PCR analyses. The results showed three lines had the high levels of expression in transgenic seedlings (Figure 1). In the present study, we mainly selected line 7, 11 and 22 for further analysis.

Germination assays of *35S::AtPP2CA2* and wild type

Progenies of *35S::AtPP2CA2* (line 7, 11 and 22) were harvested to test their sensitivity to ABA. Wild-type and *35S::AtPP2CA2* transgenic plants were plated on Murashige and Skoog (MS) media with ABA included in the media to ascertain whether *AtPP2CA2* over expression affected seed germination in response to ABA treatment.

The sensitivity of the *35S::AtPP2CA2* to inhibition of seed germination was analyzed (Figure 2). In the absence of exogenous ABA, *35S::AtPP2CA2* seeds showed a germination ratio similar to wild type. However, in the presence of different concentrations of exogenous ABA (0.3 and 1 uM) in the different treatment time (48, 72, 96 and 120 h), the average seed germination percentage of *35S::AtPP2CA2* was lower than that of wild type (Figure 2). This reduced germination in *35S::AtPP2CA2* transgenic seeds, consistent with the deeper degree of dormancy of these seeds, may be due to increased sensitivity to endogenous ABA.

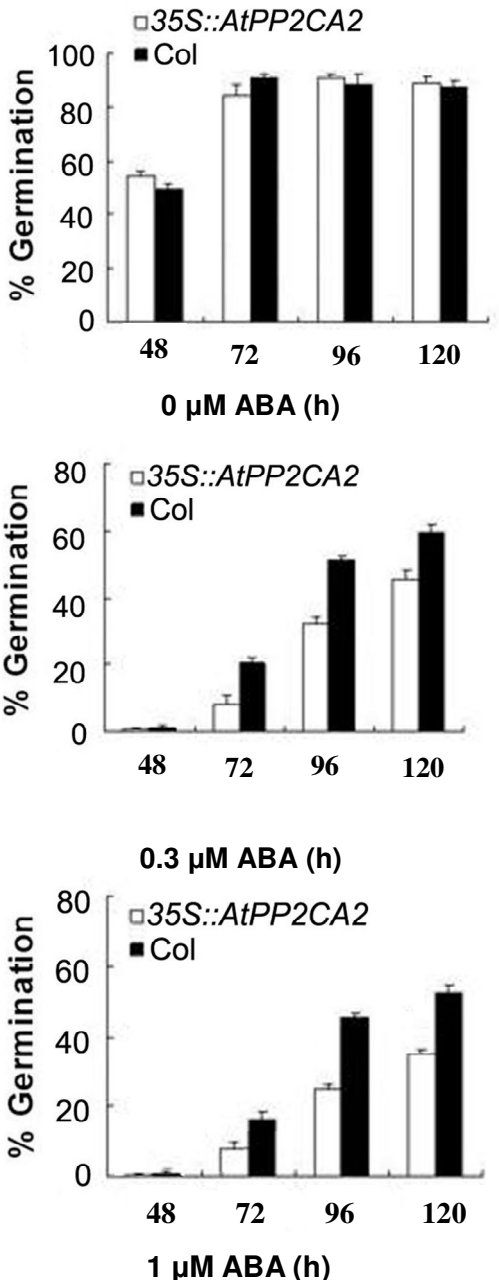

Figure 2. Effects of exogenous ABA on germination inhibition of *35s::AtPP2CA2* overexpressing plants and wild-type seeds. Percentage of seeds that germinated and developed green cotyledons in the presence of the indicated concentrations of ABA. Approximately 150 seeds of each genotype were sowed on each plate and scored for germination and early growth 5 d later. Values are averages ± SD for three independent experiments.

