

CIPK9: a calcium sensor-interacting protein kinase required for low-potassium tolerance in *Arabidopsis*

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Potassium is one of the major macro-nutrients essential for a number of cellular processes in plants. Low potassium level in the soil represents a limiting factor for crop production. Recent studies have identified potassium transporters that are involved in potassium acquisition, and some of them are critical for potassium nutrition under low potassium conditions. However, little is understood on the molecular components involved in low potassium signaling and responses. We report here the identification of a calcineurin B-like protein-interacting protein kinase (CIPK9) as a critical regulator of low potassium response in *Arabidopsis*. The *CIPK9* gene was responsive to abiotic stress conditions, and its transcript was inducible in both roots and shoots by potassium deprivation. Disruption of CIPK9 function rendered the mutant plants hypersensitive to low potassium media. Further analysis indicated that K⁺ uptake and content were not affected in the mutant plants, implying CIPK9 in the regulation of potassium utilization or sensing processes.

Keywords: calcium, calcineurin-B like protein, protein kinase, potassium nutrition, signal transduction

Cell Research (2007) 17:411-421. doi: 10.1038/cr.2007.39; published online 8 May 2007

Introduction

Plant growth requires a number of mineral nutrients, and potassium (K⁺) is the most abundant inorganic cation in plants constituting up to 5-10% of a plant's dry weight [1]. Potassium performs vital functions in metabolism, growth, and stress adaptation. On the one hand, potassium functions in the cell by directly interacting with proteins resulting in enzyme activation, stabilization of protein synthesis, and neutralization of negative charges on proteins [2-4]. In the second category of functions, K⁺ is a major driving force for osmotic regulation, for example, in stomatal movement, light-driven and seismonastic movements of organs or phloem transport [3, 5]. In cell expansion and growth,

accumulation of K⁺ (together with anions) in plant vacuoles creates the necessary osmotic potential for rapid cell enlargement. K⁺ movement also provides a charge separation across the membrane that is essential for the movement of other ions. In chloroplasts, energy production through H⁺-ATPases depends on overall H⁺/K⁺ exchange [6, 7].

K⁺ deficiency is of great agricultural importance [8]. This fact was recognized early in plant physiological research that led to an extensive description of K⁺ starvation symptoms at the physiological level [3]. It is well established that K⁺ starvation leads to growth arrest owing to the lack of the major osmoticum, impaired nitrogen balance owing to inhibition of protein synthesis, reduced levels of sugars owing to inhibition of photosynthesis, and impaired long-distance transport [3]. Low potassium content in the soil therefore severely limits crop yield, and farming practice often requires a large amount of organic and/or chemical fertilizers to overcome this condition. Use of fertilizers imposes undesirable economical and environmental problems that can be solved by genetic engineering of low-K⁺ tolerant crops based on the molecular understanding of K⁺ nutrition in plants. One important aspect of plant adaptation to low K⁺ stress is cellular and tissue homeostasis of K⁺, which involves transport of K⁺ across various membranes

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Received 8 November 2006; revised 30 January 2007; accepted 15 February 2007; published online 8 May 2007

in various tissues [9]. K^+ transport mechanisms have been studied extensively at the molecular level and many of the transporters have been cloned and functionally analyzed [10-13]. There are typically two mechanisms for K^+ acquisition, one is high-affinity and the other is low-affinity uptake from the roots [2, 14-16]. Some of the transporters such as the voltage-gated channels are generally considered as low-affinity transporters although studies also show that these channels can perform high-affinity uptake [17-21]. In addition, a single transporter can mediate both high- and low-affinity transport [20, 22, 23]. Most of the soil solutions contain less than 1 mM K^+ , thus the high-affinity uptake plays a critical role in potassium nutrition in plants [13, 24, 25]. Under potassium deficiency, high-affinity uptake is even more important for plant survival. Studies have shown that such high-affinity uptake may be inducible and tightly regulated under low potassium conditions [18, 20, 21]. However, it is unknown how plants detect or sense external K^+ concentrations and what are the signaling mechanisms that integrate physiological, biochemical, and molecular responses into a concerted adaptive response.

A recent study [26] identifies reactive oxygen species (ROS) as a critical signaling molecule for plant response to potassium-deficiency conditions. Interestingly, ROS in root cells has been shown to elicit changes in cellular calcium and is required for root hair growth and mineral uptake [27]. Together, these studies suggest that ROS signal is produced under low potassium conditions and ROS-induced calcium changes may be a crucial messenger for downstream responses. However, it is not known how low- K^+ -induced calcium signal is perceived by the cell. In addition to low- K^+ response, calcium plays multiple roles in many other cellular pathways, including signaling processes in response to pathogen, abiotic stresses, and developmental signals [28-33]. Calcium is also involved in the regulation of K^+/Na^+ homeostasis and ionic selectivity under saline conditions and exogenous Ca^{2+} can improve salt tolerance [34-37]. As a second messenger, Ca^{2+} transmits the primary signal into cellular responses through Ca^{2+} -regulated proteins that include Ca^{2+} sensors and their targets. The calcineurin B-like proteins (CBLs) are a family of recently identified calcium sensors unique to plants [38-40]. CBLs specifically target a family of protein kinases referred to as calcineurin B-like protein-interacting protein kinases (CIPKs) (CBL-interacting protein kinases) [38-40]. In *Arabidopsis*, both CBLs and CIPKs are encoded by large multi-gene families; 10 members in CBL and 25 members in CIPK gene family provide a high level of diversity and complexity in the function of the CBL-CIPK network. The CBL-CIPK network has been shown to play an essential role in several physiological processes, including abiotic stress responses [37, 41-45] and response to phytohormone

abscisic acid (ABA) [43, 45]. Recently, one of the members of CIPK gene family, CIPK23, was shown to be involved in regulation of K-nutrition by regulating the voltage-gated channel AKT1 in roots of *Arabidopsis* [46, 47]. In this study we have identified another member of the CIPK gene family, CIPK9, as a critical regulator of low potassium response in *Arabidopsis* by genetic analysis, providing a component that functions in between the low- K^+ -induced calcium signal and downstream responses.

