Two calcineurin B-like calcium sensors, interacting with protein kinase CIPK23, regulate leaf transpiration and root potassium uptake in Arabidopsis

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Summary
Calcium signalling involves sensor proteins that decode temporal and spatial changes in cellular Ca^{2+} concentration. Calcineurin B-like proteins (CBLs) represent a unique family of plant calcium sensors that relay signals by interacting with a family of protein kinases, designated as CBL-interacting protein kinases (CIPKs). In a reverse genetic screen for altered drought tolerance, we identified a loss-of-function allele of CIPK23 as exhibiting a drought-tolerant phenotype. In the cipk23 mutant, reduced transpirational water loss from leaves coincides with enhanced ABA sensitivity of guard cells during opening as well as closing reactions, without noticeable alterations in ABA content in the plant. We identified the calcium sensors CBL1 and CBL9 as CIPK23-interacting proteins that targeted CIPK23 to the plasma membrane in vivo. Expression analysis of the CIPK23, CBL1 and CBL9 genes suggested that they may function together in diverse tissues, including guard cells and root hairs. In addition, expression of the CIPK23 gene was induced by low-potassium conditions, implicating a function of this gene product in potassium nutrition. Indeed, cipk23 mutants displayed severe growth impairment on media with low concentrations of potassium. This phenotype correlates with a reduced efficiency of K^+ uptake into the roots. In support of the conclusion that CBL1 and CBL9 interact with and synergistically serve as upstream regulators of CIPK23, the cbl1 cbl9 double mutant, but not the cbl1 or cbl9 single mutants, exhibit altered phenotypes for stomatal responses and low-potassium sensitivity. Together with the recent identification of the potassium channel AKT1 as a target of CIPK23, these results imply that plasma membrane-localized CBL1– and CBL9–CIPK23 complexes simultaneously regulate K^+ transport processes in roots and in stomatal guard cells.

Keywords: calcium, calcium sensor, protein kinase, abscisic acid, drought, potassium uptake.

Introduction
Water, minerals and CO_2 are minimal requirements for plant survival in natural habitats. Water and minerals are largely acquired from the soil through roots, and CO_2 is obtained from the air by leaf cells. Both water and minerals are transported from roots to shoots via a xylem stream powered by transpiration through stomatal pores that mediate CO_2–water exchange. Therefore, stomatal movements orchestrate the transport of water and minerals throughout the plant and CO_2 uptake for photosynthesis (Blatt, 2000; Fan et al., 2004; Schroeder et al., 2001a,b). Because plants consist of at least 80% water, water availability is crucial for plant survival. As a consequence, plants have evolved effective mechanisms for detecting and responding to water deficiency. Under drought conditions, plant roots (and other tissues) produce elevated levels of abscisic acid (ABA), a phytohormone that induces stomatal closure to reduce water loss (Fan et al., 2004; Luan, 2002; Schroeder et al., 2001a,b). ABA-induced stomatal closure represents a major mechanism for plant adaptation to drought. This reaction prioritizes water preservation at a cost...
of less CO₂ uptake (or less photosynthesis) and reduced mineral transport through the transpiration stream.

Effective mineral uptake and transport not only depends on plant transpiration but also on the availability of sufficient amounts of essential ions in the soil. Lack of minerals in the soil, although not an as acute condition as water deficiency, is also detrimental for plant growth (Kochian, 2000; Reid and Hayes, 2003). Research in recent years has provided important insights into the molecular mechanisms underlying uptake and transport of major minerals such as potassium, phosphorus and nitrogen salts (Grotz and Guerinot, 2002; Reid and Hayes, 2003; Reintanz et al., 2002; Very and Sentenac, 2003). However, little is known about potential signaling mechanisms that allow plants to detect and properly respond to mineral deficiency. Interestingly, a recent study reported that low potassium levels in the soil trigger elevated production of H₂O₂ in roots, which serves as a signal to alter the expression of downstream genes (Shin and Schachtman, 2004). Remarkably, H₂O₂ also functions as a critical second messenger in guard-cell drought signaling. In these cells, ABA-induced generation of H₂O₂ results in the activation of calcium channels in the plasma membrane (Kwak et al., 2003; Pei et al., 2000). In addition, ABA-induced changes in Ca²⁺ concentration can also be driven by influx across the plasma membrane via hyperpolarization-activated Ca²⁺ channels in the plasma membrane (Hamilton et al., 2000), by cADPR- and InsP₃-mediated release from the vacuole (Leckie et al., 1998; Lee et al., 1990), and by Ca²⁺-induced Ca²⁺ release (McAinsh et al., 1995), potentially through TPC1 or other cation channels (Peiter et al., 2005). Similar to the situation in leaf guard cells, H₂O₂ production in root cells also results in specific spatial and temporal changes in Ca²⁺ concentration (Evans et al., 2005; Foreman et al., 2003).

A large number of other signals, including plant hormones, light, abiotic stress factors, and pathogenic or symbiotic elicitors, induce transient and specific changes in intracellular Ca²⁺ (Bush, 1995; Ehrhardt et al., 1996; Knight et al., 1991, 1996, 1997; McAinsh et al., 1997; Neuhaus et al., 1997; Sanders et al., 1999, 2002; Trewavas and Knight, 1994). Recent studies suggest that a Ca²⁺ signal is represented by spatial and temporal information, including Ca²⁺ localization and oscillation (Allen and Schroeder, 2001; Dolmetsch et al., 1998; Franklin-Tong et al., 1996; Holdaway-Clarke et al., 1997; Li et al., 1998; Trewavas, 1999). The combination of these parameters constitutes the ‘signature’ of Ca²⁺ changes triggered by a specific signal. In recent years, it has become increasingly appreciated that the signaling components that ‘decode’ these Ca²⁺ signatures hold the key that links the Ca²⁺ parameters to specific cellular responses (Hetherington and Woodward, 2003; Luan et al., 2002; Sanders et al., 2002).

