Arabidopsis Calcium-Dependent Protein Kinase CPK10 Functions in Abscisic Acid- and Ca\(^{2+}\)-Mediated Stomatal Regulation in Response to Drought Stress\(^1\)[W][OA]

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Plant calcium-dependent protein kinases (CDPKs) may function as calcium sensors and play important roles in the regulation of plant growth and development and in plant responses to biotic and abiotic stresses. The Arabidopsis (Arabidopsis thaliana) genome encodes 34 CDPKs, and most of them have not been functionally characterized. Here, we report the functional characterization of CPK10 in Arabidopsis response to drought stress. The cpk10 mutant, a T-DNA insertion mutant for the Arabidopsis CPK10 gene, showed a much more sensitive phenotype to drought stress compared with wild-type plants, while the CPK10 overexpression lines displayed enhanced tolerance to drought stress. Induction of stomatal closure and inhibition of stomatal opening by abscisic acid (ABA) and Ca\(^{2+}\) were impaired in the cpk10 mutants. Using yeast two-hybrid methods, a heat shock protein, HSP1, was identified as a CPK10-interacting protein. The interaction between CPK10 and HSP1 was further confirmed by pull-down and bimolecular fluorescence complementation assays. The HSPI knockout mutant (hspl) plants showed a similar sensitive phenotype under drought stress as the cpk10 mutant plants and were similarly less sensitive to ABA and Ca\(^{2+}\) in regulation of stomatal movements. Electrophysiological experiments showed that ABA and Ca\(^{2+}\) inhibition of the inward K\(^{+}\) currents in stomatal guard cells were impaired in the cpk10 and hspl mutants. All presented data demonstrate that CPK10, possibly by interacting with HSP1, plays important roles in ABA- and Ca\(^{2+}\)-mediated regulation of stomatal movements.

Plants are subjected to various environmental stresses during their growth and development and have developed various mechanisms to adapt to these stresses. As an important cytoplasmic second messenger, Ca\(^{2+}\) plays critical roles in plant responses to environmental stresses (Rudd and Franklin-Tong, 2001; Sanders et al., 2002; Kudla et al., 2010). Specific calcium signatures may be recognized by different sensor proteins. Three major families of Ca\(^{2+}\) sensors have been identified in higher plants: calmodulins (CaMs) and CaM-like proteins (McCormack et al., 2005); calciunin B-like (CBL) proteins (Kolukisaoglu et al., 2004; Luan, 2009; Weinl and Kudla, 2009); and calcium-dependent protein kinases (CDPKs; Harmon et al., 2000; Cheng et al., 2002; Harper et al., 2004; Harper and Harmon, 2005). CaMs and CBLs are small proteins and transmit the Ca\(^{2+}\) signal through interacting target proteins and regulating their activities. The CBLs not only regulate the activities of CBL-interacting protein kinases, but at least some of them are also involved in recruiting the kinases to different membranes (Luan, 2009). CDPKs are activated upon binding Ca\(^{2+}\) to their CaM-like domain and then relay the signaling to their downstream targets (Harmon et al., 2000; Cheng et al., 2002; Harper et al., 2004; Harper and Harmon, 2005).

CDPKs are found in a wide range of vascular and nonvascular plants as well as in green algae and certain protozoa (Harmon et al., 2001), suggesting their potential importance in Ca\(^{2+}\) signaling in plant cells. The CDPKs are encoded by multigene families and have been identified in various plant species, such as Arabidopsis (Arabidopsis thaliana; Harmon et al., 2001; Cheng et al., 2002), rice (Oryza sativa; Asano et al., 2005; Wan et al., 2007), cotton (Gossypium hirsutum; Huang et al., 2008), and wheat (Triticum aestivum; Li et al., 2008). Some CDPKs are expressed ubiquitously, whereas others are present in specific tissues or their expression is regulated by different stimuli (Hrabak et al., 2003). It is also known that different CDPKs have different subcellular locations, including cytosol, nucleus, the plasma membrane, endoplasmic reticulum, peroxisomes, mitochondrial outer membrane, and oil bodies (Harper et al., 2004), indicating their possible diverse functions. A number of studies have demonstrated that CDPKs play important roles in plant responses to various abiotic stresses, including cold,
salt, drought, wounding, etc. (Cheng et al., 2002; Ludwig et al., 2004; Klimecka and Muszyńska, 2007; DeFalco et al., 2010). Transcription of AtCPK10 and AtCPK11 can be rapidly induced by drought and high-salt stresses (Urao et al., 1994). Overexpression of OsCPK7 in rice plants enhanced plant tolerance to cold, salt, and drought stresses (Saijo et al., 2000, 2001). In ice plant (Mesembryanthemum crystallinum), transcription of McCPK1 was increased after exposure to high-salt and dehydration stresses (Chehab et al., 2004). Arabidopsis AtCPK32 is involved in abscisic acid (ABA)/stress responses through phosphorylating ABA-induced transcription factor ABF4 (Choi et al., 2005). Two Arabidopsis guard cell-expressed CDPK genes, CPK3 and CPK6, have been identified as important components in the regulation of guard cell ion channels and in ABA-regulated stomatal signaling (Mori et al., 2006). AtCPK23 was reported to play roles in Arabidopsis responses to drought and salt stresses (Ma and Wu, 2007). Arabidopsis CPK4 and CPK11 may be two positive regulators in CDPK/calcium-mediated ABA signaling (Zhu et al., 2007). Recently, Mehlmer et al. (2010) reported that CPK3 is involved in Arabidopsis acclimation to salt stress, and Xu et al. (2010) demonstrated that CPK6 functions as a positive regulator in Arabidopsis responses to salt/drought stress.

