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Expression of a grape calcium-dependent protein kinase ACPK1 in *Arabidopsis thaliana* promotes plant growth and confers abscisic acid-hypersensitivity in germination, postgermination growth, and stomatal movement

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Abstract Calcium is an important second messenger involved in abscisic acid (ABA) signal transduction. Calcium-dependent protein kinases (CDPKs) are the best characterized calcium sensor in plants and are believed to be important components in plant hormone signaling. However, in planta genetic evidence has been lacking to link CDPK with ABA-regulated biological functions. We previously identified an ABA-stimulated CDPK from grape berry, which is potentially involved in ABA signaling. Here we report that heterologous overexpression of *ACPK1* in Arabidopsis promotes significantly plant growth and enhances ABA-sensitivity in seed germination, early seedling growth and stomatal movement, providing evidence that ACPK1 is involved in ABA signal transduction as a positive regulator, and suggesting that the *ACPK1* gene may be potentially used for elevating plant biomass production.

Keywords Calcium-dependent protein kinase · ACPK1 · Abscisic acid signaling · Germination · Postgermination growth · Stomatal aperture

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Introduction

The phytohormone abscisic acid (ABA) regulates many aspects of plant growth and development including seed maturation and germination, seedling growth, flowering, and stomatal movement, and plays a central role in plant adaptation to environmental stresses (reviewed in Koornneef et al. 1998; Leung and Giraudat 1998; Finkelstein and Rock 2002). Numerous cellular components involved in ABA signal transduction have been identified, leading to considerable progress in understanding the ABA signaling pathway (reviewed in Finkelstein et al. 2002; Himmelbach et al. 2003; Fan et al. 2004).

Calcium is a central regulator of plant cell signaling (Hepler 2005), which has been shown to be an important second messenger involved in ABA signal transduction (reviewed in Finkelstein et al. 2002; Himmelbach et al. 2003; Fan et al. 2004). Specific calcium signatures are recognized by different calcium sensors to transduce specific calcium-mediating signal into downstream events (Sanders et al. 1999; Harmon et al. 2000; Rudd and Franklin-Tong 2001). Plants have several classes of calcium sensory proteins, including calmodulin (CaM) and CaM-related proteins (Zielinski 1998; Snedden and Fromm 2001; Luan et al. 2002), calcineurin B-like (CBL) proteins (Luan et al. 2002), and calcium-dependent protein kinases (CDPKs) (Harmon et al. 2001; Cheng et al. 2002). A CBL protein kinase CIPK15 and a CBL Ca^{++}-binding protein ScaBP5 interact with the calcium-modulated protein phosphatases (PPs) 2C ABI1 and ABI2 (Guo et al. 2002) that are two most characterized negative regulators of ABA signaling (Leung et al. 1994; Meyer et al. 1994; Leung et al. 1997; Sheen 1998; Gosti et al. 1999; Merlot et al. 2001). CIPK15 and one of its homologs CIPK3 and ScaBP5 are all involved in ABA signaling as negative

regulators (Guo et al. 2002; Kim et al. 2003), possibly by providing the PP2Cs ABI1 and ABI2 with Ca^{++}-sensors (Pandey et al. 2004) when forming a protein complex for perceiving the upstream signal Ca^{++} (Allen et al. 1999). Additionally, a link has been established between CIPK15 and an AP2 transcription factor AtERT7 that negatively regulates ABA response as a kinase substrate of CIPK5 (Song et al. 2005).