Root and hypocotyl growth analysis of *35S::AtPP2CA2* and wild type

To test whether *AtPP2CA2* gene constitutive expression in plants could affect the ABA responses, we investigated

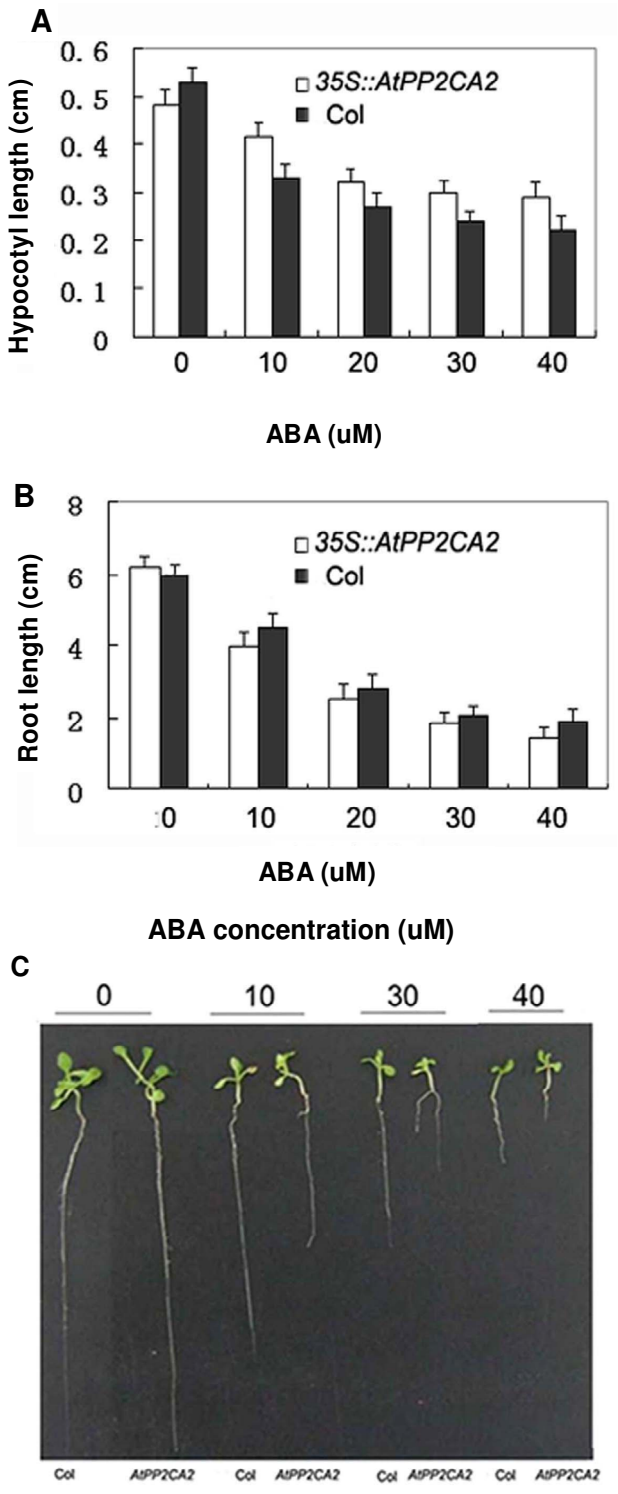

Figure 3. Comparison of hypocotyl and root length of wild-type and *35S::AtPP2CA2* seedlings. (A and B) Comparison of root length of wild-type and *35S::AtPP2CA2* seedlings. 2 day old seedlings were transferred to plates supplemented with 0, 10, 20, 30 and 40 µM ABA, and hypocotyl and root elongation was monitored after 12 day. Each data point represents the mean of three independent experiments. (C) 2 day old seedlings were transferred to plates supplemented with 0, 10, 20, 30 and 40 µM ABA, and growth of wild type and *35S::AtPP2CA2* was monitored after 12 day.

ABA inhibition of root growth by transferring 2 day old seedlings on Murashige and Skoog plates with 0, 10, 20, 30 and 40 µM ABA. The *35S::AtPP2CA2* seedlings showed an increased inhibition of root growth compared with wild-type plants (Figures 3B and C), indicating root length of transgenic plants was more sensitive to ABA than the wild type plants. However, the *35S::AtPP2CA2* seedlings showed a slightly reduced inhibition of hypocotyl growth compared with wild-type plants (Figure 3A).

