CDPKs CPK6 and CPK3 Function in ABA Regulation of Guard Cell S-Type Anion- and Ca\textsuperscript{2+}-Permeable Channels and Stomatatal Closure

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Abscisic acid (ABA) signal transduction has been proposed to utilize cytosolic Ca\textsuperscript{2+} in guard cell ion channel regulation. However, genetic mutants in Ca\textsuperscript{2+} sensors that impair guard cell or plant ion channel signaling responses have not been identified, and whether Ca\textsuperscript{2+}-independent ABA signaling mechanisms suffice for a full response remains unclear. Calcium-dependent protein kinases (CDPKs) have been proposed to contribute to central signal transduction responses in plants. However, no Arabidopsis CDPK gene disruption mutant phenotype has been reported to date, likely due to overlapping redundancies in CDPKs. Two Arabidopsis guard cell–expressed CDPK genes, CPK3 and CPK6, showed gene disruption phenotypes. ABA and Ca\textsuperscript{2+} activation of slow-type anion channels and, interestingly, ABA activation of plasma membrane Ca\textsuperscript{2+}-permeable channels were impaired in independent alleles of single and double cpk3cpk6 mutant guard cells. Furthermore, ABA- and Ca\textsuperscript{2+}-induced stomatal closing were partially impaired in these cpk3cpk6 mutant alleles. However, rapid-type anion channel current activity was not affected, consistent with the partial stomatal closing response in double mutants via a proposed branched signaling network. Imposed Ca\textsuperscript{2+} oscillation experiments revealed that Ca\textsuperscript{2+}-reactive stomatal closure was reduced in CDPK double mutant plants. However, long-lasting Ca\textsuperscript{2+}-programmed stomatal closure was not impaired, providing genetic evidence for a functional separation of these two modes of Ca\textsuperscript{2+}-induced stomatal closure. Our findings show important functions of the CPK6 and CPK3 CDPKs in guard cell ion channel regulation and provide genetic evidence for calcium sensors that transduce stomatal ABA signaling.

Introduction

Stomatal pores in the epidermis of aerial parts of plants facilitate gas exchange between plants and the atmosphere. Stomatal pores are surrounded by pairs of guard cells that mediate stomatal pore opening and closing. Guard cells respond to diverse stimuli, including blue light, CO\textsubscript{2} concentrations, drought, pathogen attack, and plant hormones, including abscisic acid (ABA) [1–3]. Stomatal movements are mediated by ion transport across the plasma membrane and vacuolar membrane of guard cells and by organic solute content changes [1,4]. Guard cell ion channels and proton pumps are regulated by the cytosolic free Ca\textsuperscript{2+} concentration ([Ca\textsuperscript{2+}]\textsubscript{cyt}) such that [Ca\textsuperscript{2+}]\textsubscript{cyt} elevation activates stomatal closing mechanisms [5–9]. These findings correlate with the Ca\textsuperscript{2+} dependence of ABA-induced stomatal closing [10,11].

ABA is a drought-inducible plant hormone. ABA regulates [Ca\textsuperscript{2+}]\textsubscript{cyt} elevations in guard cells [12–18] and other cells [19,20]. [Ca\textsuperscript{2+}]\textsubscript{cyt} elevation activates slow-type (S-type) anion efflux channels and down-regulates inward-rectifying K\textsuperscript{+} channels and proton pumps in the plasma membrane of guard cells [5,7,16,21]. The concomitant efflux of anions and K\textsuperscript{+} from guard cells results in turgor reduction and stomatal closure. Thus, Ca\textsuperscript{2+} elevation shifts ionic conductance properties in guard cells toward a stomatal closing favoring mode. Other studies indicate a possible role for Ca\textsuperscript{2+} in
mediating stomatal opening as well [22–24]. It is noteworthy that ABA signal transduction in guard cells consists of not only a Ca\(^{2+}\)-dependent response but also a Ca\(^{2+}\)-independent possibly parallel pathway [25] (for reviews: [3,4], see Discussion). Whether Ca\(^{2+}\)-independent mechanisms suffice for mediating full physiological stomatal ABA responses remains unclear. Genes encoding signal transduction proteins in both Ca\(^{2+}\)-dependent and -independent pathways are still largely unknown and molecular genetic evidence for a rate-limiting role of Ca\(^{2+}\) as a positive transducer of ABA signaling is lacking. Repression of the SOS3-like calcium binding protein 5 (SCaBP5)(Calcineurin B-like protein 1 (CBL1) and CBL9 genes result in ABA hypersensitivity, suggesting that CBL1 and CBL9 function as negative regulators of an ABA signal transduction pathway [26,27]. However, no genes encoding Ca\(^{2+}\)-sensing proteins that function as positive transducers of ABA signaling and of ion channel regulation in guard cells have been identified.

ABA regulates repetitive [Ca\(^{2+}\)]\(_{cyt}\) elevations in guard cells [12–15,17,28,29]. Experimentally imposing [Ca\(^{2+}\)]\(_{cyt}\) transients revealed two distinguishable Ca\(^{2+}\)-dependent stomatal closing responses: a rapid “Ca\(^{2+}\)-reactive” stomatal closing response, and a long-lasting “Ca\(^{2+}\)-programmed” stomatal closing response, which prevails even after Ca\(^{2+}\) transients have been terminated [29]. The long-lasting Ca\(^{2+}\)-programmed response, but not the rapid Ca\(^{2+}\)-reactive stomatal closing response, depends on the Ca\(^{2+}\) transient pattern [29–31]. However, the underlying Ca\(^{2+}\) transduction mechanisms remain unknown. In guard cells, [Ca\(^{2+}\)]\(_{cyt}\) elevation activates S-type anion channels via phosphorylation events [16,32], suggesting a role for phosphorylation events in [Ca\(^{2+}\)]\(_{cyt}\) signaling. We hypothesized that guard cell–expressed calcium-dependent protein kinases (CDPKs) [33] may function in transducing specific components of guard cell signal transduction and ion channel regulation.

CDPKs are protein kinases that are broadly distributed in the plant kingdom [34,35]. In other kingdoms, CDPKs have been found only in certain groups of protists, including Plasmodium [34]. CDPKs contain an intrinsic Ca\(^{2+}\)-activation domain with four EF hand Ca\(^{2+}\)-binding sites [33]. CDPKs have been proposed to function in multiple plant signal transduction pathways downstream of [Ca\(^{2+}\)]\(_{cyt}\) elevations, thus transducing various physiological responses [33,34]. A virus-induced gene silencing study demonstrated a role of CDPKs in tobacco defense responses [36]. Dominant constitutively active mutant forms of CPK10/AtCDPK1 (Arabidopsis gene identifier number [AGI No.: At1g74740] or CPK30/AtCDPK1a (AGI No.: At1g18890) cause constitutive stress signaling [37], dominant mutant isoforms affect pollen tube growth in Petunia [38], and antisense repression of CDPK in Medicago truncatula affected root hair development and the nodule formation rate [39]. A Ca\(^{2+}\)-dependent kinase activity phosphorylates the guard cell–expressing inward-rectifying K\(^{+}\) channel, Kat1, in vitro [40] and recombinant Arabidopsis CDPK activates Cl\(^{-}\) influx channels in Vicia faba vacuoles [41]. These results suggest functions of CDPKs in stomatal movements. Whereas many biochemical approaches show functions of CDPKs [36,37,41–50], gene disruption phenotypes of CDPKs have not yet been reported in Arabidopsis and would allow unequivocally pinpointing cellular functions of defined CDPK genes.