Materials and Methods

Plant materials, stress treatments, and RNA analysis

Arabidopsis thaliana plants (ecotype Columbia-0) were grown in the greenhouse for seed production as described previously [48]. For RNA analysis, 2-week-old seedlings grown on MS medium [49] were treated under different stress conditions. Seeds were surface sterilized as described previously [48]. For NaCl and mannitol treatment, solution was overlaid onto the 2-week-old seedlings grown on MS plates to ensure total coverage of the foliage area. Seedlings were incubated at room temperature under white light. In parallel experiments, water was overlaid as control. To perform wounding treatments, 2-week-old seedlings grown in MS medium were mechanically injured as described previously [43]. For cold treatment 2-week-old seedlings were exposed to 4 °C, and whole seedlings were harvested after a defined period of time. Total RNA was isolated with Tripure isolation reagent (Roche Diagnostics, Indianapolis, IN, USA). All experiments were repeated at least three times, and results from one representative experiment are shown.

For low- K^+ -treatment, 10-day-old *Arabidopsis* seedlings grown on ½ MS medium were transferred to modified MS (10 mM K^+) or the K^+ -deficient (0 mM K^+) liquid medium for different lengths of times as indicated in Figure 2C and 2D.

Reverse transcriptase-PCR analysis of gene expression

To examine the expression of *CIPK9*, reverse transcriptase-mediated PCR (RT-PCR) was performed as described previously [48] using full open reading frame of *CIPK9* (At1g01140) forward (5'-AAA TCTAGA ATG AGT GGA AGC AGA AGG AAG GCG ACG CC-3') and reverse (5'-AA AGGTCC TTA TTG CTT TTG TTC TTC AGC GGC TGC ATC-3') primers to amplify a PCR product of 1.344 kb. Expression levels of *Actin-2* was monitored with forward (5'-GGA AAG GAT CTG TAC GGT AAC-3') and reverse (5'-TGT GAA CGA TTC CTG GAC-3') primers to serve as a quantifying control. Aliquots of individual PCR products were resolved by agarose gel electrophoresis and visualized with ethidium bromide by Gel Doc 1000 (Bio-Rad, Hercules, CA, USA).

Isolation of T-DNA insertion mutants

The *cipk9* mutants for At1g01140 were isolated from the T-DNA insertion collections of Torrey Mesa Research Institute (*cipk9-1* is SAIL_252_F06) and SALK (<http://signal.salk.edu/>; *cipk9-2* is SALK_058629). The T-DNA borders of *cipk9-1* and *cipk9-2* alleles were defined by sequencing PCR products obtained using a T-DNA border primer and a gene-specific primer. SAILLB3 (5'-TAG CAT CTGAAT TTC ATAACCAAT CTC GAT ACA C-3') and SALKLB1 (5'-GCA AAC CAG CGT GGA CCG CTT GCT GCAACT-3') were used as T-DNA border primers for the mutant obtained from the Tor-

rey Mesa Research Institute and SALK, respectively. The T-DNA insertion in the mutants (*cipk9-1* and *cipk9-2*) was confirmed by PCR and DNA gel blot analysis, and its exact position was determined by sequencing. After selfing of heterozygous plants, homozygous *cipk9* mutants were identified by genomic PCR to confirm disruption of the endogenous gene and by RT-PCR to confirm disruption of gene expression. The expression of *Actin-2* was monitored with forward (5'-GGA AAG GAT CTG TAC GGT AAC-3') and reverse (5'-TGT GAA CGA TTC CTG GAC-3') primers to serve as a quantifying control.

Analysis of *CIPK9* promoter-*GUS* expression in transgenic plants

To generate the *CIPK9* promoter-*GUS* construct, the 5' flanking DNA of the *CIPK9* coding region was amplified with forward (5'-AAA GTCGAC ATA TAT TCA CAA TCA TCG AAT ACA AGA ACA CC-3') and reverse (5'-AAA GAATTC TTT CTT TTT CCG ATT AAG AAAATC AAC G-3') primers. The 2.58 kb of PCR fragment was cloned into *Sal*I and *Eco*RI sites (underlined in the primer sequences) in the pBI101.1 vector (Clontech, Palo Alto, CA, USA). The construct was transformed into *Arabidopsis* plants [50] and transformants were selected on 50 µg/ml kanamycin. Several independent T₁ transgenic lines were stained with 5-bromo-4-chloro-3-indolyl-D-glucuronide for 12 h followed by incubation in 80% ethanol to remove chlorophyll [51].

Growth measurement on media with variable potassium levels and different nutrient strengths

For growth measurements of wild-type and *cipk9* mutants on low potassium nutrition, seeds were plated to a modified MS medium containing various concentrations of KCl [36]. Modified MS medium contains potassium-free 1/20 strength MS major salts and 1× MS minor salts. Potassium-free medium was prepared by replacing MS salts with the following (1× MS): 1650 mg/l NH₄NO₃, 440 mg/l CaCl₂·2H₂O, 370 mg/l MgSO₄·7H₂O, 165 mg/l (NH₄)₂HPO₄, 27.8 mg/l FeSO₄·7 H₂O, 37.3 mg/l di-sodium EDTA, 0.7495 mg/l NaI, 6.3 mg/l H₃BO₃, 16.9 mg/l MnSO₄·H₂O, 8.6 mg/l ZnSO₄·7H₂O, 0.25 mg/l Na₂MO₄·2H₂O, 0.016 mg/l CuSO₄·5H₂O, and 0.0267 mg/l CoSO₄·6H₂O. For solidification of MS media, 1% ultra-pure agarose (Invitrogen, San Diego, CA, USA) was used instead of agar. Varying levels of potassium in the media were achieved by adding appropriate amounts of KCl to the K⁺-free medium. We have measured the K⁺ content in K⁺-free buffer and in agarose and found negligible levels in all buffers; however, 1% agarose media contain approximately 0.003 mM K⁺. For different nutrient strengths of MS media, 1× MS media was diluted to 1/20 to 1/10 000 times with or without addition of KCl.

