Arabidopsis CALCIUM-DEPENDENT PROTEIN KINASE8 and CATALASE3 Function in Abscisic Acid-Mediated Signaling and H$_2$O$_2$ Homeostasis in Stomatal Guard Cells under Drought Stress

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INTRODUCTION

Drought is a major threat to plant growth and crop productivity. Calcium-dependent protein kinases (CDPKs, CPKs) are believed to play important roles in plant responses to drought stress. Here, we report that Arabidopsis thaliana CPK8 functions in abscisic acid (ABA)- and Ca$^{2+}$-mediated plant responses to drought stress. The cpk8 mutant was more sensitive to drought stress than wild-type plants, while the transgenic plants overexpressing CPK8 showed enhanced tolerance to drought stress compared with wild-type plants. ABA-, H$_2$O$_2$-, and Ca$^{2+}$-induced stomatal closing were impaired in cpk8 mutants. Arabidopsis CATALASE3 (CAT3) was identified as a CPK8-interacting protein, confirmed by yeast two-hybrid, coimmunoprecipitation, and bimolecular fluorescence complementation assays. CPK8 can phosphorylate CAT3 at Ser-261 and regulate its activity. Both cpk8 and cat3 plants showed lower catalase activity and higher accumulation of H$_2$O$_2$ compared with wild-type plants. The cat3 mutant displayed a similar drought stress-sensitive phenotype as cpk8 mutant. Moreover, ABA and Ca$^{2+}$ inhibition of inward K$^+$ currents were diminished in guard cells of cpk8 and cat3 mutants. Together, these results demonstrated that CPK8 functions in ABA-mediated stomatal regulation in responses to drought stress through regulation of CAT3 activity.

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www.plantcell.org/cgi/doi/10.1105/tpc.15.00144
CPK8pro:GUS than that of the wild-type plants. Furthermore, CPK12 serves as a negative ABA-signaling regulator in seed germination and postgermination growth, which is different from the function of its homologs, CPK4 and CPK11 (Zhao et al., 2011). In addition to abiotic stress, some Arabidopsis CDPKs have been reported to be involved in the plant innate immune response (CPKs 4, 5, 6, and 11 [Boudsocq et al., 2010], CPK1 [Coca and San Segundo, 2010], and CPK5 [Dubiella et al., 2013]), herbivory-induced signaling network (CPK3 and CPK13; Kanchiwamy et al., 2010), regulation of pollen tube growth (CPK17 and CPK34 [Myers et al., 2009], CPK11 and CPK24 [Zhao et al., 2013], CPK2 and CPK20 [Gutermuth et al., 2013], and CPK32 [Zhou et al., 2014]), and stem elongation and vascular development (CPK28; Matschi et al., 2013).

We previously demonstrated that Arabidopsis CPK10 functions in ABA- and Ca2+-mediated stomatal regulation in response to drought stress (Zou et al., 2010). Here, we report that CPK8 (At5g19450) as well as its interacting protein CATALASE3 (CAT3; At1g20620) play important roles in ABA- and H2O2-mediated signal transduction and in maintaining H2O2 homeostasis in response to drought stress.

RESULTS

CPK8 Acts as a Positive Regulator in Response to Drought Stress and Involving in ABA- and H2O2-Mediated Stomatal Movement

After a 20-d period of drought stress, the CPK8 T-DNA insertion mutant plants (cpk8, SALK_036581; Supplemental Figure 1A) showed more sensitive phenotype compared with wild-type plants (Figure 1A), while there was no obvious morphological difference observed between cpk8 mutant and wild-type plants under normal growth condition (Figure 1A). When 3-week-old plants grown under normal conditions were subjected to drought stress for about 1 week, the leaf temperature of cpk8 mutant plants was lower than that in the wild-type plants (Figure 1B), suggesting that water loss of the cpk8 plants may be faster than that of the wild-type plants. β-Glucuronidase (GUS) staining of CPK8pro:GUS transgenic plants showed that the CPK8 promoter drove expression in leaves and roots (Figure 1C) and particularly in stomatal guard cells (Figure 1D), suggesting a potential role of CPK8 in regulation of stomatal movement. The results of reverse transcriptase quantitative PCR (RT-qPCR) showed that CPK8 transcript accumulation was induced by drought (Supplemental Figure 1B), ABA (Supplemental Figure 1C), and H2O2 (Supplemental Figure 1D) treatments.

To further test whether the drought sensitivity of cpk8 plants resulted from CPK8 disruption, CPK8 overexpression lines (ecotype Columbia [Col] + CPK8; 8OE and complementation lines [cpk8 + CPK8; BCOM] were generated. The RT-qPCR results showed that transcription of CPK8 was disrupted in the cpk8 homozygous plants (Figure 1E). When subjected to drought stress for 20 d, the cpk8 mutant plants were more sensitive to drought stress, whereas the CPK8 overexpression lines (8OE1 and 8OE2) showed enhanced tolerance to drought stress compared with wild-type plants (Figure 1F). The overexpression lines (8OE1 and 8OE2) grew well and the rosette leaves remained green even after 22 d without watering (Figure 1F). The cpk8 complementation lines (8COM1 and 8COM2) showed a similar phenotype as wild-type plants after the drought stress treatment (Figure 1F). These results indicate that disruption of CPK8 expression increases plant sensitivity to drought stress and that increased CPK8 expression enhances plant tolerance to drought stress.

The stomatal pores control most of the water loss via transpiration to the atmosphere (Schroeder et al., 2001). To test the hypothesis that CPK8 may be involved in regulation of stomatal movements under drought stress, water loss assays of detached leaves were conducted. As shown in Figure 1G, water loss of the detached rosette leaves of cpk8 plants was much faster than that of wild-type leaves, while water loss of the CPK8 overexpression (8OE1) leaves was slower than that of wild-type leaves under dehydration conditions.

H2O2 is one of the major reactive oxygen species (ROS) and serves as an important messenger in ABA-regulated stomatal movement (Pei et al., 2000; Murata et al., 2001; Zhang et al., 2001a; Kwak et al., 2003; Miao et al., 2006). Accumulation of H2O2 in stomatal guard cell can cause [Ca2+]cyt elevations and result in stomatal closure (Pei et al., 2000; Murata et al., 2001; Kwak et al., 2003). We further hypothesized that CPK8 may serve as a calcium sensor and function in ABA- and H2O2-mediated regulation of stomatal movement. To test this hypothesis, stomatal aperture assays were conducted to examine stomatal responses of different lines to ABA, H2O2, and Ca2+. As shown in Figures 1H to 1J, stomatal closure induced by ABA (Figure 1H), H2O2 (Figure 1I), or Ca2+ (Figure 1J) was impaired in the cpk8 mutant plants. These results suggest that CPK8 may serve as an important signaling component to mediate ABA as well as H2O2 and Ca2+ signals in regulation of stomatal movement.

