Arabidopsis CPK6 positively regulates ABA signaling and drought tolerance through phosphorylating ABA-responsive element binding factors

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Highlight:

Arabidopsis calcium-dependent protein kinase CPK6 positively regulates seed germination, seedling growth and drought tolerance via phosphorylating ABF and ABI5 transcription factors.

Abstract

Abscisic acid (ABA) regulates numerous developmental processes and drought tolerance in plants. Calcium-dependent protein kinases (CPKs) are important Ca^{2+} sensors playing crucial roles in plant growth and development as well as responses to stresses. However, the molecular mechanisms of many CPKs in ABA signalings and drought tolerance remain largely unknown. Here we combined protein interaction studies, biochemical and genetic approaches to identify and characterize substrates that were phosphorylated by CPK6 and elucidated the mechanism that underlines role of CPK6 in ABA signaling and drought tolerance. The expression of *CPK6* is induced by ABA and dehydration. Two *cpk6* T-DNA insertion mutants are insensitive to ABA during seed germination and root elongation of seedlings, in contrast, overexpression of *CPK6* showed the opposite phenotype. Moreover, *CPK6*-overexpressing lines showed enhanced drought tolerance. CPK6 interacts with and phosphorylates a subset of core ABA signaling-related transcription factors, ABA-responsive element binding factors (ABFs/AREBs) and enhances their transcriptional activities. The phosphorylation sites in ABF3 and ABI5 were also identified through mass spectrometry and mutational analyses. Taken together, we present evidences that CPK6 mediates ABA signaling and drought tolerance through phosphorylating ABFs/AREBs. This work thus uncovers a rather conserved mechanism of calcium-dependent Ser/Thr kinases in ABA signaling.

Key words: Arabidopsis, abscisic acid, basic leucine zipper transcription factor, calcium-dependent protein kinase, drought, phosphorylation

Introduction

Environmental stresses, such as salt, drought and extreme temperatures affect growth, development and yield, quality of crop plants. Plants have evolved complex networks of signal transduction pathways to adapt to environmental changes or minimize damages (Xiong *et al.*, 2002). Abscisic acid (ABA) regulates many important aspects in plants, including seed germination and dormancy, seedling growth, leaf senescence and physiological responses to environmental stresses (Finkelstein *et al.*, 2002).

Ca^{2+} is a ubiquitous second messenger involved in the signaling of a variety of endogenous and exogenous stimuli. The calcium-dependent protein kinases (CPKs) constitute an very important family of serine/threonine protein kinases that decode Ca^{2+} signals and, this family has been identified throughout the plant kingdom from algae to angiosperms and also in some protozoans (Ludwig *et al.*, 2004). CPKs are composed of four characteristic domains or motifs including a variable N-terminal domain, a catalytic kinase domain, an autoinhibitory junction domain and a calmodulin-like domain (CBD) with Ca^{2+}-binding EF-hands (Cheng *et al.*, 2002; Hrabak *et al.*, 2003). The N-terminal domain is variable with different lengths, which is important for substrate recognition and could determine substrate specificity (Asai *et al.*, 2013; Ito *et al.*, 2010). This N-terminal domain may also be modified by myristoylation or palmitoylation, two post-translational modifications (PTMs) for membrane tethering (Cheng *et al.*, 2002). The autoinhibitory junction domain contains a pseudosubstrate sequence that can interact with the active site and inhibit the kinase activity (Harmon *et al.*, 1994). The regulatory CBD contains usually four EF-hands, which can bind to Ca^{2+} and thus activate CPKs (Sanders *et al.*, 2002).

CPKs together with downstream targets have been identified to play central roles in many physiological processes, response to various abiotic stresses, e.g. cold, drought, salinity, heat and ABA, as well as biotic stresses (Boudsocq and Sheen, 2013; Reddy *et al.*, 2011; Schulz *et al.*, 2013). One of the prerequisites to understand the molecular mechanisms of CPKs is to identify their direct targets. So far, quite a few different classes of targets of CPKs in Arabidopsis, rice, tobacco, potato, etc have been identified, which include transcription factors (TFs), ion channels, ethylene biosynthesis enzymes,

plasma membrane-localized enzymes implicated in reactive oxygene species (ROS) production (Choi *et al.*, 2005; Dubiella *et al.*, 2013; Gao *et al.*, 2013; Ishida *et al.*, 2008; Kamiyoshihara *et al.*, 2010; Mori *et al.*, 2006; Wang *et al.*, 2016; Zhu *et al.*, 2007). A powerful technique to identify the target of a CPK is yeast two-hybrid (Y2H) and similar split-ubiquitin system (SUS), together with validation through coimmunoprecipitation (Co-IP) and biomolecular fluorescence complementation (BiFC) (Rodriguez Milla *et al.*, 2006; Uno *et al.*, 2009).

Arabidopsis genome contains 34 CPK genes (Cheng *et al.*, 2002; Hrabak *et al.*, 2003). In Arabidopsis alone, functional studies using T-DNA insertion mutants and overexpression technique revealed the functions of several CPK genes in ABA signalings and /or drought tolerance. For instance, AtCPK3 and AtCPK6 are involved in the regulation of guard cell ion channels and therefore in stomatal movement (Mori *et al.*, 2006). Two closely related AtCPK4 and AtCPK11 regulate ABA-related seed germination, seedling growth, stomatal movement, and tolerance to salt and drought stresses, through phosphorylating two ABA-responsive bZIP transcription factors, ABF1 and ABF4 (Zhu *et al.*, 2007). Arabidopsis CPK32 interacts with and phosphorylates ABF4 *in vitro* and, *CPK32*-overexpression affects both ABA sensitivity and the expression of a number of ABF4-regulated genes (Choi *et al.*, 2005). Arabidopsis *cpk10* mutant is more sensitive to drought stress and is impaired in ABA- and Ca^{2+}-induced stomatal closure, while *CPK10*-overexpression lines display enhanced tolerance to drought stress (Zou *et al.*, 2010). Arabidopsis *cpk23* mutant displays enhanced tolerance to drought and salt stresses with reduced stomatal apertures, while *CPK23*-overexpression lines show more sensitivity to drought and salt stresses possibly as a result of increased stomatal apertures (Ma and Wu, 2007). Furthermore, Arabidopsis *cpk21* mutant is more tolerant to hyperosmotic stress (Franz *et al.*, 2011). These reports clearly demonstrated that CPKs play essential functions to confer plants with increased resistance to abiotic and biotic stresses. However, a comprehensive interaction map between many CPKs and their substrates has not been well understood, which needs further study to clarify.

Previously, we identified, cloned and analyzed CIPK (Calcineurin B-like protein-Interacting Protein Kinase) and CPK gene families in oilseed rape (*Brassica napus* L.) and also found that a few BnaCPKs, including BnaCPK4 interacted with multiple orthologs of ABF/AREB transcription factors implicated in the core ABA signaling pathways (Wang *et al.*, 2019; Zhang *et al.*, 2014b; Zhang *et al.*, 2014a). A further exploration of ABA-inducible *BnaCPK6* revealed overexpression of it conferred drought tolerance (Zhang et al, unpublished data). To facilitate the investigation, we determined to use Arabidopsis as a model to study the molecular mechanism of AtCPK6 (At2g17290) in ABA signaling and abiotic stress response. In the present study, we showed evidences that AtCPK6 mediates ABA signaling and drought tolerance through interacting and phosphorylating multiple ABF/AREB TFs.

Materials and methods

Plant material and growth conditions

The *Arabidopsis thaliana* ecotype Columbia-0 (Col-0) was used as the wild-type. Plants were grown in a soil mix in the growth chamber at 22°C under a 14 h light/10 h dark photoperiod with a light intensity of approximately 100 μE m^{-2} s^{-1}. Mutants of T-DNA insertion were ordered from NASC (Nottingham, UK).

Stress treatments and quantitative RT-PCR (qRT-PCR)

Wild type Arabidopsis (Col-0) seedlings were surface-sterilized and grown vertically on ½×MS medium (Caisson labs, USA) with 1% sucrose solidified with 0.8% Phytoblend (Caisson labs). Ten-day-old seedlings grown on ½×MS medium were applied with abiotic stress and hormone treatments. Briefly, seedlings were transferred to ½×MS medium plates containing 50 μM ABA (Sigma), 15% PEG8000 (MP Biomedicals) and 10 μM Paraquat (Methyl viologen, Sigma), respectively. Seedlings transferred to normal ½×MS medium were used as the control. Seedlings were harvested at 1, 3 and 6 h after treatments and flash-frozen in liquid nitrogen and stored at −80°C. The planting, treatments and harvesting were repeated three times independently.

Total RNA was isolated from treated and control Arabidopsis seedlings using the

Plant RNA kit (Omega, USA). RNA was treated with DNaseI using DNA-free kit (Ambion, USA). First-strand cDNAs were synthesized from 2.5 μg of total RNA. qRT-PCR was performed using 10-fold diluted cDNAs and SYBR Green I premix (CWBio, China) on a CFX96 real-time PCR system (Bio-Rad, USA). The specificity of each pair of primers was checked by Blastn analysis in TAIR (www.arabidopsis.org) and melting curve examination before use. Three independent biological replicates were run and normalized against *Ubiquitin 10* (*UBQ10*, *At*4g05320) and *Ubiquitin-Conjugating Enzyme* (*UBC21*, *At*5g25760) cDNA levels (Czechowski *et al.*, 2005). Relative expression levels (fold changes) were calculated by comparing transcript levels under treatments (or in mutant and overexpression lines) to those under mock treatments (or in wild-type) according to the formula proposed by (Pfaffl, 2001). Significance was determined with SPSS software ($p \leq 0.05$).

Arabidopsis transformation and phenotypic assay

A 2.2-kb genomic DNA fragment of *CPK6* including the exons and introns was amplified by PCR from Arabidopsis genomic DNA and was cloned into the binary vector pYJHA. The recombinant plasmid and pYJHA-GFP vector control were individually transformed into *Agrobacterium tumefaciens* strain GV3101 and introduced into Arabidopsis wild type (Col-0) plants by the floral dip method (Clough and Bent, 1998). Transgenic seeds were selected on ½×MS medium containing 0.8% (w/v) Phytoblend (Caisson labs, USA), and 30 μg/mL hygromycin B (Roche). The resistant seedlings were transplanted to soil and grown to produce seeds. Homozygous T_3 lines were used to examine the expression level of *CPK6* or *GFP*.

For the phenotypic assay, seeds of the various genotypes were planted and harvested in the same growth chamber and at the same time. Seeds were surface sterilized in 2.65% bleach containing 0.03% Tween-20, before sown in triplicates on ½×MS medium with 1% sucrose containing no ABA or different concentrations of (±)-ABA (A1049, Sigma). After stratified at 4°C for 3 d, seed plates were transferred to a growth chamber under the same settings as described above.

For the post-germination assay, after vertically growing for 4 d, seedlings were transferred onto ½×MS medium supplemented with or without 50 μM ABA and

continued to grow vertically for another 7 d, before the root elongation was measured and plates photographed.