The CDPK gene family includes 34 members in Arabidopsis alone [35,46]. Redundancies in CDPK genes are likely to hamper molecular genetic analyses of CDPK functions. Recent studies have shown that relatively few signal transduction genes are expressed in a strictly cell-specific manner in roots, guard cells, and mesophyll cells [51–54]. However, signal transduction genes that are highly expressed in a given cell type, such as guard cells, are candidates for being incorporated into signal transduction networks within those cells. Furthermore, cell-specific signal transduction assays may allow resolution of phenotypes of single knockout mutants that global whole plant phenotypic analyses would not resolve in reverse genetic studies. Therefore, in the present study we used single cell-type gene expression analysis [54] to determine which CDPK genes are expressed in guard cells. We report phenotypes of loss-of-function mutants in two of the guard cell–expressed CDPKs and characterize functions of these CDPKs in Ca\(^{2+}\) activation of anion channels, in ABA activation of anion channels, and unexpectedly also in ABA regulation of Ca\(^{2+}\) channels as well as in Ca\(^{2+}\)-reactive and ABA-induced stomatal closing. The presented results provide molecular genetic and cell biological evidence for Ca\(^{2+}\) sensors that function as positive transducers in plant ion channel regulation and ABA- and Ca\(^{2+}\)-dependent signal transduction in guard cells.

**Results**

**Expression of CDPK Genes**

There are 34 CDPK genes in the *Arabidopsis* genome [35,46]. To investigate whether and, if so, where CDPKs may function in ion channel regulation and guard cell signal transduction branches, we first identified CDPK genes expressed in guard cells using a guard cell–enriched cDNA library and RT-PCR with degenerate oligomers [53]. Two of the guard cell–expressed CDPK genes, CPK3 (AGI No.: At4g23650) and CPK6 (AGI No.: At2g17290), showed initial insertion mutant phenotypes and were therefore further analyzed. The expression of CPK3 and CPK6 in isolated guard cell protoplasts (GCPs) was further analyzed by RT-PCR with gene-specific primers (Figure 1A) and independently later by cell type–specific genomic scale expression analyses using Affymetrix (Santa Clara, California, United States) GeneChip assays [54]. RT-PCR analysis showed that CPK3 and CPK6 are expressed in both guard cells and mesophyll cells (Figure 1A). The purity of GCPs was analyzed by RT-PCR with specific primers for the guard cell–expressed potassium channel gene, *Kat1* (Figure 1A) [56]. Guard cell preparations were further examined for contamination of mesophyll cells by analyzing mRNA abundance of a putative calmodulin-binding protein (CBP) (AGI No.: At4g33050), which was identified as being highly expressed in mesophyll cells but absent in guard cells [54]. No CBP mRNA was detected in guard cell preparations (Figure 1A), indicating that the GCP preparations had no or very little contamination. In addition to CPK3 and CPK6, several other CDPK genes were identified in guard cells by microarray experiments with guard cell RNA (Supplemental Table 1 in [54]). In this study, we focus on functional dissection of the guard cell–expressed CPK3 and CPK6 genes.

To genetically analyze functions of CPK3 and CPK6 in guard cell signal transduction, we identified T-DNA insertion mutations in *CPK3* and *CPK6* from the Salk Institute Genomic Analysis Laboratory database [57]. Homozygous T-
DNA insertion mutant lines were isolated and genomic sequences of the \textit{cpk3–1} (SALK\_107620), cpk3–2 (SALK\_023862), cpk6–1 (SALK\_093308), and cpk6–2 (SALK\_0339392) insertion mutants were determined. The T-DNA insertions in \textit{cpk3–1} and \textit{cpk3–2} are localized in the first exon and in the first intron, respectively (Figure 1B). The insertion in \textit{cpk6–1} is localized in the second exon, 60 base-pairs downstream of the translation initiation codon, and \textit{cpk6–2} is in the first intron (Figure 1B). Southern blot analyses of homozygous plants indicated only a single band in each line, suggesting a single T-DNA insertion in these mutants (data not shown). Transcripts of CPK3 or CPK6 were not detected in \textit{cpk3–1} and \textit{cpk6–1} as demonstrated by RT-PCR utilizing whole leaf RNA extracts (Figure 1C). No RT-PCR band was observed for \textit{cpk3–2} (data not shown). For \textit{cpk6–2}, no full-length cDNA was detected (Figure 1C). A faint band for transcript downstream of the T-DNA insertion was observed after 35 cycles of amplification showing substantially reduced mRNA levels (8\% or less intensity compared to wild-type level, \( n = 2 \)) (Figure 1C). Using a primer set in the first exon and the eighth exon, no RT-PCR amplification was observed (Figure 1B and 1C), showing that the first exon is missing in \textit{cpk6–2}.

We performed RT-PCR with CPK3 primers in \textit{cpk6–1} and with CPK6 primers in \textit{cpk3–1} to examine whether a compensatory expression occurs. No compensation in the wild-type transcript levels was observed (data not shown).

Homozygous \textit{cpk3} and \textit{cpk6} single and \textit{cpk3–1cpk6–1} and \textit{cpk3–2cpk6–2} double mutants were isolated and used for further analyses. Whole plant general morphological phenotypes of \textit{cpk3–1}, \textit{cpk3–2}, \textit{cpk6–1}, \textit{cpk6–2}, and the \textit{cpk3–1cpk6–1} and \textit{cpk3–2cpk6–2} double mutants were largely similar to wild-type plants (Columbia ecotype) under the standard growth conditions tested, but \textit{cpk3–1cpk6–1} and \textit{cpk3–2cpk6–2} double mutant plants showed a slight delay in growth by approximately 2 d in 4-wk-old plants compared to wild-type plants (data not shown).

**Activation of S-Type Anion Channels by Cytosolic Ca\(^{2+}\) Is Impaired in \textit{cpk3cpk6} Mutants**

S-type anion efflux channels have been proposed to play an important role as targets of ABA signal transduction in guard cells and to be regulated by upstream phosphorylation events \([5,9,16,32,55,58-62]\). To determine whether CPKFs function in the activation of S-type anion channels in guard cells, we examined Ca\(^{2+}\) activation of S-type anion channels in wild-type, \textit{cpk3}, and \textit{cpk6} single mutant and double mutant guard cells.

As illustrated in Figure 2, typical large [Ca\(^{2+}\)]\(_{\text{cyt}}\)-activated S-type anion channel currents were observed in wild-type guard cells in the presence of elevated (2 \(\mu\)M) free Ca\(^{2+}\) in the patch pipette (cytosolic solution) (Figure 2A and 2C) as previously described \([5,9]\). At 0.1 \(\mu\)M [Ca\(^{2+}\)]\(_{\text{cyt}}\) in the pipette, large S-type anion channel currents were not activated (data not shown) \([9]\). S-type anion channel currents in the presence of 2 \(\mu\)M [Ca\(^{2+}\)]\(_{\text{cyt}}\) were significantly reduced in the \textit{cpk3–1} mutant \(( n = 11 \text{ guard cells})\) compared to wild-type (Figure 2C; \( n = 17 \), \( p < 0.022 \) at -145 mV), \textit{cpk3–1} showed similar results to \textit{cpk3–1} (Figure 2C; \( n = 7 \), \( p < 0.4 \) compared to \textit{cpk3–1}). S-type anion channel currents were further reduced in \textit{cpk6–1} \(( n = 11 \) and \textit{cpk6–2} \(( n = 4 \) single mutant guard cells (Figure 2C; \( p < 0.005 \) compared to wild-type; \( p > 0.18 \) for \textit{cpk6–1} compared to \textit{cpk6–2}).

![Figure 1](https://example.com/figure1.png)

**Figure 1.** Guard Cell Expression of CPK3 and CPK6 CDPKs (A) Expression of \textit{CPK3} and \textit{CPK6} in guard cell (GC) and mesophyll cell (MC) protoplasts was examined by RT-PCR. Control amplifications of the guard cell-expressed \textit{KAT1} gene and the mesophyll-expressed \textit{CBP} marker genes \([54]\) (Leonhardt et al., 2004) were used to test the purity of cell preparations (see Results). \textit{ACTIN2} was used for an internal loading control. To amplify each CDPK-specific band, RT-PCR was performed with primer sets as indicated by arrowheads in (B) for 36 cycles. Plants were sprayed with water (-ABA) or 100 \(\mu\)M ABA (+ABA) 4 h before isolation of protoplasts and RNA extraction.