Identification of CAT3 as a CPK8-Interacting Protein

We next undertook identification of CPK8-interacting protein(s) to gain insight into CPK8 might function in ABA and H2O2 signaling. In previous studies, a number of experimental approaches had been employed to identify CDPK-interacting proteins (Patharkar and Cushman, 2000; Lee et al., 2003; Shao and Harmon, 2003; Rodríguez Millá et al., 2006; Böhmer and Romeis, 2007; Vlad et al., 2008; Uno et al., 2009). Among these different approaches, yeast two-hybrid screening with baits containing only the kinase domain of the protein had been demonstrated to be successful for identification of CDPK substrates, such as for ice plant (Mesembryanthemum crystallinum) CPK1 (Patharkar and Cushman, 2000) and tobacco (Nicotiana tabacum) CPDK1 (Lee et al., 2003). Here, using the kinase domain of CPK8 as bait, a catalase protein encoded by At1g20620 (CAT3) was identified as a potential CPK8-interacting protein (Figure 2A). There are three catalase genes in the Arabidopsis genome, including CAT71 (At1g20630), CAT72 (At4g35090), and
CAT3 (Frugoli et al., 1996). To verify the specificity of the CPK8 interaction with CAT3, three catalase isoforms were used as prey for yeast two-hybrid assays. As shown in Figure 2A, when the CPK8 bait was cotransformed with the CAT1, CAT2, and CAT3 preys individually, only the AH109 yeast cells carrying pGBK7-CPK8 and pGADT7-CAT3 grew well on the selection medium (SC/-Leu/-Trp/-His/-Ade) and exhibited β-galactosidase (β-gal) activity.

The interaction between CPK8 and CAT3 in planta was confirmed using coimmunoprecipitation (co-IP) assays. Proteins
were extracted from Arabidopsis mesophyll protoplasts harboring 35Spro:cMyc-CPK8 and 35Spro:CAT3-GFP constructs and used for co-IP assays. As shown in Figure 2B, immunoprecipitation of CPK8 with anti-cMyc agarose beads yielded a co-IP band corresponding to the tagged CAT3 that was labeled with the anti-GFP antibody.

Before we performed bimolecular fluorescence complementation (BiFC) assays (Waadt and Kudla, 2008) to further confirm the interaction between CPK8 and CAT3 in planta, we rechecked the subcellular localizations of CPK8 and CAT3. CPK8 was localized to the plasma membrane as described previously (Dammann et al., 2003) and CAT3 was localized to the peroxisome, cytoplasm, and the plasma membrane (Supplemental Figure 2). The BiFC assays showed that CPK8 could specifically interact with CAT3, and the CPK8/CAT3 complex was localized to the plasma membrane of the cotransformed Nicotiana benthamiana epidermal cells (Figure 2C). In addition, firefly luciferase complementation imaging assays confirmed that CPK8 specifically interacted with CAT3, but not with CAT1 and CAT2 (Supplemental Figure 3).

**CPK8 Can Phosphorylate CAT3 at Ser-261 and Regulate Its Activity**

Both in vitro and in planta protein phosphorylation assays were conducted to demonstrate CAT3 phosphorylation by CPK8. Strep-tagged CPK8 (Strep-CPK8) was transiently expressed in N. benthamiana and purified (Supplemental Figure 4A), and GST-CAT1, GST-CAT2, and GST-CAT3 proteins were expressed in Escherichia coli and purified (Supplemental Figure 4B). As shown in Figure 3A, CPK8 had autophosphorylation activity and could phosphorylate CAT3 but not CAT1 and CAT2.

Reiland et al. (2009) reported that CAT3 is a phosphoprotein and the phosphorylated amino acid is detected at Thr-408. When Thr-408 was mutated to Ala (CAT3 T408A), the mutant protein was still phosphorylated by CPK8 (Figure 3A), indicating that CPK8 may phosphorylate one or more other residue(s).

To determine CPK8 phosphorylation site(s) in CAT3, additional CAT3 truncated proteins were generated (Figure 3B; Supplemental Figure 4C). CAT3-N (from Met-1 to Ala-260) and CAT3-C2 (from Glu-320 to Ile-492) were not phosphorylated by CPK8, while the CAT3-C-terminal truncated proteins containing region from Ser-261 to Thr-319 could be phosphorylated by CPK8, indicating that CPK8 phosphorylation of CAT3 occurs in this region (Figure 3C; Supplemental Figures 4C and 4D). In CAT3, there are five potential phosphorylation sites in the region from Ser-261 to Thr-319 (Figure 3D; Supplemental Figure 4E). When Ser-261 in CAT3 was mutated to Ala (CAT3 S261A-C1), the mutant protein was not phosphorylated by CPK8 (Figure 3E; Supplemental Figure 4F). When Thr-273, Thr-290, Thr-311, or Thr-319 was mutated to Ala (CAT3 T273A-C1, CAT3 T290A-C1, CAT3 T311A-C1, and CAT3 T319A-C1), these mutant proteins could still be phosphorylated by CPK8 (Figure 3E; Supplemental Figure 4F). In addition, the quadruple mutant CAT3 T273A, T290A, T311A, T319A-C1 was also phosphorylated by CPK8 (Figure 3E). These results indicate that Ser-261 in CAT3 is the only residue phosphorylated by CPK8 in vitro. It should be noted that Ser-261 is not conserved in Arabidopsis CATs, and Ser-261 in CAT3 is substituted with Ala-261 in CAT1 and CAT2 (Frugoli et al., 1996).
Figure 3. CPK8 Phosphorylates CAT3 In Vitro and In Planta.

(A) CPK8 can phosphorylate CAT3 in vitro. Coomassie blue-stained recombinant proteins of CAT1, CAT2, and CAT3 proteins are indicated by the arrow in the top panel. In the bottom panel, the arrowhead shows the autophosphorylated CPK8, and the arrow shows the phosphorylated CAT3 variants.

(B) Model of CAT3 N-terminal and C-terminal deletions.

(C) Phosphorylation of CAT3 N-terminal and C-terminal deletions by CPK8 in vitro. Coomassie blue-stained recombinant proteins of CAT3 are indicated with arrows in the top panel. In the bottom panel, the arrowhead indicates the autophosphorylated CPK8, and the arrow denotes the phosphorylated CAT3-C1 terminus.

(D) Potential phosphorylation sites (indicated with triangles) in CAT3-C1 residues from Ser-261 to Thr-319.

(E) Phosphorylation of CAT3 at Ser-261 by CPK8. Coomassie blue-stained CAT3-C1 variants are indicated by the arrow in the top panel. In the bottom panel, the arrowhead indicates the autophosphorylated CPK8, and the arrow indicates the phosphorylated CAT3-C1 variants. CAT3S261A-C1, CAT3-C1 with Ser-261 mutated to Ala. CAT3T273A-C1, CAT3-C1 with Thr-273 mutated to Ala. CAT3T290A-C1, CAT3-C1 with Thr-290 mutated to Ala. CAT3T311A-C1, CAT3-C1 with Thr-311 mutated to Ala. CAT3T319A-C1, CAT3-C1 with Thr-319 mutated to Ala. CAT3S261A,T273A,T290A-C1, CAT3-C1 with Ser-261, Thr-273, and Thr-290 mutated to Ala. CAT3T273A,T290A,T311A,T319A-C1, CAT3-C1 with Thr-273, Thr-290, Thr-311, and Thr-319 mutated to Ala.