⁸⁶Rb⁺ uptake assay

For measurement of potassium uptake using ⁸⁶Rb⁺ as a tracer, we performed experiments as described [52] 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 indicated levels of KCl and 0.5 µCi/ml of ⁸⁶Rb⁺ (Amersham). The uptake was performed at room temperature under white fluorescent light. Uptake at different K⁺ concentrations was performed for 1 h. The time course of uptake at 0.02 or 20 mM K⁺ was performed for 30, 60, 90, and 120 min. At

the completion of uptake, the seedlings were rinsed three times with 50 ml of ice-cold K⁺-free media. The seedlings were blotted dry on filter paper and weighed, and the radioactivity was measured using a Beckman LS6500 scintillation counter (Beckman Instruments, Fullerton, CA, USA). Values of K⁺ uptake were obtained based on ⁸⁶Rb⁺ uptake and the external [K⁺].

Determination of K⁺ content

For the measurements of K⁺ content in plant tissues, the 7-day-old seedlings of various mutant and wild-type *Arabidopsis* were transferred from MS medium to the low potassium medium and treated for 4 days. The seedlings were then collected, rinsed 4-5 times with distilled water, and dried at 70 °C for 24 h and weighed. The samples were digested with 0.1 N HNO₃, and the K⁺ concentrations were determined with an atomic absorption spectrophotometer (model 560; Perkin-Elmer, Norwalk, CT, USA).

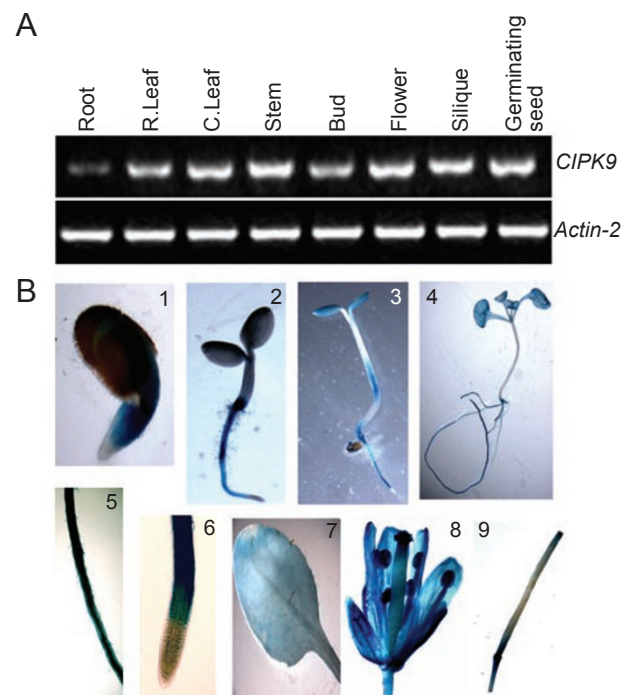


Figure 1 Expression patterns of *CIPK9* gene. **(A)** RT-PCR analysis of *CIPK9* transcripts in different organs of *Arabidopsis* plants and during seed germination. Total RNA was isolated from various tissues (root, rosette leaf, cauline leaf, stem, flower bud, opened flower, and silique) of wild-type plants (ecotype Col-0) grown under long-day conditions or from germinating seeds (2 days after sowing). Thirty-five cycles of RT-PCR were performed with *CIPK9*-specific primers (top gel) or *Actin-2*-specific primers (bottom gel). **(B)** Histochemical GUS analysis of *CIPK9* promoter-*GUS* transgenic plants. The top panel depicts from left to right a germinating seed (1), a seedling of 2 (2) and 5 (3) days after germination, and a 2-week-old plant (4). The bottom row shows root elongation and mature zone (5), root tip (6), a leaf of rosette stage plant (7), opened flower (8), and a silique (9).

Emergence of cotyledons and root elongation assays

Approximately 100 seeds each from the wild type (Col-0), *cipk9* mutant alleles (*cipk9-1* and *cipk9-2*) and 35S::*CIPK9* transgenic lines were planted in triplicate on modified MS-agarose medium with or without different concentrations of K⁺ or different nutrient strengths of MS media, and incubated at 4 °C for 6 days before being placed at 23 °C under long-day conditions. Emergence of cotyledons was scored daily for 15 days. The vertical root growth assays shown in Figures 4 and 5 were performed in a similar manner except that the plates were placed vertically on a rack and the root length was measured and recorded. Relative root growth and relative emergence of cotyledons were determined as the ratio of root growth or emergence of cotyledons for respective mutant allele to wild type at a particular concentration.

Results

CIPK9 gene is inducible by abiotic stresses and potassium deficiency

As a step towards dissecting the function of each CIPK in calcium signaling in plants, the expression patterns of these

genes were surveyed by RNA blot analyses. We noticed that the expression levels of one CIPK family member, *CIPK9*, were hardly detectable in adult plants by RNA gel blot analysis (data not shown). Therefore, we determined *CIPK9* mRNA levels using RT-mediated PCR. *CIPK9* mRNA was detected in all the tissues of the *Arabidopsis* plant by RT-PCR (Figure 1A). To analyze the expression of the *CIPK9* gene in more detail, we fused the putative promoter region of the *CIPK9* gene to the β-glucuronidase (GUS) reporter and analyzed GUS activity in transgenic plants by a histochemical procedure. As shown in Figure 1B, *CIPK9* promoter was highly active in the germinating seeds (1 day after sowing, mostly in the upper part of the radicle). A strong GUS activity was detected in the mature region of the roots, including the root hairs. However, no GUS activity was detected in the root tip and much of the elongation zone. GUS activity was also detected in most of the tissues of young rosette plants, consistent with the RT-PCR results shown in Figure 1A. A typical rosette leaf