Promoter-*β-glucuronidase* (*gusA/GUS*) fusion and histochemical staining

A 1.97 kb fragment immediately upstream of the initiation codon of *CPK6* was PCR-amplified from Col-0 genomic DNA. PCR products were restricted and inserted into the pYJGUS1 vector upstream of the *GUS* gene. The construct was mobilized into *A. tumefaciens* strain GV3101 before transformed into wild-type Arabidopsis (Col-0) (Clough and Bent, 1998). Transgenic seeds were selected on ½ x MS medium containing 1% sucrose and 30 mg/L of kanamycin (Sigma) for two generations before transplanted into soil. GUS staining of homozygous T_3 lines seedlings was performed as described previously (Jiang and Deyholos, 2009). Samples were photographed under a dissecting microscope equipped with a digital camera.

Bimolecular fluorescence complementation (BiFC) assay

The coding sequences (CDS) of *CPK6* and *ABFs/AREBs/ABI5* without stop codons were subcloned into pSPYCE(M) and pSPYNE(R)173 vector, respectively. Transient expression in tobacco leaves and microscopic observation were performed as described previously (Zhang *et al.*, 2014b).

Site-directed mutagenesis

Site-directed mutagenesis PCR was performed via overlap PCR using high-fidelity *Pfu* DNA polymerase (Bioer, China) with primers listed in Table S1. After confirmed by DNA sequencing, mutant genes were subcloned into various destination vectors.

Dual luciferase reporter assay

The promoters of *RD29B* and *Em6* were amplified from Arabidopsis genomic DNA and cloned into the pGreen II 0800-LUC vector as reporters. The effector plasmids were pYJHA-CPK6/CPK6D209A and pYJHA-GFP (control). The *Renilla* luciferase (REN) driven by 35S promoter was used as an internal control. Through *A. tumefaciens* GV3101, different combinations of reporter and effector plasmids were co-infiltrated into leaves of 30-d-old *N.benthamiana* with LUC and REN activities measures 2 d later. Three independent experiments were prepared for each combination as described previously (Chen *et al.*, 2017).

Stomatal aperture measurement

Epidermal peels were stripped from rosette leaves of four-week-old Arabidopsis plants of different genotypes, which were grown in a growth chamber at 22°C under a 8 h light/16 h dark photoperiod with a light intensity of approximately 80 μE m^{-2} s^{-1} and 60-70% relative humidity. Epidermal peels were floated in a solution of 30 mM KCl and 10 mM MES-KOH (pH 6.15) in petri dishes at 22°C for 2.5 h under light conditions. For ABA treatment, the epidermal peels were incubated for 2 h in the same solution containing different concentrations of ABA. Stomatal apertures were recorded under a Leica microscope (Leica DM5000 B) with data processed through Image J (National Institutes of Health). One hundred stomata were measured for each genotype and the whole experiment was repeated three times.

Drought tolerance assay

Seeds of various genotypes were germinated for 7 d on ½ x MS media supplemented with 1% sucrose. 7-day-old seedlings were transplanted to the soil and grew for 15 d under a short-day (8 h light/16 h dark) regime with normal watering, and then plants were subjected to drought stress by withholding watering. The plants grown under normal conditions were taken as the control. To ensure the repeatability of this test, the same numbers of seedlings were transferred and grown in different wells of the same tray. The experiments were repeated for three times.

Co-IP assay

The CDSs of *GFP*, *CPK6*, *ABF3* and *ABI5* were subcloned into pYJHA and pFXMyc vectors, respectively. These resultant constructs were infiltrated into leaves of 3-week-old *N. benthamiana* plants together with the p19 strain. After 2 d, leaves were harvested for protein extraction. Around 100 mg leaves were ground into fine powder in liquid nitrogen, then resuspended in 100 μl of extraction buffer (50 mM Tris-HCl, pH 7.5, 75 mM NaCl, 0.1% Triton X-100, 1 mM EDTA, 5% glycerol, 1 mM PMSF, 50 μM MG132, and 1x protease inhibitor cocktail). Extracts were centrifuged twice at 13,000g at 4°C for 10 min. Supernatant was aliquoted into sterile tubes as the input. Extracts were incubated with EZview Red Anti-HA M2 affinity gel (Cat#E6779, Sigma) for 4 h at 4°C. The affinity gel was washed five times with the extraction buffer. Protein

complexes were eluted by the extraction buffer containing 100 μg/μl HA peptide (Cat#I2149, Sigma) for 1 h at 4°C and then centrifuged at 6,000g at 4°C for 1 min. Proteins were separated on a 8% SDS-PAGE gel and detected by Western blot with anti-HA (Sigma) and anti-Myc (Cat# M4439, Sigma) antibodies, respectively.

Prokaryotic protein induction, purification and *in vitro* pull-down assay

ABF3 and *ABI5* were subcloned into the protein expression vector pGEX-6P-1 (GE Healthcare) and pYJFLAG (harboring 3xFLAG tag). These plasmids were transformed into BL21 (Codon Plus) strain of *E.coli* (Novagen), before induced by 0.5 mM isopropyl β-D-thiogalactoside (IPTG) for 6 h at 25 °C. Cell pellets were collected and then lysed by sonication in 1×PBS. To purify the GST-tagged protein, protein extracts were incubated with Glutathione Sepharose 4B beads (GE Healthcare) for 6 h at 4°C on a rotator, then protein elution was performed according to manufacturer's instructions. To purify the FLAG-tagged protein, the supernatant of lysate was incubated with EZview Red Anti-FLAG M2 affinity gel (Cat#F2426, Sigma) for 2 h at 4°C then eluted by 3×FLAG peptide (Cat#F4799, Sigma).

Four-week-old rosette leaves of CPK6-HA transgenic line were harvested and one gram of sample was ground into fine powder in liquid nitrogen. Protein extract was incubated in 1 ml extraction buffer (50 mM Tris-HCl, pH 7.5, 75 mM NaCl, 0.1% Triton X-100, 1 mM EDTA, 5% glycerol, 1 mM PMSF, 50 μM MG132, and 1 x protease inhibitor cocktail) for 4 h at 4°C, then centrifuged at 13,000g at 4°C for 10 min twice to remove any precipitate. The supernatant was incubated with EZview Red Anti-HA M2 affinity gel (Sigma). The CPK6-HA protein was eluted by 100 μg/μl HA peptide (Sigma).

For pull-down assay, Glutathione Sepharose 4B beads were equilibrated with 200 μL of 1×PBS three times, then incubated with equal amount of GST-tagged proteins for 1 h at 4°C. The beads were washed with 200 μL of extraction buffer (50 mM Tris-HCl, pH 7.5, 75 mM NaCl, 0.1% Triton X-100, 1 mM EDTA, 5% glycerol, 1 mM PMSF, 50 μM MG132, and 1 x protease inhibitor cocktail) three times. Beads containing GST or GST-TF were incubated with 200 μL of the *CPK6*OE20 extracts or WT extracts for 4 h at 4°C, respectively, in the absence or presence of 1 μM Ca^{2+} and/or

50 μM ABA. The beads were pelleted by centrifugation at 1,000g for 1 min and washed five times with 200 μl of extraction buffer. Protein complexes were eluted using 4×SDS-PAGE sample buffer, resolved by 8% SDS-PAGE, and analyzed by immunoblotting with anti-GST antibody (Cat# MA4004, Thermo Scientific) at 1:5,000 and anti-HA antibody (Sigma) at 1:4,000.

***In vitro* phosphorylation assay**

CPK6-HA and GFP-HA proteins purified from plants and ABF3-FLAG, ABI5-FLAG proteins purified from *E.coli* as described previously were incubated in a kinase assay buffer (50 mM Hepes, pH 7.5, 10 mM $MgCl_2$, 2 mM DTT, 5 mM EGTA, 4.6 mM $CaCl_2$ and 50 μM ATP) in triplicate for 30 min at 25°C. Syntide 2 (Sigma) was used as an artificial substrate of CPKs. Then an ADP-Glo kinase assay kit (Cat#V9101, Promega) was used to quantify ADP produced from kinase reactions following the manufacturer's manual. In brief, kinase reactions were stopped by adding ADP-Glo reagent and incubated for 1 h at 25°C. Then, kinase detection reagent was added to each reaction tube and incubated for 1 h at 25°C. Luminescence was recorded with a GloMax 20/20 luminometer (Promega). To examine the effect of ABA on kinase activity of CPK6, transgenic lines of CPK6-HA and GFP-HA were treated with 50 μM ABA for 3 h before harvested for protein extraction. To detect the kinase activity of CPK6D209A, CPK6/CPK6D209A-HA and GFP-HA proteins were purified from *N. benthamiana* leaves with EZview Red Anti-HA M2 affinity gel (Sigma).

Results

Expression pattern and promoter activity of *CPK6*

To identify the response of Arabidopsis *CPK6* to ABA and relevant stress treatments, we examined the transcript levels of it in young seedlings subjected to ABA, dehydration and oxidative stress treatments. *CPK6* expression was induced by the application of ABA, PEG8000 (induces dehydration) and methyl viologen (MV, induces oxidative stress) at 1 h after treatment, whereas at 3 h and 12 h, the three treatments did not have any effect (Fig.1A), suggesting *CPK6* may play a role in the early response to ABA and other stress treatments.

Further, to investigate the role of *CPK6*, wild-type Arabidopsis plants transformed with the *CPK6* promoter fused to the *GUS* reporter gene were used for histochemincal analysis. It can be seen that *CPK6* was highly expressed in roots of 7-d-old seedlings, cotyledons, and also in the petiole of cotyledons (Fig. 1B). It was also preferentially expressed in stomata (Fig. S1). In mature leaves, strong expression was observed in trichomes (Fig. 1D). In young siliques, *CPK6* expression was restricted to the two ends and was almost undetectable in embryos (Fig. 1C). In mature flowers, *CPK6* expression was observed in the base of sepals, as well as in filaments, stamens and stigmas (Fig. 1E).

We also analyzed the mRNA level of *CPK6* during early seedling development in wild-type plants. It was found that *CPK6* mRNA level gradually increased until 10 days post-stratification (DPS), then decreased under normal conditions (Fig. S2A). In parallel, the expression of *APETALA1* (*AP1*) as a molecular marker was monitored, which showed expected changes (Fig. S2B).