(F) CPK8 can phosphorylate CAT3 in planta. Wild-type plants (Col) or cpk8 plants expressing 35Spro:CAT3-GFP were treated with 100 μM ABA for 1, 3, and 6 h. Total proteins were extracted and CAT3-GFP proteins were immunoprecipitated using anti-GFP mAb agarose. The phosphorylated CAT3 proteins were detected by immunoblotting (IB) using biotinylated Phos-tag. Calf intestinal alkaline phosphatase (CIAP)-treated proteins were used as control.
To determine whether CAT3 is phosphorylated by CPK8 in planta, transgenic lines (Col/3SSpro:CAT3-GFP and c(pk8/3SSpro:CAT3-GFP) were generated. The CAT3 phosphorylation level was detected using Phos-Biotin technology as described previously (Kinoshita-Kikuta et al., 2007). Under normal conditions, CAT3 phosphorylation was very low in Col/3SSpro:CAT3-GFP and c(pk8/3SSpro:CAT3-GFP plants (Figure 3F). After ABA treatment, CAT3 phosphorylation was obviously enhanced in Col/3SSpro:CAT3-GFP (Figure 3F). However, this ABA-induced CAT3 phosphorylation was weakened in c(pk8/3SSpro:CAT3-GFP, indicating that ABA-induced CAT3 phosphorylation is CPK8 dependent (Figure 3F).

To study the significance of phosphorylation of CAT3, catalytic activity of the recombinant CAT3 was analyzed in the presence of CPK8. As shown in Figure 4A, CPK8 had no effect on the catalytic activity of cat3, whereas CPK8 significantly enhanced CAT3 activity in the presence of ATP. However, mutation of Thr-408 (CAT3T408A) or CAT3T408D) did not affect CPK8-activated CAT3 activity (Figure 4A). When Ser-261 was mutated to Ala (CAT3S261A), CPK8 did not stimulate catalase activity of CAT3S261A (Figure 4B), and when Ser-261 was mutated to Asp (CAT3S261D), the mutated CAT3S261D showed high catalase activity even in the absence of CPK8 (Figure 4B). These results clearly demonstrate that Ser-261 in CAT3 is an essential residue for CAT3 activation by CPK8.

The c(pk8 and cat3 Mutants Have Low Catalase Activity and High H2O2 Production

To further investigate the effect of CPK8 on catalase activity in planta, CPK8- and CAT3-related materials were used for catalase activity assays. The site of T-DNA insertion in cat3 (SALK_092911) was located in the first intron of CAT3 genomic DNA (Supplemental Figure 5A). RT-PCR analysis showed that the expression of CAT3 was completely disrupted in cat3 mutant (Supplemental Figure 5B). Transcript levels of CAT3 in Col, cat3, and CAT3 overexpression lines are presented in Figure 5A. The cat3 mutant plants had a decrease in catalase activity of ~20% compared with wild-type plants, consistent with a previous report (Figure 5B; Mhamdi et al., 2010). Similar to the cat3 mutant, the c(pk8 mutant also had decreased catalase activity (Figure 5B). When CPK8 was overexpressed in the cat3 mutant background, the catalase activity in the 8OE/cat3 line was similar as that in the cat3 mutant. Consistent with the decreased catalase activities in the c(pk8, cat3, and 8OE/cat3 plants, these plants also show greater ROS accumulation compared with wild-type plants (Figure 5C). When treated with ABA, c(pk8, cat3, and 8OE/cat3 plants accumulated more ROS compared with the ABA-treated wild-type plants (Figure 5C).

The 3,3'-diaminobenzidine (DAB) uptake method (Thordal-Christensen et al., 1997; Guan and Scandalios, 2000) was used to examine the production of H2O2 in leaves after treatment with ABA. As shown in Figure 5D, after ABA treatment, the c(pk8 and cat3 mutants as well as 8OE/cat3 plants accumulated more, and CPK8 and CAT3 overexpression lines accumulated less H2O2 in the leaves compared with wild-type plants.

The ROS production in stomatal guard cells of c(pk8, cat3, and wild-type plants was further analyzed using the dye 2',7'-dichlorofluorescein diacetate (H2DCF-DA) (Miao et al., 2006). After treatment with ABA for 5 min, the pixel intensity of fluorescence emission in guard cells of c(pk8 and cat3 mutants was higher than that of wild-type plants (Figures 5E and 5F). All of these results suggest that CPK8 can regulate H2O2 homeostasis by modulating CAT3 activity.

The cat3 Mutant Shows a Similar Phenotype as the c(pk8 Mutant in Response to Drought Stress

Previous reports revealed that CAT3 was expressed in leaves and guard cells (Zimmermann et al., 2006; Du et al., 2008;
Jannat et al., 2011), and the transcription of CAT3 was induced by ABA and drought (Xing et al., 2007), suggesting that CAT3 may function in ABA-mediated plant responses to drought stress. The transcript level of CAT3 was induced by ABA and H2O2 (Supplemental Figure 5D), consistent with the previous study (Xing et al., 2007). It was further hypothesized that CAT3, as a substrate of CPK8, may act downstream of CPK8 in ABA-mediated signal transduction in stomatal guard cells under drought stress. The cat3 mutant plants and CAT3 overexpression lines were tested for their sensitivity to drought stress. After a 20-d period of drought stress treatment (the normal drought stress treatment in this study), the cat3 mutant showed similar sensitivity to drought stress as the cpk8 mutant (Figure 6A). In addition, the cpk8 cat3 double mutants showed similar sensitivity to drought stress as cpk8 and cat3 single mutant plants (Figure 6A). When CPK8 was overexpressed in cat3 (8OE/cat3) or CAT3 overexpressed in cpk8 (3OE/cpk8), both transgenic lines were as sensitive to drought stress as cat3 and cpk8 plants (Supplemental Figure 6), which not only further demonstrates that CPK8 acts as an upstream regulator of CAT3 but also demonstrates that activation of CAT3 requires its phosphorylation by CPK8. As shown in Figure 6B, wild-type plants did not survive a 22-d period of drought stress treatment (the “severe” drought stress treatment) even after rewatering. However, the CAT3 overexpression lines not only survived, but also recovered well after rewatering. Consistent with these results, water loss assays showed that the detached leaves of cat3, similar to the cpk8, lost water faster than wild-type plants under dehydration treatment, whereas the 3OE1 leaves lost water slower than wild-type plants (Figure 6C). Furthermore, the cat3 mutant showed impaired stomatal closing induced by ABA (Figure 6D), H2O2 (Figure 6E), and Ca2+ (Figure 6F) as well as impaired ABA inhibition of stomatal opening (Supplemental Figure 7), which was similar to the observation in the cpk8 mutant. These results suggest that CPK8 and CAT3 function in the same signaling pathway in plant responses to drought stress.
CPK8 and CAT3 Modulate ABA and Ca²⁺ Inhibition of the Inward K⁺ Currents in Stomatal Guard Cells