Stomatal guard cells and water loss analysis of *35S::AtPP2CA2* and wild type

ABA signaling, by regulating stomatal aperture, plays a crucial role to reduce water loss under water shortage. Different analyses were performed to evaluate responses in *35S::AtPP2CA2* and wild type (Figure 4). In the absence of exogenous ABA, the water loss in *35S::AtPP2CA2* plants was lower than that in wild type. There is no obvious difference of water loss between *35S::AtPP2CA2* and wild type plants under 10 uM ABA treatment (Figure 4). To further analyze stomatal responses to ABA in *35S::AtPP2CA2* overexpressing plants, direct measurements of stomatal closing were performed in the *35S::AtPP2CA2* (line 7, 11 and 22) (Figure 5). Stomata of *35S::AtPP2CA2* plants had smaller stomatal apertures than did wild-type plants. In the presence of 1 and 10 µM ABA, transgenic Arabidopsis overexpressing *35S::AtPP2CA2* had a higher rate of stomatal closure than wild-type plants.

DISCUSSION

The PP2C gene family is one of the largest families identified in plants, and several PP2Cs have been described as negative regulators within the ABA mediated signaling network (Yoshida et al., 2006; Merlot et al., 2001; Tahtiharju; Palva, 2001; Gonzalez-Garcia et al., 2003; Saez et al., 2004). Recently, the 80 PP2C genes in A. thaliana (AtPP2Cs) were identified by a comprehensive computational analysis (Xue et al., 2008). The previous studies showed that the Arabidopsis HAB1 and PP2CA, homologous PP2Cs, were negative regulators involved in ABA signaling (Yoshida et al., 2006; Saez et al., 2004; Sheen, 1998; Leonhardt et al., 2004; Saez et al., 2006; Kuhn et al., 2006). Two PP2C genes (FsPP2C1 and FsPP2C2) were isolated from beech seeds (Fagus sylvatica). Constitutive expression of FsPP2C1 under the cauliflower mosaic virus 35S promoter confers ABA insensitivity during seed dormancy and germination in Arabidopsis seeds (Gonzalez-Garcia et al., 2003), which indicate that FsPP2C1 also acts as a negative regulator of ABA signaling. However, constitutive expression of FsPP2C2 under the cauliflower mosaic virus 35S promoter in Arabidopsis resulted in enhanced sensitivity to ABA and abiotic stress in seeds