(B) Cartoon showing the T-DNA insertion positions in \textit{cpk3} and \textit{cpk6} T-DNA insertion alleles. PCR was performed with a left boarder primer of the T-DNA and a gene-specific primer, and the PCR products were sequenced to determine the T-DNA insertion positions. Arrowheads indicate primer locations for RT-PCR in (A) and (C). ATG and TGA indicate start and stop codons. White boxes indicate exons.

(C) RT-PCR confirmed that \textit{cpk3–1} and \textit{cpk6–1} alleles are disruption mutants. PCRs (32 cycles) were performed with primer sets as indicated in (B) (black arrowheads) in the left three panels. Transcripts of wild-type (WT) and \textit{cpk6–2} were examined with two sets of primers [white and black arrowheads in (B)] showing that \textit{cpk6–2} lacks exon 1 and that the \textit{cpk6–2} has 8\% or less the mRNA level of wild-type based on densitometry analyses \(( n = 2 \) ). RNA was extracted from leaves of WT, homozygous \textit{cpk3–1}, \textit{cpk6–1}, and \textit{cpk6–2} single mutants, and the \textit{cpk3–1cpk6–1} double mutant.

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![Figure 2](https://example.com/figure2.png)

**Figure 2.** Impairment in [Ca\(^{2+}\)]\(_{\text{cyt}}\)-Dependent Activation of S-Type Anion Channel Currents in Guard Cells of \textit{cpk3} and \textit{cpk6} Single and \textit{cpk3cpk6} Double Mutants

(A and B) Typical S-type anion channel current traces in wild-type (WT) \( (\text{A and B}) \) guard cells were shown in response to 2 \(\mu\)M free Ca\(^{2+}\) in the patch-clamp pipette solution that dialyzes the cytoplasm.

(C) Average current-voltage curves of wild-type \(( n = 17 \text{ cells})\), \textit{cpk3–1} \(( n = 11 \) and \textit{cpk3–2} \(( n = 7 \) ), \textit{cpk6–1} \(( n = 11 \) and \textit{cpk6–2} \(( n = 4 \) ), \textit{cpk3–1cpk6–1} \(( n = 17 \) ), and \textit{cpk3–2cpk6–2} \(( n = 11 \) ). Error bars show SEM. DOI: 10.1371/journal.pbio.0040327.g002
Similar results were obtained in CPK6

\[ p \]

regulation of S-type anion currents was impaired (Figure 3C, guard cells [3], CDPKs mediate an important Ca\(^{2+}\) complex network of ion channel regulation mechanisms in wild-type controls (Figure 2B and 2C; channel currents were substantially reduced compared to disruptions compared to and 2C). The data showed stronger effects of CPK6 anion currents did remain in the double mutants (Figure 2B and 2C). ABA activation in wild-type guard cells and (Figures 2 and 3). ABA activation in wild-type guard cells and in the absence of ABA (A) and in the presence of 50 \( \mu \text{M} \) ABA (B). (C and D) Whole-cell S-type anion channel current traces in \( \text{cpk3-1cpk6–1} \) double mutant guard cells in the absence of ABA (C) and in the presence of ABA (D). (E) Average current-voltage curves of wild-type and \( \text{cpk3-1cpk6–1} \) and \( \text{cpk3-2cpk6–2} \) double mutant guard cells in the absence and presence of ABA (n = 8 WT; n = 7 \( \text{cpk3-1cpk6–1} \); n = 3 \( \text{cpk3-2cpk6–2} \) guard cells). GCPs were treated with 50 \( \mu \text{M} \) ABA or solvent control (0.1% ethanol) for 2 h prior to establishing g2 seals. Open and closed symbols indicate –ABA and +ABA, respectively.

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1cpk6–1 double mutant guard cells (n = 17), S-type anion channel currents were substantially reduced compared to wild-type controls (Figure 2B and 2C; p < 0.0005). Essentially similar results were obtained in \( \text{cpk3-1cpk6–2} \) and \( \text{cpk3-2cpk6–2} \) double mutant guard cells in the absence and presence of ABA (n = 8 WT; n = 7 \( \text{cpk2-1cpk6–1} \); n = 3 \( \text{cpk3-2cpk6–2} \) guard cells). The data showed stronger effects of CPK6 disruptions compared to CPK7 disruptions. Thus CPK3 and CPK6 are important for Ca\(^{2+}\) activation of S-type anion channels.

Impairment in ABA Activation of S-Type Anion Channels in \( \text{cpk3cpk6} \) Mutants

Previous studies have shown that guard cell signal transduction is mediated by a network of events, which includes parallel Ca\(^{2+}\)-dependent and -independent signaling branches (see Discussion; for reviews: [3,4]). Therefore, experiments were pursued to analyze ABA activation of S-type anion channels. These experiments were performed under different conditions than those shown in Figure 2, such that cytosolic Ca\(^{2+}\) elevation alone would not fully activate S-type anion currents (see Materials and Methods) [9]. As shown in Figure 3, with preincubation of guard cells in low extracellular Ca\(^{2+}\), 2 \( \mu \text{M} \) cytosolic Ca\(^{2+}\) in the pipette activated S-type anion currents in guard cells of only intermediate amplitudes (Figure 3A). Under these conditions, ABA up-regulated S-type anion current activities in wild-type protoplasts (Figure 3A, 3B, and 3E; n = 8). In \( \text{cpk3-1cpk6–1} \) (n = 7) and \( \text{cpk3-2cpk6–2} \) (n = 7) double mutant guard cells, ABA regulation of S-type anion currents was impaired (Figure 3C, 3D, and 3E; p < 0.01). These data show that, despite the complex network of ion channel regulation mechanisms in guard cells [3], CDPKs mediate an important Ca\(^{2+}\)-decoding transduction step in ABA regulation of S-type anion channels (Figures 2 and 3). ABA activation in wild-type guard cells and the impairment in ABA activation of S-type anion channels at 2 \( \mu \text{M} \) cytosolic Ca\(^{2+}\) in \( \text{cpk3cpk6} \) mutants (Figure 3) further provide evidence for a recently proposed hypothesis in which stomatal closing signals (i.e., ABA) mediate priming of guard cell Ca\(^{2+}\) sensors, such that they can respond to elevated cytosolic Ca\(^{2+}\) levels [63].

Impairment in ABA activation of \( \text{ICa} \) Channels in \( \text{cpk3cpk6} \) Mutants

ABA activates plasma membrane Ca\(^{2+}\)-permeable (\( \text{ICa} \)) channels [64–66]. Combined physiological, molecular genetic, and cell biological analyses have shown that \( \text{ICa} \) channels function in the guard cell ABA signal transduction network at hyperpolarized voltages [9,53,64–66]. We examined whether CPK3 and CPK6 function in the regulation of \( \text{ICa} \) channels.

Typical \( \text{ICa} \) currents were activated by extracellular application of ABA to patch-clamped wild-type guard cells (Figure 4A and 4B; n = 13). Unexpectedly, ABA activation of \( \text{ICa} \) channels was not observed in \( \text{cpk3-1cpk6–6} \) and \( \text{cpk3-2cpk6–2} \) double mutant guard cells (Figure 4C–4E; n = 14, p = 0.56 for \( \text{cpk3-1cpk6–6} \); n = 8, p = 0.47 for \( \text{cpk3-2cpk6–2} \) when comparing before and after ABA treatment). Blind patch-clamp experiments in which the genotype of protoplasts was unknown (n = 2 for wild-type, n = 2 for \( \text{cpk3-1cpk6–1} \)), and similar findings by Y.M., I.C.M., Y.W., and S.M. in this study, further confirmed the impairment of ABA activation of \( \text{ICa} \) channels in \( \text{cpk3cpk6} \) mutant guard cells. Next we analyzed whether only one of the two CDPKs might affect ABA activation of \( \text{ICa} \) channels. Defects in the ABA activation of \( \text{ICa} \) channels were observed in the \( \text{cpk3–1; cpk3–2; cpk6–1; and cpk6–2} \) single mutants (Figure 4F–4I; n = 9, p = 0.38 for \( \text{cpk3–1} \); n = 11, p = 0.47 for \( \text{cpk6–1} \); n = 3, p = 0.96 for \( \text{cpk3–2} \); n = 5, p = 0.30 for \( \text{cpk6–2} \), when comparing before and after ABA treatment). Together these data show that CPK3 and CPK6 function in ABA regulation of \( \text{ICa} \) channels (Figure 4) and Ca\(^{2+}\) and ABA activation of S-type anion channels (Figures 2 and 3).