ABA inhibition of the inward K⁺ currents in stomatal guard cells has been well documented (Blatt, 1990; Schwartz et al., 1994; Zhang et al., 2001b). Elevation of [Ca²⁺]_{cyt} in guard cells inhibits the inward K⁺ currents (Schroeder and Hagiwara, 1989; Kelly et al., 1995; Grabov and Blatt, 1997, 1999; Ivashikina et al., 2005). Some studies have demonstrated that CDPKs function in the regulation of ion channel activities in stomatal guard cells (Pei et al., 1996; Li et al., 1998; Mori et al., 2006; Zou et al., 2010; Geiger et al., 2010, 2011; Brandt et al., 2012; Scherzer et al., 2012). We found that ABA- and Ca²⁺-induced stomatal closing and ABA-inhibited stomatal opening was impaired in the cpk8 and cat3 mutants (Figures 1H to 1J and 6E to 6G; Supplemental Figure 7). Using patch-clamp whole-cell recording techniques, we tested the effects of CPK8 and CAT3 on ABA- and Ca²⁺-mediated regulation of the inward K⁺ channels in guard cells. Inhibition of the inward K⁺ currents by ABA (Figures 7A and 7B) and Ca²⁺ (Figures 7C and 7D) was observed in wild-type plants. However, inhibition of the inward K⁺ currents by ABA (Figures 7A and 7B) and Ca²⁺ (Figures 7C and 7D) was impaired in the cpk8 and cat3 mutants. These results suggest that CPK8 and CAT3 may function downstream of ABA and Ca²⁺ in regulation of inward K⁺ channel activities in stomatal guard cells.

DISCUSSION

CDPKs have been predicted to function in response to [Ca²⁺]_{cyt} elevations in many physiological processes in plants (Cheng et al., 2002; Boudsocq and Sheen, 2013; Romeis and Herde, 2014). Considering their diverse expression profiles as well as subcellular localizations together with their different responses to various environmental stimuli, each CDPK may have a specific function (Boudsocq and Sheen, 2013). Arabidopsis CPK7 shares 90% protein sequence identity with CPK8 (Cheng et al., 2002); however, the expression pattern of CPK7 is different from that of CPK8. Expression driven by the CPK8 promoter was abundant in stomatal guard cells (Figure 1D), whereas GUS activity driven by the CPK7 promoter was not evident in stomatal guard cells (Supplemental Figure 8). Li et al. (2014) showed that CPK7 functions in root water transport, revealing different roles for CPK7 and CPK8 in spite of their high protein

Figure 6. The cat3 Mutant Shows a Similar Phenotype to the cpk8 Mutant in Response to Drought Stress and Stomatal Movement.

(A) Phenotype comparison of wild-type, cpk8, cat3, and cpk8 cat3 plants placed under drought stress for 20 d and then rewatered for 3 d. The experiments were repeated three times with similar results.

(B) Phenotype comparison of wild-type, cat3, and 3OE1 plants under drought stress for 22 d and then rewatered for 3 d. The experiments were repeated three times with similar results.

(C) Time courses of water loss from detached leaves of wild-type, cat3, and 3OE1 plants. The experiments were repeated three times with similar results. Each data point represents mean ± se (n = 3).

(D) to (F) ABA-, H₂O₂-, and Ca²⁺-induced stomatal closing in cat3 mutant. The experiments were repeated three times. Each data point represents mean ± se (n = 50). Asterisk indicates significant difference relative to Col (Student’s t test, *P < 0.05 and **P < 0.01).
identity. Previous studies have shown that several CDPKs play important roles in plant responses to drought and salt stresses (Saijo et al., 2000; Zhu et al., 2007; Xu et al., 2010; Zou et al., 2010; Latz et al., 2013). In this study, our results support the notion that CPK8 serves as a positive regulator in ABA-mediated stomatal movement in plant responses to drought stress (Figure 1). Hubbard et al. (2012) reported that ABA-induced stomatal closure is not significantly impaired in cpk10, cpk4 cpk11, and cpk7 cpk8 cpk32 compared with wild-type plants, which was different from the findings of ABA insensitivity for cpk4 cpk11 (Zhu et al., 2007), cpk10 (Zou et al., 2010), and cpk8 in this study (Figure 1H). The difference between these results may be due to the different protocols for stomatal aperture assay (Zhu et al., 2007; Zou et al., 2010; Hubbard et al., 2012).

Arabidopsis CDPKs display highly variable calcium dependences for their activities, and six CPKs (including CPK8) from subgroup 3 exhibit low or no calcium sensitivity (Boudsocq et al., 2012). CPK24 activity is independent of calcium, whereas Ca2+-activated CPK11 subsequently phosphorylates CPK24, transducing the calcium signals and regulates SPIK activity during pollen tube growth (Zhao et al., 2013). CPK8 has weak calcium sensitivity (Boudsocq et al., 2012) and may

Figure 7. Inhibition of the Inward K+ Currents in Stomatal Guard Cells by ABA and Ca2+ Is Impaired in cpk8 and cat3 Mutants.

(A) Representative whole-cell recordings of inward K+ currents in guard cell protoplasts isolated from different materials (Col, cpk8, and cat3) with or without addition of 50 μM ABA in the bath solution. The voltage protocols, as well as time and current scale bars for the recordings, are shown inside the figure. Each trace represents the K+ currents at the indicated voltage.

(B) Current density-voltage curves of steady state whole-cell inward K+ currents in guard cell protoplasts isolated from different plant materials with or without the addition of 50 μM ABA in the bath solution. The data are derived from the recordings as shown in (A) and are presented as means ± se (Col, n = 12; cpk8, n = 11; cat3, n = 13).

(C) Representative whole-cell recordings of inward K+ currents in guard cell protoplasts isolated from different plant material (Col, cpk8, and cat3) with or without the addition of 2 μM free Ca2+ in pipette solution. The voltage protocols, as well as time and current scale bars for the recordings, are shown inside the figure. Each trace represents the K+ currents at the indicated voltage.

(D) Current density-voltage curves of the steady state whole-cell inward K+ currents in guard cell protoplasts isolated from different plant materials with or without the addition of 2 μM free Ca2+ in the pipette solution. The data are derived from the recordings as shown in (C) and are presented as means ± se (Col, n = 14; Col+Ca2+, n = 21; cpk8, n = 13; cpk8+Ca2+, n = 22; cat3, n = 15; cat3+Ca2+, n = 20).
phosphorylate its substrate CAT3 even without addition of calcium in the reaction buffer (Figure 3A). However inhibition of the inward K⁺ currents by Ca²⁺ (Figures 7C and 7D) was impaired in the cpk8, suggesting that CPK8 may function downstream of Ca²⁺. These results indicate that CPK8 may be indirectly regulated by Ca²⁺ signals and that other Ca²⁺-dependent proteins may transduce Ca²⁺ signals to CPK8. One example of a similar mechanism is Ca²⁺-independent CPK24, which is activated (phosphorylated) by Ca²⁺-dependent CPK11 (Zhao et al., 2013).