CPK6 positively regulates ABA sensitivity during both seed germination and post-germination growth

To characterize the function of *CPK6* in ABA signaling and drought tolerance, we searched the publicly available T-DNA collections and obtained two T-DNA insertion mutants (SALK_025460c and SALK_034156c). The two mutants were designated as *cpk6-3* and *cpk6-4* (Fig 2A), respectively, compared to the two other mutants reported previously (Mori *et al.*, 2006). Plants homozygous for the T-DNA insertion were identified by PCR, and sequencing of the T-DNA flanking region indicated *cpk6-3* harbors the T-DNA inserted 112 bp downstream of the translational start codon while *cpk6-4* in the leader intron (171 bp upstream of the translational start codon) of *CPK6* gene (Fig. 2A). RT-PCR analysis showed that *CPK6* transcript was absent in the two T-DNA lines, indicating they are knock-out mutants (Fig. 2B). We firstly examined the ABA-related phenotype during seed germination and seedling growth. The results showed that both *cpk6-3* and *cpk6-4* mutants were highly insensitive to ABA treatment compared to WT, which was also confirmed by quantitative comparison (Fig. S3). In parallel, we generated overexpression (OE) lines of *CPK6* driven by CaMV35S

promoter and selected three high expression lines, which are OE9, OE17 and OE20 from over 30 independent lines at T3 generation (Fig. 2C). Besides, the binary vector, which harbors *GFP* expression cassette was also transformed into wild-type Arabidopsis plants and homozygous T3 seeds were used as the vector control (VC).

Although we did not observe obvious difference in growth between WT, VC, *cpk6* loss-of-function mutants and overexpresssion lines on the normal MS medium, *cpk6-3* mutants were more insensitive to ABA treatment than WT and VC at the germination level, whereas the OE lines were more sensitive to ABA than the WT and VC on MS medium containing 0.5 μM ABA (Fig. 2D-E). Similarly, the phenotypes of various genotypes were assayed and compared at the post-germination level, and the results showed that no significant difference in the terms of root elongation and cotyledon growth was observed among the mutants, WT and overexpression lines on normal media; in contrast, the two mutants were more insensitive to ABA while *CPK6*-overexpression lines were more sensitive to ABA treatment than the WT control on MS medium containing 50 μM ABA (Fig. 2F–G). These results demonstrated that CPK6 plays a positive role in ABA signaling in Arabidopsis.

Overexpression of *CPK6* enhances drought tolerance

Since the above results demonstrated *CPK6*-overexpressing lines were more sensitive to ABA treatment, we inferred *CPK6* overexpression might confer drought tolerance. To test this, identical numbers of WT, *cpk6-3* mutant and 35S::*CPK6* transgenic plants were grown for three weeks in soil mix before water was withheld. We confirmed through semi qRT-PCR that *CPK6* transcripts were absent in the *cpk6-3* mutant, whereas more were accumulated in the two overexpression lines, especially in the OE20 line, compared to the WT (Fig. 3A).We observed that rosette leaves of WT are slightly larger than the other genotypes under well-watered conditions (Fig. 3B). After drought stress, WT and *cpk6-3* mutant plants wilted and their leaves became curled, whereas the 35S::*CPK6* transgenic plants remained turgid and their leaves remained rather flat (Fig. 3B). After rewatered, the *CPK6*OE20 line completely recovered from drought stress and a small portion of *CPK6*OE17 plants also survived (Fig. 3B). However, WT and *cpk6-3* plants were all dead (Fig. 3B). These results

suggested that both WT and *cpk6* mutant lost water more rapidly than 35S::*CPK6* transgenic plants, and thus wilted more quickly. To investigate this, we measured the stomatal apertures of rosette leaves from WT, VC, *cpk6-3* and 35S::*CPK6* plants grown in soil under normal and ABA treatment conditions. Under normal conditions, the average stomatal aperture indices of WT and control transgenic line were 0.61 and 0.59, respectively and that of *cpk6-3* was 0.60; in contrast, those of 35S::*CPK6*OE17 and 35S::*CPK6*OE20 plants were significantly lower than those of WT, VC and *cpk6-3*, being 0.54 and 0.51, respectively (Fig. 4A). Under 5 μM ABA treatment, the average stomatal aperture indices of all the five genotypes decreased and those of 35S::*CPK6*OE17 and 35S::*CPK6*OE20 plants decreased to 0.25 and 0.29, respectively, which were significantly smaller than those of WT and VC plants (Fig. 4B). Under 10 μM ABA treatment, the stomatal aperture indices of all the five genotypes decreased further compared to the 5 μM ABA treatment (Fig. 4C). Specifically, the stomatal aperture index of *cpk6-3* was the biggest among all the genotypes, which was 0.31 whereas that of *CPK6*OE20 was the smallest, being 0.17. No significant difference was observed in the stomatal aperture indices among WT, VC and *CPK6*OE17 plants (Fig. 4C). These results support the inference that 35S::*CPK6* transgenic plants have the ability to efficiently close stomata and thus reduce transpiration, which confers tolerance to drought stress.

Identification of interacting proteins of CPK6

To further explore the molecular mechanism of CPK6, we determined to screen and identify its interacting proteins, especially the substrates. ABF/ARBE TFs are core TFs involved in ABA signaling (Choi *et al.*, 2000; Uno *et al.*, 2000) (Fig. S4) and these six TFs belong to Clade A of bZIP family showing conserved domain structures and high similarity (Fig. S5). They can bind to different ABRE-containing promoters in late embryogenesis abundant (LEA) genes, which are thought to participate in the acquisition of desiccation tolerance (Jakoby *et al.*, 2002). Surprisingly, we failed to detect any interaction between CPK6 and any of the six ABF/AREB TFs through yeast two-hybrid screening (data not shown). This phenomenon was also observed for interaction study of BnaCPKs and BnaABFs in a previous report (Zhang *et al.*, 2014b).

Similar scenarios have also been reported in several other studies (Uno *et al.*, 2009; Zhu *et al.*, 2007), possibly because of low expression levels of plant-source TF genes in yeast or a lack of scaffold proteins mediating CPK-substrate interactions in yeast, an inference awaiting further exploration.

To solve this, we therefore used BiFC technique to examine the protein-protein interactions *in planta*. The expression of various proteins in leaves of *N.benthamiana* was confirmed through immunoblotting (Fig. S6). Strong fluorescent signals were detected in nuclei of leaf cells upon co-expression of *CPK6-YFP*$_C$ and *YFP*$_N$*-ABF3*, *YFP*$_N$*-ABI5* and *YFP*$_N$*-AREB3*, respectively (Fig. 5). Weak YFP signals were found in nuclei of leaf cells when *CPK6-YFP*$_C$ was co-expressed with *YFP*$_N$*-ABF2* or *YFP*$_N$*-ABF4* (Fig. 5). No YFP fluorescence was detected in three negative controls (Fig. S7). These results indicate that CPK6 interacts strongly with ABF3, ABI5 and AREB3, suggesting CPK6 might regulate ABA signaling and drought tolerance through a subset of ABF/AREB TFs to activate the expression of downstream responsive genes.

To further investigate how ABA affects the protein level of CPK6 *in vivo*, accumulation of CPK6 tagged with 3xHA (hemagglutinin) epitope was determined via immunoblotting. The results showed that under mock treatment, CPK6-HA protein level did not show any significant change across different time points (Fig. S8A), however, abundance of CPK6-HA increased gradually and significantly after treated with ABA (Fig. S8B). Therefore, these results suggest that ABA treatment increases CPK6 accumulation *in vivo*, which is consistent with a positive role of CPK6 in ABA signaling.

Since previous reports have shown that *ABF3* and *ABI5* are highly induced by ABA, compared to the other four ABF/AREB genes (Choi *et al.*, 2000; Lopez-Molina *et al.*, 2001; Sirichandra *et al.*, 2010; Uno *et al.*, 2000), we determined to focus on ABF3 and ABI5. We then performed co-immunoprecipitation (co-IP) assay to confirm the interactions between CPK6 and ABF3, ABI5 *in vivo*. *CPK6-HA* was co-expressed with *Myc-ABF3* or *Myc-ABI5* in *N. benthamiana* leaves and epitope tag-specific antibodies were used in immunoblottings. As a control, *GFP-HA* was also expressed in parallel.

The results indicated that Myc-tagged ABF3 and ABI5 co-immunoprecipitated CPK6-HA, but not GFP-HA (Fig. 6A, B).

Next, we performed a modified pull-down assay to analyze the physical interaction of CPK6 with ABF3 and ABI5. CPK6-HA was expressed and purified from *CPK6*OE20 transgenic plants. ABF3 and ABI5 fused with GST tag was expressed in and purified from *E. coli*. Calcium and/or ABA were also added to the system to test their effect on the kinase-TF interactions. The results indicated that GST-ABI5 was able to pull down CPK6-HA from the total protein extract of *CPK6*OE plants, but not from the WT control (Fig. 6C). Moreover, addition of calcium and/or ABA did not significantly change the results. Similarly, GST-ABF3 was able to pull down CPK6-HA from the total protein extract of *CPK6*OE plants, but not from the WT control (Fig. 6D). Moreover, addition of calcium and/or ABA did not show any significant effect on the pull-down assay. These results indicate that CPK6 physically interacts with ABI5 and ABF3 both in plant cells and *in vitro*.

CPK6 phosphorylates ABF3 and ABI5

To test whether CPK6 can phosphorylate ABF3 and ABI5 *in vitro*, a luminescence-based kinase assay was performed. In this luminescence assay, the amount of ADP formed from ATP after phosphorylation was measured. Two types of control were included in this assay. One is a synthetic peptide Syntide 2, which is an artificial substrate of CPKs (Hashimoto and Soderling, 1987). The other is GFP-HA purified in parallel from plants. We also tested the phosphorylation level with or without ABA and calcium treatments. The specific activity of CPK6–HA was first evaluated *in vitro* on syntide-2 substrate in the presence of increasing concentration of Ca^{2+}, using CPK6-HA purified from transgenic Arabidopsis. The results showed that CPK6 displayed enhanced activity with increasing [Ca^{2+}] (Fig. S9A, B). However, when the aspartic acid (D) residue at 209 site of CPK6 was changed to alanine (A), CPK6D209A became inactive and lost the ability to phosphorylate Syntide 2 (Fig. S10), indicating D209 is an important site for the kinase activity.

Similarly, incubation of ABF3-Flag or ABI5-Flag with CPK6-HA resulted in a significantly increased activity in the presence of calcium, although the overall

phosphorylation level is lower than with syntide-2. In contrast, GFP-HA did not show any phosphorylation activity on either Syntide 2 or ABF3-Flag and ABI5-Flag even in the presence of 1 μM [Ca^{2+}], indicating the reliability of this assay. On the other hand, when ABA was present in the reactions, the phosphorylation level between CPK6 and ABF3 or ABI5 was significantly decreased, regardless of the presence of [Ca^{2+}] in the reactions (Fig. S9A, B).