Although many previous studies have shown the importance of CDPKs for plant signaling in response to various environmental stresses, biological functions of most CDPKs have not been characterized so far. Obviously, to identify potential targets of CDPKs is becoming an important task for further understanding of the CDPK-involved plant signaling mechanisms. Various approaches have been employed to screen CDPK-interacting proteins, such as a phage display library screening (Shao and Harmon, 2003), yeast two-hybrid screening (Patharkar and Cushman, 2000; Lee et al., 2003; Choi et al., 2005; Rodriguez Milla et al., 2006; Uno et al., 2009), and a chemical-genetic approach (Böhmer and Romeis, 2007).

Here, we report that CPK10 (At1g18890) is involved in plant responses to drought stress via modulation of ABA- and Ca\(^{2+}\)-regulated stomatal movements. Following the observation that plants of the CPK10 T-DNA insertion mutant (cpk10) were much more sensitive to drought stress, we have identified a heat shock protein, HSP1 (At4g14830), as a CPK10-interacting protein. Functions of CPK10 and HSP1 in plant responses to drought stress via modulation of ABA and Ca\(^{2+}\) signaling are discussed.

RESULTS

Disruption of CPK10 Transcription in the cpk10 Mutants Increased Arabidopsis Sensitivity to Drought Stress

In total, 23 T-DNA insertion lines corresponding to 19 Arabidopsis CDPK genes (expressed in guard cells from GUS staining results; data not shown) were obtained from the Arabidopsis Biological Resource Center (ABRC; http://www.arabidopsis.org/abrc/) and were tested for their sensitivity to the drought stress treatment. Among several CDPK knockout mutants that showed sensitivity to drought stress (data not shown), the cpk10 mutants (SALK_082441) showed a much more sensitive phenotype compared with wild-type plants (Fig. 1A, middle panel). There was no obvious morphological or developmental difference between the cpk10 plants and wild-type plants under normal growth conditions (Fig. 1A, top panel). After 1 week of growth in a growth chamber, both the wild-type plants and mutants had irrigation withheld for drought stress treatment. After a 20-d period of drought treatment, the cpk10 mutants showed a much more sensitive phenotype compared with the wild-type plants. After drought stress treatment, the cpk10 plants wilted and the rosette leaves became chlorotic, whereas the wild-type plants were turgid and their leaves remained green (Fig. 1A, middle panel). After rewettering for 3 d, the wild-type plants quickly recovered, while the cpk10 mutants could not survive (Fig. 1A, bottom panel).

To confirm the disruption of CPK10 transcription in the cpk10 mutants, reverse transcription (RT)-PCR experiments were conducted. The T-DNA insertion in the cpk10 mutant was located in the fourth intron of CPK10 genomic DNA (Fig. 1B). Although the partial CPK10 transcripts (777 bp, encoding the kinase domain) in the cpk10 mutant can be detected when using primers to amplify the cDNA fragment upstream of the insertion site (Fig. 1C, middle panel), no transcript was observed in the homozygous cpk10 plants when using specific primers to amplify the full-length cDNA (1,638 bp) of CPK10 (Fig. 1C, top panel).

Urao et al. (1994) showed that CPK10 transcription was rapidly induced by drought and salt stress. In contrast with this previous report, our quantitative real-time (qRT)-PCR results presented in Figure 1D showed that the transcription of CPK10 was quickly induced by a dehydration treatment within 30 min and gradually decreased to the initial level after 3 h.

Overexpression of CPK10 Enhanced Arabidopsis Tolerance to Drought Stress

To further confirm that the increased sensitivity of the cpk10 mutants to drought stress resulted from the disruption of CPK10 transcription, the CPK10 overexpression lines (ecotype Columbia [Col] + CPK10; OE) and the complementation lines (cpk10 + CPK10; COM) were generated and analyzed. The expression levels of CPK10 in different plant materials were analyzed by qRT-PCR (Fig. 2A). The CPK10 transcription was much higher in line OE2 than in the wild-type plants, and the CPK10 transcription in the complementation line (COM2) was comparable to that in wild-type plants (Fig. 2A). When subjected to the drought stress, the COM2 plants showed a similar phenotype as the wild-type plants (Fig. 2B), indicating a full complementation of the cpk10 mutant. The
CPK10-overexpressing plants did not show any difference in their phenotype compared with wild-type plants under control conditions, but they did show much stronger tolerance to drought stress than wild-type plants under the drought stress (Fig. 2B).