**Ca²⁺-Mediated Protein Phosphorylation: A Universal Regulatory Mechanism for Plant Ion channels and Transporters**

A number of CDPKs have been reported to regulate ion channel activities in stomatal movement (Pei et al., 1996; Li et al., 1998; Mori et al., 2006; Geiger et al., 2010, 2011; Zou et al., 2010; Brandt et al., 2012; Scherzer et al., 2012), plant responses to salt stress (Latz et al., 2013), and pollen tube growth (Gutermuth et al., 2013; Zhao et al., 2013; Zhou et al., 2014). In this report, the plasma membrane localized-CPK8 showed abundant expression in stomatal guard cells (Figure 1D; Supplemental Figure 2), indicating its potential role in regulation of stomatal movement and ion channel activity. As shown in Figure 7, ABA and Ca²⁺ inhibition of inward K⁺ currents were impaired in guard cells of cpk8 mutant plants. So far, at least 11 Arabidopsis CDPKs have been reported to function in regulation of ion channel activity in plant cells (Hwang et al., 2000; Mori et al., 2006; Geiger et al., 2010, 2011; Zou et al., 2010; Brandt et al., 2012; Scherzer et al., 2012; Latz et al., 2013; Gutermuth et al., 2013; Zhao et al., 2013; Zhou et al., 2014). The previous reports have revealed that plant ion channels and transporters are regulated by members of the CIPK (CBL-interacting protein kinase) family, another Ca²⁺-regulated protein kinase family, such as AKT1 regulation by CIPK23 (Xu et al., 2006; Wang and Wu, 2013) and Na⁺/H⁺ antiporter (SOS1) regulation by CIPK24 (Xiong et al., 2002). Together with the results in this study, these findings support the notion that regulation of various plant ion channels and transporters via their Ca²⁺-mediated phosphorylation represents a universal mechanism.

**Identification of a Downstream Target of CPK8**

Identification and functional characterization of the specific target (or substrate) of a CDPK is a key step to understand CDPK functions in plant signaling (Boudsocq and Sheen, 2013). Some substrates of CDPKs have been identified in the last decade, revealing their different roles in plant development and immune and stress signaling (Boudsocq and Sheen, 2013; Schulz et al., 2013). For example, Arabidopsis CPK4 and CPK11 phosphorylate ABF1 and ABF4 during ABA signal transduction (Zhu et al., 2007). Arabidopsis CPK5 induces ROS production by directly phosphorylating the NADPH oxidase RBOHB during innate immune responses (Dubiella et al., 2013). Arabidopsis CPK11 and CPK24 modulate the activity of shaker pollen inward K⁺ channel (SPIK, also named AKT6) during pollen tube growth (Zhao et al., 2013). In this study, yeast two-hybrid, co-IP, BiFC, and protein phosphorylation assays demonstrated specific interaction between CPK8 and CAT3 (Figures 2 and 3A). In addition, CPK8 specifically enhanced CAT3 activity through phosphorylation at Ser-261 (Figures 2 to 4). The cpk8 cat3 double mutants showed similar drought stress sensitivity as the cpk8 and cat3 single mutants (Figure 6A). Similar to CPK8, CAT3 functions in the regulation of stomatal movement, most likely through regulation of the inward K⁺ channel activities in stomatal guard cells (Figures 6D to 6F and 7). Our data reveal important roles of CPK8 and CAT3-mediated ABA stomatal signal transduction in response to drought stress.

**Roles of CPK8-CAT3 Interaction in H₂O₂ Homeostasis**

Excess ROS accumulation in living plant cells is toxic to cellular activities, so the cytosolic concentration of ROS must be stringently regulated (Mittler, 2002; Apel and Hirt, 2004). ROS play a dual role in plant responses to abiotic stresses, acting as toxic by-products of aerobic metabolism and as key regulators of growth, development, and defense pathways (Mittler et al., 2004; Miller et al., 2010). ROS-scavenging pathways are responsible for maintaining a low steady state level of ROS on which the different signals can be registered (Mittler et al., 2004; Miller et al., 2010). Rice (Oryza sativa) CPK12-overexpressing plants exhibit enhanced tolerance to salt stress, possibly as a result of decreased ROS accumulation (Asano et al., 2012). Arabidopsis glutathione peroxidase GPX3 plays dual roles, the first in the general control of H₂O₂ homeostasis and the second in relaying ABA and H₂O₂ signaling in stomatal movement (Miao et al., 2006).

As an important H₂O₂-scavenging enzyme, the expression or activity of Arabidopsis catalase is also regulated by other components. The expression profiles and cellular localizations of Arabidopsis catalases are varied, suggesting their potential roles in response to environmental stimuli in addition to H₂O₂ decomposition (Zimmermann et al., 2006; Bueso et al., 2007; Xing et al., 2007; Du et al., 2008). ABA-induced CAT1 expression depends on the production of H₂O₂, which is triggered by MKK1-MPK6 signaling pathway, revealing the roles of CAT1 in ROS scavenging and its feedback regulation of the H₂O₂ signaling (Xing et al., 2007, 2008). CAT2 has been reported to be involved in crosstalk between oxidative stress, cation homeostasis, and ethylene (Bueso et al., 2007). Arabidopsis CAT3 can interact with several proteins, such as CaM PCM6 in potato (Solanum tuberosum) (Yang and Poovaiah, 2002) and NDK1 (Fukamatsu et al., 2003), SOS2 (CIPK24; Verslues et al., 2007), and LSD1 (Li et al., 2013) in Arabidopsis, consistent with roles for CAT3 in regulating H₂O₂ homeostasis and signaling in response to ROS stress. This work showed that H₂O₂ can induce the expression of CPK8 and CAT3 (Supplemental Figures 1D and 5D). The cpk8 and cat3 mutants had lower catalase activities and accumulated more ROS in leaves and stomatal guard cells compared with wild-type plants when treated with ABA (Figures 5C to 5F), whereas CPK8 and CAT3 overexpression lines had lower ROS accumulation compared with wild-type plants when treated with ABA (Figures 5C and 5D).

In summary, the results presented in this study demonstrate that CPK8 can specifically phosphorylate CAT3 and regulate its activity. The CPK8-CAT3 interaction not only acts as a positive
regulatory component in ABA-mediated regulation of stomatal movement in plant responses to drought stress, but also plays important role in maintaining H2O2 homeostasis in living plant cells.

METHODS

Plant Materials and Growth Conditions
Arabidopsis thaliana ecotype Columbia and Nicotiana benthamiana were used in this study. The T-DNA insertion lines cpk8 (SALK_036581) and cat3 (SALK_002911) were obtained from the ABRC (http://www.arabidopsis.org/abrc/).