The preferred phosphorylation motif of a few plant CPKs was previously studied using synthetic peptides and it was found that LXRXXpS/T sequence is preferred, in which X represents any amino acid residue (Loog *et al.*, 2000; Vlad *et al.*, 2008). We therefore surveyed the protein sequences of ABF3 and ABI5. As shown in Fig S5, we detected three putative phosphorylation sites in ABF3 and they are S32, S126, S134 and one site, T169 that is highly similar to the preferred motif. As for ABI5, a total of three possible phosphorylation sites were identified, which are S41, S42 and S145. Meanwhile, in ABI5, S182 is located in a context similar to the preferred LXRXXpS/T motif. To test the role of the identified phosphorylated sites in ABF3 and ABI5 by CPK6, point mutations were created, that is from serine (S) or threonine (T) to alanine (A) for both ABF3 and ABI5 proteins. A total of four Ser or Thr residues were mutated to be Ala for both ABF3 and ABI5. Similarly, the mutated proteins tagged with GST were expressed and purified and subjected to phosphorylation assays. The results showed that phosphorylation levels of ABF3S32A, ABF3S126A, ABF3S134A and ABF3T169A were significantly lower than that of native ABF3 protein (Fig. 7A). As a control, GFP-HA showed only background signals when incubated with ABF3 and its mutated versions after phosphorylation reactions. For ABI5, ABI5S41A, ABI5S42A and ABI5S145A were significantly less phosphorylated by CPK6, compared to native ABI5 protein (Fig. 7B). However, ABI5S182A did not change the phosphorylation level mediated by CPK6, suggesting S182 is not a phosphorylation residue by CPK6. As expected, the control GFP-HA did not show any significant phosphorylation activity toward the ABI5 and its mutated forms.

To identify the possible phosphorylation sites in ABF3 and ABI5, CPK6-HA purified from plants and GST-ABF3/ABI5 purified from *E.coli* were used for LC–

MS/MS assay. Firstly, *in vitro* kinase assay was performed to confirm the phosphorylation of ABF3 and ABI5 by CPK6 (Fig. 7C-D). It can be seen that quantitative comparison of the phosphorylation level showed that incubation of the ABF3 with CPK6 resulted in a significantly increased ADP production in the presence of calcium, as a more than two-fold greater luminescence signal was observed compared with control reactions containing CPK6 and GST or ABF3 and GFP (Fig. 7C). Similarly, incubation of ABI5 with CPK6 also significantly increased the luminescence signal by five folds (Fig. 7D). Secondly, the end products from the above phohphorylation reactions were separated. Following proteolytic digestion and purification, the protein samples were analyzed with LC–MS/MS to map the possible phosphorylation sites using neutral loss of the phosphate group (98 Da) during fragmentation of precursor ions. The results revealed that the serine (S) at residue 126 in ABF3 (Fig. S11), and the serine (S) at residues 16, 41, 138, 145, 418 and threonine (T) at residue 156 in ABI5 exhibited much stronger phosphorylation signals (Fig. S12A-F). These results suggest that CPK6 is indeed able to phosphorylate ABF3 and ABI5, and CPK6 is also able to phosphorylate Ser or Thr residues in suboptimal sites besides the preferential Ser or Thr in the LXRXXpS/T motif in substrates.

CPK6-mediated phosphorylation of ABF3 and ABI5 enhances transcriptional activities

To explore how CPK6-mediated phosphorylation affects ABF3 and ABI5 function and regulates the expression of target genes, that is, *RD29B* (*Responsive to Dehydration 29B*) and *Em6* (*Late Embryogenesis Abundant 6*), we performed transient transactivation assays using the *RD29B* and *Em6* promoters fused to the reporter gene coding for firefly luciferase (LUC) (Fig. 8A, B). Effector constructs in which *ABF3*, *ABI5* and *CPK6* were expressed under control of the 35S promoter were co-transfected with the *ProRD29B*/*ProEm6*::*LUC* reporter constructs into *N. benthamiana* leaves. As another type of control, *GFP* expressed under CaMV35S was also used. Moreover, based on the previous findings, we generated both phosphomimic and inactive forms of ABF3 and ABI5, and obtained ABF3S32A, ABF3S32D, ABI5S41/42/145A, and ABI5S41/42/145D. Further, these mutated versions were included in dual LUC assays,

together with both native CPK6 and inactive CPK6D209A. Consistent with expectation, co-expression of *CPK6* and *ABF3* or *ABI5* obviously enhanced ABF3/ABI5-activated *RD29B* and *Em6* expression, compared to the GFP control (Fig. 8C-D). Moreover, native CPK6 had a stronger effect on the transcriptional activity of ABF3 than the inactive CPK6D209A, which was comparable to the GFP control (Fig. 8C). Further, when the phosphomimic ABF3S32D was used as the effector, the transcriptional activity was higher than native ABF3 and inactive ABF3S32A (Fig. 8C). A similar result was observed with the native, inactive and phosphomimic ABI5 (Fig. 8D). Besides, co-infiltration of 50 μM ABA significantly attenuated ABF3/ABI5-activated *RD29B* and *Em6* marker gene expression, but had no little effect when the inactive CPK6D209A and GFP control were included in the assay (Fig. 8C-D). The robustness of this assay was supported by immuoblotting assay of various proteins (bottom panel of Fig. 8C-D).

Disruption or overexpression of *CPK6* alters the expression of a few LEA-like genes

Previous studies showed that *RD29B*, promoter of which contain ABRE element is a direct target gene of ABF1-4 and AREB3 TFs (Choi *et al.*, 2000; Uno *et al.*, 2000), while *Em1* and *Em6*, promoter of which also contain ABRE element are direct targets of ABI5 (Carles *et al.*, 2002; Nakamura *et al.*, 2001). We therefore tested the expression of these three LEA-like genes in the *cpk6-3* mutant and transgenic *CPK6*-overexpression lines as well as double mutants under mock and ABA treatment conditions. Double mutants were generated through crossing single mutants. For *ABI5*, we screened out homozygous lines from SALK_013163, which was previously identified to be a loss-of-function mutant (Zheng *et al.*, 2012). For *ABF3*, a knock-out line was identified from SALK_096965. RT-PCR analysis indicated the absence of corresponding transcripts in the individual mutants (Fig. S13A-B). As reported previously, the expression of *RD29B* and *Em6* was strongly stimulated by ABA while *Em1* showed only marginal up-regulation upon ABA stimulus (Fig. 9). Disruption of *CPK6* down-regulated expression of *RD29B* and *Em6* in the presence of ABA, but had slight effect on *Em1* transcription, except under mock condition. Mutation of *ABF3* or

ABI5 significantly down-regulated the expression of *RD29B*, *Em1* and *Em6* compared to WT, especially in the presence of ABA. Double disruption of *CPK6* and *ABF3* or *ABI5* genes had stronger inhibiting effects on expression of these three target genes compared to *cpk6-3* single mutant, which was true both in the absence and presence of the ABA treatments (Fig. 9). In contrast, overexpression of *CPK6* increased the expression levels of *RD29B*, *Em1* and *Em6*, which was especially true for the high-expression *CPK6*OE20 line (Fig. 9).

Genetic Interaction between CPK6 and interacting ABI5 and ABF3

Since the results and previous reports on ABF3 and ABI5 indicate they are positive regulators of ABA signaling, we were curious to know if there existed additive effect between them in terms of the phenotype under ABA treatment. To investigate this, wild type, single mutants, and double mutant (dm) seeds were sown on 1/2 x MS medium without or with different concentrations of ABA. On the MS medium, there was no significant difference between the WT and various mutants; almost 100% of the seeds germinated and produced seedlings with green cotyledons and true leaves (Fig. 10A). On the MS medium supplemented with either 0.25 or 0.5 μM ABA, *cpk6* and *abi5* mutants were insensitive to ABA than WT, and *cpk6abi5* double mutant was a little more insensitive to ABA than *abi5* mutant only on the medium supplemented with 0.25 μM ABA, which is also supported by the quantitative comparison (Fig. 10B). Similarly, the responses of these materials to ABA treatment were also evaluated in root elongation assay (Fig. 10C), with similar phenomenon observed as supported by statistical analysis of primary root length (Fig. 10D). This phenotypic assay showed that loss-of-function mutations of *CPK6* and *ABI5* have limited additive effect at the root elongation level, but subtle or very small difference at the germination level.

As for *ABF3*, phenotypic assays of seed germination and root elongation were also performed with the single and double mutants as compared to WT. It can be seen that *cpk6* and *abf3* mutants were more insensitive to ABA, and *cpk6abf3* double mutant was a little more tolerant of ABA treatment compared to WT only on the medium containing 0.5 μM ABA, at the germination level (Fig. S14A-B). In the root elongation assay, there is no significant different among the four different genotypes on 1/2 MS or 1/2 MS

containing 0.25 μM ABA (Fig. S14C-D). On 1/2 MS containing 0.5 μM ABA. the roots of *cpk6abf3* double mutant were slightly longer that those of *abf3* mutant (Fig. S14C-D). Together with the finding that both ABI5 and ABF3 are substrates of CPK6, these results of phenotypic assays suggest that CPK6 plays a positive role in ABA signaling at least in part through its interaction with and phosphorylation of ABI5 and ABF3.

Discussion

Calcium plays an essential role in cell signalings and is an important second messenger implicated in ABA signaling and abiotic stress tolerance (Kudla *et al.*, 2010). CPKs, being Ser/Thr protein kinases are important calcium sensors in plants (Cheng *et al.*, 2002). CPKs are unique in that Ca^{2+}-sensor domain and kinase effector domain are combined within the same molecule. So far functions of a few members of AtCPKs have been reported; however, the underlying mechanisms of these CPKs, especially their substrates, and the functions of many other CPKs remain to be identified.

ABA is a key phytohormone involved in many biological processes, including seed dormancy and germination, plant development and responses to biotic and abiotic stresses (Finkelstein *et al.*, 2002). Endogenous ABA levels in plant cells are increased in response to drought and high salinity, leading to expression of stress-responsive genes, which are mainly regulated by six Clade A bZIP TFs, ABF1, ABF2/AREB1, ABF3, ABF4/AREB2, AREB3 and ABI5 (Cutler *et al.*, 2010). Among these, ABI5 was identified genetically while the others identified by yeast one-hybrid screening of ABRE binding proteins (Choi *et al.*, 2000; Finkelstein and Lynch, 2000; Uno *et al.*, 2000). ABI5 and AREBs/ABFs are considered to have a redundant function and similar binding properties. ABI5 is able to form homo- or heterodimers with other ABF/AREB proteins. For instance, ABI5 was shown to interact with ABF3 and ABF1 (Finkelstein *et al.*, 2005; Kim *et al.*, 2002). Research on the four ABF/AREB TFs show overall, ABF2, ABF4 and ABF3 are master TFs in ABA signaling involved in drought stress tolerance (Yoshida *et al.*, 2014). ABF2, ABF3 and ABF4 were previously reported to play important roles in drought tolerance mediated by ABA (Fujita *et al.*, 2005; Kang *et al.*, 2002). Upon challenge by abiotic stress such as drought, elevated ABA levels regulates water balance and physiological water deficit to confer drought tolerance

(Finkelstein *et al.*, 2002; Zhu, 2002). The role of ABA in water balance is mainly through guard cell conductance, whereas the latter role is associated with induction of lots of genes that encode upstream regulatory proteins or downstream LEA-like proteins (Cutler *et al.*, 2010; Xiong *et al.*, 2002). Previously, loss-of-function mutant analysis of *AtCPK3* and *AtCPK6* indicate they regulate stomatal aperture through positively regulating activation of S-type anion channels (Mori *et al.*, 2006). Later, it was found that AtCPK6, different from AtCPK3, functions as a positive regulator of methyl jasmonate (MeJA) signaling in guard cells through activating nonselective Ca^{2+}-permeable cation channels (Munemasa *et al.*, 2011). AtCPK6 phosphorylates and activates SLOW ANION CHANNEL-ASSOCIATED 1 (SLAC1) expressed in *Xenopus* oocyte (Brandt *et al.*, 2012; Scherzer *et al.*, 2012).