Several families of Ca²⁺ sensors contribute to decoding the intracellular calcium changes in higher plants. Ca²⁺-dependent protein kinases (CDPKs) contain calmodulin-like Ca²⁺-binding domains and a kinase domain in a single protein, and have therefore been classified as ‘sensor responders’ (Cheng et al., 2002; Harmon et al., 2000; Sanders et al., 2002). With regard to their function in stomatal regulation, a recent reverse genetic analysis has uncovered a critical role for CPK3 and CPK6 in regulating guard-cell aperture during responses to environmental stimuli (Mori et al., 2006). Among the Ca²⁺ ‘sensor relay’ proteins, which upon Ca²⁺ binding interact with their target proteins and thereby regulate their activity, members of the calcineurin B-like (CBL) protein family have been recognized as fulfilling critical functions in diverse Ca²⁺-dependent processes in plants (Cheong et al., 2003; Hwang et al., 2005; Kolukisaoglu et al., 2004; Kudla et al., 1999; Liu and Zhu, 1998; Pandey et al., 2004). Protein interaction studies have revealed that these calcium sensors (with at least 10 members in Arabidopsis) target a specific family of at least 25 serine/threonine protein kinases, designated as CBL-interacting protein kinases (CIPKs) (Albrecht et al., 2001; Hrabak et al., 2003; Kim et al., 2000; Shi et al., 1999), forming a network-like signaling system for specific and synergistic stimulus–response coupling (Albrecht et al., 2001; Batistic and Kudla, 2004; Luan et al., 2002; Zhu, 2003). Reverse genetics analyses of single components of this signaling network have greatly advanced our understanding of their physiological functions and uncovered diverse signal–response reactions as mediated by CBLs and CIPKs. For example, the calcium sensor CBL1 mediates multiple stress responses by interacting with several CIPKs (Albrecht et al., 2003; Cheong et al., 2003). CBL9 is closely related to CBL1 but fulfils a unique role in distinct signaling processes including response to ABA and osmotic stress (Pandey et al., 2004). A recent reverse genetics analysis of the protein kinase CIPK1 revealed that alternative complex formation of this kinase with either CBL1 or CBL9 controls abscisic acid-dependent and -independent stress responses in Arabidopsis (D’Angelo et al., 2006). Meanwhile, the SOS3/CBL4-SOS2/CIPK24 pathway has been shown to be involved in mediating salt tolerance (reviewed in Zhu, 2003). These data suggest that, depending on its CBL interaction partner, a kinase can regulate distinct signal–response pathways. Consequently, CBL–CIPK-mediated decoding of Ca²⁺ signatures represents a major paradigm in plant signaling that involves a high level of functional specificity and flexibility in information processing.

In order to understand the functional significance of each module of the CBL–CIPK network, we have taken a systematic reverse genetics approach to investigate the function of the CBL and CIPK genes. Here we report the identification of CIPK23 as a major component controlling ABA-regulated drought tolerance in Arabidopsis. Our genetic, physiological and cell biological studies indicate that CIPK23 fulfils this function by interaction with the calcium sensors CBL1 and CBL9, which appear to synergistically regulate this kinase. We also found that CBL1–CIPK23 and CBL9–CIPK23...
complexes regulate K⁺ uptake into roots, consistent with recent reports that CIPK23 and its interacting calcium sensors (CBL1 and CBL9) regulate the potassium channel AKT1 (Li et al., 2006; Xu et al., 2006). Taken together, our results suggest that a CBL–CIPK pathway regulates both drought and low-potassium responses in Arabidopsis, and thereby functions as a calcium-decoding mechanism regulating distinct but closely related physiological processes.

Results

Characterization of cipk23 as a drought-tolerant mutant

In a systematic effort to dissect the potential function of the CBL–CIPK network in plant drought-stress tolerance, we characterized all available single mutant alleles for CBL and CIPK genes in comparative drought-stress assays using adult plants cultivated in soil (for details, see Experimental procedures). This approach resulted in the identification of one mutant line with significantly enhanced drought tolerance when compared to the other T-DNA mutant and wild-type lines. This mutant line represented a T-DNA insertional allele in the CIPK23 gene (At1g30270), which was isolated from the Salk T-DNA mutant collection (http://signal.salk.edu/; cipk23-1 is SALK_032341). To further investigate the role of the CIPK23 gene in the regulation of drought tolerance, we isolated and characterized a second independent mutant allele of CIPK23 from the Torrey Mesa Research Institute T-DNA mutant collection (cipk23-2 is SAIL402F05). Both alleles were in the Col-0 background. Sequence analysis of both flanking regions of the T-DNA revealed that the T-DNA insertion site of cipk23-1 was located in the 4th intron (962 bp downstream from the ATG), and that the insertion site of cipk23-2 was located in the 7th intron (1644 bp downstream from the ATG) (Figure 1a). CIPK23 mRNA was not detectable by RT-PCR in homozygous plants of either T-DNA allele (Figure 1b), indicating that T-DNA insertions inside the gene disrupted the expression of CIPK23 transcripts. When grown under well-watered conditions, neither cipk23 mutant displayed significant phenotypic alterations. We therefore comparatively analysed cipk23-1 and cipk23-2, as well as wild-type plants, in drought assays, and found that both mutant lines exhibited enhanced drought tolerance compared to wild-type plants (Figure 1c). The collective results from three independent experiments are shown in Figure 1(d). Under the drought conditions used in this study, approximately 70% of cipk23 mutants survived, whereas wild-type plants had a much lower survival rate (approximately 20%). The consistent phenotypes of the two independent mutant lines (cipk23-1 and cipk23-2) demonstrate that increased drought tolerance in cipk23 mutants is caused by loss of CIPK23 gene function.

Stomatal movements in cipk23 mutants are hypersensitive to ABA

The degree of drought tolerance is determined by at least three molecular and cellular parameters in plants. Several
studies have correlated drought tolerance with the level of stress-induced gene expression (Cheong et al., 2003; Shinozaki and Yamaguchi-Shinozaki, 2000; Thomashow, 1999). Other studies have established that high levels of ABA may lead to increased drought tolerance (reviewed in Shinozaki and Yamaguchi-Shinozaki, 2000). Alternatively, or in addition, the ABA responsiveness of stomatal opening or closure movements, which control water loss through transpiration, can modify the drought tolerance of plants (Allen et al., 1999; Schroeder et al., 2001a,b). To determine which of these processes is altered in the drought-tolerant cipk23 mutants, we first analysed the expression levels of several stress gene markers in wild-type and cipk23 mutant plants. As shown in Figure 2(a), these analyses did not show significant differences in the level of marker gene expression in response to ABA and drought-stress conditions, indicating that alterations in stress gene expression may not be the major reason for altered drought tolerance in cipk23 mutant plants. Subsequently, we measured the ABA content of wild-type and mutant plants under normal and drought conditions. Again, the mutant and wild-type appeared to have similar ABA levels under both normal and drought treatment conditions (Figure 2b). When we compared the rates of water loss from rosette leaves, we observed that both cipk23-1 and cipk23-2 mutant leaves lost water significantly more slowly than the wild-type leaves (Figure 2c), suggesting that the increased tolerance to drought might result from altered leaf transpiration.

To determine whether the cipk23 mutations affect the responsiveness of guard cells to ABA, stomatal responses to various concentrations of ABA were investigated. When wild-type, cipk23-1 and cipk23-2 plants were incubated under light at high humidity for 2.5 h to allow stomata to open fully, no significant difference was detected among the various plant lines in the size of stomatal pores (WT, 3.37 ± 0.34 μm; cipk23-1, 3.11 ± 0.31 μm; cipk23-2, 3.01 ± 0.45 μm; n = 90, P < 0.05). However, after treatment with 5 μM ABA for 2.5 h, the size of stomatal pores was reduced more dramatically in cipk23-1 and cipk23-2 plants than in wild-type plants (Figure 3a). Stomatal apertures of wild-type plants were decreased to 81% (2.74 ± 0.30 μm) of that without ABA treatment, and in cipk23-1 and cipk23-2 plants, apertures were decreased to 70% (2.19 ± 0.29 μm) and 66% (1.98 ± 0.25 μm), respectively. The stomatal apertures in cipk23-1 and cipk23-2 were reduced to 50% (1.58 ± 0.21 μm) and 52% (1.56 ± 0.22 μm), respectively.