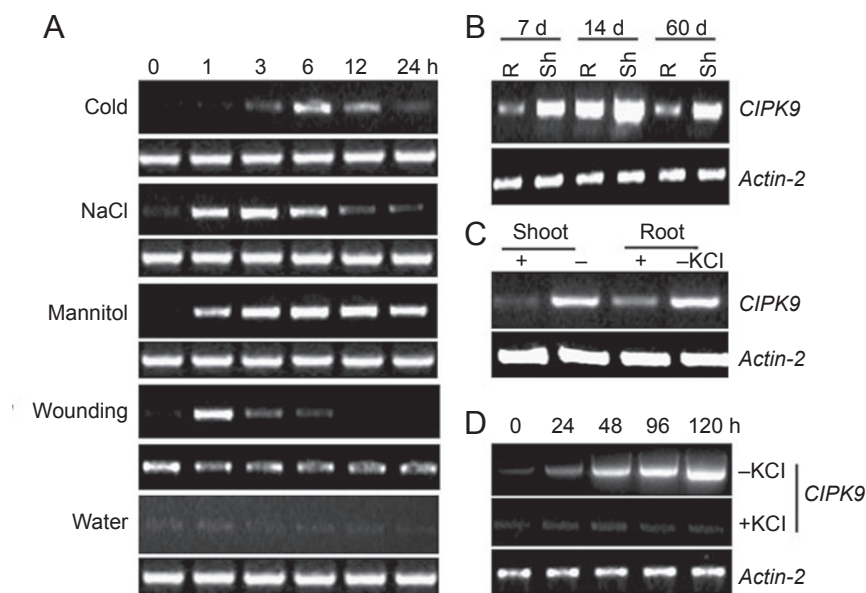


Figure 2 Induction of *CIPK9* transcripts by stress and low-K⁺ conditions. **(A)** RT-PCR analysis of *CIPK9* mRNA levels under various conditions. 14-day-old seedlings grown on MS medium were treated with cold (4 °C), NaCl (300 mM), mannitol (400 mM), and wounding (mechanical injury). Semi-quantitative RT-PCR analyses (25 cycles) were performed with *CIPK9*-specific primers. **(B)** Differential expression of *CIPK9* transcript at different developmental stages. Wild-type *Arabidopsis* seedlings were grown on MS-agar plate at standard long-day growth conditions. Total RNA was isolated from roots and shoots harvested at different developmental stages (7, 14 and 60 days). Semi-quantitative RT-PCR (35 cycles) was conducted by using *CIPK9*-specific primers. **(C)** Induction of *CIPK9* mRNA in roots and shoots under potassium-deficient conditions. Wild-type *Arabidopsis* seedlings were grown on ½ MS-agar media for 10 days before transfer to modified MS with K⁺ (10 mM K⁺) or without K⁺ (0 mM K⁺) for 48 h. Total RNA was isolated from roots and shoots and semi-quantitative RT-PCR (28 cycles) was performed. **(D)** Time course of *CIPK9* expression under potassium-deficient condition. 10-day-old *Arabidopsis* seedlings grown on ½ MS medium were transferred to liquid-modified MS media containing 10 or 0 mM K⁺. Samples were collected at 0, 24, 48, 96, and 120 h after transfer and total RNA was extracted from whole seedlings for semi-quantitative RT-PCR analysis using *CIPK9*-specific primers. *Actin-2* was used as a loading control in **(A-D)**.

exhibited low detectable GUS activity whereas anthers and stigma in the flower were stained strongly. Significant GUS activity was also detected in the petals, sepals, and siliques (Figure 1B).

As adult plants expressed *CIPK9* at low levels, we suspected that *CIPK9* expression may be regulated by environmental factors. Indeed, stress signals (including cold, hyper-osmotic stress, and wounding) strongly induced the expression of the *CIPK9* gene (Figure 2A). Induction by osmotic stress (mannitol) was the strongest, followed by high salt, cold, and wounding. The induction of *CIPK9* transcript by abiotic stress conditions was transient as shown for the induction of many other signaling components such as CBL1, CBL9, and CIPK3 in the CBL-CIPK network [42, 43, 45]. For example, in the cold treatment, the *CIPK9* transcript increased after 3 h and reached a maximum at 6 h followed by a gradual decline at 12 and 24 h. In NaCl treatment, *CIPK9* mRNA levels rapidly increased after 1 h and reached a maximum at 3 h followed by a decline. Under mannitol treatment, *CIPK9* transcript levels also increased rapidly at 1 h and retained a maximum at 6 and 12 h and were reduced at the 24 h time point. *CIPK9* was also expressed at different levels in the roots and shoots at different developmental stages of plant growth (Figure 2B).

Interestingly, *CIPK9* mRNA levels were upregulated under potassium-deficient growth conditions (Figure 2C

and 2D). Such induction was observed in both roots and shoots although a stronger induction was observed in the shoots (Figure 2C). The mRNA levels of *CIPK9* increased significantly, as the low potassium treatment continued for more than 2 days and further increased within the 5-day period (Figure 2D). This result was consistent with the data in the TAIR (The *Arabidopsis* Information Resource) micro-array database [53]. Although induction by abiotic stress conditions is a common property of several genes in the CBL and CIPK families, strong induction by low-potassium condition is rather unique to *CIPK9* and may indicate a unique function of *CIPK9* in low potassium responses.

The cipk9 mutant was specifically hypersensitive to low potassium conditions

To examine the function of *CIPK9* gene, we have isolated two T-DNA insertion alleles of this gene, *cipk9-1* and *cipk9-2*, from the collections of T-DNA-transformed *Arabidopsis* lines (SAIL252_F06 and SALK_058629, ABRC). Homozygous mutant lines were established after selfing. Sequence analysis indicated that the insertion site in the *cipk9-1* allele is located in the 5' UTR (un-translated region) approximately at 100 bp upstream of ATG and the T-DNA in the *cipk9-2* allele is inserted in the fifth exon following the 1211th bp after ATG (Figure 3A). RT-PCR analysis showed that both insertions abolished the expression of *CIPK9* in the mutant plants (Figure 3B).