The assays of water loss from detached leaves showed that the cpk10 plants lost water much more quickly than the wild-type plants and that the OE2 plants lost water much more slowly than both wild-type and cpk10 plants (Fig. 2C). These results suggest that CPK10 may function as a positive regulator in plant responses to drought stress.

Expression Patterns of CPK10

The qRT-PCR results showed that CPK10 mRNA was detectable in all tested tissues or organs, including seedling, root, leaf, stem, flower, and silique (Fig. 3A). To get a further insight into the expression patterns of CPK10, transgenic plants harboring a GUS reporter gene fusion with the CPK10 promoter were generated. GUS staining of the CPK10::GUS transgenic lines confirmed the universal expression patterns of the CPK10 gene (Fig. 3B). High GUS activities were detected in the stomata of leaf epidermis (Fig. 3B), suggesting the potential importance of CPK10 in the regulation of stomatal movements.

The transient expression of CPK10-GFP in the mesophyll protoplasts of Arabidopsis was tested to determine the subcellular localization of CPK10 proteins. As shown in Figure 3C, CPK10 proteins were localized in the plasma membrane, consistent with the prediction that CPK10 has an N-terminal myristoylation site that can promote membrane association (Cheng et al., 2002).

ABA- and Ca\(^{2+}\)-Induced Stomatal Closure and Inhibition of Stomatal Opening Were Impaired in the cpk10 Mutant

Stomatal movements are finely regulated to control water loss through transpiration in response to environmental changes. It is well known that ABA and Ca\(^{2+}\) play essential roles in the regulation of stomatal

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**Figure 1.** Experimental analysis of the cpk10 mutants. A, Phenotype analysis of Arabidopsis cpk10 mutants (SALK_082441) compared with wild-type plants under normal and drought stress conditions. Wild-type (Col) and cpk10 mutant plants were grown in pots containing soil mixture (rich soil:vermiculite, 2:1, v/v) and kept in a growth chamber at 22°C with illumination at 120 \(\mu\)mol m\(^{-2}\) s\(^{-1}\) for a 16-h daily light. For the drought stress treatment (middle panel), the photograph was taken after withholding water for 20 d. For the rewatering treatment (bottom panel), the photograph was taken 3 d after rewatering the same plants as shown in the middle panel for drought stress. The experiments were repeated seven times with similar results. B, T-DNA insertion site in the cpk10 mutant. The T-DNA was inserted in the fourth intron of the CPK10 genomic DNA. Black boxes, solid lines, and diagonal striped boxes denote exons, introns, and untranslated regions, respectively. Solid and dashed arrows indicate the primer locations for CPK10 full transcript and CPK10 kinase domain transcript, respectively. C, RT-PCR analysis of CPK10 full transcript and CPK10 kinase domain transcript in wild-type plants and the cpk10 mutant. Four-week-old leaves were used for RNA extraction. EF1a was used as a loading control. The primer sequences are described in “Materials and Methods.” D, qRT-PCR analysis of CPK10 transcription under drought stress. Total RNA was extracted from the detached leaves placed on the laboratory bench for the indicated times. The 18S rRNA was used as an internal control. The primer sequences are described in “Materials and Methods.” Each data point represents the mean ± se (n = 3).
movements (Blatt, 2000; Evans et al., 2001; Schroeder et al., 2001; Fedoroff, 2002; Desikan et al., 2004). Addition of 10 μM ABA in the incubation medium induced the closure of the opened stomata for wild-type plants, which is consistent with numerous previous reports, whereas ABA-induced stomatal closure was remarkably impaired in the cpk10 mutant (Fig. 4A). ABA is a drought-inducible plant hormone and can induce cytoplasmic Ca2+ elevation in guard cells, and Ca2+, as an essential second messenger in stomatal regulation, can induce stomatal closure (McAinsh et al., 2000). Addition of 5 mM Ca2+ in the incubation medium induced the closure of the opened stomata for wild-type plants, whereas Ca2+-induced stomatal closure was impaired in the cpk10 mutant (Fig. 4C). Furthermore, ABA or Ca2+ inhibition of stomatal opening was also remarkably impaired in the cpk10 mutants (Fig. 4, B and D). These results demonstrated that CPK10 plays an important role in transducing ABA and Ca2+ signals in the regulation of stomatal movements.