In this study, we found that AtCPK6 is induced ABA and dehydration stress and, phenotypic assay of mutants and overexpression lines of *CPK6* showed it positively regulates pleiotropic ABA signalings including seed germination, seedling growth, and stomatal apertures (Figs 2-5). Reduced stomatal apertures and increased expression of *RD29B*, *Em1* and *Em6* in *CPK6*-overexpresion lines may account for its function in improved drought tolerance compared to wild-type under drought stress (Figs. 3, 4 and 9). In another word, enhanced drought tolerance of *CPK6*-overexpresion plants could be resulted from higher efficiency in conserving water, which is similar to *AtCPK4* and *AtCPK11* (Zhu *et al.*, 2007).

AtCPK6 was identified to localize in the nucleus (Kawamoto *et al.*, 2015). This localization in cells may facilitate phosphorylation of nuclear-localized regulators, such as TFs that mediate gene expression. Previous reports have demonstrated that several ABA-responsive TFs, including the six members of the bZIP protein family, ABF1, ABF2, ABF3 and ABF4, AREB3 and ABI5 have been shown to be phosphorylated by different classes of protein kinases. For instance, SNF1-related kinase 2s (SnRK2s) such as SnRK2.2, -2.3 and -2.6 (OST1) and CIPK11 (PKS5), CIPK26 as well as CPK4, CPK11 and CPK32 have been reported to mediate ABA signaling through phosphorylating one or more of the six TFs (Choi *et al.*, 2005; Fujii *et al.*, 2007; Furihata *et al.*, 2006; Lyzenga *et al.*, 2013; Wang *et al.*, 2013; Zhou *et al.*, 2015).

However, whether there exist other CPKs phosphorylating any of these six ABF/AREB/ABI5 TFs remain elusive. In this report, we provide evidences that AtCPK6 mediate ABA signaling and drought tolerance through interacting and phosphorylating a subset of these six core TFs (Figs 5-7, S9-S12). These findings suggest that multiple protein kinases may share common substrates in ABA signalings. Further, we showed that phosphorylation of ABF3 and ABI5 by CPK6 significantly increased the transcriptional activities of both TFs (Fig. 8), suggesting CPK6-mediated phosphorylation plays a positive role in ABA signaling. Previously, it was shown that phosphorylation of ABF1 and ABF3 by ABA-activated SnRK2.6/OST1 enhances stability of these two ABFs and regulates the activation of ABFs, which is required for ABA-responsive gene expression (Furihata *et al.*, 2006; Sirichandra *et al.*, 2010). In this sense, CPK6 is highly similar to OST1 in activating downstream ABA-responsive gene expression.

ABF/AREB/ABI5 proteins contain three conserved N-terminal (C1-C3) domains and one conserved C-terminal (C4) domain (also called bZIP domain) (Fig. S5), each of which harbors putative phosphorylation sites by different kinases (Fujita *et al.*, 2009; Furihata *et al.*, 2006). It should be noted that other Ser or Thr residue outside of C1, C2, C3 and C4 domains may also be phosphorylated. For instance, it is recently identified that Thr411 existing in apple AREB2 (ABF4) protein is a novel phosphorylation site by MdCIPK22 (Ma *et al.*, 2017). This indicates that although the CPK–SnRK kinases have related phosphorylation preferences, i.e. LXRXXpS/T motif, and therefore may share common substrates (Vlad *et al.*, 2008), Ser or Thr residues in other contexts in the ABF/AREB/ABI5 proteins could also be potential phosphorylation sites. Further, the phosphorylation sites of ABF3 and ABI5 identified in this study were compared with those of previous reports. For ABF3 protein, we found that three of the phosphorylation sites we identified (S32, S126, S134) were similarly identified to be phosphorylated by OST1(SnRK2.6) in a previous report (Sirichandra *et al.*, 2010). However, we found that S126 site in ABF3 is not an important site for CPK6 compared to OST1, as mutation of this site only produced a subtle change in the kinase assay (Fig. 7A). For ABI5, S42 has been reported to be an important site phosphorylated by

CIPK11 (PKS5) (Zhou *et al.*, 2015). This comparison indicates that a Ser or Thr site could be phosphorylated by more than one kinase and the activities of ABF3 and ABI5 are thus fine-tuned by multiple protein kinases.

It is also noteworthy that the double mutations in the *CPK6* and interacting *ABF3* or *ABI5* resulted in a stronger induction of ABA-responsive gene expression, especially under the ABA condition (Fig. 9). This suggests that phosphorylation of ABF3 and ABI5 by CPK6 plays a substantial role in regulating their activities and transcription of target genes. Being the best-studied key regulator in ABA signaling, ABI5 is modulated at both transcriptional and post-translational levels (Skubacz *et al.*, 2016). However, double mutants of *cpk6abi5* and *cpk6abf3* only showed a subtle or small difference compared to the single mutants in seed germination and seedling growth assays (Figs 10 and S14), suggesting other CPKs (such as reported CPK4, CPK11) and interesting ABF/AREB TFs (such as ABF1 and ABF4) play redundant roles in these two processes (Zhu *et al.*, 2007). Lastly, we provided a simplified working model of CPK6-ABF/AREB/ABI5 cascade in seed germination, seedling growth and drought tolerance (Fig. S15), which shows the multi-layer regulations of ABA-signaling-related core ABF/AREB/ABI5 by different upstream protein kinases. Our identification of ABF3 and ABI5 as novel interaction partners of CPK6 is interesting and provides new insight into mechanism of CPK6 and its homologs. Taken together, our data presented here indicate that AtCPK6 regulates ABA signaling at least partly through the functions of its direct targets ABF3 and ABI5.

Competing Interests

The authors have declared that no competing interests exist.

Acknowledgements:

This work is supported in part by the National Natural Science Foundation of China (31471153) and the Fundamental Research Funds for the Central Universities [2452017025] and, the Open Project from CAU [SKLPPBKF1403]. We thank NASC for providing the mutant seeds, Prof. Jörg Kudla (Universität Münster, Germany) for

providing the BiFC vectors, Drs. Jian-Ye Chen (South China Agricultural University) for providing the LUC vector and Xiayan Liu (NWAFU) for the transient mCherry vector.

Author contributions

YQJ and BY conceived the project. YQJ, BY and HZ designed the experiments. HZ, DL, WZL, BM, HS performed the experiments. BC, YL, DR, HD contributed research materials. YQJ, BY and HZ wrote the manuscript. YQJ, BY and DR acquired the fundings. All authors read and approved the manuscript.

Supplementary data

Table S1: Primers used in this study

Fig. S1. Promoter-driven GUS reporter activity in stomata.

Fig. S2. Expression analysis of *CPK6* during early seedling development.

Fig. S3. Phenotypic assay of two mutants of *CPK6* gene during seed germination and post-germination root elongation.

Fig. S4. Phylogenetic analysis of Clade A bZIP transcription factors in Arabidopsis and rice.

Fig. S5. Multiple alignment and motif analysis of Clade A bZIP transcription factors in Arabidopsis.

Fig. S6. Immunodetection of various proteins expressed in leaves of *N.benthamiana*.

Fig. S7. Assay of interaction between CPK6 and bZIP15 through BiFC.

Fig. S8. Accumulation of CPK6 proteins upon ABA treatment.

Fig. S9. Kinase activity assay of CPK6 and its constitutively inactive form.

Fig. S10. Kinase assay of CPK6-mediated phosphorylation of ABF3 and ABI5.

Fig. S11. Annotated fragmentation spectra of tryptic phosphopeptides derived from ABF3 phosphorylated *in vitro* by CPK6.

Fig. S12. Annotated fragmentation spectra of tryptic phosphopeptides derived from ABI5 phosphorylated *in vitro* by CPK6.

Fig. S13. RT-PCR analysis of homozygous mutant lines.

Fig. S14. Examination of relationship between CPK6 and ABF3 during the ABA-induced inhibition of seed germination.

Fig. S15. A simplified working model of CPK6-ABF/AREB/ABI5 cascade in seed germination, seedling growth and drought tolerance.

Supplemental methods

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Figures and legends

Fig. 1. The response of *CPK6* to ABA, dehydration and oxidative stresses. (A) Relative expression of *CPK6* following hormone and stress treatments was determined by qRT-PCR at 1, 3 and 12 h after treatment. Data are relative to basal expression in mock-treated plants. ABA, abscisic acid; PEG, PEG8000; MV, methyl paraquat. Error bars show the SE (n = 3). (B-E) Histochemical staining of transgenic line expressing a *CPK6* promoter driven-*GUS* reporter gene. (B) 7-day-old seedlings with a cotyledon shown in a larger view at the right side. (C) Siliques of a 5-week-old plant. (D) Rosette leaf of a 5-week-old plant showing the trichomes. (E) Flower of a 6-week-old plant with a close view shown at the right side.

Fig. 2. Arabidopsis *cpk6* mutant is more tolerant to ABA while overexpression more sensitive to ABA. (A) A schematic diagram of the T-DNA insertion sites in the *CPK6* locus. Triangles, black rectangles, grey rectangles and lines represent T-DNA insertions, exons, untranslated region (UTR) and introns, respectively. (B) Detection of *CPK6* mRNA by RT-PCR. *ACT2* was used as an internal control. (C) Detection of *CPK6* mRNA in overexpression lines. qRT-PCR quantification was normalized to the expression of *UBC21* and *UBQ10* and also compared to endogenous transcript of *CPK6* in the vector control (VC) line. Error bars represent SE (n = 3). (D) Germination assay of WT (Col-0), *cpk6-3* mutant and overexpression lines on normal medium or medium supplemented with 0.5 μM ABA. (E) Quantitative data from (D). Germination was assessed at different days after the end of stratification. Data represent means ±SE, from three biological replicates. (F) Root elongation assay of WT, *cpk6-3* and *cpk6-4* mutants and two overexpression lines on normal medium or medium supplemented with 50 μM ABA. Growth was evaluated 11 d after the end of stratification. (G) Quantitative data from (F). Error bars represent the standard errors of 30 seedlings from three independent experiments.

Fig. 3. Overexpression of *CPK6* increases drought tolerance in Arabidopsis.

(A) RT-PCR detection of *CPK6* transcript level in WT, *cpk6-3* mutant, and two overexpression lines grown under normal conditions. *ACT2* was amplified as a control.

(B) Growth status of WT (Col-0), *cpk6-3* and 35S::*CPK6* transgenic plants (OE17 and OE20) in soil before water withheld, after water withheld for 2 weeks and three days after rewatered, respectively. The entire experiment was repeated three times and 16 plants were tested for each genotype grown in an 8-well-tray in each experiment.