Figure 2. Analyses of stress gene marker expression, ABA content and transpirational water loss in the wild-type and cipk23-1 and cipk23-2 mutants.
(a) Expression of stress-responsive gene markers in wild-type (Col-0), cipk23-1 and cipk23-2 plants after drought and ABA (100 μM) treatment.
(b) ABA levels in wild-type (Col-0), cipk23-1 and cipk23-2 plants with or without drought treatment. The ABA level was determined in 3-week-old plants grown in the greenhouse. Drought treatment was conducted by dehydrating the leaves for 5 h. The results shown are the mean and SD of two independent experiments.
(c) Kinetics of water loss from detached leaves of wild-type (open circles), cipk23-1 (closed circles) and cipk23-2 (open squares). Water loss is presented as the percentage weight loss versus initial fresh weight. Values are means ± SD of three independent experiments. Asterisks represent statistically significant differences.
by treatment with 20 µM of ABA, whereas in the wild-type they was only reduced to 70% (2.36 ± 0.18 µm). Similarly, the cipk23 mutants were also hypersensitive to ABA inhibition of light-induced stomatal opening (Figure 3b). These data indicate that loss of function of CIPK23 alters the ABA responsiveness of guard cells during their opening and closure, leading to reduced water loss under drought conditions.

In contrast to the results obtained by the investigation of stomatal aperture regulation, the cipk23 mutations did not affect the degree of seed dormancy or the sensitivity to exogenous ABA during seed germination (data not shown), indicating that CIPK23 does not function (or its functional contribution is redundant) in ABA responsiveness in developmental processes other than stomatal movements.

**CBL1 and CBL9 interact with and recruit CIPK23 to the plasma membrane**

Each CIPK is regulated by interaction with one or more CBL-type calcium sensors (Albrecht et al., 2001; Ishitani et al., 2000; Kudla et al., 1999; Luan et al., 2002; Shi et al., 1999). In our previous yeast two-hybrid assays, CBL1 and CBL9 both interacted with CIPK23 (Kolukisaoglu et al., 2004). It therefore appeared conceivable that both CBL1 and CBL9, which exhibit a very high sequence similarity, interact with and regulate the function of CIPK23.

To determine whether CBL1 and CBL9 interact with CIPK23 in plant cells, we first examined the cellular localization of the CBLs and CIPK23 using a GFP fusion in transiently transformed *Nicotiana benthamiana* protoplasts (Figure 4a). Localization of CBL1–GFP or CBL9–GFP fusion protein was observed at the plasma membrane. In contrast, CIPK23–GFP or CIPK23 lacking the CBL-interacting NAF domain were localized to the cytosol and nucleus. CBL1 and CBL9 both contain a myristoylation site at their N-terminus, a lipid modification that enhances the association of modified proteins with cell membranes. We predicted that, by interaction with either CBL protein, CIPK23 may be recruited to the plasma membrane. We tested this idea by using bimolecular fluorescence complementation (BiFC) in *N. benthamiana* protoplasts (Walter et al., 2004). In these assays, both CBL1–CIPK23 and CBL9–CIPK23 complexes were localized to the plasma membrane, as indicated by the production of fluorescence upon protein–protein interaction (Figure 4b).

It is known among researchers using the BiFC procedure that sometimes ‘empty splits’ (without fusion to the interacting proteins) produce certain levels of fluorescence when expressed in various systems. Therefore, the best negative control is the fusion of the YFP splits with mutant versions of the ‘test’ proteins that can no longer interact. In this case, CBL–CIPK23 interaction depends on the presence of the NAF domain in the CIPK protein. We therefore performed a negative control experiment using the NAF-deletion form of CIPK23 fused to the YFP split, and did not observe significant fluorescence signals in cells co-expressing CIPK23DNAF together with CBL1 or CBL9 (Figure 4b). The expression of all YFP fusion proteins in these assays was confirmed by Western blot analyses using monoclonal antibodies against the fusion tags (Walter et al., 2004) (data not shown). These data demonstrate that CBL1 and CBL9 interact with CIPK23 and recruit the kinase to the plasma membrane where the substrate(s) of CIPK23 may reside (Li et al., 2006; Xu et al., 2006).
The expression profile of CIPK23 overlaps with that of CBL1 and CBL9

The protein–protein interaction studies suggested that CBL1, CBL9 and CIPK23 may function together in similar pathways. To gain further insights into the function of these proteins, we examined the expression pattern of all three genes in plant tissues. Earlier results on CBL1 and CBL9 genes revealed that these two calcium sensors were strongly expressed in plant seedlings and some adult organs including leaves and flowers (Cheong et al., 2003; Pandey et al., 2004). RT-PCR analysis of CIPK23 transcripts in various tissues indicated expression of CIPK23 in roots, leaves, stems, flowers and siliques of adult Arabidopsis plants.

Figure 4. CBL1 and CBL9 interact with and recruit CIPK23 to the plasma membrane. (a) Fusions of GFP with CBL1, CBL9, CIPK23 or CIPK23::NAF (CIPK23 without the NAF domain) were localized in the protoplasts. Bright-field (left) and fluorescence images (right) were taken from the same protoplast for each protein localization assay. (b) BiFC images of CBL1–CIPK23 and CBL9–CIPK23 interactions. CIPK23 without the NAF domain did not interact with CBL1 or CBL9. The same protoplasts are shown as bright-field images on the left.

Figure 5. Histochemical analysis of CIPK23, CBL1 and CBL9 promoter–GUS expression in transgenic plants. (a) RT-PCR analysis of CIPK23 transcripts in various organs of Arabidopsis plants. Total RNA was isolated from various tissues (root, leaf, stem, flower and siliques) of 4-week-old wild-type plants grown under long-day conditions. RT-PCR was performed with either CIPK23-specific primers (top gel) or Actin2-specific primers (bottom gel). (b) Expression of CIPK23 (left), CBL1 (middle) and CBL9 (right) promoter–GUS fusions in leaf guard cells. (c) Expression of CIPK23, CBL1 or CBL9 promoter–GUS fusions in the vascular system of a rosette leaf (left), a seedling (middle) and the roots (right). (d) RT-PCR analysis of CBL1, CBL9 and CIPK23 gene expression under control (10 mM KCl) and low-potassium (20 μM KCl) conditions in 2-week-old seedlings.
(Figure 5a). Based on these results, CBL1, CBL9 and CIPK23 genes appeared to be ubiquitously expressed throughout plants at various developmental stages. To further compare the expression pattern of CIPK23 with that of CBL1 and CBL9 genes, we analysed transgenic plants harbouring β-glucuronidase (GUS) reporter constructs whose expression was driven by each of the gene promoters using five independent lines of each transgenic construct. Because CIPK23 regulates transpiration and the ABA response in guard cells, we first focused on GUS expression in stomatal guard cells. As shown in Figure 5(b), CIPK23, CBL1 and CBL9 promoters were all active in guard cells. We subsequently observed other tissues and organs and found that the activity of the CIPK23 gene promoter was also very high in roots and vascular tissues of leaves (Figure 5c), overlapping with the expression pattern of the CBL1 and CBL9 genes (Cheong et al., 2003; Pandey et al., 2004). It is noteworthy that CIPK23 and CBL9 (but not CBL1) also appeared to be expressed in the root hairs. It is possible that CBL1 is also expressed in root hairs but at levels beyond the limits of detection by the promoter–GUS approach. Alternatively, the CBL1 promoter construct used here may not include the necessary cis-acting element, which may be located in the 5′ region of the gene.