Identification of CPK10-Interacting Proteins

Yeast two-hybrid methods were further applied to screen a cDNA library for the identification of candidate proteins that interact with CPK10. The full length and kinase domain (KD) of CPK10 were constructed as baits and then transformed into Y187 yeast strain. The cDNA library prepared from Arabidopsis leaves of drought-stressed plants was constructed and used for yeast mating with CPK10 baits according to the manufacturer’s protocol (Clontech). Among several positive clones identified from yeast two-hybrid experiments, one cDNA clone, encoding heat shock protein 20-like protein 1 (At4g14830 [HSP1]; Uno et al., 2009), showed strong interaction with CPK10 (Fig. 5A). However, HSP2 (At3g22530; Uno et al., 2009), as a homolog of HSP1, did not interact with CPK10 (Fig. 5A). The GUS-staining assays showed that the expression pattern of HSP1 (Fig. 5B) was similar to that of CPK10 (Fig. 3B). Subcellular localization analysis showed that HSP1 proteins are located in cytosol and nucleus (Fig. 5C; Supplemental Fig. S1). The interaction between CPK10 and HSP1 was further tested using in vitro pull-down assay with His-CPK10 and glutathione S-transferase (GST)-HSP1. As shown in Figure 5D, His-CPK10 interacted specifically with HSP1 in the presence of Ca2+, whereas His-CPK10 signal was undetectable with the anti-His antibody on the resulting western blot in the absence of Ca2+ (Fig. 5D). This finding indicates that CPK10 interaction with HSP1 is Ca2+ dependent. The bimolecular fluorescence complementation (BiFC) assays (Fig. 5E) showed that the interaction between CPK10 and HSP1 occurred in the plasma membrane, suggesting that CPK10 may recruit HSP1 to the plasma membrane and form a functional complex.
The hsp1 Mutant Plants Showed Similar Sensitivity to Drought Stress

The HSP1 T-DNA insertion mutant (hsp1; SALK_017461) was obtained from the ABRC and tested for its sensitivity to drought stress. There is only one exon in HSP1 genomic DNA, and the T-DNA insertion site is indicated in Figure 6A. As shown in Figure 6B, either the full length of HSP1 transcript or a 300-bp fragment upstream of the T-DNA insertion site of HSP1 was not detectable in the hsp1 homozygous mutants by RT-PCR analysis, indicating that the expression of HSP1 was completely disrupted in the mutant.

After a 20-d period of drought treatment, the hsp1 mutant plants exhibited a similar sensitive phenotype under the drought stress as the cpk10 plants (Fig. 6C), and the leaves of the hsp1 mutants wilted and their overall growth was inhibited. The water-loss assays showed that the detached leaves of the hsp1 plants lost water faster than leaves of wild-type plants did (Fig. 6D). Similar to the CPK10 mutants shown in Figure 4, the hsp1 mutants also showed impaired ABA- and Ca²⁺-induced stomatal closure (Fig. 6E) and inhibition of stomatal opening (Fig. 6F). These results further support the notion that HSP1 may function together

Figure 3. Expression patterns and subcellular localization of CPK10. A, qRT-PCR analysis of CPK10 expression in different tissues as indicated. The 18S rRNA was used as an internal control. The primer sequences are described in “Materials and Methods.” Each data point represents the mean ± se (n = 3). B, Expression patterns of CPK10 as determined by CPK10::GUS transgenic plants. Transgenic plants were stained with 5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid solution for 12 h. GUS staining is shown in a 2-d-old seedling (a), a 10-d-old seedling (b), a mature rosette leaf (c), stomata (d), and flowers (e). C, Subcellular localization of CPK10. Confocal images of Arabidopsis mesophyll protoplasts transiently expressed with CPK10-GFP construct are shown. The protoplast expressed with pUC-EGFP was used as the control. Bars = 10 μm.

Figure 4. ABA- and Ca²⁺-induced stomatal closing and inhibition of stomatal opening are impaired in the cpk10 mutant. Stomatal apertures were measured on epidermal peels of the wild-type plants, the cpk10 mutant, and the complementation line COM2. The experiments were repeated three times. Each data point represents the mean ± se (n = 50). A, ABA-induced stomatal closing measurements. Stomata were pre-opened in the light for 2.5 h and then incubated for 2 h with or without the addition of 10 μM ABA in the incubation medium. B, ABA inhibition of light-induced stomatal opening. The epidermis was pretreated in darkness and then incubated for 2 h in the light with or without the addition of 10 μM ABA in the incubation medium. C, Extracellular Ca²⁺-induced stomatal closing measurements. Stomata were pre-opened in the light for 2.5 h and then incubated for 2 h with or without the addition of 5 mM Ca²⁺ in the incubation medium. D, Extracellular Ca²⁺ inhibition of light-induced stomatal opening. The epidermis was pretreated in darkness and then incubated for 2 h in the light with or without the addition of 5 mM Ca²⁺ in the incubation medium. The detailed procedures are described in “Materials and Methods.”
with CPK10 in response to drought stress by the regulation of stomatal movements.