Seeds were surface sterilized and placed in the Petri dishes containing Murashige and Skoog agar (0.8%, v/v) medium and incubated for 2 d at 4°C before transfer to 22°C for germination. After 7 d under constant illumination at 60 μmol m⁻² s⁻¹, the seedlings were transplanted to pots containing soil mixture (rich soil: vermiculite, 2:1, v/v) and kept in growth chambers at 22°C with illumination at 120 μmol m⁻² s⁻¹ for a 16-h daily light period. The relative humidity was approximate 70% (±5%).

N. benthamiana seeds were planted in the potting soil mixture (rich soil: vermiculite, 2:1, v/v) and kept in growth chambers at 23°C with illumination at 120 μmol m⁻² s⁻¹ for a 16-h daily light period. After 3 weeks growth, the plants were used for transformation.

Vector Constructions and Generation of Transgenic Plants
The CPK8-GUS construct was generated by introducing the CPK8 promoter fragment (1.96 kb) in front of the GUS coding sequence in the EcoRI and SalI sites of pCAMBIA1381 vector. The GUS staining assays were performed as described previously (Xu et al., 2006).

For Superpro-CPK8 and Superpro-CAT3 constructs, the coding sequence of CPK8 was introduced into the KpnI and XhoI sites of pCAMBIA1300 vector under the Super promoter, and the coding sequence of CAT3 was introduced into the XbaI and XhoI sites of pCAMBIA1300 vector under the Super promoter (Chen et al., 2009), respectively. Primers for Superpro-CPK8 and Superpro-CAT3 constructs are listed in Supplemental Table 1. The Superpro-CPK8 and Superpro-CAT3 constructs were introduced into wild-type plants for generation of CPK8 and CAT3 overexpression lines, respectively. To generate the construct for complementation of cpk8, a genomic DNA fragment of CPK8 was amplified and cloned into SacI and KpnI sites of pCAMBIA1300 vector.

Arabidopsis transformation with Agrobacterium tumefaciens (strain GV3101) was performed by the floral dip method (Clough and Bent, 1998). Homozygous T3 transgenic lines were used for further analyses.

RT-qPCR
Total RNA was extracted from plants with Trizol reagent (Invitrogen) following the manufacturer’s protocols. For RT-qPCR analyses, total RNA treated with DNase I (Takara) was used for CDNA synthesis by SuperScript II reverse transcriptase (Invitrogen). The quantitative PCR analysis was performed using an Applied Biosystems 7500 real-time PCR system. The SYBR Premix Ex Taq kit (Applied Biosystems) was used for reaction according to the manufacturer’s instruction. Arabidopsis 18S rRNA was quantified as an internal control. The primer sequences of CPK8, CAT3 and 18S rRNA for RT-qPCR are listed in Supplemental Table 1.

Drought Treatment and Water Loss Measurements
For drought treatment, plants grown on Murashige and Skoog medium for 7 d were transplanted into soil for about 1 week with sufficient watering followed by a 20-d drought stress (withholding irrigation) as described before (Zou et al., 2010), with slight modifications. The seedlings were grown in a growth room with 12-h daily light. Normally watered plants were used as the control. For drought stress treatment, water was withheld for 20 d (normal drought stress) or 22 d (severe drought stress). Two independent transgenic lines were used for drought stress.

For water loss measurement, rosette leaves were detached from 4-week-old plants, weighed, and placed on the laboratory bench (the relative humidity was between 40 and 50%) at 22°C (±1°C). Weight loss of the detached leaves was monitored at the indicated time intervals. Water loss was expressed as the percentage of initial fresh weight.

Thermal Imaging
Thermal imaging of plants was performed as described previously (Hua et al., 2012). Three-week-old plants grown under normal conditions were subjected to drought stress for about 1 week. Thermal images were obtained using VarioCAM HD and leaf temperature was calculated using IRBIS 3 software.

Stomatal Aperture Measurements
Stomatal closure assays were conducted as described before (Zou et al., 2010). After treatment, the abaxial epidermis was peeled and photographed using a Leica microscope (Leica DFC320). Stomatal apertures were measured using ImageJ software (National Institutes of Health).

Yeast Two-Hybrid Assay
The GAL4-based two-hybrid system was used for yeast two-hybrid screening (Clontech). The CPK8-pGBK77 construct was generated by fusing the sequence encoding the CPK8 kinase domain (from 169 to 945 bp of the coding sequence) with that encoding the GAL4 binding domain in the EcoRI and SalI sites of pGBK77, and the resulting construct was transformed into the AH109 yeast strain. For screening, the reporter yeast was transformed with library plasmid DNA from CD4-22 library (ABRC). Yeast transformation, growth conditions, and assays for β-gal activity were performed according to the manufacturer’s instructions (Clontech).

Positive clones were selected for sequencing. Three coding sequences of catalases were inserted into pGADT7 prey plasmid containing the GAL4 activating domain and cotransformed into AH109 with CPK8 bait for β-gal activity assays, respectively. The primer sequences and restriction sites are listed in Supplemental Table 1.

Co-IP Assays
The coding sequence of CPK8 with a cMyc tag (cMYC-CPK8) was cloned into the SpeI and KpnI sites of pCAMBIA1307 vector under control of the 35S promoter. The coding sequence of CAT3 with a GFP tag (GFP-CAT3) was cloned into the XbaI and SalI sites of pSAT6-GFP-N1 vector under control of the 35S promoter. The primer sequences and restriction sites are listed in Supplemental Table 1. The combinations of cMyc-CPK8 and GFP-CAT3 were cotransformed into Arabidopsis mesophyll protoplasts as described before (Ren et al., 2013). After incubation overnight at 4°C, the protoplasts were lysed, sonicated, and centrifuged at 12,000g for 10 min at 4°C. The supernatant was incubated with 10 μL anti-cMyc agarose conjugate (Sigma-Aldrich) for 6 h at 4°C. The co-IP products were washed briefly with extraction buffer for five times at 4°C and then detected via immunoblot analysis. Both anti-GFP (Sigma-Aldrich) and anti-cMyc (Sigma-Aldrich) antibodies were used at 1:5000 dilutions, and chemiluminescence signals were detected using Fusion Solo.

Subcellular Localizations of CPK8 and CAT3
For generation of GFP-fusion protein, the coding sequence of CPK8 was fused with GFP in the XbaI and KpnI sites of pCAMBIA1300 vector. The
coding sequence of CAT3 was fused with GFP in the Ascl and PacI sites of pCM1205-C-GFP vector (Zhou et al., 2012). The coding sequence for potassium transporter AKT1 (Xu et al., 2006) was fused with mCherry in the SUPERrexWEmCherry_C-Bar vector (Schücking et al., 2013) and used as the plasma membrane marker. PX-RK (CD3-83; ABRC) was used as peroxisome marker. All constructs were transformed into the Agrobacterium strain GV3101. The Agrobacterium lines were infiltrated into leaves of N. benthamiana. Plants were grown at 23°C and allowed to recover for 3 d. The fluorescence of GFP or mCherry in the leaves was imaged using a confocal laser scanning microscope (Leica sp5).