CDPKs are the best characterized calcium sensor in plants, which have both kinase and CaM-like domain (Harper et al. 1991, 1994; Harmon et al. 2001; Cheng et al. 2002). CDPKs are encoded by large multigene family with possible redundancy and/or diversity in their functions (Harmon et al. 2001; Cheng et al. 2002), and they are believed to be important components in plant hormone signaling (Cheng et al. 2002; Ludwig et al. 2004). Two Arabidopsis CDPKs, AtCPK10, and AtCPK30, have been demonstrated to activate a stress and ABA-inducible promoter, showing the connection of CDPKs to ABA signaling pathway (Sheen 1996). In addition, the expression of some members of CDPK family was shown to be stimulated by exogenous ABA in rice (Li and Komatsu 2000) and in tobacco (Yoon et al. 1999). One member of the Arabidopsis CDPK family, AtCPK32, has been shown to be involved in ABA-regulated seed germination (Choi et al. 2005). More recently, Mori et al. (2006) showed the Arabidopsis CDPKs CPK3 and CPK6 are regulators in ABA-mediated stomatal closure. However, evidence has been lacking to link CDPK with ABA-regulated, broader, biological functions with pleiotropic effects such as seed maturation and germination, seedling growth, stomatal movement and plant stress tolerance. We previously identified an ABA-stimulated CDPK, ACPK1, from grape berry, which may be potentially involved in ABA signaling (Yu et al. 2006). Because of the difficulties of genetic manipulations in the perennial woody plant, we generated the *ACPK1*-overexpressing Arabidopsis transgenic lines. Here we report that over-expression of *ACPK1* in Arabidopsis promotes plant growth and enhances ABA-sensitivity in seed germination, early seedling growth and stomatal movement, indicating that ACPK1 is involved in ABA signal transduction as a positive regulator, and that the *ACPK1* gene could be potentially used for elevating plant biomass production.

Materials and methods

Plant materials, constructs, and Arabidopsis transformation

Arabidopsis (*Arabidopsis thaliana*) plants (Col ecotype) were used for the generation of the transgenic plants. To generate the construction, the full-length cDNA of *ACPK1* (GenBank accession number AY394009) were amplified by polymerase chain reaction (PCR) using the forward primer 5′-GCCTCTAGAATGAAGAAATCGTCCGCAG-GAGC-3′ and reverse primer 5′-GCTGGTACCGGTTTG-TCAAGCGCATATCTGGTA-3′ from the cDNA synthesized from total RNA of grape berries (see Yu et al. 2006). The forward primers contain *Xba*I restriction site and reverse primers contain *Kpn*I restriction site, which are underlined. The full-length cDNA of *ACPK1* were cloned into the vector of a pCAMBIA-1300-based Super Promoter (Ni et al. 1995) by the *Kpn*I and *Xba*I sites under the control of the Super Promoter. The Super Promoter is a hybrid promoter combining a triple repeat of the *Agrobacterium tumefaciens* octopine synthase (*ocs*) activator sequences along with the mannopine synthase (*mas*) activator elements fused to the *mas* promoter, termed $(Aocs)_3Amas Pmas$ (Ni et al. 1995). The over-expression construct of *ACPK1* was introduced into *Agrobacterium tumefaciens* GV3101 and transformed into wild-type Arabidopsis Columbia plants by floral dip method (Clough and Bent 1998). Transgenic plants were grown on Murashige–Skoog (MS) agar plates containing hygromycin (50 μg/ ml) in order to screen the positive seedlings. Six homozygote lines containing single insert were obtained. Plants were grown in a growth chamber at 20–21°C on MS medium at about 80 μmol photons m^{-2} s^{-1}, or in compost soil at about 120 μmol photons m^{-2} s^{-1} over a 16-h photoperiod.

RNA gel blotting and immunoblotting

RNA gel blotting was performed to analyze the expression of *ACPK1* in the transgenic plants. Total RNA was isolated using Trizol reagent (Invitrogen, Carlsbad, CA) from leaves of two-week-old Arabidopsis seedlings. Twenty micrograms of total RNA was subjected to electrophoresis on formaldehyde/agarose gels and transferred to nylon membranes (Hybond-N^+, Amersham Pharmacia Biotech) according to standard protocols. RNA blots were probed with the ^{32}P-labeled cDNA fragments corresponding to the N-terminal variable domain of *ACPK1* (see Yu et al. 2006). Hybridization was performed according to the method of Church and Gilbert (1984).