**ABA and Ca\(^{2+}\) Inhibition of the Inward K\(^{+}\) Currents Was Impaired in the cpk10 and hsp1 Mutants**

During stomatal closure, the ion channel-mediated inward K\(^{+}\) currents were inhibited by elevations of ABA and cytosolic Ca\(^{2+}\) in stomatal guard cells (Pandey et al., 2007). Increases in cytosolic Ca\(^{2+}\) concentration in guard cells inhibit inward K\(^{+}\) currents and activate anion channels, facilitating solute efflux and stomatal closure (Blatt, 2000). In contrast to the response of wild-type plants, the cpk10 and hsp1 mutants showed decreased sensitivity to ABA- and Ca\(^{2+}\)-induced stomatal closure (Figs. 4 and 6, E and F). It was further hypothesized that CPK10 and HSP1 may function in the regulation of stomatal movements by modulation of the inward K\(^{+}\) currents in stomatal guard cells. To test this hypothesis, the patch-clamp method was...
applied to analyze inward K\textsuperscript{+} currents in guard cell protoplasts. As shown in Figure 7, the basal (control) inward K\textsuperscript{+} currents displayed no difference between wild-type plants and the \textit{cpk10} and \textit{hsp1} mutants. In contrast to wild-type plants, ABA and Ca\textsuperscript{2+} inhibition of the inward K\textsuperscript{+} currents was not observed in guard cell protoplasts of the \textit{cpk10} and \textit{hsp1} mutants. These results demonstrated that ABA and Ca\textsuperscript{2+} inhibition of the inward K\textsuperscript{+} channels requires the presence of functional CPK10 as well as HSP1, and CPK10 and HSP1 function as important regulatory components involved in ABA- and Ca\textsuperscript{2+}-mediated regulation of the inward K\textsuperscript{+} channels in stomatal guard cells.
DISCUSSION

In this study, CPK10 was identified and characterized as an important regulatory component involved in plant response to drought stress through the modulation of ABA- and Ca²⁺-mediated stomatal movements (Figs. 1, 2, and 4). HSP1 was identified as a CPK10-interacting protein (Fig. 5), and disruption of HSP1 expression in the hsp1 mutants resulted in similar effects on plant responses to drought stress as disruption of the CPK10 gene in the cpk10 mutants (Fig. 6). Furthermore, ABA and Ca²⁺ inhibition of the inward K⁺ currents was similarly impaired in both the cpk10 and hsp1 mutants (Fig. 7). The presented results demonstrate that CPK10 and HSP1 function in the regulation of stomatal movements via ABA and Ca²⁺-signaling pathways. The results also indicate that CPK10 and HSP1 may play important roles in plant responses to drought stress.

Regulation of Ion Channels by CDPKs

A previous study reported that Arabidopsis CPK1 (AK1) activated a tonoplast Cl⁻ channel in isolated vacuoles from Vicia faba guard cells (Pei et al., 1996). The CDPK from Vicia guard cells phosphorylated the K⁺ channel KAT1 (At5g46240) protein in a Ca²⁺-dependent manner (Li et al., 1998). Guard cells of Arabidopsis cpk3/cpk6 double mutants showed impaired Ca²⁺-induced activation of S-type anion currents and reduced sensitivity to ABA regulation of these channels (Mori et al., 2006). The cpk3/cpk6 double mutants also displayed reduced sensitivity of stomatal closure to ABA and Ca²⁺ (Mori et al., 2006). The guard cell anion channel SLAC1 is regulated by CPK21 and CPK23 with distinct Ca²⁺ affinities (Geiger et al., 2010). The results in this study demonstrated that CPK10 as well as its interacting protein HSP1 are involved in ABA and Ca²⁺ inhibition of the inward K⁺ channels (Fig. 7). All these results indicate that the CDPKs, at least some of them predominantly expressed in stomatal guard cells, may function as Ca²⁺ sensors and play important roles in the regulation of stomatal movements by the regulation of ion channels.

Figure 7. Impairment of ABA and Ca²⁺ inhibition on the inward K⁺ currents in the cpk10 and hsp1 mutants. A, Patch-clamp whole-cell recordings of the inward K⁺ currents in guard cell protoplasts isolated from different plant materials (Col, cpk10, and hsp1) with or without the addition of 50 μM ABA in the bath solution. B, 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 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 = 11; Col + ABA, n = 11; cpk10, n = 10; cpk10 + ABA, n = 10; hsp1, n = 8; hsp1 + ABA, n = 8). C, Patch-clamp whole-cell recordings of the 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 solutions. D, 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 = 11; Col + Ca²⁺, n = 12; cpk10, n = 13; cpk10 + Ca²⁺, n = 13; hsp1, n = 10; hsp1 + Ca²⁺, n = 13).
CPKs, as Ca\(^{2+}\) Sensors, Play Important Roles in Ca\(^{2+}\)-Mediated Stomatal Regulation

As discussed previously, several CDPKs have been shown to be involved in Ca\(^{2+}\)-mediated stomatal regulation, such as AtCPK1 (Pei et al., 1996), AtCPK3 and AtCPK6 (Mori et al., 2006), and AtCPK4 and AtCPK11 (Zhu et al., 2007). This study demonstrates that CPK10 functions in the regulation of stomatal movements via ABA- and Ca\(^{2+}\)-mediated signaling. Although many previous studies have shown Ca\(^{2+}\) regulation of ion channel activity in guard cells (Pei et al., 2000; Mori et al., 2006; Geiger et al., 2010), our study provides direct molecular-genetic (Figs. 2 and 4) and electrophysiological (Fig. 7) evidence for CPK10 involvement in ABA- and Ca\(^{2+}\)-mediated stomatal signaling.