**BiFC Assays**

BiFC assays were performed as described previously (Waadt and Kudla, 2008). For generation of the BiFC vectors, the coding region of CPK8 was cloned via BamHI-SalI into pSPYCEM, resulting in CPK8-YC, and the coding region of CAT1, CAT2, or CAT3 was cloned via BamHI-SalI into pSPYNE173, resulting in CAT1-YN, CAT2-YN and CAT3-YN, respectively. The gene-specific primer pairs for CPK8 and CATs are listed in Supplemental Table 1. All constructs were transformed into the Agrobacterium strain GV3101. An equal volume of Agrobacterium harboring CPK8-YC, CAT1-YN (or CAT2-YN, CAT3-YN) and P19 was mixed to a final concentration of OD600 = 0.8. Agrobacterium lines were infiltrated leaves of N. benthamiana. Plants were grown at 23°C and allowed to recover for 3 d; then, the fluorescence of YFP in the leaves was imaged using a confocal laser scanning microscope (Leica sp5).

**Firefly Luciferase Complementation Image Assay**

Firefly luciferase complementation imaging assays were performed as described before (Chen et al., 2008). For generation of the assay vectors, the coding region of CPK8 was cloned via BamHI-SalI into pCAMBIA1300-cluc, resulting in CPK8-CLuc, and the coding region of CAT1, CAT2, or CAT3 was cloned via BamHI-SalI into pCAMBIA1300-nLuc, resulting in CAT1-nLuc, CAT2-nLuc, or CAT3-nLuc, respectively. The gene-specific primer pairs for CPK8 and CATs are shown in Supplemental Table 1.

All constructs were transformed into the Agrobacterium strain GV3101. An equal volume of Agrobacterium harboring CPK8-YC, CAT1-YN (or CAT2-YN, CAT3-YN) and P19 was mixed to a final concentration of OD600 = 0.8. Three different combinations of Agrobacterium were infiltrated into three different positions at the same leaves of N. benthamiana. Plants were grown at 23°C and allowed to recover for 3 d. A low-light cooled CCD imaging apparatus (fluorescence/chemiluminescence imaging system) was used to capture the LUC image.

**In Vitro Kinase Assay**

To prepare recombinant CPK8, the full-length coding sequence of CPK8 was amplified and cloned into the XcmI sites of pOXSN vector, resulting in pCXSN-Strep-CPK8. The gene-specific primer pairs of CPK8 are listed in Supplemental Table 1. The Strep-CPK8 was purified as described previously (Witte et al., 2004).

To prepare recombinant CAT1, CAT2, and CAT3 truncated proteins, the corresponding coding sequences of CATs were amplified and cloned into BamHI and SalI sites of pGEX-4T-1 vector, resulting in pGEX-4T-1-CATs constructs, respectively. CAT3 mutant proteins were made by site-directed mutagenesis using a QuickChange kit (Agilent Technologies; the primer are listed in Supplemental Table 1). The constructs were then introduced into BL21 cells by transformation and used to purify GST-CAT proteins.

**In vitro kinase assays were performed as described previously (Rodriguez Milla et al., 2006), with slight modification.** In brief, phosphorylation was initiated by adding 0.5 μCi [γ-32P] to reaction buffer containing 25 mM Tris-HCl (pH 7.5), 10 mM MgCl2, 1 mM DTT, and 10 μM ATP, followed by incubation for 20 min at 25°C. The proteins were separated by 10% SDS-PAGE, and the phosphorylated proteins were visualized by autoradiography.

**In Plant Kinase Assay**

In planta kinase assays were used to test the phosphorylation of CAT3. Total proteins in Col/35Spro:CAT3-GFP or cpk8/35Spro:CAT3-GFP before and after treatment with ABA were extracted. CAT3-GFP proteins were immunoprecipitated using Anti-GFP mAb agarose (MBL). CAT3-GFP proteins were then separated by 10% SDS-PAGE, and phosphorylated proteins were detected by immunoblotting using Biotinylated Phos-tag as described previously (Kinosita-Kikuta et al., 2007). Calf intestinal alkaline phosphatase-treated (Takara) proteins were used as control.

**Measurements of H2O2 Production**

H2O2 was detected by DAB staining as described previously (Thordal-Christensen et al., 1997; Guan and Scandalios, 2000). Fully expanded leaves were excited and incubated in water containing 100 μM ABA for 30 min in dark at 28°C. The leaves were then incubated in DAB solution (1 mg mL−1; pH 3.8; Sigma-Aldrich) for 8 h in dark at 28°C. The leaves were immersed in boiling 80% (v/v) ethanol for 10 min to terminate the staining and to decolor the leaves (except for the deep brown polymerization product by the reaction of DAB with H2O2). After cooling, the leaves were extracted with 80% (v/v) ethanol and preserved at 4°C in 80% (v/v) ethanol before photographed.

ROS production in guard cells was examined by loading abaxial epidermal strips with H2DCF-DA (Miao et al., 2006). The detached leaves were floated in incubation buffer for 2.5 h to induce stomatal opening, and the epidermal strips were peeled and placed into loading buffer (10 mM Tris and 50 mM KCl, pH 7.2) containing 50 μM H2DCF-DA in the dark for 10 min. Excess dye was removed by washing the samples three times with distilled water. Epidermal tissues were then incubated in loading buffer for 5 min with 50 μM ABA or with an equal volume of ethanol added as a control. Examinations of peel fluorescence were performed with a confocal laser scanning microscope (Nikon TE2000-E). To quantitatively analyze the data, pixel values were averaged over rectangular regions (4 μm2) manually located on each image. The pixel intensity from at least 150 guard cells was recorded.

**Measurement of Catalase Activity**

The activity of CAT was determined with a CAT assay kit according to the manufacturer’s instructions (Beyotime). Briefly, samples were treated with excess H2O2 for decomposition by catalase for 5 min, and the remaining H2O2 coupled with a substrate was treated with peroxidase to generate a red product, N-4-antipyryl-3-chloro-5-sulfonate-p-benzoquinonemonoimine, which absorbs maximally at 520 nm. Catalase activity was thus determined by measuring the decomposed H2O2. All values were normalized by the total protein concentration of the same sample.

**Patch-Clamp Experiments**

Arabidopsis guard cell protoplasts were isolated as described previously (Pei et al., 1997; Wang et al., 2001). Standard whole-cell recording techniques were applied in this study (Hamill et al., 1981). All experiments were conducted at room temperature (~22°C) under dim light. The whole-cell currents were recorded as described before (Zou et al., 2010). The pCLAMP software (version 6.0.4; Axon Instruments) was used to acquire and analyze the whole-cell currents. SigmaPlot software was used to draw current-voltage plots and for data analysis.
Accession Numbers
Sequence data from this article can be found in the Arabidopsis Genome Initiative or GenBank/EMBL databases under the following accession numbers: At5g19450 for CPK8, At1g20630 for CAT1, At4g35090 for CAT2, and At1g20620 for CAT3.