We did not observe any significant phenotypic changes in *cipk9* mutant plants compared with wild-type plants under normal growth conditions in the growth chamber or greenhouse. As *CIPK9* is highly inducible under some abiotic stress conditions and was responsive to potassium deficiency, we speculated that it may function under these stress conditions. We performed phenotypic analysis of both seedlings and adult plants under different abiotic stress conditions, such as cold, salinity, osmotic stress, and in the presence of several plant hormones including ABA, auxin, cytokinin, and GA. No significant changes were scored between the mutants and the wild-type plants under these conditions (data not shown).

When we grew the seedlings on the modified MS media with low potassium levels (in the micro-molar range), the growth of *cipk9* mutant plants was significantly more inhibited as compared to the wild type. As shown in Figure 4, both roots and shoots in the mutants were more sensitive to the low potassium condition than the wild type. Such hypersensitivity was consistently observed in both the *cipk9-1* and *cipk9-2* alleles. The growth of wild-type plants (Col-0) was also reduced under low potassium media, but the growth of the mutant alleles was clearly hypersensitive to low potassium conditions. In 10 mM KCl (equivalent to the K⁺ level of half-strength MS salts), *cipk9* mutant

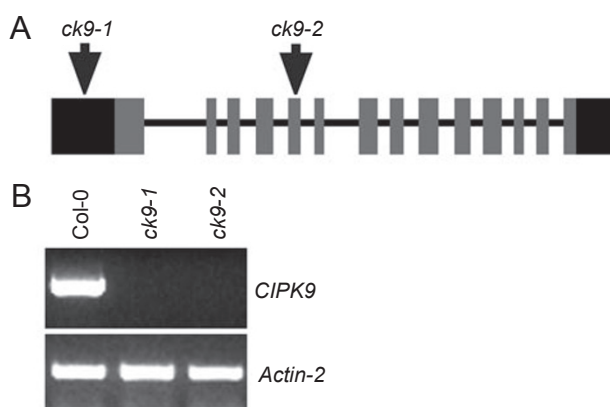


Figure 3 Isolation of the *CIPK9* T-DNA insertion mutants. (A) Intron-exon organization of the *Arabidopsis CIPK9* gene and T-DNA locations. Solid black boxes indicate un-translated regions (UTRs), and gray shaded boxes and lines indicate coding regions and introns, respectively. The positions of the T-DNA insertion in two alleles, *cipk9-1* and *cipk9-2*, are indicated by arrowhead (not to scale). (B) Thirty-five cycles of RT-PCR analysis of *CIPK9* transcripts in wild-type (Col-0) and mutant alleles (*cipk9-1* and *cipk9-2*). Expression of *Actin-2* was analyzed as a loading control.