To gain insight into the question whether activation of phosphorylation events are required before or after ABA application and during ABA activation of \( \text{ICa} \) channels in patch-clamped Arabidopsis guard cells, wild-type guard cells were pretreated with the broad serine/threonine kinase inhibitor, K252a ([8R*,9S*,11S*)–(–)9-hydroxy-9-methoxy-carbonyl-8-methyl-2,3,9,10-tetrahydro-8,11-epoxy-1H,8H,11H-2,7b,11a-triazadibenzo[a,g]cyclo-octa[e,d]-trinden-1-one], 20 min prior to, and continuously during, patch clamping. Moreover, the cytoplasm of whole cells was diazylated in the absence of ATP in whole cell recordings for longer than 15 min prior to extracellular ABA exposure. Ionic currents were recorded in the same guard cells prior to and after exposure to ABA. In negative controls, treatment with K252a alone did not cause constitutive activation of \( \text{ICa} \) channels without ABA treatment (n = 3). Interestingly, pretreatment with K252a (2 \( \mu \text{M} \)) did not disrupt ABA activation of \( \text{ICa} \) channels (Figure 4) and 4K; n = 6, p < 0.04 at ~180 mV). These findings indicate that the effect of the \( \text{cpk3} \) and \( \text{cpk6} \) mutations on ABA activation of \( \text{ICa} \) channel currents are most likely not caused by direct ABA-induced upstream CDPK activation in the NADPH-dependent activation branch of \( \text{ICa} \) channels [66], but possibly by prior CDPK action (see Discussion).

We further examined whether the defect in ABA activation of \( \text{ICa} \) channels is due to impairment in reactive oxygen species (ROS) activation of \( \text{ICa} \) channels, as found for the ABA-insensitive mutants, \( \text{gca2} \) and \( \text{abi2–1} \) [65,66]. As shown in
Figure 4. Impairment in ABA Activation of $I_{\text{Ca}}$ Channel Currents in $cpk3$ and $cpk6$ Single Mutants and $cpk3\text{-}cpk6$ Double Mutants

(A and B) ABA (50 μM) activated $I_{\text{Ca}}$ channel current in wild-type (WT) guard cells. (A) Current traces before (−ABA) and after ABA (+ABA) activation of $I_{\text{Ca}}$ channels are shown in a representative cell. (B) Average current-voltage curves ($n = 13$) are shown. (C and D) ABA failed to activate $I_{\text{Ca}}$ channel currents in $cpk3$-$1cpk6$–1 double mutant guard cells. (C) A response in a representative cell is shown. (D) Average current-voltage curves ($n = 14$) are shown. Traces before and after ABA overlap in (C). (E–H) Averages of current-voltage curves in guard cells isolated from (E) WT, (F) $cpk3$-$1cpk6$–1, (G) $cpk6$–1 ($n = 9$), (H) $cpk3$–2 ($n = 3$), and (I) $cpk6$–2 ($n = 5$) are shown. (J and K) ABA activation of $I_{\text{Ca}}$ channel currents in wild-type (WT) guard cells pretreated with the protein kinase inhibitor K252a (2 μM) for 15 min prior to and during patch clamping and with no ATP added in the patch pipette solution. Response in a guard cell is shown in (J), and average current-voltage curves ($n = 6$) are shown in (K). Open symbols indicate −ABA and closed symbols indicate +ABA. Error bars represent SEM.

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Figure 5, hydrogen peroxide activation of $I_{\text{Ca}}$ channels was observed in $cpk3$-$cpk6$ double mutant and wild-type guard cells (Figure 5; $n = 7$, $p < 0.001$ for $cpk3$-$1cpk6$–1; $n = 4$, $p < 0.05$ for $cpk3$-$2cpk6$–2; and $n = 7$, $p < 0.01$ for wild-type; when comparing before and after $H_2O_2$ treatment).

ABA- and $Ca^{2+}$-Induced Stomatal Closure

The findings that cytosolic $Ca^{2+}$ and ABA activation of $S$-type anion channels and ABA activation of $I_{\text{Ca}}$ channels are impaired in $cpk3$-$cpk6$ mutants led us to examine ABA-induced stomatal closure in these $cdpk$ mutants. Application of 1 and 10 μM ABA induced a decrease in stomatal aperture in wild-type (Figure 6A, $n = 15$ experiments, 260 stomata). In contrast, $cpk3$-$1cpk6$–1 and $cpk3$-$2cpk6$–2 double mutant stomata showed reduced ABA responses (Figure 6A, Table 1, $n = 7$ experiments, 140 stomata; at 10 μM ABA: $p < 0.01$ for $cpk3$-$1cpk6$–1 versus wild-type; $n = 4$ experiments, 120 stomata, $p < 0.011$ for $cpk3$-$2cpk6$–2). Furthermore, ABA-induced stomatal closure was partially impaired in all of the $cpk3$ and $cpk6$ single mutant alleles ($p < 0.05$) (Table 1). These results show roles for $CPK3$ and $CPK6$ in ABA-induced stomatal closure.

Extracellular $Ca^{2+}$ causes stomatal closing, by initiating repetitive cytoplasmic $Ca^{2+}$ elevations in guard cells [67–69]. Application of 100 μM $CaCl_2$ to intact leaves closed wild-type stomata (Figure 6B). However, in the $cpk3$-$1cpk6$–1 and $cpk3$-$2cpk6$–2 double mutants, stomatal closure was significantly attenuated compared to the wild-type response (Figure 6B, $p < 0.01$ for $cpk3$-$1cpk6$–1 and $p < 0.05$ for $cpk3$-$2cpk6$–2 at 100 μM external $Ca^{2+}$). External $Ca^{2+}$-induced stomatal closing was also impaired in all four $cpk3$ and $cpk6$ single mutants ($n = 60$ to 100 stomata; $p < 0.05$, data not shown).

To further evaluate $Ca^{2+}$ regulation of stomatal closure, we examined the effect of experimentally imposed [$Ca^{2+}]_{\text{cyt}}$ oscillations on stomatal closure in $cpk3$-$cpk6$ double mutant plants [29–31,68]. A $Ca^{2+}$ oscillation pattern was applied to guard cells with a similar time pattern to those that cause long-term programmed stomatal closure (i.e., inhibition of stomatal reopening) in Arabidopsis [29]. Hypothesizing a contribution of additional CDPKs or other $Ca^{2+}$ transducers in these experiments, we applied a hyperpolarizing buffer with lower extracellular $Ca^{2+}$ elevations in guard cells [67–69]. Application of 100 μM $CaCl_2$ to intact leaves closed wild-type stomata (Figure 6B). However, in the $cpk3$-$1cpk6$–1 and $cpk3$-$2cpk6$–2 double mutants, stomatal closure was significantly attenuated compared to the wild-type response (Figure 6B, $p < 0.01$ for $cpk3$-$1cpk6$–1 and $p < 0.05$ for $cpk3$-$2cpk6$–2 at 100 μM external $Ca^{2+}$). External $Ca^{2+}$-induced stomatal closing was also impaired in all four $cpk3$ and $cpk6$ single mutants ($n = 60$ to 100 stomata; $p < 0.05$, data not shown).