The results of our pull-down assay (Fig. 5D) showed that CPK10 can recruit HSP1 in a Ca\(^{2+}\)-dependent manner. The plasma membrane localization of CPK10 (Fig. 3C) and interaction between CPK10 and HSP1 (Fig. 5, A and E) suggest that CPK10 may recruit HSP1 to the plasma membrane. Furthermore, our patch-clamp data (Fig. 7) revealed the important roles of CPK10 and HSP1 in the ABA and Ca\(^{2+}\) regulation of inward K\(^{+}\) channels in stomatal guard cells.

CPK10 Functions Together with HSP1 in Drought Stress Signaling

Several Arabidopsis CDPKs have been functionally characterized as important regulators involved in plant responses to drought and salt stresses (Ma and Wu, 2007; Zhu et al., 2007; Mehmer et al., 2010; Xu et al., 2010), in ABA signaling (Choi et al., 2005; Mori et al., 2006; Zhu et al., 2007), and in the regulation of pollen tube growth (Myers et al., 2009). Obviously, to identify targets or substrates of these CDPKs becomes an important task for further understanding the molecular mechanisms of CDPK functions. Increasing evidence for the identification of CDPK substrates supports the notion that CDPKs are multifunctional kinases involved in the regulation of diverse cellular functions (Harper and Harman, 2005). Efforts have been made to identify CDPK-interacting proteins, such as ABF4 for AtCPK32 (Choi et al., 2005), AtDI19-1 for CPK11 (Rodriguez Milla et al., 2006), StrBOHB for StCDPK5 (Kobayashi et al., 2007), ABF1 and ABF4 for AtCPK4 and AtCPK11 (Zhu et al., 2007), and RSG for NtCDPK1 (Ishida et al., 2008). In this study, HSP1 was identified and also functionally characterized as CPK10-interacting protein.

HSP1 was previously demonstrated to interact with CPK4 and CPK11 (Uno et al., 2009). The interactions between HSP1 and other CDPKs as CPK10 homologs (Supplemental Fig. S2) indicate that HSP1 may interact with different CDPKs. To further validate these interactions in vivo and investigate the functions of these protein-protein interactions in a particular biological process may reveal diverse functions of HSP1. HSP1 belongs to class I or II of small heat shock proteins (sHSPs) for its low molecular mass (16.9 kD) and subcellular localization (cytosol and nucleus; Sun et al., 2002). The HSP1 mRNA was highly expressed in stomatal guard cells (Leonhardt et al., 2004). Similar to the transgenic CPK10::GUS plants, high GUS activity was also detected in stomatal guard cells of the transgenic HSP1::GUS plants (Fig. 5B). The results from GST pull-down and BiFC assays (Fig. 5, D and E) indicated that CPK10 may recruit HSP1 to the plasma membrane and form a complex to regulate stomatal movements under drought stress.

Considering the fact that the sHSPs play important roles in plant tolerance to environmental stresses (for review, see Sun et al., 2002; Wang et al., 2004), it is not surprising that the HSP1 functions together with CPK10 in plant responses to drought stress. Several studies have demonstrated that sHSPs may be involved in plant responses to water stress, such as the induction of sHSPs by water stress in sunflower (Helianthus annuus; Almoguera et al., 1993) and the increased Arabidopsis osmotolerance by overexpression of a class II sHSP (Sun et al., 2001). Desiccation tolerance of the resurrection plant (Craterostigma plantagineum) seems related to high and continuous expression of sHSPs in all vegetative tissues (Alamillo et al., 1995).

Although the molecular mechanisms by which sHSPs are involved in plant responses to abiotic stresses remain unclear, a number of findings support the notion that these small proteins may function as molecular chaperones to prevent their partners (such as CPK10 proteins in this study) from deactivation or degradation under various stresses (for review, see Sun et al., 2002; Wang et al., 2004). Alternatively, phosphorylation of sHSPs has been demonstrated before, such as phosphorylation of a class I heat shock protein by a SNF1-related protein kinase (Slocombe et al., 2004) and in vivo-detected phosphorylation of a maize (Zea mays) mitochondrial HSP22 at its N terminus (Lund et al., 2001), suggesting possible HSP1 phosphorylation by CPK10.

Considering all the previously reported results together with the evidence presented in this study, we conclude that the interaction between CPK10 and HSP1 is physiologically important in plant responses to drought stress, particularly in stomatal regulation under water stress.