Fig. 4. Stomatal aperture assay of WT and *CPK6*-related genotypes under normal and ABA-treated conditions. (A), (B) and (C) indicate the assay performed under normal, 5 μM ABA and 10 μM ABA treatment conditions, respectively. Values are mean ratios of width to length. Error bars represent SE of three independent experiments (n = 10 for each replicate). Different letters above bars indicate significant differences.

Fig. 5. Assay of interactions between CPK6 and ABFs/AREBs/ABI5 *in planta*. *N.benthamiana* leaves were co-infiltrated with constructs encoding the indicated fusion proteins with N- or C-terminal half of YFP. Yellow fluorescence reconstructed from YFP as a result of interaction was examined. Images from left to right are YFP field, NLS-mCherry fluorescence, bright field and a merge of all the fields. NLS, nuclear localization sequence. Scale bars, 50 μm.

Fig. 6. Protein–protein interactions between CPK6 and ABF3, ABI5. (A)-(B) Co-IP confirmation of interactions between CPK6 and ABF3, ABI5. The total protein extracts from *N. benthamiana* leaves transfected with 35S::CPK6-HA/35S::Myc-ABF3 or 35S::Myc-ABI5 were immunoprecipitated with anti-HA Sepharose beads. The proteins from crude lysates (left, input) and immunoprecipitated proteins (right) were detected with anti-Myc antibody. (C)-(D) Pull-down confirmation of interactions between CPK6 and ABI5, ABF3. CPK6-HA was expressed and purified from transgenic plants. GST-ABI5/ABF3 were expressed and purified from *E. coli*. CPK6-HA and GST-ABI5/ABF3 were detected with anti-HA and anti-GST antibodies, respectively. The relative intensity of each band of interest is indicated below each band (normalized to the 2nd lane, which is set as 1.00).

Fig 7. *In vitro* kinase assay of CPK6 phosphorylating ABF3 and ABI5. (A)–(B) *In vitro* phosphorylation assay of CPK6 towards native and mutated ABF3 (A), and native and mutated ABI5 (B). GST is used as the control for substrate while GFP used as the kinase control. RLU, relative luminescence units. Data shown are the means ±SE of three independent replicates. Different letters indicate significant differences among reactions (P<0.05). (C)-(D) Phosphorylation assay of CPK6 towards ABF3 (C) and ABI5 (D) subjected to the LC-MS/MS assay. Consumption of ADP is expressed as luminescence signal. Data shown are the means ±SE of three independent replicates. Asterisks indicate significant difference from Student's *t*-test (***, P<0.001).

Fig. 8. Dual luciferase assay of CPK6-mediated phosphorylation on transcriptional activities of ABF3 and ABI5.

(A)-(B) Schematic diagrams of reporter and effector constructs used in the assay. LUC, firefly luciferase; REN, relinna luciferase; TL, translational leader sequence; Ter, terminator sequence. HA and cMyc indicate epitope tag sequences fused in-frame with the coding regions of different genes. (C)-(D) Luciferase activities were measured in *N.benthamina* co-infiltrated with different combinations of effectors and reporters without or with 50 μM ABA. Values are means of four replicates ± SE. Different letters indicate significant differences (P<0.05). Bottom panel, immunodetection of various proteins extracted from leaves infiltrated with agrobacteria harboring different effector and reporter plasmids, with anti-HA and anti-cMyc antibodies. Ponceau S staining of the abundant RbcL (large subunit of Rubisco) was used to verify equal loading.

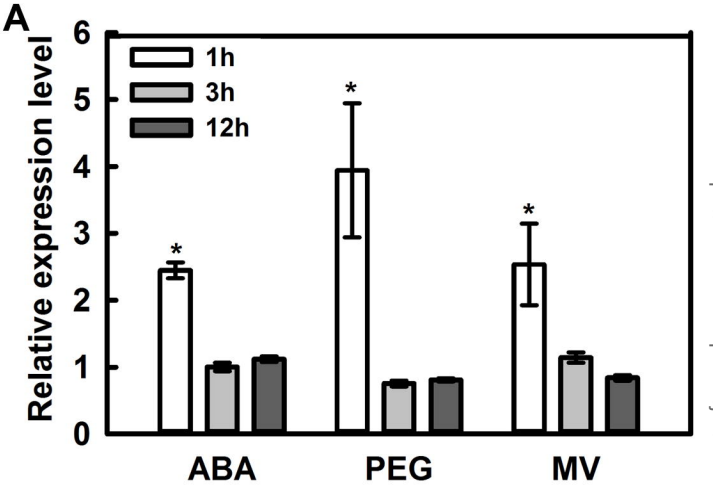
Fig. 9. Expression analysis of target genes in *CPK6*-related mutants and overexpression lines.

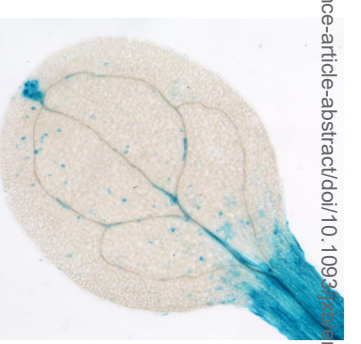
Seedlings of different genotypes were treated without or with 50 μM ABA for 3 h, before used for qRT-PCR assay. Mock, ABA-free treatment; ABA, 50 μM ABA treatment. The expression levels are presented as relative units with the levels in ABA-treated Col-0 leaves being taken as 1. Each value is the mean± SE of three independent biological replicates. Different letters above bars indicate significant differences in the

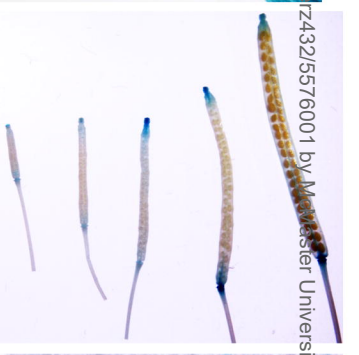
same group (P<0.05).

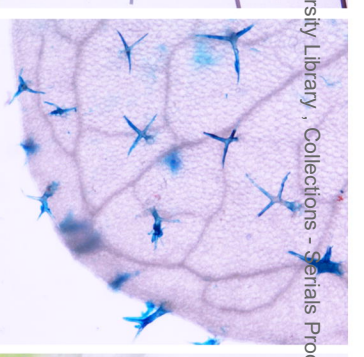
Fig. 10. Examination of relationship between CPK6 and ABI5 during ABA-induced inhibition of seed germination and root elongation.

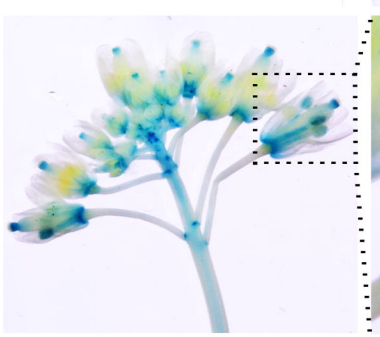
(A) Seeds of wild type (WT, Col-0), *cpk6-3* (termed as *cpk6* in the figure), *abi5*, and *cpk6abi5*(dm) were germinated on 1/2 x MS medium without (top) and with 0.25 μM ABA (middle) or 0.5 μM ABA (bottom). Photographs were taken after 5 d of growth. Three independent experiments were performed, and one representative result is presented. (B) Quantification of the percentages of germination from (A) from day 3 through day 7. Data shown are averages ± SE (n=3). (C) Seeds of WT, *cpk6*, *abi5*, and *cpk6abi5* (dm) were germinated vertically on 1/2 x MS medium without (top) and with 0.25 μM ABA (middle) or 0.5 μM ABA (bottom). Photographs were taken after 7 d of growth. Three independent experiments were performed, and one representative result is presented. (D) Quantification of primary root length after 7 d of growth. Data shown are averages ± SE (n=3). ANOVA test was used to determine statistical significance and different letters mean significant difference (P<0.05).