The overlapping expression profiles of CBL1, CBL9 and CIPK23 are consistent with the interaction between the two CBLs and their target kinase CIPK23. Moreover, the expression of all three genes is not restricted to guard cells, strongly suggesting that these genes may fulfil critical functions in other physiological processes in various tissues of the plant.

As CIPK23 plays a role in drought stress and ABA responses, we examined the expression of CIPK23 under drought, salt, cold and ABA treatment. Unlike CBL1 and CBL9, which are induced by ABA and stress conditions (Cheong et al., 2003; Pandey et al., 2004), these conditions did not appear to alter the expression level of CIPK23 transcripts or promoter–GUS activity (data not shown). In addition, we investigated the expression of all three genes in response to several adverse stress conditions that may relate to the strong expression of these genes in roots. Interestingly, we found that expression of the CIPK23 gene, but not of CBL1 or CBL9, was induced under conditions of low-K⁺ nutrition (Figure 5d). This could suggest that the amount of CIPK23 may be a limiting factor under low-K conditions. As K⁺ transport is a major function of roots and a component of stomatal movement, expression of all three genes in both guard cells and roots appears to be functionally relevant. This prompted us to investigate the respective mutants under conditions of low-K⁺ availability.

cipk23 mutants are hypersensitive to low potassium levels and defective in K⁺ uptake

When grown on media containing low levels of KCl (micromolar range), cipk23 mutants displayed drastically reduced stature compared to the wild-type. In particular, the roots of mutant plants were significantly shorter than those of wild-type plants (Figure 6). With 20 mM KCl (equivalent to the K⁺ concentration of full-strength MS salts), cipk23 mutant seedlings were indistinguishable from the wild-type.
However, under lower potassium concentrations (micromolar to low millimolar levels), the growth of cipk23 plants was inhibited to a significantly greater extent than the wild-type (Figure 6a). Figure 6(b) shows that root growth in the cipk23 mutant seedlings was significantly more inhibited under low-potassium conditions until the potassium level reached 5 mM. These results demonstrate that CIPK23 plays a critical role in plant growth under low K⁺ concentrations. In most soils, the potassium concentration is below 5 mM (Maathuis and Sanders, 1996), suggesting that CIPK23 functions in potassium nutrition under physiological conditions.

Potassium nutrition is regulated at multiple levels, including acquisition and utilization (Horie and Schroeder, 2004; Very and Sentenac, 2003). If K⁺ acquisition is altered in cipk23 mutant plants, the K⁺ content in these plants may be different from that in the wild-type. Indeed, lower K⁺ contents were observed in mutant seedlings when compared with the wild-type (about 25% less, Figure 6c). This result suggests that K⁺ uptake and accumulation from the medium are less efficient in cipk23 mutant plants. We determined the capacity for K⁺ uptake by measuring ⁸⁶Rb⁺ accumulation into cipk23 mutants and wild-type seedlings (see Experimental procedures). An important consideration here is that plants must be cultured in full-strength MS medium so that mutant and wild-type plants grow equally well (with the same stature) before performing uptake assays. The ⁸⁶Rb⁺ tracer experiments demonstrated that K⁺ uptake in cipk23 mutant seedlings was significantly reduced (Figure 7). The uptake rate for K⁺ in cipk23 mutants was only 60–70% of that in the wild-type when the external K⁺ concentration was in the micromolar range (20 and 200 μM; Figure 7a,c). Similar differences were seen at various time points during a 2 h period. However, the difference in K⁺ uptake rate between cipk23 and the wild-type became insignificant when high external K⁺ (20 mM) was used (Figure 7b). These results are consistent with the data from the root growth assay in Figure 6, and illustrate that cipk23 mutants are defective in K⁺ uptake, especially under low-potassium conditions. These findings are consistent with results from earlier studies (Li et al., 2006; Xu et al., 2006).

CBL1 and CBL9 function synergistically in regulating stomatal movements and potassium nutrition

Having established that CBL1 and CBL9 can interact with CIPK23 in plant cells and that both calcium sensors mediate CIPK23 plasma membrane recruitment, we sought to investigate the functional importance of these protein interactions. Earlier genetic analysis of cbl1 and cbl9 single mutants showed that CBL1 and CBL9 each have unique functions in stress and ABA responses (Albrecht et al., 2003; Cheong et al., 2003; Pandey et al., 2004). Particularly relevant is the finding that CBL1 regulates drought stress without changing the stomatal response to ABA. Similarly, the cbl9 single mutant does not show any altered stomatal response to ABA despite its strong ABA hypersensitivity during seed germination (Pandey et al., 2004). Our observation that CBL1 and CBL9 both interact with CIPK23, a regulator of stomatal responses, suggested that CBL1 and CBL9 may synergistically regulate CIPK23 function in ABA-dependent stomatal closure. To address this hypothesis, we generated a cbl1 cbl9 double mutant from cbl1
(Cheong et al., 2003) and cbl9 (Pandey et al., 2004) single mutants (Figure 8a). In homozygous plants of the cbl1 cbl9 double mutant, transcripts of CBL1 and CBL9 were not detectable by RT-PCR, indicating disruption of both CBL1 and CBL9 expression.

We analysed the phenotypic consequences of the loss of CBL1 and CBL9 functions using drought tolerance and water-loss assays. The cbl1 and cbl9 single mutants were in the Ws and Col-0 ecotype backgrounds, respectively, and different ecotypes may contain genetic variations leading to phenotypic changes in physiological processes. For example, the Ler ecotype contains a mutated ERECTA gene that functions in regulating transpiration efficiency (Masle et al., 2005). As a result, Ler and Col-0 display significant difference in the transpiration efficiency (Masle et al., 2005). Therefore, in order to compare the wild-type and the double mutant constructed from different ecotype backgrounds, we performed comparative control analyses to ensure that the phenotypes of interest are not significantly variable among the two ecotypes and their hybrid. We constructed hybrid lines of the two wild-types (Ws x Col), and included these ecotype hybrid plants, the cbl1 cbl9 double mutant, the Ws

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and Col-0 single wild-types, and cbl1 and cbl9 single mutants in the phenotypic analysis. In addition, we also included an alternative cbl1 cbl9 double mutant that was created using single mutants from the same Col-0 background.