MATERIALS AND METHODS

Plant Materials and Growth Conditions

Arabidopsis (Arabidopsis thaliana) T-DNA insertion mutants of CPK10 (cpk10; SALK_02441) and HSP1 (hsp1; SALK_017461) were obtained from the ABRC. The seeds were surface sterilized with mixed solutions of NaClO (0.5%, w/v) and Triton X-100 (0.01%, v/v) for 10 min followed by washing with sterile water four times. Seeds were placed in petri dishes containing Murashige and Skoog agar (0.8%, w/v) medium and incubated for 2 d at 4°C before being transferred to 22°C for germination. After 7 d of growth on plates under constant illumination at 60 μmol m\(^{-2}\) s\(^{-1}\) at 22°C, the seedlings were transferred to pots containing soil mixture (rich soil:vermiculite, 2:1, v/v) and kept in a daily light period. The relative humidity was approximate 70% ± 5%.
To obtain the homozygous mutant lines, the individual plants were identified by PCR using T-DNA left border primer Lba1 (5'-TGGTTCAAGTGGCCCATG-3') and CPK10 gene-specific primers (forward primer 5'-ATCCGTAGCCGACTAAGACGCCG-3' and reverse primer 5'-CCAAACAGTACACTGAGAAGT-3') or HSP1 gene-specific primers (forward primer 5'-CTTAAACAGGAACAGTTTGTCCA-3' and reverse primer 5'-ATGGGTAACTGTAACGCCTGT-3').

Plasmid Construction and Arabidopsis Transformation

CPK10::GUS and HSP1::GUS were generated by fusing the promoter fragments of CPK10 (1.57 kb) or HSP1 (0.27 kb) in front of the GUS coding sequence in pCAMBIA1381 vector, respectively. The sequences for CPK10 promoter-specific primers are 5'-AAGCACCATAAGCTCTGTG-3' and 5'-TTAGTCTATATGGACGCCG-3', and the sequences for HSP1 promoter-specific primers are 5'-CGCGCATATACAAATAGGTTT-3' and 5'-GTTGTA-GAGTGATAAGCTCACCACGGG-3'. The GUS staining assays were carried out as described previously (Xu et al., 2006).

For the EGFP constructs, the coding sequence of CPK10 was amplified by the forward primer 5'-TTTCTAATTAAATCAAGTTGAGTCCACCAGTT-3' (Xhol site underlined) and the reverse primer 5'-TTTGATCAACACCGAACCATGAGAACCT-3' (KpnI site underlined), and the HSP1 coding sequence was amplified by the forward primer 5'-TTATAGTCCAGAACAAATCCACCAGTTAC-3' (Xhol site underlined) and the reverse primer 5'-TAATCTCCAGCTGAAATCCATATCT-3' (Xhol site underlined). The PCR products of CPK10 and HSP1 were verified by DNA sequencing and cloned into pU6-EGFP vector (Xu et al., 2006). To generate the CPK10 overexpression plants, the coding sequence of CPK10 was cloned and introduced into Super1300 vector (Chen et al., 2009). For the generation of complementation lines of the cpk10 mutant, a 4,682-bp genomic DNA sequence of CPK10 (including 1,690 bp upstream of the ATG codon and 723 bp downstream of the stop codon) was amplified by PCR with CPK10-specific primers (forward primer 5'-TTGGACCTCATTAAATCCACAGTCTATT-3' and reverse primer 5'-TTTGCACGCTATGATGCTGCTATTGGAATG-3'). The PCR product was verified by DNA sequencing and cloned into pCAMBIA1300 vector.

The constructs were introduced into Agrobacterium tumefaciens (strain GV3101) and transferred into Arabidopsis plants (wild type or mutant) by the floral dip method (Clough and Bent, 1998). All transgenic lines used in this study were T3 homozygous plants with single copy insertion.

Drought Stress and Water Loss Experiments

For the phenotype tests under drought stress, seedlings grown on Murashige and Skoog medium for 7 d were transferred to mixed soil (rich soil:vermiculite, 2:1, v/v) and grown for 1 week with sufficient watering. Each pot had nine seedlings planted. Then the plants were subjected to drought stress treatment by withholding irrigation, and the well-watered plants were used as the control. To ensure reproducibility for the phenotype tests, three or six pots of plants were grown for each line (genotype) and each treatment in six pots. For qRT-PCR analysis, total RNA extraction and treatment were performed as described above. The cDNA was synthesized from total RNA by SuperScript II reverse transcriptase (Invitrogen) using oligo(dT)15 primer (Promega). The full transcript of CPK10 was amplified using the forward primer 5'-ATGGGAATCTTAACGCCC-3' and the reverse primer 5'-TAAACAGGAACAGTTTGTCCA-3'. The transcript of CPK10::KD was amplified using the forward primer 5'-TACATCTTAACTGAATGACTGACT-3' and the reverse primer 5'-TATCCTAATGGAATGACTGACT-3'. The full transcript of HSP1 was amplified using the forward primer 5'-AGAATCCTTTCAATACCAG-3' and the reverse primer 5'-TATCTTGTACCAAGCACATTACCT-3' and the transcript of HSP1 fragment was amplified using the forward primer 5'-AGAATCCTTTCAATACCAG-3' and reverse primer 5'-AGCGCTTGGTTTCTTGCCCC-3' using the 2 s (A656000J) transcript served as a control and was amplified by EfiI-specific primers 5'-ATGCCCAAGGACATGTTAATCCAT-3' and 5'-TTGGCCGCACTTAAATGACTGACT-3'. The total RNA extraction and treatment were performed as described above. The cDNA was synthesized from total RNA by SuperScript II reverse transcriptase (Invitrogen) using Random Hexamer Primer (Promega). qRT-PCR was performed as described previously (Chen et al., 2009). Relative quantitative results were calculated by normalization to 18S rRNA. RT-qPCR was conducted with CPK10-specific primers (forward primer 5'-GGCTCAAGAAGTGCTGCTAC-3' and reverse primer 5'-AGCTTCGGCTAGCTCATACCA-3').