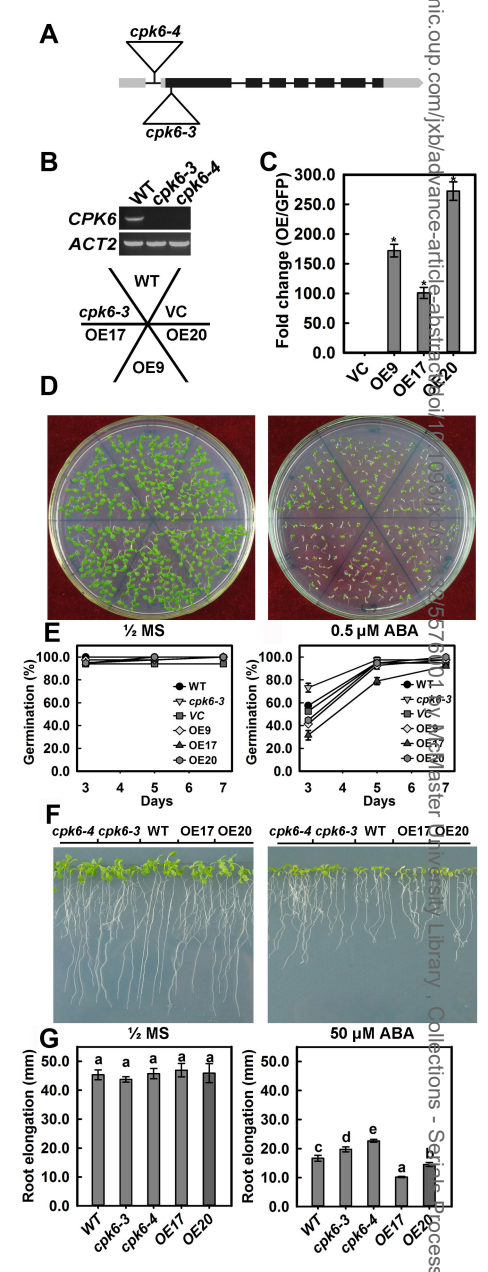


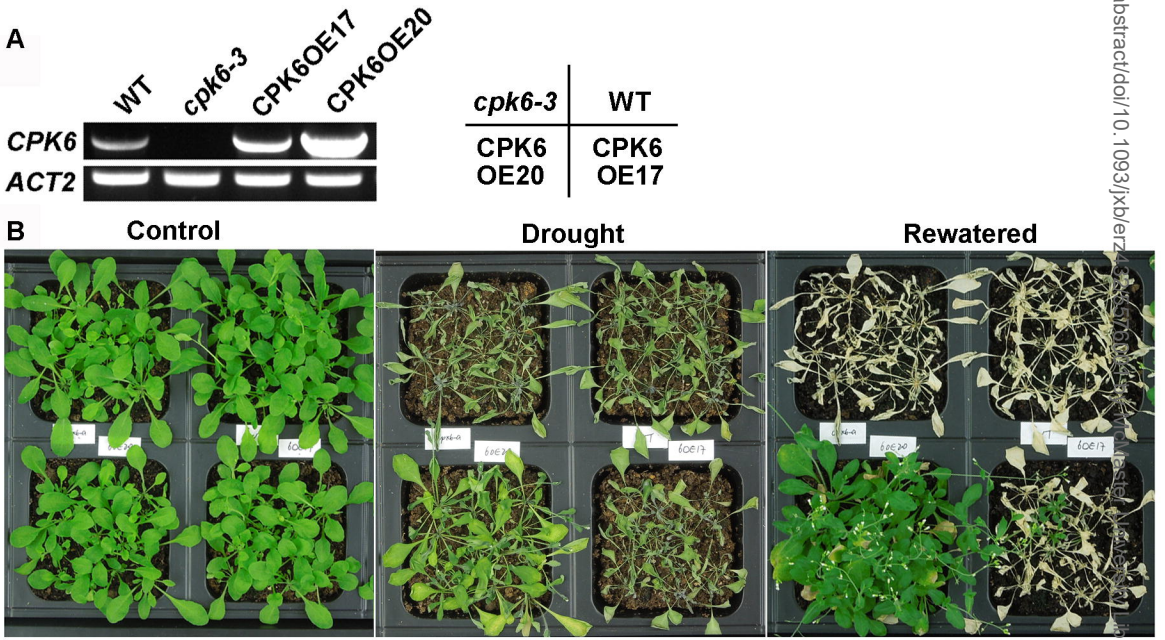


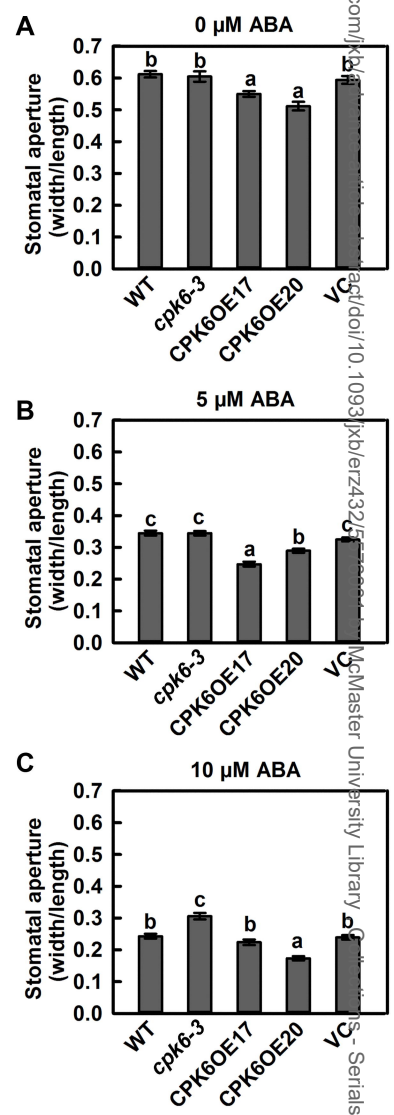


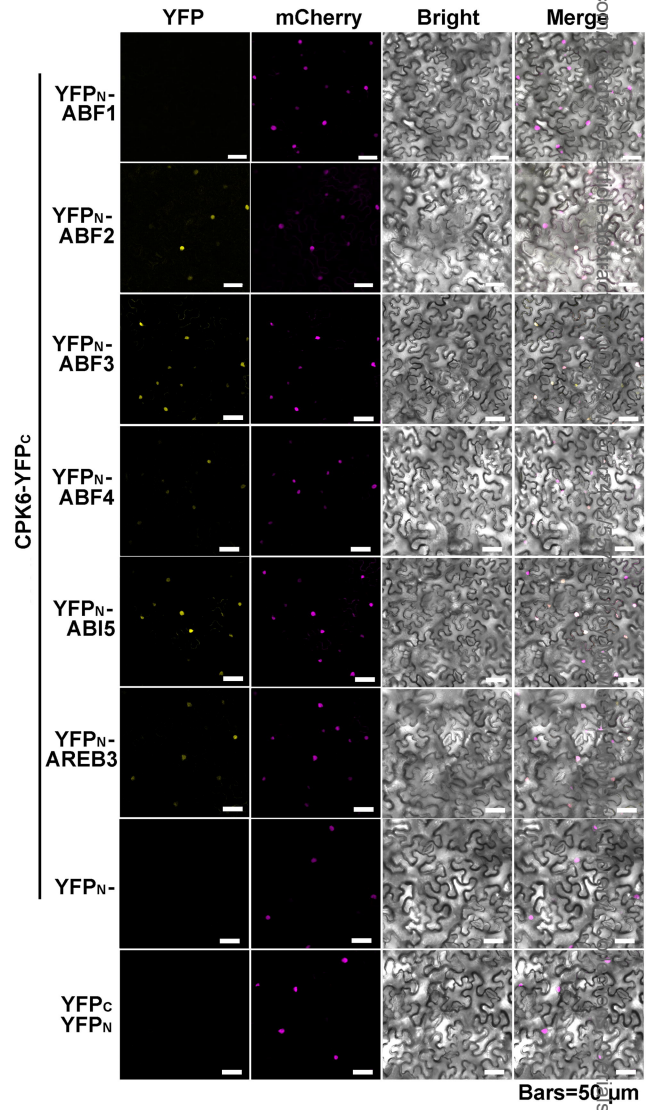


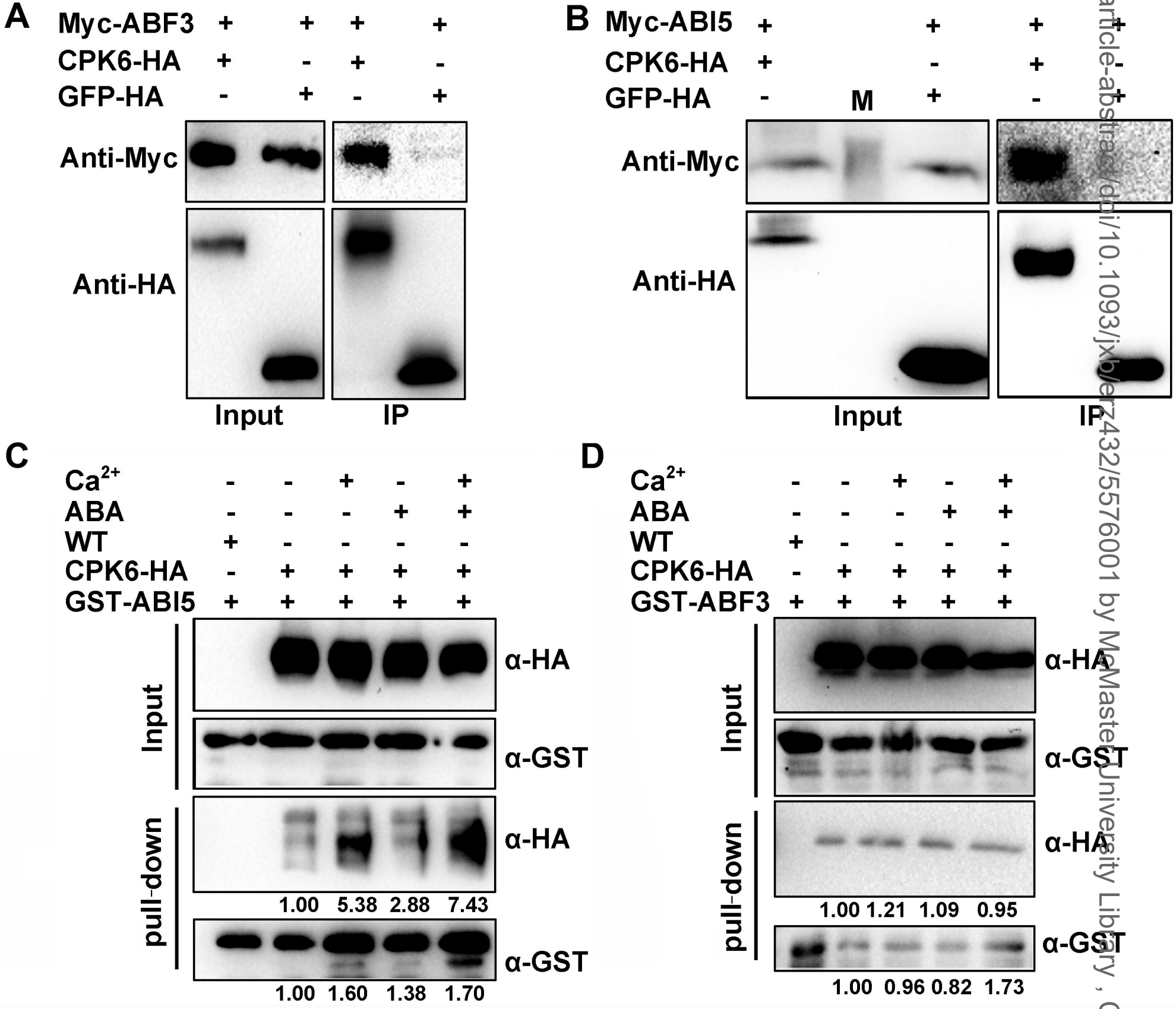


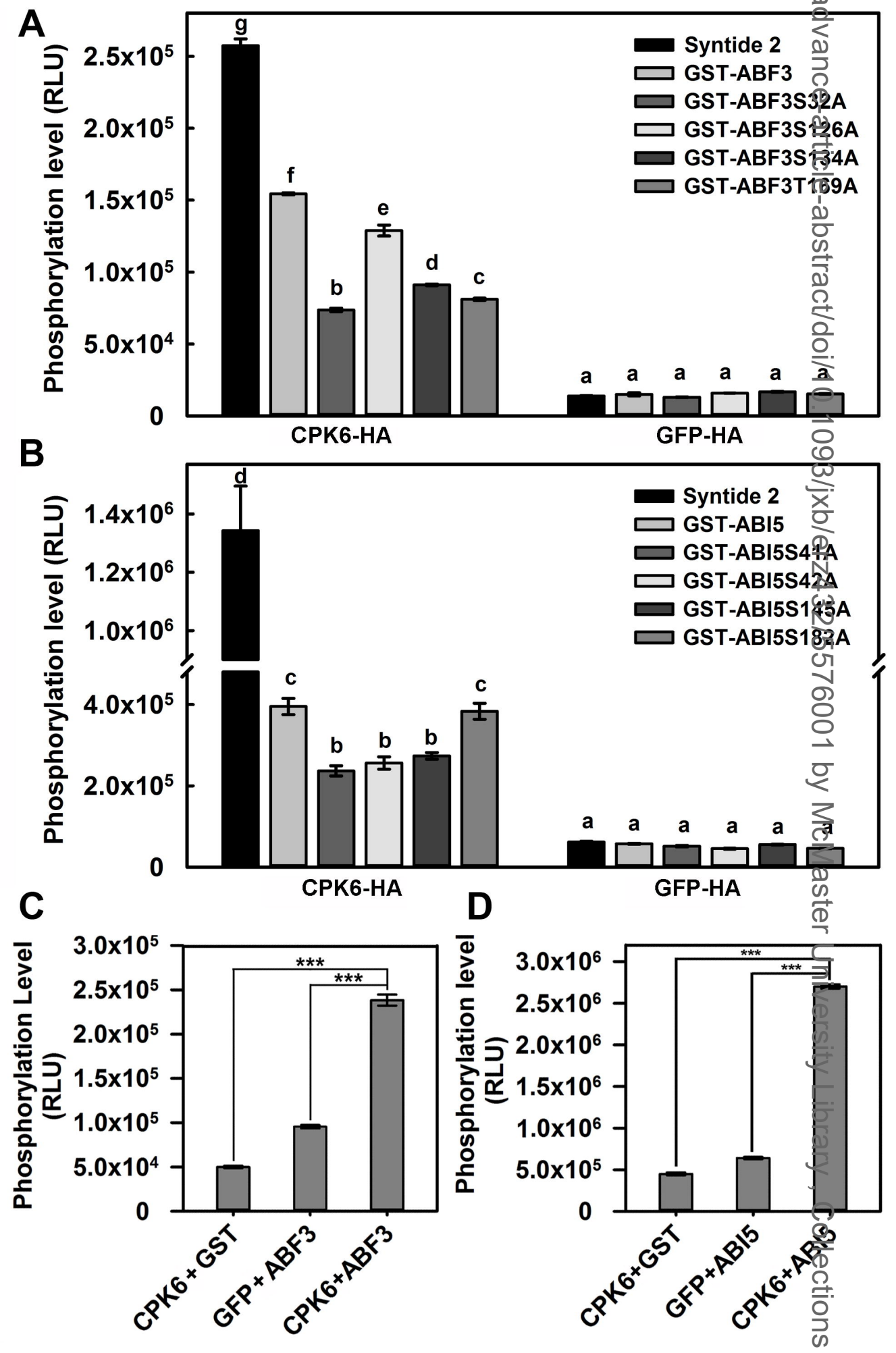


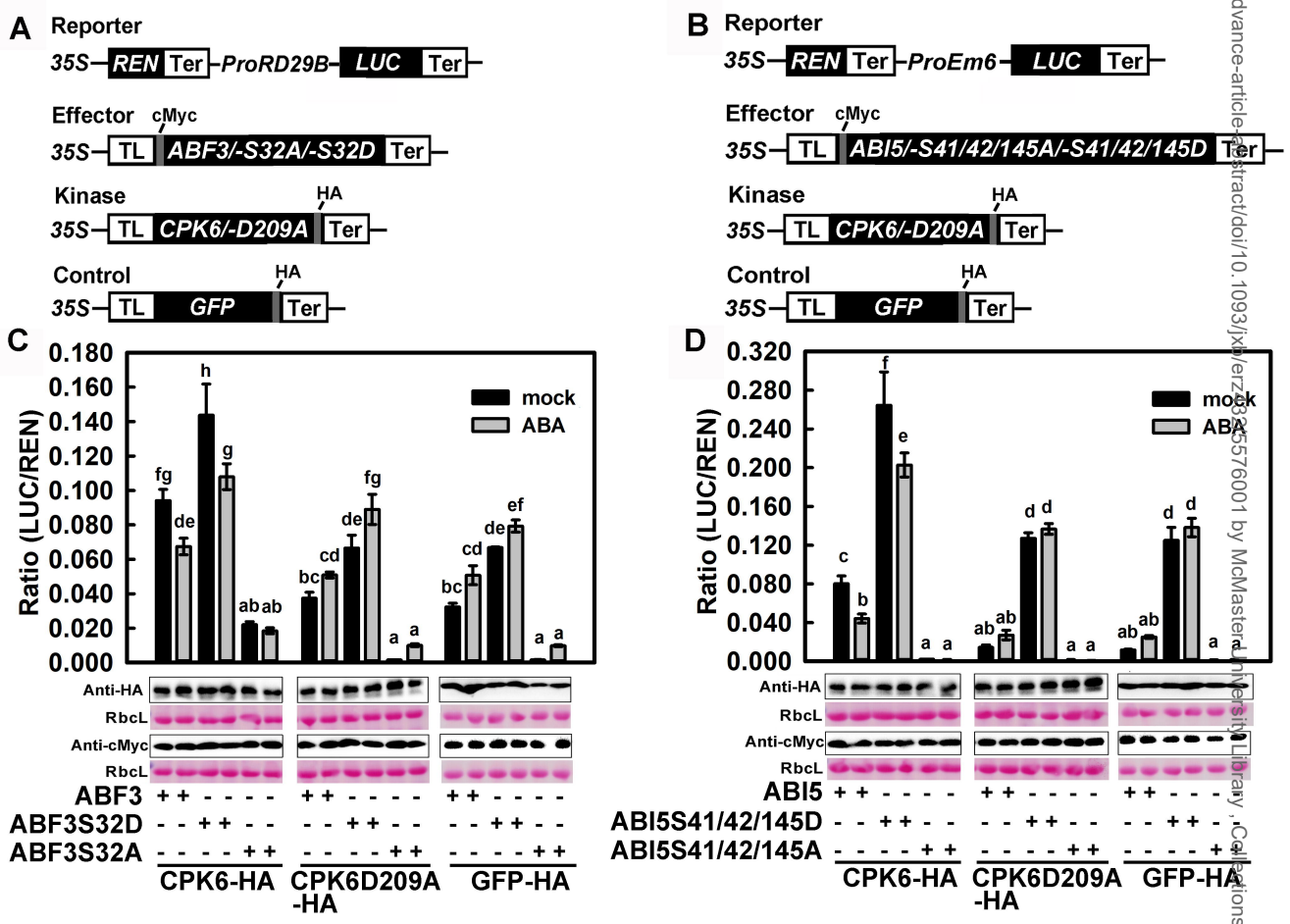


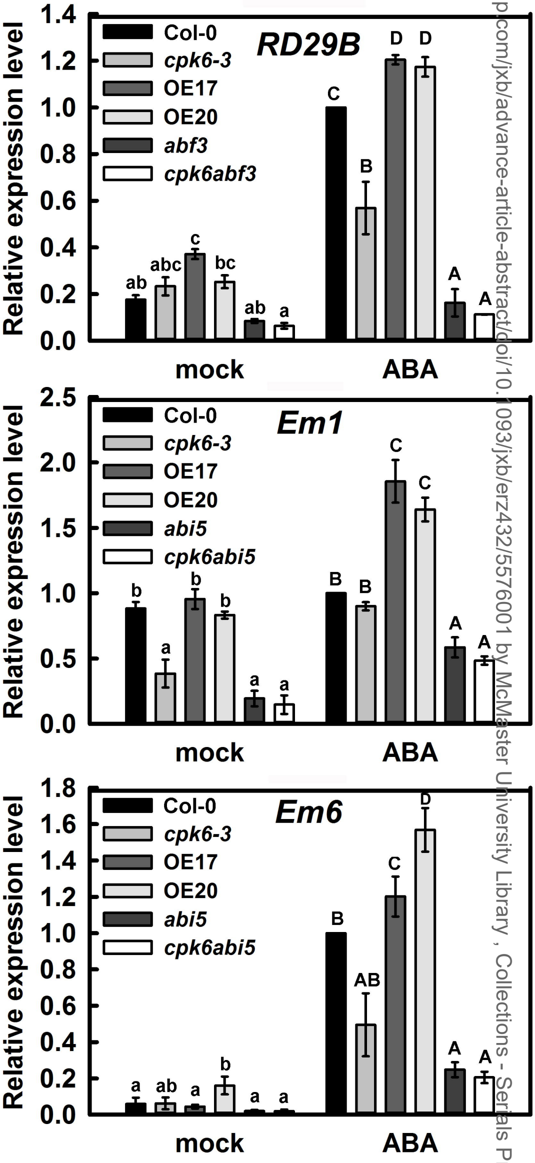