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cells; cpk3-1cpk6–1: 8.456 RU * 0.1 min ± 0.517, n = 24 cpk3-1cpk6–1 cells). These data are consistent with findings that external Ca\(^{2+}\)-induced [Ca\(^{2+}\)]\(_{cyt}\) elevations in guard cells include intracellular Ca\(^{2+}\) release from guard cell organelles [67,69].

Four Ca\(^{2+}\) transients with a 10-min period, which induce long-lasting “Ca-programmed” stomatal closure [29], were applied to wild-type and cpk3cpk6 double mutant stomata. Wild-type stomata started closing immediately after the first Ca\(^{2+}\) elevation was imposed and continued to show progressive Ca\(^{2+}\)-reactive closing for longer than 40 min (Figure 7; n = 6 experiments, 47% closure). In contrast, Ca\(^{2+}\) transient-induced closure of cpk3-1cpk6–1 and cpk3-2cpk6–2 double mutant stomata was reduced (14% and 22% closure, respectively) (Figure 7; n = 6 experiments, p < 0.01 for cpk3-1cpk6–1 versus wild-type and n = 3 experiments, p < 0.05 for cpk3-2cpk6–2 versus wild-type, p > 0.60 cpk3-1cpk6–1 versus cpk3-2cpk6–2). Stomata of both wild-type and cpk3cpk6 double mutant guard cells remained closed during the ensuing 2 h and 20 min measurements, even though cells were extracellularly bathed in a typical “stomatal opening” solution containing 50 mM KCl and 0

![Diagram](image)
Table 1. ABA-Induced Stomatal Closure in cpk3 and cpk6 Mutants

| Aperture Width (µm) | % Closure | n | p  |
|---------------------|-----------|---|----|
|                     | 0 µM ABA  | 10 µM ABA |     |    |
| Wild-type           | 3.13 ± 0.15 | 1.67 ± 0.05 | 47 | 260 |
| cpk3-1              | 3.04 ± 0.03 | 1.98 ± 0.12 | 35 | 60  |
| cpk3-2              | 3.10 ± 0.02 | 2.17 ± 0.08 | 30 | 60  |
| cpk6-1              | 3.17 ± 0.11 | 1.99 ± 0.09 | 38 | 60  |
| cpk6-2              | 3.01 ± 0.12 | 1.84 ± 0.05 | 39 | 120 |
| cpk3-1cpk6-1        | 3.06 ± 0.16 | 2.52 ± 0.18 | 17 | 140 |
| cpk3-2cpk6-2        | 3.22 ± 0.21 | 2.91 ± 0.21 | 9  | 80  |

Data are presented as mean ± SEM.

n indicates number of stomata.

*p values were calculated with Student’s t-test (unpaired, double-tailed) by comparing closure with 10 µM ABA of the mutants against that of wild-type.

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Discussion

CPKs have been predicted to function in response to cytoplasmic Ca^{2+} elevations in many physiological processes in plants [33,34]. Many biochemical studies have suggested roles of CPKs in plant biology [36,40,41,43,45,48–50,74]. A dominant mutant study [37] and a biochemical study of CPK32 [49] have provided evidence for functions of CPKs in ABA signaling, and gene-silencing/antisense cdpk mutant phenotypes were demonstrated for a plant defense response in tobacco [36] and for nodule formation in Medicago truncatula root hairs [39]. The relative dearth of genetic or reverse genetic CDPK loss-of-function mutation phenotypes may be attributed to partial redundancies in the functions of the large CDPK gene family. Furthermore, cell-specific and mechanistic protein regulation (e.g., ion channel) analyses allow resolution of quantitative single cpk gene disruption mutant phenotypes, as demonstrated here.

Our findings do not rule out the possibility that the CPKs analyzed here may function in other pathways in other plant tissues. Single cell-type microarray studies have shown that relatively few genes are expressed in a strictly cell-specific manner in Arabidopsis root cell types, guard cells, and mesophyll cells [52,54]. Combinatorial usage of a single protein in different signaling pathways was initially documented in yeast [75]. Furthermore, studies of mitogen-activated protein kinase and protein phosphatase genes in plants have suggested that individual mitogen-activated protein kinases and protein phosphatases function in different signal transduction cascades in different tissues and under different conditions [55,76–79] (for review: [80]), and therefore the present study does not exclude that CPK3 and

Figure 7. Ca^{2+}-Reactive Closure Is Impaired in cpk3cpk6 Double Mutant Stomata

Four [Ca^{2+}]_{cyt} transients (inset) were imposed in wild-type (WT) (n = 6 experiments) and cpk3-1cpk6-1 double mutant (n = 6 experiments) stomata. Individually mapped stomatal apertures were measured for the last 30 min before imposed [Ca^{2+}]_{cyt} transients (before Time = 0) and for the ensuing 180 min at the indicated time points. Error bars represent SEM. Inset (top) shows imposed [Ca^{2+}]_{cyt} transients in wild-type (blue trace) and in cpk3-1cpk6-1 (red trace) guard cells expressing Yellow Cameleon 3.6 (RU: ratio units).

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CPK6 have additional functions in other tissues. The present study demonstrates important functions of the CPK3 and CPK6 CDPKs in control of stomatal movements and in ABA and Ca\(^{2+}\) regulation of guard cell S-type anion channels and unexpectedly also in ABA regulation of Ca\(^{2+}\)-permeable IC\(_{Ca}\) channels.

Although many studies have shown Ca\(^{2+}\)-dependent steps in guard cell signal transduction and in plant ion channel regulation [81], Ca\(^{2+}\) sensor-encoding genes that function as positive transducers in these responses have remained unknown. A calcineurin-like Ca\(^{2+}\) sensor, CBL1/SCaBP5, has been reported to function as a negatively regulating Ca\(^{2+}\) sensor in ABA signaling in guard cells ([26], but see [82]). Knock-out mutation of another CBL, CBL9, resulted in ABA hypersensitive phenotypes in seed germination, root and shoot growth, and ABA-inducible gene expression in Arabidopsis, indicating its function as a negative regulator of ABA signaling [27]. In the present study, guard cell expression analyses of CDPK genes aided in narrowing the number of candidate genes that may function in this system. Furthermore, the guard cell system has also allowed us to pursue detailed mechanistic and cellular analyses revealing functions of specific CDPKs in planta.

**CDPKs Function in Ca\(^{2+}\)-Reactive Stomatal Closure and S-Type Anion Channel Activation**

[Ca\(^{2+}\)]\(_{cv}\) elevation in guard cells causes a rapid “Ca\(^{2+}\)-reactive” stomatal closure response, irrespective of the above threshold Ca\(^{2+}\) elevation pattern [29]. In contrast, a long-term “Ca\(^{2+}\)-programmed” stomatal closure is modulated by imposed Ca\(^{2+}\) transient parameters [29-31]. Ca\(^{2+}\)-dependent stomatal movements were analyzed in response to imposed Ca\(^{2+}\) transients. In cpk3cpk6 double mutant plants, Ca\(^{2+}\)-reactive stomatal closure was significantly reduced compared to wild-type (Figure 7). However, the partial Ca\(^{2+}\) transient-induced stomatal closing response of cpk3cpk6 double mutant plants was maintained in white light more than 2 h after Ca\(^{2+}\) transients were terminated. Thus, the imposed long-term Ca\(^{2+}\)-programmed stomatal closure response [29] appears to be functional in the cpk3cpk6 double mutant, whereas the rapid Ca\(^{2+}\)-reactive stomatal closure response is clearly impaired (Figure 7). The presented data provide genetic evidence for a mechanistic separation of Ca\(^{2+}\)-reactive and Ca\(^{2+}\)-programmed stomatal closure. Several other CDPK transcripts are expressed in guard cells as determined by oligonucleotide-based microarray experiments [54]. It is possible that other guard cell–expressed CDPKs function in Ca\(^{2+}\)-programmed stomatal closure.