To examine drought tolerance of the cbl1 cbl9 double mutant, we compared the wild-type Col-0 and cbl1 cbl9 double mutant (in Col-0 background, Xu et al., 2006) under drought-stress treatment. Upon withholding of water, the wild-type plants became more severely wilted compared with the cbl1 cbl9 double mutant (Figure 8b). In the three experiments conducted in parallel, the wilting phenotype was observed in 44 out of 46 plants for wild-type Col-0, and eight out of 48 for the cbl1 cbl9 double mutant. In the water-loss assays, we found that the cbl1 cbl9 double mutant lost water significantly more slowly than the ecotype hybrid plants, single mutants and single ecotype wild-type (Figure 8c,d). The wild-types Ws and Col-0, the Ws and Col-0 ecotype hybrid, and the single mutants did not show significant differences in the water-loss assays (Figure 8c), suggesting that genetic variations in the two ecotypes may not affect the water-loss process. Furthermore, at a later stage of our investigations, we corroborated these results with another cbl1 cbl9 double mutant (Xu et al., 2006). This line, which was generated using different single mutant alleles from the Col-0 background, exhibited a similar difference from wild-type plants (Figure 8f). Although the actual values for water loss cannot be compared directly between the Figure 8f and d as the experiments were performed separately using different plant materials, the difference between the wild-type and mutant plants was consistent. These data clearly indicate that the reduced water-loss rate in the cbl1 cbl9 double mutant is a result of disruption of both CBL1 and CBL9.

We also examined stomatal responses to ABA. When the ecotype hybrid control and cbl1 cbl9 plants were incubated under light at high humidity for 2.5 h to allow stomata to open fully, the size of stomatal pores was similar among all plant lines (Ws × Col-0, 3.14 ± 0.41 μm; cbl1 cbl9, 3.05 ± 0.38 μm; n = 90, P < 0.05, respectively). However, after 2.5 h incubation with 5 μM ABA, the stomatal pore width was reduced more in the cbl1 cbl9 plants than in the control plants (Figure 8e). The stomatal apertures of wild-type plants were decreased to 81% (2.57 ± 0.35 μm) of the initial opening in the presence of 5 μM ABA, and in cbl1 cbl9 plants they were decreased to 71% (2.13 ± 0.35 μm). Experiments with the second cbl1 cbl9 double mutant also showed an ABA-hypersensitive response in the double mutant (Figure 8g), supporting the conclusion that CBL1 and CBL9 function synergistically in stomatal responses to ABA. This provides genetic evidence for the functional significance of the physical interaction of CBL1 and CBL9 with CIPK23.

As our analyses had established a synergistic function of CBL1 and CBL9 in guard cells, a function similar to their target kinase CIPK23, we subsequently investigated whether cbl1 cbl9 double mutants display a low-K⁺-sensitive phenotype as observed in the cipk23 mutants. As shown in

![Figure 9](image-url)
Figure 9, the phenotype of the cbi1 and cbi9 single mutants was similar to the wild-type under either high- or low-potassium conditions. In contrast, the growth of the cbi1 cbi9 double mutant was significantly more inhibited than the growth of wild-type roots under low-K+ conditions (Figure 9a–c). As in the water-loss assay, the ecotypes Ws and Col-0, their hybrid and the single mutants did not show a significant difference in their response to low K concentrations (Figure 9c), suggesting that ecotype backgrounds did not contribute to the phenotypic changes observed in the double mutant. In addition, the K+ contents in the seedlings of cbi1 cbi9 mutants were significantly lower (about 30% less) than those in the control plants when cultured under low-K+ conditions (Figure 9d), indicating that CBL1 and CBL9, like CIPK23, participate in the regulation of K+ nutrition, especially under low-K conditions. While this paper was in preparation, Xu et al. (2006) reported that their double mutant cbi1 cbi9 constructed from cbi1 and cbi9 single mutants different from those used in our study also displayed low-K sensitivity and low K content, consistent with the observations reported here. Taken together, the results from the genetic analysis of the cbi1 cbi9 double mutant support the conclusion that CBL1 and CBL9 both act as upstream regulators of CIPK23 in both stomatal movement and potassium uptake.

Discussion

Our study has revealed that the protein kinase CIPK23 is expressed in diverse cell types and tissues, and regulates distinct physiological processes, including stomatal opening/closure in leaves and potassium uptake in roots. Furthermore, the kinase CIPK23 interacts with and functions together with the two calcium sensors CBL1 and CBL9, providing a molecular link between intracellular calcium fluctuations and the regulation of transpiration and mineral nutrition. Both CBL1 and CBL9 can recruit CIPK23 to the plasma membrane, suggesting that plasma membrane-associated CIPK23–CBL complexes modulate membrane-localized target proteins, including the AKT1 potassium channel (Li et al., 2006; Xu et al., 2006) by protein phosphorylation.

Dynamic and variable CBL–CIPK complex formation enables regulation of diverse Ca2+-dependent processes

Our study provides new insights into mechanistic aspects of plant calcium signaling. Despite recent studies suggesting that calcium changes induced by different signals are distinct in their temporal and spatial patterns, the 'specificity' of calcium codes remains largely a mystery. Recent studies on the CBL–CIPK network have provided new insights into the functional specificity of calcium fluctuations: calcium signals are decoded by a specific set or combination of calcium sensors and effectors that are present in a specific cell type, leading to specific responses in these cells (Cheong et al., 2003; Luan et al., 2002). Consequently, even though fluctuations in calcium concentration may be identical in cell A and cell B, the specificity of the response reaction in these two cell types depends on the specification of calcium sensors and effectors. Identical responses only occur if the two cell types experience the same calcium changes and contain exactly same set of calcium signaling components. The differences and similarities in these components in various cell types result in differences or overlaps in responses. Accordingly, the results from this study corroborate the principle that the identity of response reactions depends on the repertoire of signaling components and the complexity of their interactions. The same component of calcium signaling such as CIPK23 may be present in multiple cell types (guard cells and root hairs), but its 'combination' with other, cell-specific signaling components or alternative phosphorylation substrates determines the specificity of the responses in each cell type. Combination of CIPK23 with a specific set of other components in guard cells results in regulation of the stomatal response to ABA, whereas CIPK23 and another set of components in root tissues participate in regulation of potassium uptake. As CIPK23 is also present in other tissues such as the vascular tissues of roots, stems and leaves, we speculate that it may function together with other components in those tissues as well, for example in the long-distance transport and distribution of K+ throughout the whole plant.