To analyze the expression of *ACPK1* in the transgenic plants at the protein level, immunoblotting was done essentially as described by Yu et al (2006). After SDS-polyacrylamide gel electrophoresis of the total proteins extracted from leaves of Arabidopsis plants, the proteins on gels were electrophoretically transferred to nitrocellulose membranes (0.45 μm, Amersham Pharmacia). The membranes were blocked for 2 h at room temperature with 3% (w/v) bovine serum albumin and 0.05% (v/v) Tween 20 in a Tris-buffered saline (TBS) containing 10 mM Tris-HCl

(pH 7.5) and 150 mM NaCl, and then were incubated with gentle shaking for 2 h at room temperature in the rabbit ACPK1-specific polyclonal antibodies (against the N-terminal fragment of forty amino acids covering N-terminal variable domain and 11 amino acids in its adjacent kinase domain, see Yu et al. 2006) (diluted 1:1000 in the blocking buffer). After being washed three times for 10 min each in the TBS containing 0.05% (v/v) Tween 20, the membranes were incubated with the alkaline phosphatase-conjugated antibody raised in goat against rabbit IgG (diluted 1:1000 in the blocking buffer) at room temperature for 1 h, and then washed three times for 10 min each with 50 mM Tris-HCl (pH 7.5) buffer containing 150 mM NaCl and 0.1% (v/v) Tween 20. Protein bands were visualized by incubation in the color-development solution using a 5-bromo-4-chloro-3-indolyl-phosphate/nitroblue tetrazolium substrate system according to the manufacturer's protocol.

Reverse transcriptase-mediated PCR and real-time PCR analysis

To analyze expression of *AtCPK4* (Arabidopsis genomic locus tag number At4g09570) and *AtCPK11* (At1g35670), two homologs of *ACPK1*, in the transgenic plants, reverse transcriptase-mediated PCR analysis was performed. Total RNA was isolated form leaves of 2-week-old Arabidopsis seedlings with the RNeasy Plant Mini Kit (Qiagen, Valencia, CA) supplemented with an on-column DNA digestion (Qiagen RNase-Free DNase set) according to the manufacturer's instructions, and then the RNA sample was reverse transcribed with the Superscript II RT kit (Invitrogen, Carlsbad, CA). PCR was conducted at linearity phase of the exponential reaction for each gene. The gene-specific primer pairs were: for *AtCPK4:* 5′-CTAGCCGACCCTCAAACAGTG-3′ (forward primer) and 5′-GCTTAGCATCATCACTGGGAC-3′ (reverse primer), and for *AtCPK11:* 5′-CACCACGATTAAGAGATCATTACC-3′ (forward primer) and 5′-CTGGCTTATAAGCTTAGCATCAT-3′ (reverse primer). Actin gene (At5g09810) expression level was used as a quantitative control.

To assay the expression of ABA-responsive genes in the transgenic plants, quantitative real-time PCR analysis was done with the RNA samples isolated from two-week-old seedlings harvested 5 h after the treatments with or without 100 μM ABA (mixed isomers; Sigma, St. Louis, MO). PCR amplification was performed with primers specific for various ABA-responsive genes: *RD29A* (At5g52310) forward 5′-ATCACTTGGCTCCACTGTTGTTC-3′ and *RD29A* reverse 5′-ACAAAACACACATAAACATCCAAAGT-3′; *MYB2* (At2g47190) forward 5′-TGCTCGTTGGAACCACATCG-3′ and *MYB2* reverse 5′-ACCACCTATTGCCCCAAAGAGA-3′; *MYC2* (At1g32640) forward 5'-TCATACGACGGTTGCCAGAA-3' and *MYC2* reverse 5′-AGCAACGTTTACAAGCTTTGATTG-3′; *RAB18* (At5g66400) forward 5′-CAGCAGCAGTATGACGAGTA-3′ and *RAB18* reverse 5′-CAGTTCCAAAGCCTTCAGTC-3′; *KIN1* (At5g15960) forward 5′-ACCAACAAGAATGCCTTCCA-3′ and *KIN1* reverse 5′-CCGCATCCGATACACTCTTT-3′; *KIN2* (At5g15970) forward 5′-ACCAACAAGAATGCCTTCCA-3′ and *KIN2* reverse 5′-ACTGCCGCATCCGATATACT-3′. Amplification of *ACTIN2/8* (forward primer 5′-GGTAACATTGTGCTCAGTGGTGG-3′ and reverse primer 5′-AACGACCTTAATCTTCATGCTGC-3′) genes was used as an internal control (Charrier et al. 2002). The suitability of the primers sequences in term of efficiency of annealing was evaluated in advance using the Primer 5.0 program. Real-time quantitative PCR experiments were repeated thrice independently, and the data were averaged. For real-time quantitative-PCR, the cDNA was amplified by using SYBR Premix Ex Taq^{TM} (TaKaRa) with a DNA Engine Opticon 2 thermal cycler (MJ Research, Watertown, MA).