Previous studies have provided evidence that phosphorylation events function in Ca\(^{2+}\) activation of S-type anion channels [16,32] (see Introduction). The cytosolic Ca\(^{2+}\) activation of S-type anion channels requires the presence of hydrolyzable ATP in the patch-clamp electrode, and this channel activation is abolished by the general serine/threonine protein kinase inhibitors K252a and staurosporine [16]. The finding that Ca\(^{2+}\) activation of S-type anion channels is impaired in cpk3 and cpk6 gene disruption mutants provides molecular genetic evidence for this model and suggests that CDPKs function in [Ca\(^{2+}\)]\(_v\) perception upstream of S-type anion channel activation (Figure 9). The cpk6–1 and cpk6–2 alleles exhibit similar defects in all of the phenotypes that were examined, suggesting that both alleles are strong alleles. Although both alleles show no expression of full-length cDNA, a faint band representing residual transcript of the CPK6 open reading frame was amplified in the cpk6–2 allele (8% or less of wild-type level). However, the cpk6–2 transcript is lacking the wild-type origin of transcription. Furthermore, this transcript also lacks the first exon of the wild-type cDNA, and 5’ UTRs have been shown to play roles in enhancing translational efficiency [83]. Together, these data indicate that the greatly reduced level of cpk6–2 transcripts and the absence of the first exon are sufficient to cause the strong phenotypes observed in this study. Future direct investigation of the model that CDPKs phosphorylate important targets during S-type anion channel activation will require identification of the molecular components that encode these ion channels or upstream regulators. CPK3 and CPK6 may have distinct functions in the upstream Ca\(^{2+}\) channel regulation pathways. The present findings point to a model in which Ca\(^{2+}\) activation of S-type anion channels functions in the rapid Ca\(^{2+}\)-reactive stomatal closure response (Figures 2, 7, and 9) [29].

**CDPKs, R-Type Anion Channels, and Ca\(^{2+}\)-Independent Signaling**

A Ca\(^{2+}\)-independent ABA signal transduction branch has been implicated in guard cells, based on data in which ABA-induced cytosolic Ca\(^{2+}\) increases were not observed in guard cells [13,16,25,28,84-86] (for reviews: [2-4,87]). Ca\(^{2+}\)-dependent or -independent ABA signaling pathways have been reported to be emphasized depending on physiological conditions in Commelina communis [25]. The abolishment of ABA-induced stomatal closing by injection of the Ca\(^{2+}\)-chelator BAPTA into guard cells [86,88] indicates that a Ca\(^{2+}\)-independent branch within the ABA signaling network would interact with Ca\(^{2+}\)-dependent mechanisms. The present study provides direct molecular genetic evidence that Ca\(^{2+}\) sensors function within the Arabidopsis guard cell ABA signaling network (Figures 3, 4, and 6A) and provides a genetic basis to analyze interactions with a Ca\(^{2+}\)-independent pathway.

Several studies have indicated a role for R-type anion channel regulation [81], Ca\(^{2+}\) (black trace) and cpk3-1cpk6–1 double mutant (gray trace).

**Figure 8. R-Type Anion Channel Currents in cpk3cpk6 Double Mutant and Wild-Type Guard Cells**

(A) Representative traces of R-type anion channel current in wild-type (black trace) and cpk3-1cpk6–1 double mutant (gray trace).

(B) Averages of peak currents of R-type anion channel currents in wild-type (white bar, n = 7) and cpk3-1cpk6–1 double mutant (black bar, n = 7 ± SEM) guard cells.

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CDPKs in Guard Cell Signaling

Figure 9. Simplified Models for Early ABA Signal Transduction Network and CPK3 and CPK6 Functions in Guard Cells

Plasmalemma 

Channels are regulated in a parallel CPK3-CPK6-dependent signal transduction pathway. The arrow connecting R-type and S-type anion channels indicates that these channels may share molecular components [89]. See Discussion for details.

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Residual small S-type anion channel currents were consistently observed in cpk3cpk6 double mutant guard cells (Figures 2B, 2C, 3C, and 3D), which should also contribute to partial ABA- and Ca2+-induced stomatal closing. The rate of anion efflux from Arabidopsis guard cells can be estimated, to predict whether the residual anion currents in cpk3cpk6 double mutants can close stomata within a physiological response. Cell volume of Arabidopsis GCPs was estimated at 0.11 pL according to the average diameter of protoplasts (6 μm). An estimated Cl− concentration decrease from 400 mM to 100 mM during ABA-induced stomatal closure would correspond to Cl− efflux of 33.9 fmol/cell. The membrane potential of guard cells treated with ABA was approximately −50 mV [73]. Cl− efflux currents in wild-type and cpk3cpk6 double mutant guard cells at −50 mV were approximately −60 pA and −25 pA, respectively (Figure 3E). These S-type anion currents correspond to Cl− efflux rates of 37.3 fmol/min for wild-type and 15.54 fmol/min for the double mutant. This estimate shows that during the slow onset of drought that occurs over several days in intact plants, the residual S-type and R-type anion currents in cpk3 cpk6 double mutants should be sufficient for a physiological stomatal closing response. Similarly, previous estimates have predicted that ion channel activities are over 10-fold higher in biological membranes than required for a typical response, due to their high transport rate [90]. This estimate also highlights the power of analyzing individual ion channel targets for characterizing CDPK functions in plants.

It is possible that additional CDPKs contribute to activation of the residual S-type anion current activity. The partial Ca2+-induced stomatal closure in the cpk mutants (Figures 6B and 7) can be explained by Ca2+-activated residual S-type anion channel currents. In summary, the lack of R-type anion channel activity alteration and the residual S-type anion current in cpk3 cpk6 mutant guard cells (Figures 2, 3, and 8) correlate with the partial ABA- and Ca2+-induced stomatal closing responses found here (Figures 6, 7, and 9) and may also reflect a contribution of parallel Ca2+-independent and/or pH-dependent (91,92) signaling mechanisms.

CDPK Mutants Impair ABA Activation of Ca2+-Permeable Channels

Unexpectedly, ABA activation of Ica channel currents was impaired in cpk3cpk6 double mutant guard cells (Figure 4). Impairment in ROS signaling partially impairs rather than abolishes ABA-induced stomatal movement responses, consistent with their activity mainly at hyperpolarized voltages [53]. The present findings are consistent with a model in which additional signaling branches function in the ABA signal transduction network parallel to the ABA → ROS → Ica signaling branch (Figure 9) [65]. Several parallel pathways function in the ABA signaling network, including intracellular Ca2+-release mechanisms (Figure 9) [15,17,18,81].

Interestingly, in wild-type guard cells, ABA could activate Ica channels even when cells were preexposed to the general serine/threonine kinase inhibitor K252a and the cytosol of guard cells was dialyzed with an ATP-free solution for longer than 15 min in whole-cell recordings prior to ABA exposure (Figure 4) and 4K). K252a inhibits CDPK activities in diverse plants [45,93–95] and abolishes Ca2+-activated activity of S-type anion channels in guard cells [16,32]. These data indicate the following possible mechanisms by which the cpk3cpk6 mutant.
alleles may impair ABA activation of \( I_{\text{Ca}} \) channels in guard cells (Figure 4).

1. CPK3 and CPK6 may not be directly activated in response to exogenous ABA within the ABA → NADPH oxidase → ROS → \( I_{\text{Ca}} \) channel signaling branch (Figure 9). But these CDPKs are necessary for maintaining \( I_{\text{Ca}} \) channel activation in a parallel regulation pathway of \( I_{\text{Ca}} \) channels [96] that also regulates S-type anion channels (Figure 9B). In this model, ABA regulation of S-type anion channels is not strictly downstream of \( I_{\text{Ca}} \) channels (Figure 9B).

2. CPKs may regulate \( I_{\text{Ca}} \) channels in a feedback loop by which cytosolic Ca\(^{2+}\) elevations regulate \( I_{\text{Ca}} \) channels (Figure 9A).