Our study showed that the other components that interact with CIPK23 include the calcium sensors CBL1 and CBL9 that functionally overlap in regulating stomatal movement and K+ nutrition. It is possible that other CBLs may also interact with CIPK23 in the regulation of K+ nutrition. Such selective and overlapping interactions may code for unique responses that are different from any single CBL–CIPK interaction. Among the CBLs that regulate a specific CIPK in the same process, some may play a more dominant role than others. For instance, the function of CIPK23 in the stomatal response and K+ nutrition appear to be mainly regulated by CBL1 and CBL9, each of which functions in other processes by regulating other CIPKs (Cheong et al., 2003; D’Angelo et al., 2006; Pandey et al., 2004). The functions of CBL1 and CBL9 appear to be overlapping in the processes of stomatal regulation and K+ acquisition, but their functions in other processes such as abiotic stress responses and ABA sensitivity during germination do not seem to overlap (Albrecht et al., 2003; Cheong et al., 2003; Pandey et al., 2004). Importantly, alternative complex formation of either CBL1 or CBL9 with the protein kinase CIPK1 controls ABA-dependent and -independent abiotic stress response reactions (D’Angelo et al., 2006). Taken together, the CBL–CIPK complexes represent a versatile network for signaling of defined cellular processes depending on the combination of CBLs and CIPKs.
Different CBL–CIPK complexes regulate ABA responses in different cell types

This study shows that CIPK23 regulates drought tolerance, at least in part by modifying ABA sensitivity in guard cells. In the context of the ABA response and calcium signaling specificity, it is interesting to compare the finding on CIPK23 with the results of an earlier study on CIPK3. Both CIPK3 and CIPK23 are protein kinases regulated by calcium via interaction with CBL calcium sensors, and both regulate ABA signaling. However, their modes of action are clearly different. CIPK3 regulates ABA sensitivity during seed germination but not in guard cells (Kim et al., 2003), whereas CIPK23 regulates ABA sensitivity in guard cells but not during seed germination. Such differences provide important clues on ABA signaling in various tissues. The fact that different CIPKs participate in ABA signaling in different tissues indicates that the ‘wiring’ of the ABA signaling pathways in different cell types is distinct. One aspect contributing to such differences could be the expression pattern of particular components of the ABA signaling network. Indeed, CIPK3 is expressed in germinating seeds and young seedlings (and functions in ABA responses in germination), whereas CIPK23 is expressed in guard cells and roots (and is shown here to regulate stomatal movements and potassium uptake). However, also important for the generation of specificity is the repertoire of interacting CBLs that regulate CIPK activity. CIPK3 interacts with CBL9, and CIPK23 interacts with both CBL9 and CBL1 (Kolukisaoglu et al., 2004; Figure 4). As the cbl9 single mutant displayed a similar phenotype to the cipk3 mutant during seed germination, it is proposed that CIPK3 interaction with CBL9 controls the functionality of CIPK3 in the ABA response during seed germination. This idea is supported by our recent double mutant analyses of CBL9 and CIPK3 (G.K.P., Y.H.C. and S.L., unpublished results). The fact that CIPK23 interacts with CBL9 and yet the cipk23 mutant is not altered in seed germination responses could be explained by the absence of CIPK23 expression in the seeds or the presence of other CIPKs that can functionally substitute loss of CIPK23 function in these tissues. Both CBL1 and CBL9 are expressed in guard cells. However, neither the cbl1 mutant nor cbl9 single mutants showed any change in ABA sensitivity in guard cells (Cheong et al., 2003; Pandey et al., 2004). This suggests an overlapping function of these two CBLs and/or other CBLs that are also involved in regulation of the guard-cell response to ABA. These results again support the same principle: the possibly similar changes in calcium elicited by the same signal (in this case ABA) are decoded by a different combination of sensors and effectors in different cell types leading to distinct responses (Batistic and Kudla, 2004; Luan et al., 2002). In this regard, it is important to note that CBL–CIPK complexes are not the only components decoding calcium signals in guard cells. A recent study has reported the importance of the CDPKs CPK3 and CPK6 in the positive regulation of ABA-dependent responses in guard cells. Remarkably, as reported here for the cbl1 cbl9 double mutant, only a cpk3 cpk6 double mutant exhibited discernable alterations in ABA responses. These similar findings may indicate the robustness of the protein network decoding Ca²⁺ signals in guard cells (Hetherington and Woodward, 2003). While CDPKs appear to be positive regulators of ABA signaling, our results indicate a negative regulatory function of CBL1/CBL9 and CIPK23, suggesting a yet to be investigated additional level of complexity and interconnection in calcium-decoding systems in guard cells (Figure 10).

Identical CBL–CIPK complexes enable the regulation of distinct Ca²⁺-dependent physiological processes

Despite the fact that CIPK23 regulates distinct processes in various organs, it is important to consider the connection between these processes. The uptake of potassium and other minerals from the soil by plant roots provides mineral nutrition to the whole plant. In order to distribute minerals to the upper parts of plants, long-distance transport of the minerals must take place through the xylem stream, powered by transpiration through stomatal pores. Coordination between transpiration and potassium uptake and transport has long been recognized (Very and Sentenac, 2003), but the underlying mechanism(s) remains unknown. It is interesting to note that the two processes are both regulated by a calcium-regulated protein kinase, because both ABA and low K⁺ concentrations have been shown to

Figure 10. The CBL–CIPK pathway regulates both stomatal movement and low-K tolerance. Drought/ABA and low-K concentrations may produce reactive oxygen species (ROS) that trigger calcium fluctuations. CBLs bind calcium and interact with CIPK23 that activates AKT1 and possibly other channels (?) involved in K uptake and turgor regulation. Other CIPKs may also be involved in the regulation of AKT1. The other branch of calcium signaling in guard cells involves CDPKs that activate anion channels and inhibit stomatal opening.
elicit production of H₂O₂ (and other second messengers in guard cells), which activates calcium influx (Foreman et al., 2003; Pei et al., 2000; Shin and Schachtman, 2004). It is therefore reasonable to propose that calcium changes elicited by ABA and low K⁺ concentrations are sensed by CBL1 and CBL9, and these in turn activate CIPK23. The myristoylation sites in the CBL1 and CBL9 proteins are capable of targeting CBL–CIPK complexes to the plasma membrane where K⁺ transporters (and other transporting proteins) reside. Potential phosphorylation of membrane transporters may be critical for K⁺ acquisition under low-potassium conditions. Because guard cell turgor is regulated by K⁺ fluxes, potential targets of CIPK23 in the guard cells may also be K⁺ transporters. A number of K⁺ transporter genes, such as the CBL and CIPK genes investigated in this study, are expressed in multiple cell types. For example, the genes for some voltage-dependent K⁺ channels, including KAT1, AKT1 and AKT2/3, are expressed in both roots and guard cells, although their functions have not been studied in both cell types. It has been recently reported that K⁺-channel activity in roots may be controlled by phosphorylation (e.g. Michard et al., 2005), but very little is known about how potassium transporters in guard cells are regulated by protein phosphorylation. While this study was under preparation, two studies (Li et al., 2006; Xu et al., 2006) reported that CIPK23 interacts with and activates the AKT1K⁺ channel, providing a mechanism for CBL–CIPK regulation of the low-K⁺ response and K⁺ uptake in roots (Hedrich and Kudla, 2006). It is possible that AKT1 may also serve as a target for CIPK23 in the regulation of transpiration. Alternatively, different or additional ion transporters may be regulated by CIPK23 in stomatal guard cells (Figure 10). These possibilities remain to be investigated in future studies.