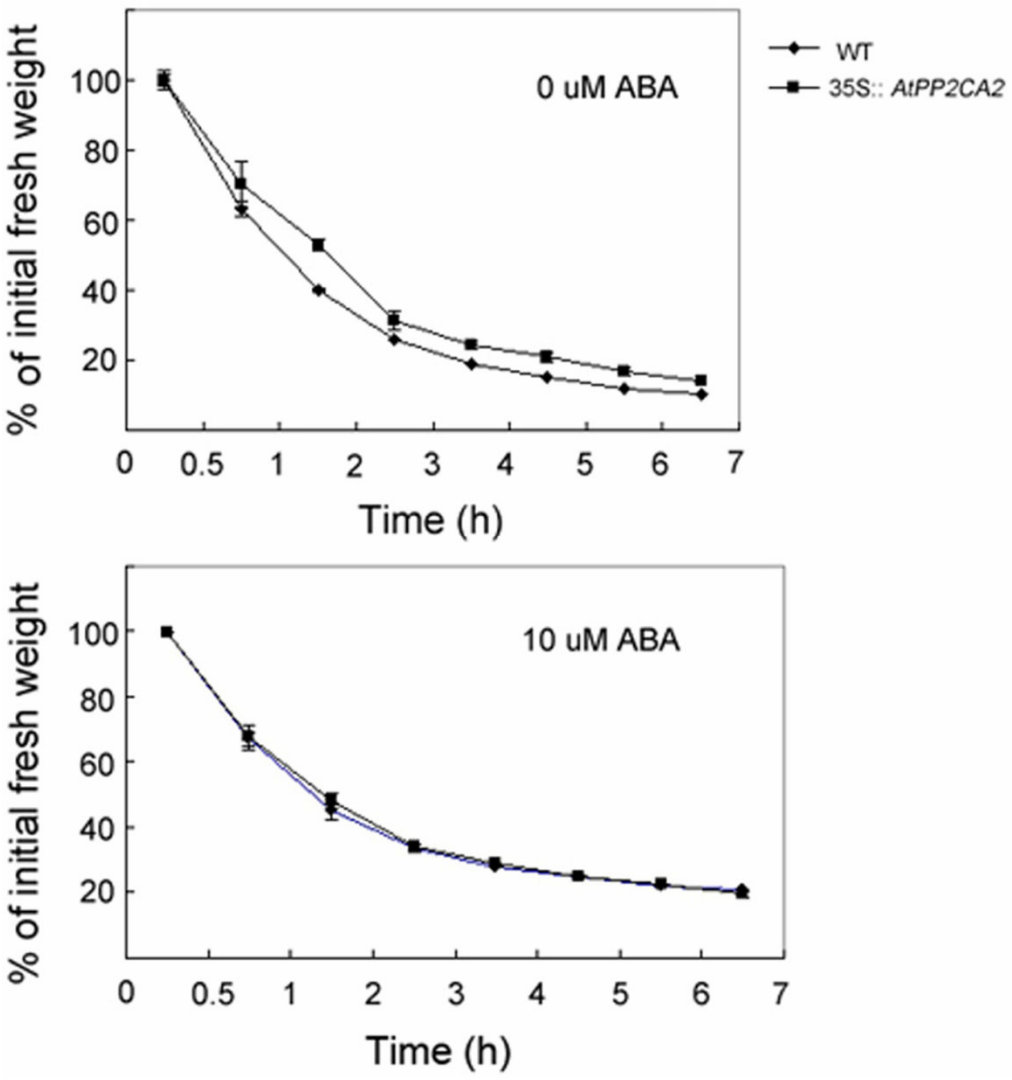

Figure 4. Loss of fresh weight of detached rosette leaves of 3-week-old plants was measured at the indicated time points. Data represent the mean of three independent experiments.

and vegetative tissues and caused a dwarf phenotype and delayed flowering, which shows that FsPP2C2 is a positive regulator of ABA (Reyes et al., 2006).

In this study, we used an over expression approach in Arabidopsis to further investigate *AtPP2CA2* function. We constructed transgenic plants possessing the *35S::GFP:AtPP2CA2* fusion gene, and studied *AtPP2CA* gene over expression phenotypes in Arabidopsis. In the presence of different concentrations of exogenous ABA (0.3 and 1 uM) in the different treatment time (48, 72, 96 and 120 h), the average seed germination percentage of *35S::AtPP2CA2* was lower than that of wild type (Figure 2). Root assays showed the *35S::AtPP2CA2* seedlings had an increased inhibition of root growth compared with wild-type plants in Murashige and Skoog plates with 0, 10, 20, 30 and 40 µM ABA (Figures 3B and C). Furthermore, in the absence of exogenous ABA, the water loss in *35S::AtPP2CA2* plants was lower than that in wild type. There is no obvious difference of water loss between *35S::AtPP2CA2* and wild type plants under 10 uM ABA treatment (Figure 4), as expected for an ABA sensitive phenotype. Together, these results point to an important function of *AtPP2CA2* as a positive regulator of ABA signal transduction events.