3. The present findings can also be explained by an alternative model in which \( eph3\) mutations affect gene expression in such a manner that ABA activation of \( I_{\text{Ca}} \) channels is impaired. Similarly, the effects of the dominant \( abi1-1 \) and \( abi2-1 \) protein phosphatase 2C (PP2C) mutants on ABA responses could in principle result from indirect effects of these mutations on these ABA signal components, because these are dominant mutations and no pharmacological PP2C inhibitors are available to test effects of short-term PP2C impairment on channel regulation, as previously discussed [66]. This model in which \( eph3\) may affect transcriptional or translational responses would be consistent with the activation of \( I_{\text{Ca}} \) currents in patch-clamped wild-type Arabidopsis guard cells without added cytoplasmic ATP and in the simultaneous presence of K252a (Figure 4f and 4k).

Experiments in V. \textit{faba} guard cells show different responses than wild-type \textit{Arabidopsis} guard cells that may shed light on the impairment in \( I_{\text{Ca}} \) channel activation in \( eph3\) guard cells. In \textit{V. fava} guard cells, K252a inhibits ABA activation of \( I_{\text{Ca}} \) currents and the protein phosphatase inhibitors calyculin A and okadaic acid activate \( I_{\text{Ca}} \) channel currents, suggesting that phosphorylation/dephosphorylation events regulate \( I_{\text{Ca}} \) channels in a parallel pathway in V. \textit{faba} [96] (Figure 9). The present findings in \textit{Arabidopsis} guard cells would predict a requirement for CDPK-dependent phosphorylation, prior to ABA activation of the ABA → NADPH oxidase → ROS → \( I_{\text{Ca}} \) channel regulation branch (Figures 4 and 9B). Previous analyses have indicated that \textit{Arabidopsis} and \textit{Vicia} guard cells emphasize different components of the guard cell signaling network, including differential phosphorylation-dependent responses [32,53,59,86,97,98]. In this respect, further comparative analyses among species should be useful in illuminating new signaling network mechanisms and branches. Further studies will be necessary to distinguish the above models. The present study, however, provides a first characterization of a genetic mutation that impairs both Ca\(^{2+}\) activation of S-type anion channels and ABA activation of Ca\(^{2+}\)-permeable \( I_{\text{Ca}} \) channels, indicating closer interactions among mechanisms that regulate these two classes of ion channels in guard cells than previously modeled.

Conclusions

In summary, in this study, we provide direct molecular genetic evidence for Ca\(^{2+}\) sensors that function as positive transducers in stomatal ABA signaling and demonstrate functions of CPK6 and CPK3 in plant ion channel regulation in defined guard cell signal transduction elements. We functionally characterize disruption mutations in the two guard cell–expressed CDPK genes, CPK3 and CPK6. These CDPKs function in ABA and Ca\(^{2+}\) activation of S-type anion channels and in ABA regulation of Ca\(^{2+}\)-permeable \( I_{\text{Ca}} \) cation channels but not in R-type anion channel activity in guard cells. Furthermore, \textit{eph3}\(\textit{hph6}\) double mutants partially impair ABA-induced stomatal closure and Ca\(^{2+}\)-reactive stomatal closure but not long-term Ca\(^{2+}\)–programmed stomatal closure. Partial stomatal closing responses and differential regulation of R- and S-type anion current activities in \textit{eph3}\(\textit{hph6}\) mutant guard cells are consistent with models proposing parallel signaling mechanisms in a branched guard cell signal transduction network. Future cell-type–specific analyses of CDPKs may illuminate further cellular functions of this protein kinase family in plants.

Materials and Methods

Plant growth. \textit{Arabidopsis thaliana} plants (Columbia ecotype) were grown in soil (Sungro Special blend Professional Growing Mix; Seha Beach, Alberta, Canada) in a growth room under a 16-h-light/8-h-dark cycle at a photon fluence rate of 75 µmol m\(^{-2}\) s\(^{-1}\) and a temperature of 20 °C.

Identification of CDPK genes expressed in guard cells. Degenerate oligomer-based RT-PCR was used to identify guard cell–expressed CDPK genes from guard cell–enriched cDNA libraries [55]. Degenerate oligomers were designed from two highly conserved regions that were selected from aligned CDPK peptide sequences: HRDLKPFENF and DGE(K/R)(D/N)(Y/F)/(E/S)EF. The degenerate oligomers used to amplify guard cell–expressed CDPK genes are as follows: 5’-cayggyggyiataacgagary-3’ and 5’-caattacttiytiikicrtc-3’. Total RNA was extracted from guard cell–enriched epidermal strips as described [55] with the TRIzOL reagent (Invitrogen, Carlsbad, California, United States). cDNA was synthesized using the First-Strand cDNA Synthesis Kit (Amersham-Pharmacia Biotech, Little Chalfont, United Kingdom). PCR was performed as described [53]. PCR products were cloned into the pGEM-T Easy vector (Promega, Madison, Wisconsin, United States). The sequences of the cloned PCR products were determined by regular sequencing reactions. Homozygous \( eph3 \) and \( ephh \) single and double mutants were identified using gene-specific primers and primers that match T-DNA sequences as described previously [53,55].

Cell-type–specific expression analyses and RT-PCR. GCPs and mesophyll cell protoplasts were isolated by enzymatic digestion from 4-wk-old \textit{Arabidopsis} plants as described in [54]. Total RNA was extracted from GCPs and mesophyll cell protoplasts and reverse-transcribed with the TRIzOL reagent. Gene-specific primers for CPK3 and CPK6 were localized downstream of the DNA insertion sites as indicated (Figure 1B). For analyses of \textit{KATI} (AGI No.: At1g46240), CBP (AGI No.: At4g3950), and \textit{ACTIN2} (AGI No.: At3g18780) mRNAs, gene-specific primers were used for amplification.

Patch-clamp analyses. S- and R-type anion and \( I_{\text{Ca}} \) channel currents in \textit{Arabidopsis} guard cells were recorded as published previously and described below (S-type; [9], R-type and \( I_{\text{Ca}} \); [65]). Patch-clamp data were recorded using an Axopatch 200 amplifier and pClamp software and analyzed with Axograph software (Axon Instruments, Union City, California, United States).

To measure whole-cell S-type anion currents, the pipette solution contained 150 mM CaCl\(_2\), 2 mM MgCl\(_2\), 6.7 mM EGTA, 5 mM MgATP, 10 mM HEPES-Tris (pH 7.1), and 100 mM CaCl\(_2\) to result in 2 mM free Ca\(^{2+}\). The bath solution contained 30 mM CaCl\(_2\), 2 mM MgCl\(_2\), 1 mM CaCl\(_2\), and 10 mM MES-Tris (pH 5.6). Guard cells were extracellularly preincubated in the same solution with 40 mM CaCl\(_2\) added prior to patch-clamping (Figure 2), as previously described [9]; except for in ABA regulation analyses in which guard cells were preincubated in a solution containing 1 mM Ca\(^{2+}\) (Figure 3). Pretreatment of protoplasts with 40 mM CaCl\(_2\) allows analyses of Ca\(^{2+}\) activation of S-type anion channel currents in \textit{Arabidopsis} (see [9] for details), and pretreatment with 1 mM extracellular Ca\(^{2+}\) allows analyses of ABA regulation of S-type anion channels while cytosolic Ca\(^{2+}\) is elevated, as previously shown [55]. Osmolarity of the solutions was adjusted with sorbitol to 500 and 485 mmol kg\(^{-1}\), respectively. The liquid junction potential was 0.5 mV. The membrane voltage was stepped from the holding potential of +35 mV to −145 mV for 40 s in −30-mV decrements [5]. The interpulse period was 12 s. No leak.
subtraction was performed. Recordings were made 7 to 10 min after access to the whole-cell configuration. For ABA activation of S-type channels, ABA was added to the bath solution prior to patch-clamping and seal formation. To test the robustness of ABA insensitivity in cpk mutant guard cells, 50 μM ABA was applied to guard cells.