Phenotype analysis

For the assays of germination and seedling growth, the seeds were surface sterilized in 5% (v/v) hypochlorite, and then rinsed five times with sterile water. For the assays of germination, approximately 100 seeds each from wild type (Colombia) and different transgenic lines were planted on MS medium (Sigma, product#, M5524) with or without different concentrations of ABA and incubated at 4°C for 3 days before being placed at 22°C under light conditions, and germination was scored at the indicated times. For seedling growth experiment, seeds were sowed on common MS medium and 48 h later transferred to MS medium supplemented with different concentrations of ABA in the vertical position. Seedling growth was investigated two weeks after the transfer.

For stomatal aperture assays, detached rosette leaves from 3-week-old plants were floated in the buffer containing 50 mM KCl and 10 mM Mes-Tris (pH 6.15) under a halogen cold-light source (Colo-Parmer) at 200 μmol m^{-2} sec^{-1} for 2 h, which was followed by addition of different concentrations of (±)-ABA. Apertures were recorded on epidermal strips under a microscope after 2 h of further incubation to estimate ABA-induced closure. To study inhibition of opening, leaves were floated on the same buffer in the dark for 2 h before they were transferred to the cold-light for 2 h in the presence of ABA, and then apertures were determined. The experiments were repeated thrice, and about 200 stomata were analyzed for each treatment.

For water loss measurement, rosette leaves of sample plants were detached from their roots and placed in weighing dishes and incubated at the room temperature.

Loss in fresh weight was monitored at the indicated times. For dehydration tolerance experiment of the whole plants, plants were grown aseptically in Petri dishes containing selective agar germination medium for 2 weeks, and then transferred to 8-cm compost-soil-filled pots. About 15 d later when plantlets reached the stage of five to six fully expanded leaves, drought was imposed by withdrawing irrigation. The status of the plants was investigated 15 days after the drought treatment.

Results

Molecular analysis of the ACPK1-transgenic lines

We screened six *ACPK1*-overexpressing Arabidopsis transgenic lines (2, 3, 6, 12, 24, and 31). Molecular analysis showed that *ACPK1* was constitutively expressed in these lines at both mRNA and protein levels, whereas no expression was detected in wild-type plants, as expected (Fig. 1A, and data not shown). We further analyzed the expression of two Arabidopsis closer homologues of ACPK1, *AtCPK4* and *AtCPK11*, to investigate the possible influence of the heterologous expression of *ACPK1* on its homologues in the transgenic lines. The results showed that the expression of the two Arabidopsis CDPKs was not affected in any transgenic line (Fig. 1B, and data not shown), which ensures the specificity and reliability of the expressed-*ACPK1*-induced phenotypes. The ABA-responsive phenotypes of all the six transgenic lines were similar, so, we show only the results form the line 3 (OE3) or line 6 (OE6). It should be noted that, in addition to the transgenic plants generated with the pCAMBIA-1300 vector under the control of the Super Promoter (see Materials and methods), which is much more active than the cauliflower mosaic virus (CaMV) 35S promoter (Ni et al. 1995), we generated also the transgenic *ACPK1*-overexpressing plants under the control of CaMV 35S promoter. The *ACPK1*-overexpressing plants under the control of two different kinds of promoters gave the similar results (data not shown).