Yeast Two-Hybrid and GST Pull-Down Assays

Bait cloning and yeast two-hybrid screening were performed following the manufacturer’s instructions (Clontech). The coding sequences of CPK10 and CPK10-KD were cloned into pGBK7 (Clontech) as baits and transformed into Y187 yeast strain. The cDNA library was constructed using total RNA isolated from local 4-week-old plants after a dehydration treatment and transformed into AH109 yeast strain for yeast mating. The putative positive clones from yeast two-hybrid screening were further confirmed by yeast two-hybrid tests and assayed for β-galactosidase activity according to the manufacturer’s instructions (Clontech).

For GST pull-down assays, the coding region of CPK10 cDNA was amplified by using the forward primer 5'-TTGTTCAAGTGGCCCATG-3' and the reverse primer 5'-AGAATCCTTTCAATACCAG-3' cloned into KpnI and SfII sites of pet-30a (+) vector. HSP1 was amplified by using the forward primer 5'-TACATCTTAACTGAATGACTGACT-3' and the reverse primer 5'-TATCTTGTACCAAGCACATTACCT-3' and cloned into SfII and NsiI.
sites of pGEX-4T-1 vector. Fusion protein was expressed in the BL21 (DE3) strain of Escherichia coli by induction with 0.5 mM isopropyl β-D-thiogalacto-
side. GST-HSP1 fusion protein was immobilized on Glutathione-Sepharose 4B beads and incubated with His-tag CPK10 extracts (soluble fraction; Shi et al., 1999). After 2 h of incubation at 4°C on a rotary incubator, the beads were washed five times with ice-cold phosphate-buffered saline, resuspended in SDS gel-loading buffer, and then loaded on SDS-PAGE (10%, w/v) gels. For two SDS-PAGE gels in each experiment, one of them was stained with Commassie Brilliant Blue R 250 and the other was analyzed by protein gel blotting against anti-His antibody.

**BiFC Assays**

The cDNAs of CPK10 and HSP1 were cloned into vector kan-SPY-PRC (Mer) and vector hyg-SPY-PYR(R)173, respectively, as described by Waadt and Kudla (2008). The sequences for the CPK10-specific primers are 5′-TATAACATGTTATGGAATCCTGGAT-3′ (Spel site underlined) and 5′-TATACGCAAGACAGAAGATTTGCGAT-3′ (Xhol site underlined), and the sequences for the HSP1-specific primers are 5′-TATAACATGTT-
ATGAAAAATCCACCAAGTTTGCACG-3′ (Spel site underlined) and 5′-TATAACACGCTACCATACGACATAAATCTCCTA-3′ (Xhol site underlined). Following transient expression A. thaliana strain G3101 carrying the BiFC constructs was used to infiltrate 5-week-old Nicotiana benthamiana leaves. The photographs were taken after 24 to 72 h of transformation using a confocal laser-scanning microscope (Nikon EZ-C1).

**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 (approximately 22°C) under dim light. The bath solutions contained 30 mM KCl, 2 mM MgCl₂, 10 mM MES-Tris (pH 5.6), and 1 mM CaCl₂ with osmolality of 485 mmol kg⁻¹. The pipette solutions contained 3.35 mM CaCl₂, 6.7 mM EGTA, 2 mM MgCl₂, 10 mM HEPES-Tris (pH 7.1), 70 mM K-Glu, and 30 mM KCl with osmolality of 500 mmol kg⁻¹. The final osmolalities in both bath solution and pipette solution were adjusted with ≥-sorbitol. The final free Ca²⁺ concentration in the pipette solution was 200 nM, calculated using Chelator software Max Chelator version 5.60 (developed by Dr. Chris Patton at Stanford University). The fresh ATP (Mg-ATP; 5 mM) was calculated using Chelator software Max Chelator version 5.60 (developed by Waadt and Kudla (2008)). The sequences for the CPK10-specific primers are 5′-TATAACATGTTATGGAATCCTGGAT-3′ (Spel site underlined) and 5′-TATAACATGTTATGGAATCCTGGAT-3′ (Spel site underlined) and 5′-TATACGCAAGACAGAAGATTTGCGAT-3′ (Xhol site underlined). Following transient expression A. thaliana strain G3101 carrying the BiFC constructs was used to infiltrate 5-week-old Nicotiana benthamiana leaves. The photographs were taken after 24 to 72 h of transformation using a confocal laser-scanning microscope (Nikon EZ-C1).

**Supplemental Data**

The following materials are available in the online version of this article.

**Supplemental Figure S1.** Subcellular localization of CPK10 and HSP1.

**Supplemental Figure S2.** Yeast two-hybrid assays for the identification of HSP1-interacting CDPKs.

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