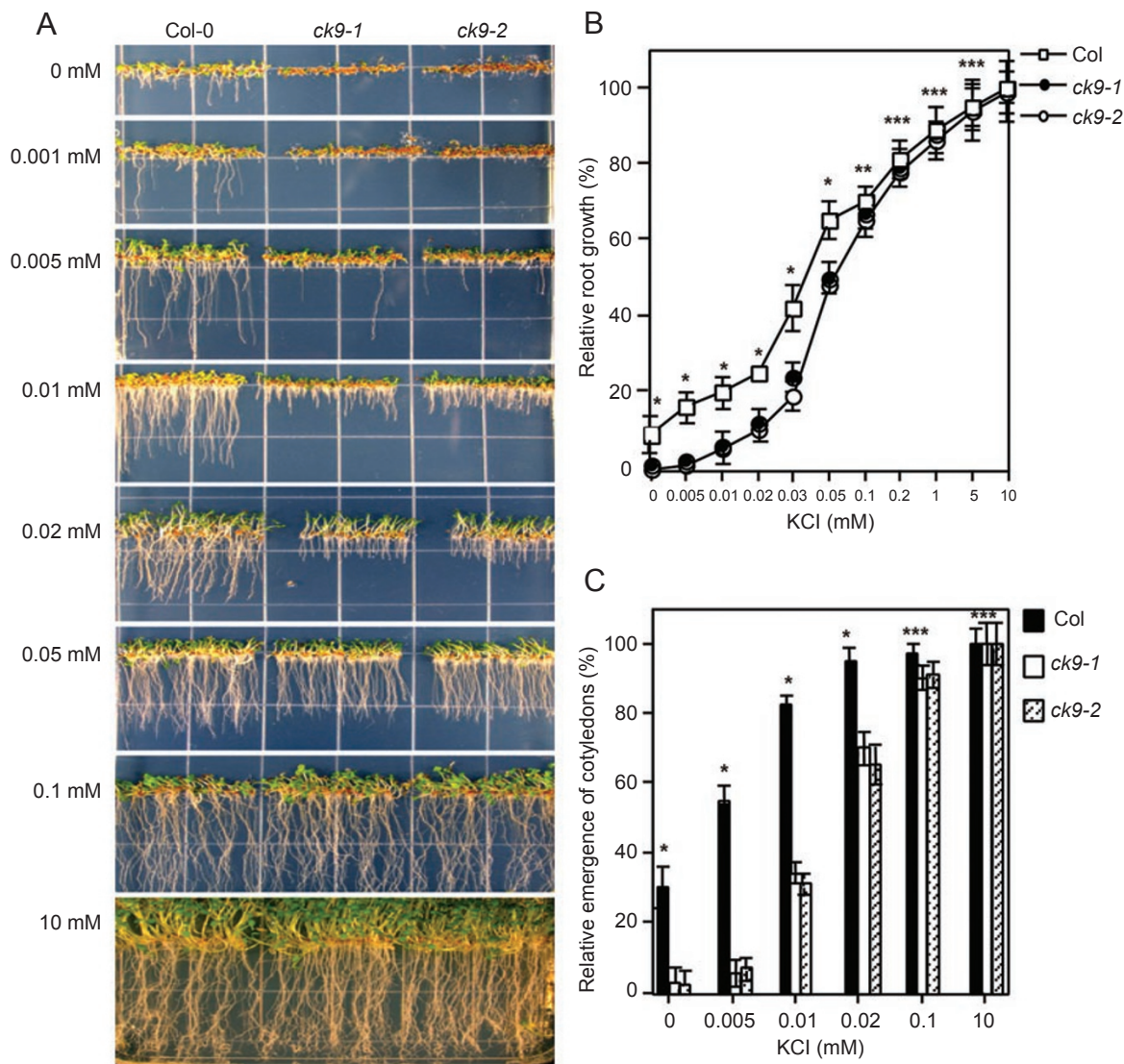


Figure 4 Hypersensitive growth of *cipk9* mutants under low potassium media. **(A)** Seedlings were plated on vertical MS-agarose plates (½ MS; 10 mM K⁺), or modified MS (see Materials and Methods) with 0, 0.001, 0.005, 0.01, 0.02, 0.05, 0.100, or 10 mM K⁺ for 10 days before taking the photographs. **(B)** Measurement of root growth under various K⁺ levels. Root length of 7-day-old seedlings was measured (n = 95 for each condition). Three independent experiments were performed and values are means ± SD. **(C)** Cotyledon emergence under various K⁺ levels. Emergence of cotyledons was scored after 9 days of growth on the modified MS media containing various levels of K⁺ (n = 100 for each condition). Three independent experiments were performed and values are means ± SD. Student's *t* test was performed to determine the significance, in **(B)** and **(C)** **p* < 0.0001, ***p* = 0.07, and ****p* > 0.5.

seedlings were indistinguishable from the wild type. Results of more detailed analyses of root growth under different concentrations of K⁺ are depicted in Figure 4B. The root growth in low potassium media (from 0 to 0.05 mM K⁺) is strongly inhibited in both wild type and mutant. However, the *cipk9* mutant seedlings were significantly more inhibited by the low potassium level. When the K⁺ concentration in the MS media is equal to or higher than

0.1 mM, root growth of wild type and *cipk9* mutants was not significantly different. At 0.02 mM K⁺, the root length of both *cipk9* mutant alleles was about 50% of wild type seedlings. Similarly, cotyledon emergence and expansion were also strongly inhibited in the low potassium media and *cipk9* mutants again displayed hypersensitivity (Figure 4C). At 0.01 mM K⁺, 82 % wild-type seedlings expanded cotyledons whereas only 34% and 31% of mutant *cipk9*

alleles (*cipk9-1* and *cipk9-2*, respectively) did so after 9 days of growth at 23 °C ($p < 0.0001$). These results demonstrated that CIPK9 plays a critical role in plant growth under low potassium concentrations. Because $[K^+]$ in most of the soils is in the micro-molar range [24, 25], CIPK9 may function to regulate potassium nutrition under physiological conditions.

Hypersensitivity to low potassium in the *cipk9* mutant may be specific to potassium or *cipk9* mutant may also be hypersensitive to deficiency of other nutrients or simply

hypersensitive to overall low nutrition status in the media. To test whether *cipk9* mutants are hypersensitive to deficiency of other nutrients, we grew wild type and *cipk9* mutant plants in modified MS media depleted in each of the following nutrients (calcium, magnesium, iron, phosphorus, nitrate and ammonia and some micronutrients) and found no significant difference between the wild type and mutant plants (data not shown). To test whether *cipk9* mutant is hypersensitive to a general low nutrition status, we grew the wild type and *cipk9* mutants in different dilu-