Expression of ACPK1 results in a higher vigor of plant growth and ABA hypersensitivity in seed germination, seedling growth and stomatal regulation

The *ACPK1*-overexpressing seeds germinated normally as the wild-type seeds did in the ABA-free media, but in the media supplemented with different concentrations of (±)-ABA (0.1, 0.5 or 1 μM), their germination rate was significantly more reduced than that of the wild-type seeds (Fig. 2).

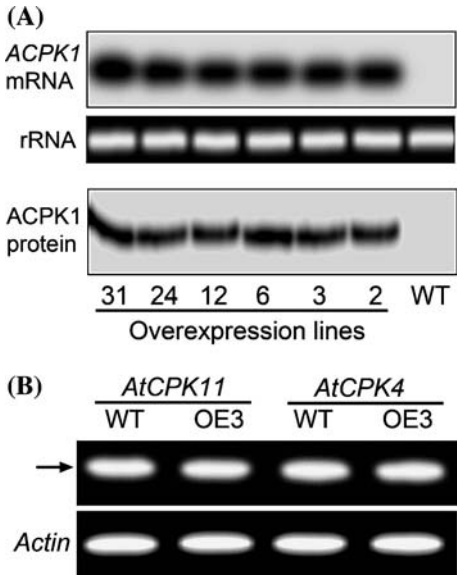

Fig. 1 Molecular analysis of the *ACPK1*-overexpressing Arabidopsis transgenic lines. (**A**) RNA gel blot (indicated by *ACPK1* mRNA) and immunoblot (indicated by ACPK1 protein) of ACPK1, showing that *ACPK1* was over-expressed in the transgenic lines (2, 3, 6, 12, 24, and 31). Neither *ACPK1* mRNA nor ACPK1 protein was detected in wild-type (WT) plants (Col). The ethidiumbromide-stained gel demonstrates equivalent RNA quantities loaded in each line. (**B**) The expression of *AtCPK4* and AtCPK11, two closer homologues of ACPK1, was not affected in the *ACPK1*-transgenic Arabidopsis lines (taking the over-expression line 3, OE3, as an example) in comparison with the wild-type plants (WT). The expression levels were estimated by semi-quantitative reverse transcriptase-mediated PCR as described in METERIALS AND METHODS using Actin gene expression (indicated by *Actin*) as a quantitative control

The *ACPK1*-overexpressing plants grew faster, and have a final size significantly larger than the wild-type plants (Fig. 3A and B). All the six *ACPK1*-transgenic lines exhibited the significantly higher vigor of plant growth (data not shown). However, the early growth of the *ACPK1*-overexpressing seedlings was much more reduced by ABA treatments than that of the wild-type seedlings: the growth of the transgenic seedlings was completely inhibited in the media containing more than 1 μM ABA, whereas the wild-type seedling grew more or less in the same media (Fig. 3C). It should be noted that the phenotypes in ABA-responsive seedling growth were observed only if the seedlings were transferred to the ABA-containing medium less than 48 h after stratification, but they were not observed when the transfer was done more than 48 h after stratification (data not shown). We have also observed the same phenomenon in the ABA receptor ABAR-regulated seedling growth (Shen et al. 2006), which may be associated with mechanisms like the postgermination developmental arrest checkpoint mediated by temporal expression of *ABI5* (Lopez-Molina et al. 2001).