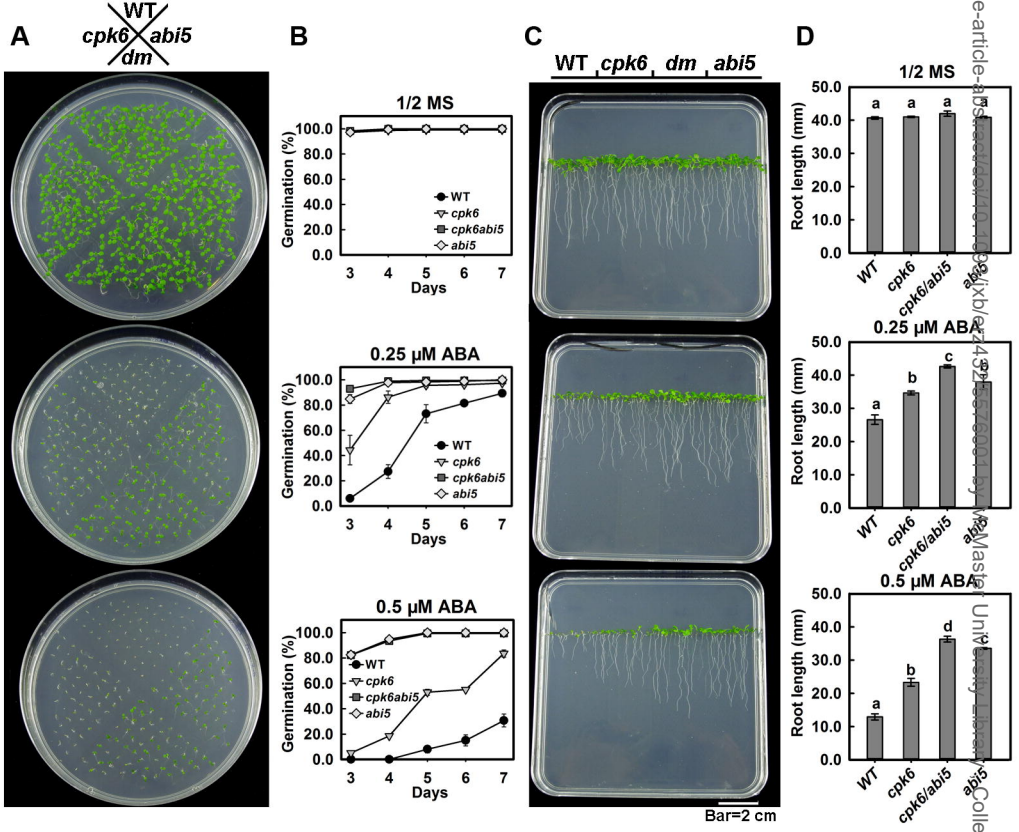

YFP
mCherry
Bright
Merge
CPK6-YFPc
YFPN-ABF1
YFPN-ABF2
YFPN-ABF3
YFPN-ABF4
YFPN-ABI5
YFPN-AREB3
YFPN-
YFPc YFPN
Bars=50 μm










A
WT
cpk6
abi5
dm
B
1/2 MS
Germination (%)
Days
WT
cpk6
cpk6abi5
abi5
0.25 μM ABA
0.5 μM ABA
C
WT
cpk6
dm
abi5
Bar=2 cm
D
1/2 MS
Root length (mm)
0.25 μM ABA
0.5 μM ABA