Supplemental Data
Supplemental Figure 1. Position of T-DNA Insertion in cpk8 and Expression Levels of CPK8 in Response to Abiotic Stresses.
Supplemental Figure 2. Subcellular Localizations of CPK8 and CAT3.
Supplemental Figure 3. Firefly Luciferase Complementation Image Assay of CPK8 Interaction with CAT3 in N. benthamiana Leaves.
Supplemental Figure 4. Identification of CPK8 Phosphorylation Sites in CAT3.
Supplemental Figure 5. Position of T-DNA Insertion in CAT3 and Expression Levels of CAT3 in Response to ABA and H2O2.
Supplemental Figure 6. Phenotype Observation of Various CPK8- and CAT3-Related Plant Materials under Drought Stress.
Supplemental Figure 7. ABA Inhibition of Stomatal Opening Is Impaired in the cpk8 and cat3 Mutants.
Supplemental Figure 8. Expression Patterns of CPK7 as Determined from CPK7pro::GUS Transgenic Plants.
Supplemental Table 1. Primer Sequences Used in This Study.

ACKNOWLEDGMENTS
This work was financially supported by grants from the “973” Project (2011CB915401 and 2012CB114203 to W.-H.W.), the “111” Project (No. B06003 to W.-H.W.), and the National Natural Science Foundation of China (31000119 to J.-J.Z.). We thank Jörg Kudla (Institut für Botanik, Universität Münster, Germany) for providing the vectors SUPERR:sXVE:mCherry:c:Bar for AKT1-mCherry construct and pSPYCEM and pSPYNE173 for BiFC assays. We thank Yan Guo (China Agricultural University) for providing pCM1205-C-GFP and pSAT6-GFP-N1 vectors.

AUTHOR CONTRIBUTIONS

REFERENCES


In this article, Figure 1, Figure 6, and Supplemental Figure 6 show photographs of pots containing plants corresponding to various genotypes and treatments. To create these images, multiple photographs of different groupings of pots were taken. Several mistakes were made in the physical placement of pots for Figure 1F and Supplemental Figure 6, and the images did not clearly show how the original photographs were cropped and pieced together to create each figure. We apologize for these mistakes and present the following corrected figures, legends, and explanations.

(1) In Figure 1F of the original publication, the photograph shown in the bottom panel contained mistakenly placed pots, which confused the identity of two independent transgenic lines, 8OE1 and 8OE2, for 22-d drought treatment. In addition, the 8OE1 and 8OE2 plants appear “greener” in the figure at 22 d relative to 20 d drought treatment. This was due to a difference in camera settings and light conditions for the image taken at 22 d relative to 20 d (the images are presented unaltered) but does not affect the overall results or conclusions. Figure 1F is reproduced here, with images unaltered, but marked to show which images correspond to distinct photographs and the correct genotypes for 8OE1 and 8OE2 pots at 22-d drought. The legend has been amended accordingly and to include the number of replicate pots per treatment/genotype per experiment. The conclusions of our article are unaffected by this correction.

(2) In Supplemental Figure 6 of the original publication, pots containing cpk8/cat3 plants (the same pots shown in Figure 6 corresponding to cpk8/cat3 control and rewatered) were mistakenly placed in the photograph, instead of pots containing the 8OE/cat3 line (control and rewatered). We apologize for this error. Here, we present a revised Supplemental Figure 6 with a different photograph (taken at the same time) showing the correct grouping of pots corresponding to the stated genotypes and treatments. We further note that some of the pots shown in this figure are the same pots that were used in the photograph presented in other figures of the original article. Thus, the Col and cpk8 control pots were also placed in the grouping for the Figure 1F photograph, and cat3 rewatered pot was also placed in the grouping for the Figure 6A photograph (i.e., they are the same pots, but different photographs). The figure legend was amended to include the number of replicate pots per treatment/genotype. The conclusions of our article are unaffected by this correction.

Figure 1. Original: Arabidopsis CPK8 Acts as a Positive Regulator in ABA- and H$_2$O$_2$-Mediated Stomatal Movement in Response to Drought Stress.

(F) Drought stress sensitivity of the wild type, cpk8, 8COM1, 8COM2, and 8OE1 in soil after water was withheld for 20 d (normal drought stress) or 22 d (severe drought stress). The experiments were repeated three times with similar results.

Figure 1. Corrected: Arabidopsis CPK8 Acts as a Positive Regulator in ABA- and H$_2$O$_2$-Mediated Stomatal Movement in Response to Drought Stress.

(F) Drought stress sensitivity of the wild type, cpk8, 8COM1, 8COM2, 8OE1, and 8OE2 in soil after water was withheld for 20 d (normal drought stress) or 22 d (severe drought stress). The experiments were repeated three times with similar results; for each experiment, there were three to six pots per genotype/treatment, and a representative pot from one experiment is shown. The control and 20-d drought rows are from the same photograph taken on day 20, whereas the 22-d drought row is from a separate photograph taken on day 22 (images from different photographs separated by a red line). The plants in 22-d drought pots appear a slightly different color due to different lighting and camera settings.
Supplemental Figure 6. Original: Phenotype Observation of CPK8- and CAT3-Related Plant Materials under Drought Stress.

The Superpro:CPK8 was introduced into cat3 mutant to generate 8OE/cat3 lines. The Superpro:CAT3 was introduced into cpk8 mutant to generate 3OE/cpk8 lines. For the drought stress treatment, the photographs were taken after withholding water for 20 d.

Supplemental Figure 6. Corrected: Phenotype Observation of CPK8- and CAT3-Related Plant Materials under Drought Stress.

The Superpro:CPK8 was introduced into cat3 mutant to generate 8OE/cat3 lines. The Superpro:CAT3 was introduced into cpk8 mutant to generate 3OE/cpk8 lines. Plants grown on Murashige and Skoog medium for 7 d were transplanted to soil for ~1 week with sufficient watering followed by continued watering (control) or 20 d of withholding water (drought stress) followed by two more days of drought stress and then 3 d of regular watering (rewatered). Images of control and drought stress pots are from one photograph taken after 20 d of drought, and images of rewatered pots are from a separate photograph taken after rewatering (images from different photographs separated by a red line). The experiments were repeated three times with similar results; for each experiment there were three to six pots per genotype/treatment, and a representative pot is shown.

Editor’s note: the corrected figures and accompanying text were reviewed by members of The Plant Cell editorial board. Both the original and corrected figures are shown for ease of comparison. The supplemental figures PDF file available for downloading online was replaced with the corrected figure.
Arabidopsis CALCIUM-DEPENDENT PROTEIN KINASE8 and CATALASE3 Function in Abscisic Acid-Mediated Signaling and H₂O₂ Homeostasis in Stomatal Guard Cells under Drought Stress

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Plant Cell 2015;27;1445-1460; originally published online May 12, 2015;
DOI 10.1105/tpc.15.00144

This information is current as of July 15, 2017

Supplemental Data
/content/suppl/2015/04/29/tpc.15.00144.DC1.html

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