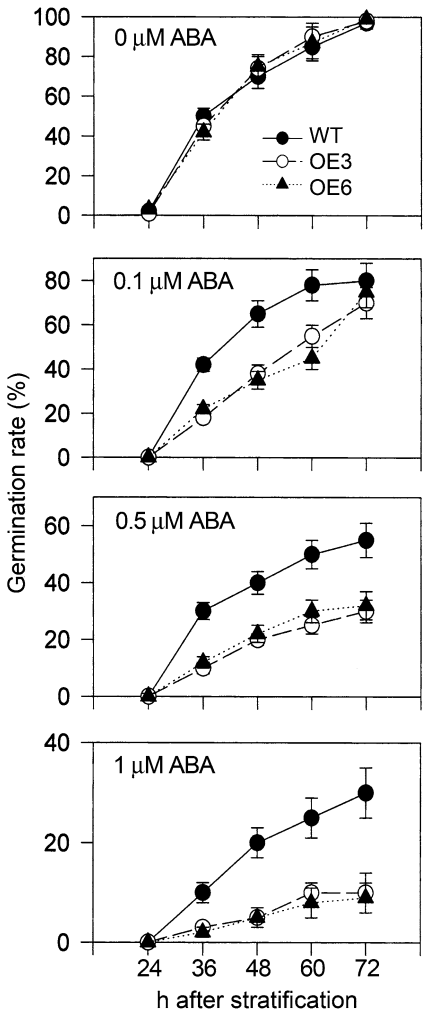

Fig. 2 Over-expression of *ACPK1* in Arabidopsis enhances the sensitivity of seed germination to ABA. The germination of the *ACPK1*-overexprressing (taking the lines OE3 and OE6 as examples) and wild-type (WT) seeds was scored in the MS media supplemented with different concentrations of (±)-ABA (0, 0.1, 0.5, and 1 μM) from 24 to 72 h after stratification

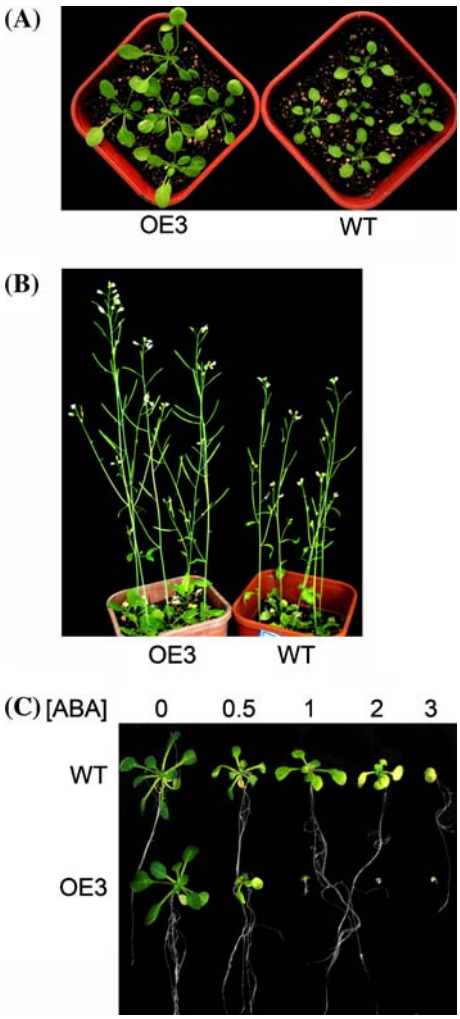

Fig. 3 Over-expression of *ACPK1* in Arabidopsis promotes plant growth and enhances the sensitivity of early seedling growth to ABA. (**A**) and (**B**) Status of growth of the *ACPK1*-overexpressor (taking the line 3, OE3, as an example) and wild-type (WT) plants in different growing stages. (**C**) The early growth of *ACPK1*-overexpressor seedlings (taking the line 3, OE3, as an example) are much more sensitive to ABA than that of the wild-type seedlings (WT). The seedlings were transferred to the MS media containing different concentrations of (±)-ABA ([ABA]: 0, 0.5, 1, 2 and 3 μM) less than 48 h after stratification

The overexpression of *ACPK1* in Arabidopsis results also in the ABA-hypersensitive phenotypes in ABA-induced promotion of stomatal closure and inhibition of stomatal opening (Fig. 4A). The detached leaves of the *ACPK1*-overexpressing plants were more tolerant to dehydration than those of the wild-type plants (Fig. 4B), which may be due to their hypersensitivity of stomatal closure to ABA (Fig. 4A). We did not, however, observed the obvious difference in the whole-plant drought tolerance between the *ACPK1*-overexpressing and wild-type plants. This may be due partly to the complexity of the drought tolerance mechanism (Xiong et al. 2006). However, the plants of bigger size generally transpire more water, and frequently more susceptible to water stress. In this regard, that the bigger *ACPK1*-overexpressing plants grew as well as the smaller wild-type plants in the same drought conditions (Fig. 4C) suggests a substantially higher dehydration tolerance of the *ACPK1*-transgenic lines.