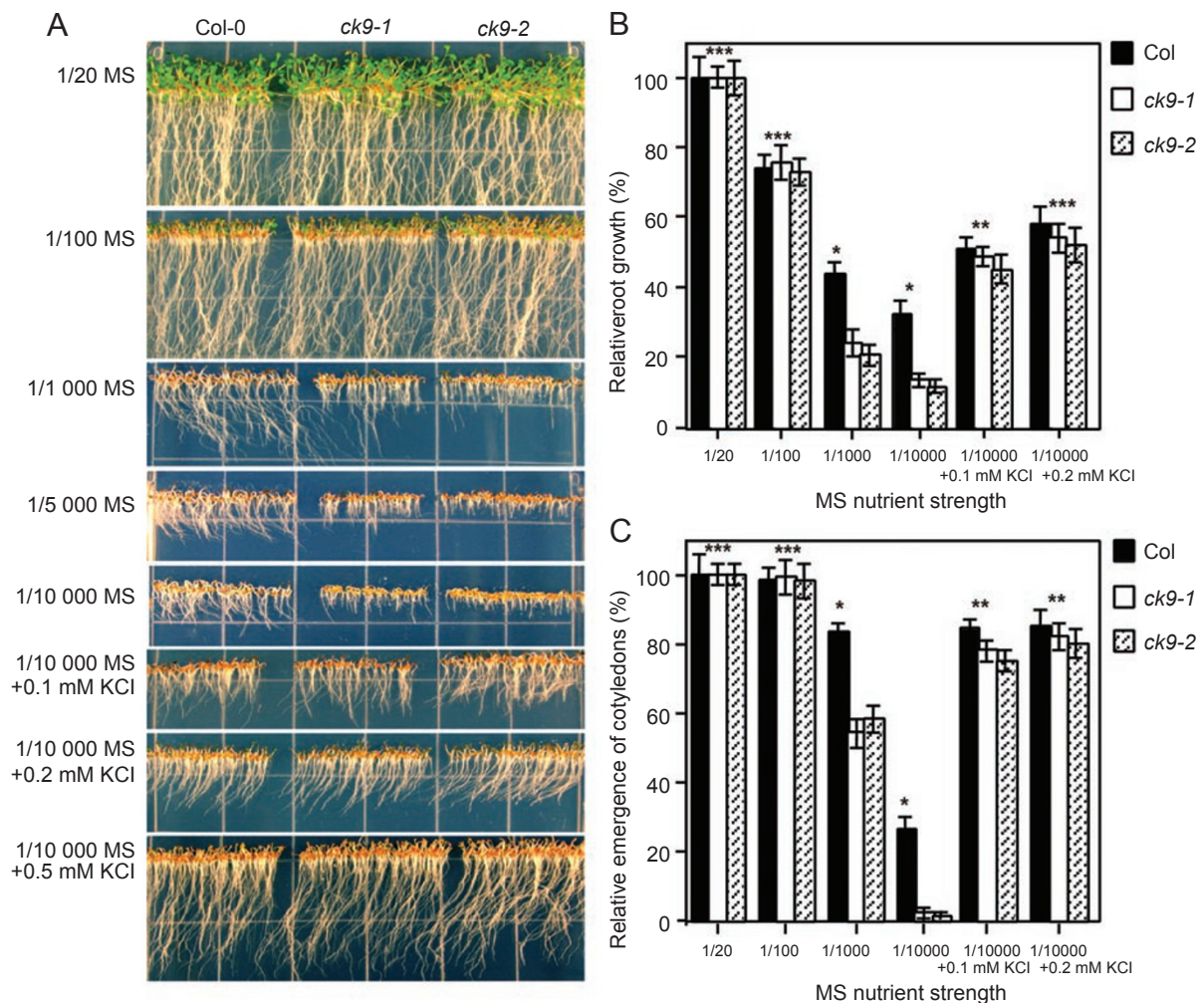


Figure 5 Phenotypic analysis of *cipk9* mutants grown in diluted MS media. **(A)** Seedlings of Col-0, *cipk9-1*, and *cipk9-2* were grown vertically in different strengths of MS-agarose media (1/20, 1/100, 1/1000, 1/5 000 and 1/10 000 dilutions) with or without supplementing K^+ . Photographs were recorded after 12 days of growth at standard growth conditions. **(B)** Measurement of root growth under different strengths of MS media. Root length of 12-day-old seedlings was measured ($n = 25$ for each condition). Three independent experiments were performed and values are means \pm SD. **(C)** Measurement of cotyledon emergence under different strengths of MS media. Emergence of cotyledons was scored after 12 days of growth on the different strengths of MS media ($n = 100$ for each condition). Three independent experiments were performed and values are means \pm SD. Student's t test was performed to determine the significance, in **(B)** and **(C)**, $*p < 0.0003$; $**p > 0.15$; and $***$ denotes $p > 0.60$.

tions of the MS media (including 1/20, 1/100, 1/1 000, 1/5 000 and 1/10 000 dilutions). The growth of both wild type and *cipk9* mutant seedlings was drastically inhibited as the MS media was diluted progressively from 1/20 to 1/10 000. Again, the *cipk9* mutant plants were more inhibited than wild type in root growth and emergence of cotyledons (Figure 5A). A detailed analysis of the root growth and emergence of cotyledons (Figure 5B and 5C) clearly showed that both *cipk9* mutant alleles were strongly more sensitive than wild-type seedlings. At 1/1 000 dilution of MS media where the K^+ concentration is calculated to be 0.02 mM, the root growth of both the *cipk9* mutant alleles was approximately 50% of wild type. The emergence of cotyledons in wild type was 83% compared to 54% and 58% for *cipk9-1* and *cipk9-2*, respectively ($p < 0.0001$). Such hypersensitive response of *cipk9* mutant alleles to the dilution of MS medium was eliminated by addition of 0.1 mM K^+ in the diluted MS media (Figure 5A-C), confirming that CIPK9 specifically regulates potassium nutrition in *Arabidopsis*. We did not observe a significant difference in growth improvement for over-expression lines of CIPK9

under low low- K^+ growth conditions when compared to wild type (data not shown).

K^+ -uptake and content are not affected in the *cipk9* mutant

From earlier studies, it is conceivable that potassium nutrition can be regulated at multiple levels including acquisition and utilization [11, 54]. To identify possible mechanisms for the low-potassium hypersensitivity in *cipk9* mutants, we examined the potassium acquisition in the mutants and wild-type plants by measuring K^+ uptake and content. Using $^{86}Rb^+$ tracer experiments described in the Materials and methods, we measured the uptake in the wild type and *cipk9* mutant seedlings under low (0.02 mM) and high (20 mM) K^+ conditions, and did not find significant differences (Figure 6A). We also measured K^+ contents in both wild type and mutant plants (seedlings and adult plants) by the atomic absorption spectrophotometry (see Materials and Methods). Again there was no difference in K^+ content between wild type and *cipk9* mutants under low and optimal levels of K^+ (Figure 6B). These