To measure ICa channel currents, the pipette solution contained 10 mM BaCl2, 0.1 mM DTT, 4 mM EGTA, and 10 mM HEPES-Tris (pH 7.1), and the bath solution contained 100 mM BaCl2, 0.1 mM DTT, and 10 mM MES-Tris (pH 5.6). Guard cells were not preinoculated in 40 mM CaCl2 in these experiments. Osmolarity of the solutions was adjusted with sorbitol to 500 and 485 mmol kg⁻¹, respectively. NADPH (5 mM) was added to the pipette for the measurement of ABA-activated ICa currents. ABA was added extracellularly 16 min after whole-cell recordings began. In typical experiments, activation of ICa was observed within 1 min of ABA addition and continued for longer than 30 min. The voltage was ramped from 0 to -198 mV (after liquid junction potential correction) with a ramp speed of 180 mV s⁻¹. In a standard measurement, the ramp protocol was applied 16 times to obtain an average current for a cell. The interpulse period was 1 min. When H₂O₂ (5 mM) activation of ICa was measured, the same procedures, analysis of average currents, and timing of application were used as for ABA regulation experiments.

To measure R-type anion channel currents, the pipette solution contained 75 mM K₂SO₄ [99], 2 mM MgCl₂, 5 mM EGTA, 2.5 mM CaCl₂, and 10 mM HEPES-Tris (pH 7.2) and the osmolarity was adjusted to 500 mmol kg⁻¹. The bath solution contained 50 mM CaCl₂, 2 mM MgCl₂, and 10 mM MES-Tris (pH 5.6), and the osmolarity was adjusted to 485 mmol kg⁻¹ with sorbitol. The voltage was ramped from the holding potential of 0 mV to -200 mV with a ramp speed of -20 mV s⁻¹. Significance of differences between data sets was assessed by noncoupled double-tailed Student’s t-test analysis.

Stomatal movement analyses. To measure changes in stomatal aperture, excised rosette leaves from 3- to 4.5-week-old Arabidopsis plants were floated on 3 ml of opening buffer (5 mM KCl, 50 μM CaCl₂, and 10 mM MES-Tris [pH 5.6]) for 2.5 h at 20°C under light (photon flux rate of 125 μmol m⁻² s⁻¹), so that the opening solution was taken up through petioles. After 2.5 h, ABA or CaCl₂ was added to the opening buffer. For Ca²⁺-induced stomatal closing assays, opening solution without added CaCl₂ was used. Leaves were incubated for an additional 3 h, and in the case of cpk3-2 cpk6-2, for 3 h and for 45 min (Figure 6), after addition of Ca²⁺. Similar impairment in Ca²⁺-induced stomatal closure was found in cpk3-2 cpk6-2 double mutant plants compared to wild-type after both 45 min. and 3 h. Furthermore, similar stomatal responses were observed in cpk3-2 cpk6-2 plants 45 min. and 3 h after stimulation in parallel experiments (p > 0.50 at 0 μM CaCl₂, p > 0.50 at 0 μM CaCl₂, p > 0.45 at 100 μM CaCl₂ (45 min versus 3 h cpk3-2 cpk6-2 data). Leaves were blended in opening solution in a Waring commercial blender (Waring Commercial, Torrington, Connecticut, United States) for 30 s. The blended material was filtered on a 100-μm nylon mesh, and epidermal strips were placed onto a microscope slide with a glass coverslip. The ratio of stomatal width to length was measured with ImageJ (National Institutes of Health, Bethesda, Maryland). Stomatal length was measured as the distance between the inner walls of the stomata, and length was measured as the length of the guard cells [100,101]. Data from stomatal ratios mirrored stomatal width data (Figure 6). For each sample, 20 to 30 stomata were measured. Blind experiments were also conducted, in which the genotype of leaves (wild-type or cpk3 cpk6 mutant and the added stimuli) was unknown to the experimenter.

Imposed Ca²⁺ oscillation responses. To measure imposed Ca²⁺ oscillation–induced stomatal closure, epidermal peels were prepared by using Hollister medical adhesive (Stock No. 7777; Hollister Inc., Libbyville, Illinois, United States) to attach a leaf abaxial side down onto a coverslip. A razor blade was used to carefully remove the cuticle and mesophyll layers of the leaf, leaving the lower leaf epidermal layer containing stomatal complexes intact. The coverslip was then sealed with glue to the bottom of a custom microscope slide with a 2-cm-diameter hole cut in the center, creating a 200-μl well. The well was then filled with depolarizing buffer (50 mM KCl and 10 mM MES-Tris [pH 5.6]), and the leaf segment was incubated for 2.5 h at 20°C to open stomata. [Ca²⁺]cyt transients were imposed by rapid exchange of the chamber buffer between depolarizing buffer and hyperpolarizing buffer (1 mM KCl, 1 mM CaCl₂, 10 mM MES-Tris [pH 5.6]). Depolarizing buffer decreases [Ca²⁺]cyt levels, whereas hyperpolarizing buffer increases [Ca²⁺]cyt levels [68]. For each experiment, four separate transients with a 0.5 μM CaCl₂ concentration of 1, respectively. The time of onset of imposed oscillations was known to the experimenter.

Cytosolic Ca²⁺ concentration changes during imposed [Ca²⁺]cyt transient experiments were confirmed by using the improved Yellow Cameleon 3.6 (YC3.6) fluorescence reporter [102]. Col-0 and cpk3-1 cpk6-1 plants were transfected with YC3.6. Based on calibration measurements, the imposed Ca²⁺ transients using YC3.6 corresponded to average cytoplasmic Ca²⁺ baseline levels of approximately 0.15 μM and average peak levels of approximately 0.6 μM. The imposed oscillation protocol that was applied subjects cells to large changes in both the external Ca²⁺ and Cl⁻ concentrations. Earlier versions of cameleon were sensitive to the Cl⁻ concentration, as Cl⁻ affects the pH sensitivity of the yellow fluorescent protein chromophore [103]. In the present study, YC3.6 was used, which also has substantially reduced sensitivity to the interacting Cl⁻ ions and a greatly enhanced ratio changes in response to physiological [Ca²⁺]cyt changes [102]. Previous work with YC2.1 showed that the ratio changes observed during imposed oscillations are due to the changes in calcium, not changes in Cl⁻, as imposing the same chloride changes while removing extracellular Ca²⁺ does not produce any YC2.1 ratio changes (supplemental data to [29]; http://www.nature.com/nature/journal/v411/n6841/extref/4111053aa.html). In these experiments, extracellular Ca²⁺ was chelated with 10 mM EGTA, which lowers extracellular Ca²⁺ levels, to physically inhibit Ca²⁺ influx that would otherwise occur at strongly hyperpolarized potentials. In further controls, replacing CaCl₂ with MgCl₂ in the bath did not produce cameleon ratio changes [106]. Further control experiments were performed with the new reporter YC3.6 in which Ca²⁺ changes were imposed but not Cl⁻ changes, by using the impermeant counteranion iminodiacetate (Figure 4). Stomata were extracellularly perfused alternately with solutions containing either 100 mM K⁺ iminodiacetate (no added CaCl₂) (pH 6.13) or 1 mM K⁺ iminodiacetate and 1 mM Ca²⁺ iminodiacetate (pH 5.6). These controls showed YC3.6 changes were imposed at the absence of imposed Cl⁻ changes, demonstrating that Ca²⁺ changes are being reported (Figure 10), as previously shown for YC2.1 [29,104,105].

Germination assays. Germination assays were performed as described previously [107]. Mutant and wild-type seeds were harvested at the same time and then sterilized using chlorine. Sterilized seeds were plated on 1/2 MS medium containing MES (0.5 g/L) (pH 5.7), supplemented with 0, 0.3, 1, and 5 μM ABA. Plates were
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