Expression of some ABA-responsive genes was altered in the ACPK1-overexpressing plants

ACPK1-overexpresssion in Arabidopsis was shown to up-regulate the expression of the positive regulators of ABA signaling *MYB2* (Abe et al. 2003), *RAB18* (Lang and Palva 1992), *KIN1* and *KIN2* (Kurkela and Borg-Franck 1992) in leaves, but the levels of other two positive regulators

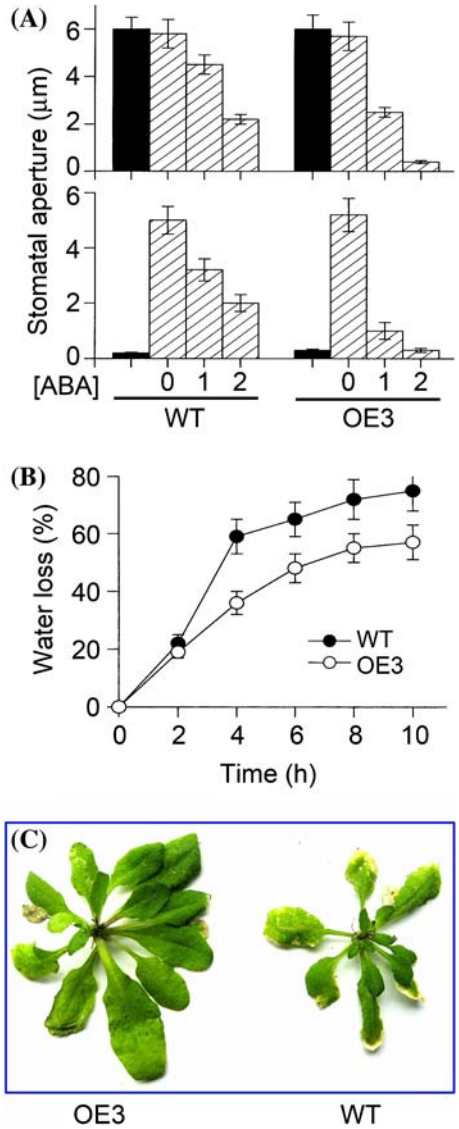

Fig. 4 Over-expression of *ACPK1* in Arabidopsis enhances the sensitivity of stomatal movement to ABA. (**A**) ABA-induced stomatal closure (above) and inhibition of opening (below). ABA-induced stomatal closure was assayed by floating detached rosette leaves on the ABA-free buffer under cold-light for 2 h and further on the ABA-containing buffer for 2 h. Inhibition of opening was assayed by floating the leaves on the ABA-free buffer in the dark for 2 h before they were transferred to the cold-light for 2 h in the presence of ABA. Apertures were recorded on epidermal strips. Black columns, initial stomatal apertures; grey columns, apertures after ABA treatment. ABA concentrations ([ABA]) are in μM. (**B**) Water loss from detached leaves. Rosette leaves were placed in weighing dishes and incubated at the room temperature. Loss in fresh weight was monitored at the indicated times. (**C**) Plant status after drought treatment. Plants were grown in Petri dishes containing germination medium for 2 weeks before transferred to soil. 15 d later, drought was imposed by withdrawing irrigation. The status of the plants was investigated 15 days after the drought treatment. WT, wild-type (Col); OE3, overexpressor line 3