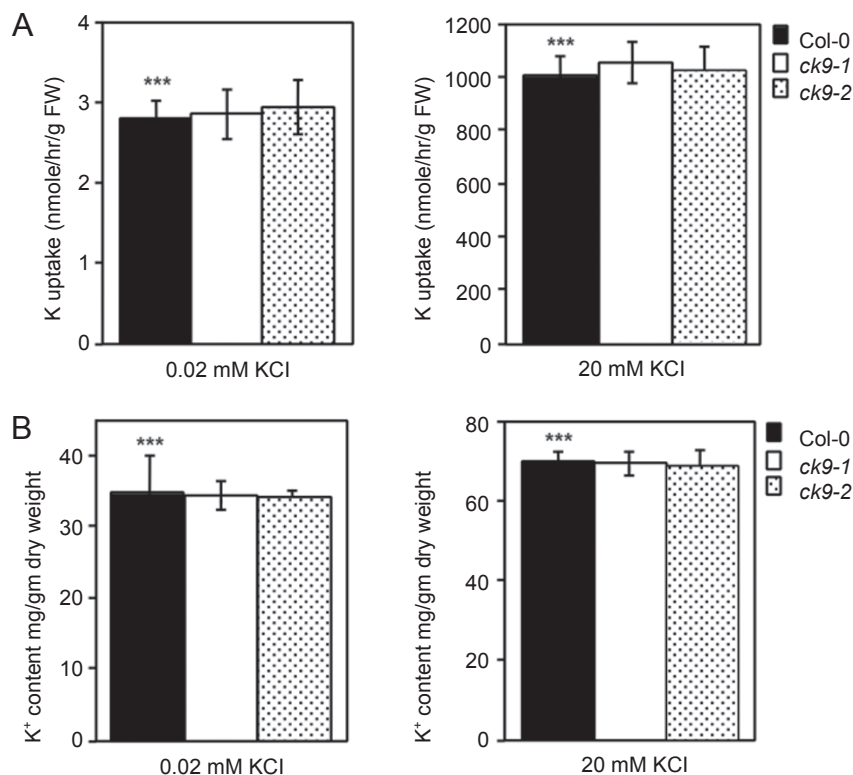


Figure 6 Potassium uptake and content in *cipk9-1*, *cipk9-2*, and the wild-type (Col-0) plants. **(A)** $^{86}Rb^+$ -uptake at 0.02 and 20 mM external K^+ . The uptake time was set at 1 h. **(B)** Potassium contents in the wild-type (Col-0), *cipk9-1*, and *cipk9-2* seedlings treated at 0.02 and 20 mM external K^+ . Three independent experiments were performed and values are means \pm SD. Student's *t* test was performed to determine the significance, in **(A)** and **(B)**, *** $p > 0.60$.

results indicate that CIPK9 may regulate processes other than K^+ acquisition.

Discussion

Potassium is a key element for plant growth and low- K^+ levels in the soil present a serious problem for agricultural practices. Understanding the molecular mechanism underlying low- K^+ response and adaptation in plants will provide a platform for improving crop tolerance to low- K^+ conditions. We show here that CIPK9, a calcium sensor-interacting protein kinase, functions as an essential component for plant growth under low- K^+ conditions. Disruption of *CIPK9* gene function in the *cipk9* mutants rendered the plants hypersensitive to low- K^+ media.

It is well established that the capability of high-affinity K^+ -uptake in plants plays a pivotal role in low- K^+ adaptation [10-13]. As a result, disruption of *AKT1*, a gene coding for a K^+ channel involved in the high-affinity uptake of K^+ from the soil or growth medium, resulted in poor growth of *Arabidopsis* seedlings under low- K^+ conditions [18, 20]. Another high-affinity K^+ transporter, *HAK5*, is also important for potassium nutrition [21]. Studies have also demonstrated that the activity and/or expression of a number of K^+ -transporters including *AKT1* and *HAK5* are often highly induced by the low- K^+ conditions. This finding identifies a natural mechanism for plants to adapt to a low- K^+ environment and establishes a “signaling pathway” starting with the low- K^+ as a “signal” and increased transport as a “response”. What components are in between the signal and response? In a recent study, Shin and Schachtman [26] have shown that K^+ -deficient condition leads to generation of H_2O_2 and changes in gene expression, with the ROS production being the early root response that might modulate the overall physiology of the seedling development. Such a finding is consistent with an earlier study that demonstrated a link between H_2O_2 production, calcium changes, and root hair development [27]. It is shown that a mutation in the NADPH oxidase reduced H_2O_2 production leading to stunted root growth and shorter root hairs in the mutant plants. The reduced H_2O_2 production resulted in low calcium channel activity in the root cells. It is believed that H_2O_2 is required for the activation of calcium channels that in turn trigger the cellular calcium changes essential for cell growth in root hairs and possibly also in other root cells. Under low- K^+ conditions, production of H_2O_2 may also trigger calcium changes required for further signaling steps leading to expression of a number of low- K^+ -induced genes. Therefore, our finding of a calcium-regulated protein kinase in this study provides a possible molecular link between calcium signals and further cellular regulatory steps. The kinase CIPK9 is a member of the CIPK family, a subfamily

of the extended SNF1-like protein kinases (or SnRKs). The activity of CIPKs is regulated by interactions with calcium sensors called CBLs [38-40]. We hypothesize that calcium changes in the cytoplasm alter the conformation of CBLs that interact with and regulate the activity of CIPKs. In this case, CIPK9, after being activated by a CBL, may regulate the activity of other protein components involved in low- K^+ responses. These could be transcriptional factors required for low- K^+ -induced gene expression, metabolic enzymes involved in osmotic regulation, or K^+ transporters that are activated under low- K^+ condition. It is noteworthy that K^+ content and uptake capability were not altered in the *cipk9* mutants, indicating that the low- K^+ hypersensitivity phenotype may not be caused by less K^+ acquisition from the medium. In other words, the downstream targets for CIPK9 may not be directly related to K^+ uptake. In addition to participation of CIPK9 in low- K^+ tolerance, other members of the CIPK family have been shown to function in other cellular processes including abiotic stress responses and responses to plant hormones such as ABA and GA during seed germination [38-40, 55]. One member of CIPKs, *SOS2/CIPK24*, was shown to be essential for salt tolerance and K^+ nutrition by excluding Na^+ from the cell [37]. Unlike *sos2/cipk24* mutant, *cipk9* mutant did not show any phenotypic changes under salt or osmotic stress (data not shown), suggesting that CIPK9 functions specifically in K^+ nutrition and is not involved in salt tolerance. Taking together, the members in the CBL-CIPK network may function as major components for the regulation of ionic homeostasis including salt tolerance and potassium nutrition in plants. Recently, one of the CIPK members, *CIPK23*, has been shown to form complexes with *CBL1* and *CBL9* and regulate potassium nutrition [46, 56]. A detailed mechanistic analysis has revealed that *CIPK23* physically interacts with and phosphorylates *AKT1*. The *AKT1* channel activity is regulated by *CIPK23-CBL1* or *CIPK23-CBL9* complexes, thereby regulating potassium nutrition [46, 47]. In contrast, *CIPK9* was not found to interact with any potassium transporter in the yeast two-hybrid system and did not regulate *AKT1* function (data not shown). Future analysis of downstream targets of *CIPK9* will add more information to the molecular mechanisms underlying low- K^+ response and adaptation.

Acknowledgments

We are grateful to the ABRC (Ohio State University, Columbus, OH) for *Arabidopsis* seeds and Syngenta Research and Technology, Torrey Mesa Research Institute (San Diego, CA, USA) for providing the T-DNA insertion lines. This work was supported by a grant from the National Science Foundation (USA) (to SL).

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