**Experimental procedures**

**Plant materials and RNA analysis**

Plants (Arabidopsis thaliana ecotype Columbia-0) were grown in a greenhouse under long-day conditions (16 h light/8 h dark cycle) to flowering stage for plant transformation and RNA analysis. For RNA isolation from plants under various stress conditions, 3-week-old seedlings grown on MS medium (Murashige and Skoog, 1962) were used. Sterilized seeds were plated on MS medium solidified with 0.8% agar. For abscisic acid (ABA) treatment, 100 μM (±)-cis-trans-ABA was sprayed on the seedlings to ensure total coverage of the foliage area. The plants were incubated at room temperature under white light. For analysis of drought-induced gene expression, 4-week-old plants grown in soil were carefully removed and dehydrated on filter paper as described by Yamaguchi-Shinozaki and Gooden (1994). For low-K⁺ treatment, 10-day-old Arabidopsis seedlings grown on MS medium were transferred to MS medium (10 mM K) or the low-K⁺-containing liquid medium (20 μM K) for various times as indicated in Figure 6(c). Total RNA was isolated from Arabidopsis tissues using the TRIzol reagent (Roche Applied Science; http://www.roche-applied-science.com). Aliquots of individual PCR products were resolved by agarose gel electrophoresis and visualized with ethidium bromide under UV light.

**Isolation of T-DNA insertion mutants**

The cipk23 mutants were isolated from the SALK (http://signal.salk.edu; cipk23-1 is SALK_033241) and Torrey Mesa Research Institute (cipk23-2 is SAIL420F05) T-DNA insertion collections. The T-DNA borders of cipk23-1 and cipk23-2 alleles were defined by sequencing PCR products obtained using T-DNA border primers, and gene-specific primers. The T-DNA insertion in the mutants (cipk23-1 and cipk23-2) was confirmed by PCR and DNA gel-blot analysis, and its exact position was determined by sequencing. After selfing of heterozygous plants, homozygous cipk23 mutants were identified by genomic PCR to confirm disruption of the endogenous gene and by RT-PCR to confirm disruption of gene expression. To obtain the cbl1 cbl9 double mutant, cbl1 in the Ws background (Cheong et al., 2003) and cbl9 in the Col-0 background (Pandey et al., 2004) were crossed. We identified cbl1 cbl9 double mutants by antibiotic selection and RT-PCR analysis using CBL9-specific forward (5′-GATGATGGGGAGTGAGTAATATCAGAA-3′) and reverse (5′-GTCACCCACTGGAATATACGAATC-3′) primers or CBL1 gene-specific forward (5′-GGGCTGCGGATGTAATATCAGA-3′) and reverse (5′-GCAATCTCAGATCGAATGC-3′) primers. The expression of Actin-2 was monitored using forward (5′-GGGAAAGATCGTGACTGC-3′) and reverse (5′-GTCACCCACTGGAATATACGAATC-3′) primers to serve as a quantifying control. Hybrids ofWs and Col-0 wild-type plants were produced along the course of cbl1 cbl9 double mutant construction. The second cbl1 cbl9 double mutant in the Col-0 background was provided by Dr. W. Wu (China Agricultural University, China) and is described by Xu et al. (2006).

**Drought treatments, water-loss assays and ABA measurements**

For the drought assays, watering was withheld from 3-week-old soil-grown plants for specified time periods. To minimize experimental variations, the same number of plants in each comparison group was grown in the same tray or pot. Drought treatment was performed in a greenhouse under long-day conditions (16 h light/8 h dark cycle, 100 μmol m⁻² sec⁻¹, 21–23°C, 70% relative humidity). Watering was withheld from 3-week-old plants for 18 days before resuming watering. Photographs were taken, and the survival rate was determined on the 7th day after resuming watering unless specified otherwise. To determine the water loss, six leaves from 3-week-old plants grown in the greenhouse were detached from each plant and placed in a weighing dish. Dishes were kept on the laboratory bench (light intensity 100 μmol m⁻² sec⁻¹, 21–22°C, approximately 40% relative humidity) for various time periods, and the loss of fresh weight was determined. All stress assays were performed in three independent experiments, and mean values and
standard errors are presented. Three-week-old plants grown on soil were used for measurement of plant ABA levels with or without drought treatment. Plant tissues were then harvested and immediately frozen in liquid nitrogen and ground to powder. ABA was extracted and measured as described by Xiong et al. (2001). Powdered tissue (1 g) was suspended in 15 ml of extraction buffer containing 80% methanol, 100 mg l\(^{-1}\) of butylated hydroxytoluene and 0.5 g l\(^{-1}\) of citric acid monohydrate. The suspension was stirred overnight at 4°C, and centrifuged at 1000 g for 20 min. The ABA concentration in the solution was determined using the Phytodetek ABA immunoassay kit (Agdia; http://www.agdia.com).

### Analysis of CIPK23 promoter–GUS expression in transgenic plants

To generate the CIPK23 promoter–GUS construct, the 5′ flanking DNA of the CIPK23 coding region was amplified using forward (5′-CCGGGGAATATACAAGCTTGTAAGAAAAGTC-3′) and reverse (5′-GATCCTGGATACGTCGTTTCAGAGAAAGGAGCCTCA-3′) primers. A 1770 bp PCR fragment was cloned into the HindIII–BamHI sites (underlined in the primer sequences) in the pB101.1 vector (Clontech, http://www.clontech.com/). The construct was transformed into Arabidopsis plants (Clough and Bent, 1998), and transformants were selected on 50 μg ml\(^{-1}\) kanamycin. 70 transgenic seedlings were stained with 5-bromo-4-chloro-3-indolyl-D-glucuronide for 12 h, followed by incubation in 80% ethanol to remove chlorophyll (Jefferson et al., 1987).

### Protein interaction and localization studies in planta

Deletion of the NAF domain in the CIPK23 cDNA was achieved using a procedure described by Higuchi et al. (1989). Briefly, two standard PCRs were performed using the primer pairs CIPK23f for/CIPK23naf2 and CIPK23naf1/CIPK23rev with CIPK23 cDNA as a template. Both PCR products were purified using the E.Z.N.A. Cycle Pure kit from Peqlab (http://www.peqlab.de) and mixed 1:1 (mol mol\(^{-1}\)). The mixed PCR products were then used as template for a second PCR reaction using primers CIPK23f for and CIPK23rev. The PCR product was purified and inserted into pHBluescript plasmid using the SpeI–KpnI sites, and the correct deletion was confirmed by sequencing.

For in planta localization and BiFC studies, constructs CIPK23 and CIPK23nafN were amplified by PCR using primers CIPK23f for and CIPK23rev, and cloned into pSPYNE–35S and/or pGPTVII.GFP–Kan. CBL1 and CBL9 were amplified by PCR using primers CBL1f for/CBL1revGFP and CBL9f for/C176, respectively, and cloned via BamHI–XmaI sites into pSPYCE–35S and pGPTVII.GFP–Kan as described by Walter et al. (2004). All fusion constructs were verified by sequencing.