Stomatal size change has been observed in many transgenic or mutant Arabidopsis plants. For example, overexpression of AtMYB61 (Liang et al., 2005), which controls dark-induced stomatal closure, resulted in smaller stomatal apertures in transgenic Arabidopsis. Mutations on AtMYB60, OST1 (Xie et al., 2006) and HT1 (Hashimoto et al., 2006) resulted in smaller stomatal apertures, respectively. Therefore, overexpression or mutation of the genes involved directly or indirectly in structural movements of the stomata might affect

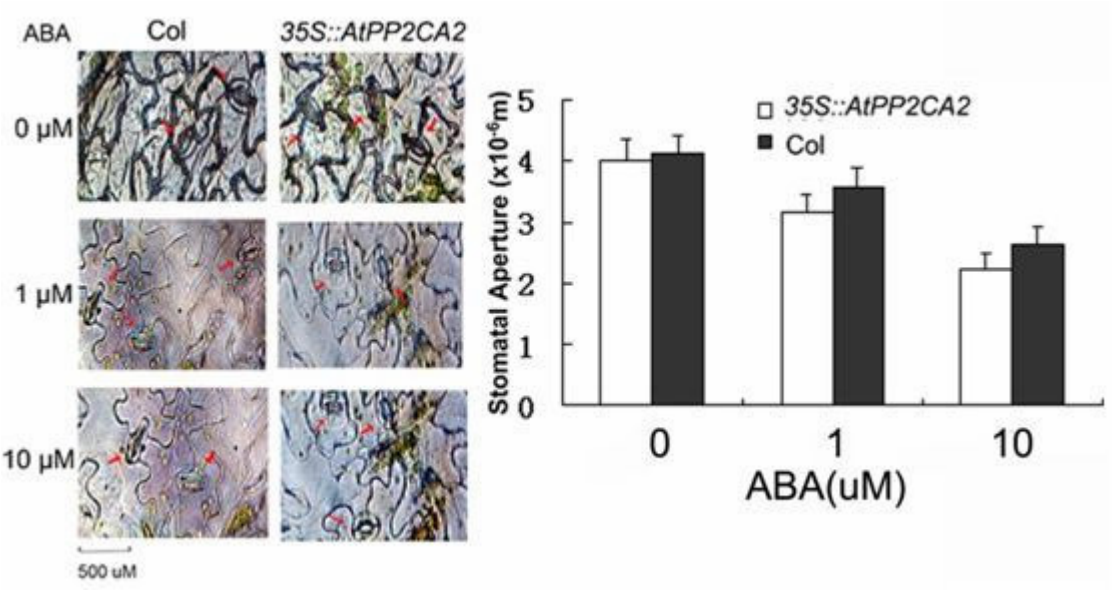

Figure 5. Measurement of stomatal aperture of wild type and *35S::AtPP2CA2* from 5-week-old plants in response to 0, 1, and 10 μM ABA. Stomas are indicated by red arrows. Data represent the mean of three independent experiments.

morphology of the guard cells in transgenic plants. However, we found the *35S::AtPP2CA2* plants had smaller stomatal apertures compared with wild type in the presence of 1 and 10 μM ABA (Figure 5). Thus, transgenic Arabidopsis overexpressing *35S::AtPP2CA2* was more sensitive to ABA and had a more rapid ABA-induced stomatal closure response than wild-type plants. In conclusion, we demonstrate that the protein phosphatase AtPP2CA2 acts as a positive regulator of ABA signal transduction during seed germination, root growth, water loss and stomatal closure. An independent screen for ABA signaling components in Arabidopsis was conducted by Yoshida et al. (2006). Their studies demonstrated the ectopic expression of a *35S::AtPP2CA* fusion caused ABA insensitivity, disruption mutants displayed the opposite phenotype, namely, strong ABA hypersensitivity. Although, there are large numbers of PP2C genes in the Arabidopsis genome, this study shows that loss- and gain-of-function of AtPP2CA causes strong modulation of ABA responses. With the positive or negative regulatory roles of PP2CA in ABA signal transduction, further studies will be to discover the interacting proteins of PP2CA.

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