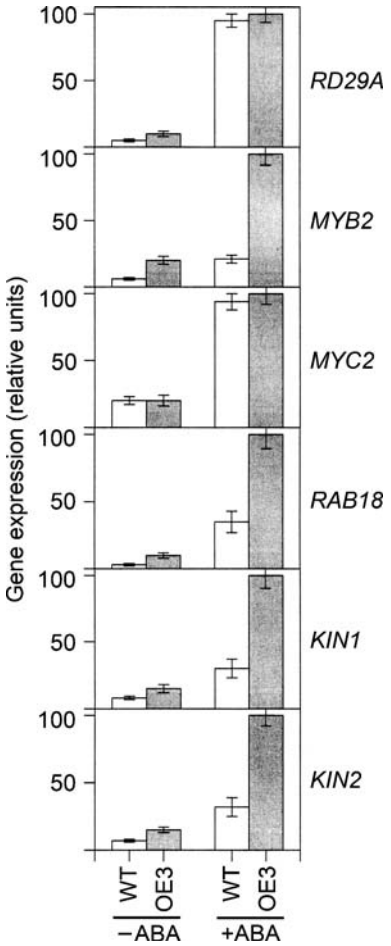

Fig. 5 Expression of ABA-responsive genes in the *ACPK1*-overexpressor plants. The gene expression was assayed by real-time PCR analysis in the overexpressor lines (taking the line OE3 as an example) and wild-type plants (WT) in the absence (–ABA) or presence (+ABA) of ABA

RD29A (Yamaguchi-Shinozaki and Shinozaki 1994) and *MYC2* (Abe et al. 2003) were not altered in the *ACPK1*-transgenic lines (Fig. 5). As reported previously (Kurkela and Borg-Franck 1992; Lang and Palva 1992; Yamaguchi-Shinozaki and Shinozaki 1994; Abe et al. 2003), the expression of all these ABA-responsive genes was strongly stimulated by ABA (Fig. 5). The overexpression of *ACPK1* also amplified the ABA-induced stimulating effects on *MYB2*, *RAB18*, *KIN1*, and *KIN2* (Fig. 5).

Discussion

We previously showed that ABA specifically stimulates both the expression and enzymatic activities of the grape ACPK1, strongly suggesting that ACPK1 may be involved

in ABA signal transduction (Yu et al. 2006). In the present experiments, the heterologous expression of *ACPK1* in Arabidopsis confers the ABA-hypersensitive phenotypes in seed germination, early seedling growth and ABA-induced stomatal closure, and inhibition of stomatal opening (Figs. 2–4), and alters the expression of some ABA-responsive genes (Fig. 5), providing in planta genetic evidence that ACPK1 positively regulates the plant cell responses to ABA. This is also a line of further supporting evidence for important roles of calcium messenger in ABA signaling pathway.

Interestingly, the overexpression of *ACPK1* significantly promoted plant growth, but enhanced, or at least did not affect plant dehydration tolerance (Figs. 3 and 4). This feature of the *ACPK1* gene could be used for improving plant biomass production. The *ACPK1* gene was shown to be specifically expressed in the seeds and fleshy portion of grape berries, which suggests that the functions of ACPK1 may be closely linked with berry growth and development. It would be of great interest to improve grape yield and quality through genetic engineering of *ACPK1* if the *ACPK1*-overexpression in grapevine gives the results similar to those in Arabidopsis. The genetic approaches would be also hopeful in other crop plants.

The previous experiments of the transient expression of the ACPK1-green fluorescent protein fusion in Arabidopsis protoplasts showed that ACPK1 localizes predominantly in chloroplasts and also in plasma membranes (Yu et al. 2006). An ABA receptor, ABAR, was also shown to reside predominantly in chloroplasts to perceive intracellular ABA signal (Shen et al. 2006). The same cellular compartmentation would facilitate the relay of ABA signal from ABAR to ACPK1 if they function in the same signaling pathway. It will be of interest to explore the functions of the Arabidopsis homologues of ACPK1 such as AtCPK4 and AtCPK11 in ABA signal transduction to elucidate how CDPK works in ABA signaling pathways.

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