Infiltration of N. benthamiana leaves was performed as described previously (Walter et al., 2004). Protoplasts were prepared 3 days after infiltration by incubating leaf discs in 500 mM mannitol, 10 mM CaCl\(_2\), 5 mM MES/KOH (pH 5.5), 3% cellulase and 0.75% Mazerzyme. For documentation of the results, pictures were taken either with an inverted fluorescence microscope (Leica DMI6000B, Leica Microsystems; http://www.leica-microsystems.com), equipped with a Hamamatsu Orca AG camera (Hamamatsu Photonics; http://www.hamamatsu.com) and processed by Openlab Software (Improvision; http://www.improvision.com), or with an inverted microscope (Leica DMRE2) equipped with a Leica TCS SP2 laser scanning device.

In Figure 4, a typical single protoplast is shown for each localization pattern. In the transformed protoplast population (typically about 50 cells were examined in detail for each construct in each experiment), about 90% of the protoplasts (in the case of CBL1–CIPK23 or CBL9–CIPK23) showed the fluorescence pattern depicted in Figure 4. In the NAF deletion control experiment, 97% protoplasts did not show any detectable fluorescence. The remaining 3% of protoplasts showed traces of fluorescence. The intensity of the fluorescence in this 3% of cells was about 5% of that for the CBL–CIPK combination, i.e. very weak but detectable.

### BiFC analysis

The primers used in the cloning processes for BiFC analysis are shown in Table 1.

### Stomatal aperture bioassays

To measure stomatal closing, four rosette leaves from 4-week-old plants (grown under 8 h light/16 h dark at 22°C; 70% relative humidity) were detached and floated in stomatal opening solution (SOS: 50 mM KCl and 10 mM MES-KOH, pH 6.15, 10 μM CaCl\(_2\)) in the light (100 μmol m\(^{-2}\) s\(^{-1}\)) as described by Pei et al. (1997) with some modifications. After 2.5 h, buffer was replaced with fresh SOS containing ABA of varying concentrations. After 2.5 h further incubation, leaves were blended for 20 sec using a Waring blender in 100 ml of SOS solution containing the same concentration of ABA as used for incubation. The resulting epidermal fragments were filtered with a 30 μm nylon mesh, placed on a microscope slide, and covered with a cover slip. Stomatal opening experiments were performed in a similar fashion, except that leaves were harvested in darkness, and then incubated in SOS for 2.5 h in the dark. Leaves were then incubated in fresh SOS solutions containing the relevant concentration of ABA in the light (100 μmol m\(^{-2}\) s\(^{-1}\)) for a further 2.5 h before blending. Approximately 30 stomata were measured in each individual sample, and each experiment was performed in triplicate. A Zeiss Axiopt microscope (http://www.zeiss.com/) at 40× magnification was used to visualize samples, and the width of individual stomata was recorded. Standard errors were calculated relative to the square

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence</th>
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<tr>
<td>CIPK23for</td>
<td>TTTGGGCCCACTAGTATGGCTTCTCGAACAACGCTTC</td>
</tr>
<tr>
<td>CIPK23rev</td>
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</tr>
<tr>
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<tr>
<td>CIPK23naf2</td>
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</tr>
<tr>
<td>CBL1f for</td>
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<tr>
<td>CBL1revGFP</td>
<td>TTTCGCGGTTGGCAATCTCAGGCCAC</td>
</tr>
<tr>
<td>CBL9f for</td>
<td>TTCTAGAGGATCTCGAATGGGTTGTTCTCATTCCA</td>
</tr>
<tr>
<td>CBL9revGFP</td>
<td>TTTCGCGGCTGCAATCTCGTCCA</td>
</tr>
</tbody>
</table>

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root of the number of stomatal aperture experiments. Statistical significance was determined using Student’s t test and Excel software (version 2003; Microsoft; http://www.microsoft.com).

Growth measurement on media with variable potassium concentrations

For growth measurements of wild-type and cipk23 mutants with low potassium nutrition, seeds were plated onto a modified MS medium containing various concentrations of KCl (Liu and Zhu, 1997). Modified MS medium contains potassium-free 1/20th strength MS major salts and 1× MS minor salts. Potassium-free medium was prepared by replacing MS salts with the following: 1650 mg l⁻¹ NH₄NO₃, 440 mg l⁻¹ CaCl₂·2H₂O, 370 mg l⁻¹ MgSO₄·7H₂O, 165 mg l⁻¹ (NH₄)₂HPO₄, 27.8 mg l⁻¹ FeSO₄·7H₂O, 37.3 mg l⁻¹ disodium EDTA, 0.7495 mg l⁻¹ NaCl, 6.3 mg l⁻¹ H₃BO₃, 16.9 mg l⁻¹ MnSO₄·H₂O, 8.6 mg l⁻¹ ZnSO₄·7H₂O, 0.25 mg l⁻¹ Na₂MoO₄·2H₂O, 0.016 mg l⁻¹ CuSO₄·5H₂O and 0.0267 mg l⁻¹ CoSO₄·6H₂O. Varying levels of potassium in the medium were achieved by adding appropriate amounts of KCl to the K⁺-free medium. Ultra-pure agarose containing a negligible level of potassium was used to solidify the medium.

³⁶Rb⁺ uptake assay

For measurement of potassium uptake using ³⁶Rb⁺ as a tracer, we performed experiments as described by Wu et al. (1996) with some modifications. Seven-day-old seedlings from MS liquid cultures were transferred to K⁺-free medium for 2 days. Seedlings were collected, rinsed briefly in K⁺-free medium, and then placed into a 10 ml uptake solution containing K⁺-free medium supplemented with the indicated levels of KCl and 0.5 µCi ml⁻¹ ³⁶Rb⁺ (Amersham, http://www6.amershambiosciences.com/). Uptake was performed at room temperature under white fluorescent light at various K⁺ concentrations for 1 h. Determination of the time course of uptake at 20 µM or 200 µM K⁺ was performed for 30, 60, 90 and 120 min at completion of uptake, the seedlings were rinsed three times with 50 ml of ice-cold K⁺-free medium. The seedlings were blotted dry on filter paper and weighed, and the radioactivity was measured using a Beckman LS6500 scintillation counter (Beckman Instruments; http://www.beckman.com). Values for K⁺ uptake were obtained based on ³⁶Rb⁺ uptake and the external K⁺ concentration.

Determination of K⁺ content

For measurements of K⁺ content in plant tissues, 4-day-old seedlings of various mutants and wild-type Arabidopsis were transferred from MS medium to low-K⁺ medium and treated for 4 days. The seedlings were then collected, rinsed briefly with distilled water, dried at 65°C for 24 h, and weighed. The samples were digested with 1 N HNO₃ and then boiled for 30 min. The K⁺ concentrations were determined using an atomic absorption spectrophotometer (model 560, Perkin-Elmer).

Acknowledgements

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