Science in China Series C: Life Sciences

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Regulation of salt and ABA responses by CIPK14, a calcium sensor interacting protein kinase in *Arabidopsis*

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Calcium and protein kinase serve as the common mediators to regulate plant responses to multiple stresses including salt and ABA stimulus. Here we reported a novel protein kinase (CIPK14) that regulated the responses to ABA treatment and salt stress in *Arabidopsis*. *CIPK14* transcripts, capable been checked in roots, stems, leaves and flowers, were highly expressed in flowers and roots. *CIPK14* was induced by ABA and salt treatments. The disruption of *CIPK14* altered the transcriptional pattern of a gene marker line related to ABA and salt responses, and the results suggested that *CIPK14* probably was responsible to the control of the salt and ABA responses. Comparing with wild types, the lines inserted with the T-DNA in which *CIPK14* gene expression was knocked out were also more sensitive to ABA and salt stimulus, showing low germination rate and the less root elongation. While, when these conditioned seeds were treated with norflurazon, their germination percentages could recover to a certain extent. We also found that exogenous calcium could have an effect on the transcription of *CIPK14* under ABA and salt treatments, and it seemed that calcium ion might work upstream *CIPK14* to regulate the plant response to ABA and salt response.

calcium, *CIPK14*, ABA, salt, stress response, germination

When plant coped with various forms of abiotic stresses such as salt, drought, flooding, temperature and oxidative stress throughout their life, the temporal and spatial expression patterns of specific stress genes is an important part of the plant stress response. CIPK is a group of protein kinases designated as a calcineurin B-like protein (CBL)-interacting protein kinase[1−3]. These kinases form a novel plant-specific family of serine-threonine kinases, structurally quite similar to the SNF1 (sucrose non-fermenting) kinase from yeast and AMPK (AMP-activated protein kinase) from animals[4,5]. Because of this structural similarity, the CIPKs have also been assigned to the SnRK3 (SNF1-Related Protein Kinase-3) subgroup of plant SNF-like kinases[6]. The *Arabidopsis* genome contains 25 putative *CIPK* genes. The presence of multi-gene families of CBLs and CIPKs in *Arabidopsis* suggests that CBL-CIPK network may be involved in a number of signaling processes in plants[7−9]. For example, mutations reducing the Ca^{2+}-binding capability of SOS3 results in a salt hypersensitive phenotype[10]. SOS3 was found to interact with SOS2, a member of the Snf1-related SnRK3/CIPK kinase family[6,11]. *CIPK3*, a homolog of *CIPK15*, associated via its NAF domain with a CBL calcium sensor and negatively regulated ABA-induced gene expression[12]. Interestingly, interaction between *CBL1* and *CIPK1* required micromole levels of Ca^{2+}. This Ca^{2+}-dependent interaction is consistent with the general paradigm established for Ca^{2+}sensor interactions with target proteins in animals[1].

Received September 18, 2007; accepted January 24, 2008
doi: 10.1007/s11427-008-0059-z
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Supported by the "985" Program (China) for the higher education enhancement fund to Hunan University, Hunan Natural Science Foundation (Grant No. 05JJ30038) and National Natural Science Foundation of China (Grant No. 30600368)

Using a genetics approach to dissecting the function of the *CIPK* genes, we identified *CIPK14* that play specific roles in stress responses and response to abscisic acid (ABA). Here, we report the studies on the regulation of *CIPK14* of the responses to salt and ABA treatment. Loss-of-function studies were carried out to reveal the *CIPK14*-specific regulation pathways leading to stress gene expression. Exogenous calcium was also investigated in controlling the transcription of *CIPK14* under ABA and salt treatments for better understanding the probable role of calcium ion in regulation of plant responses to ABA and salt stresses.

1 Materials and methods

1.1 Plant materials, stress treatments and germination assay

Three-week-old *Arabidopsis thaliana* seedlings grown on MS medium[13] were treated with 100 μmol/L ABA or 300 mmol/L NaCl for 6 h. The seedlings were sprayed thoroughly to ensure the total coverage of the foliage with the chemicals. Approximately 100 seeds, treated with 70% ethanol for 1 min, then with 30% household bleach for 10 min, and washed five times with sterile water, were plated on MS medium solidified with 0.8% agar incubated at 4℃ for 4 d before being placed at 23℃ under long-day conditions. Different concentrations of ABA, NaCl, mannitol, and glucose were added to the medium to investigate their effects on seed germination rates (emergence of radicals). Germination rate was recorded daily for 6 d. Results were presented as average values ± SE from three experiments.

1.2 Reverse transcriptase-mediated PCR for expression analysis of *CIPK14* and stress marker gene

To analyze the expression of *CIPK14* by reverse transcriptase–mediated (RT) PCR, total RNA was extracted from *Arabidopsis* tissues with EasyWay RNA Plant Mini Kit (Ambiogen Life Science Technology Ltd., China). Total RNA (2 μg) was heated to 65℃ for 7 min and then subjected to reverse transcription reaction using *Moloney murine leukemia virus* reverse transcriptase (200 units per reaction; MBI) for 60 min at 42℃. PCR amplification was performed with initial denaturation at 95℃ for 5 min followed by different (20－27) cycles of incubations at 95℃ for 30 s, 55℃－60℃ for 30 s, and 72℃ for 30 s, and a final extension at 72℃ for 5 min using the *CIPK14* specific primers (F: 576 bp－599 bp; R: 1038 bp－1017 bp) or stress key gene primers (Table 1). Actin gene expression level was used as a quantitative control. Sixteen μL of individual PCR products were separated by 1.5% agarose gel electrophoresis and visualized with ethidium bromide under UV light.

Table 1 Primers used in RT-PCR

Gene name	Primer sequence
Actin	F: 5′-CACTGTGCCAATCTACGAGGGT-3′ R: 5′-CACAAACGAGGGCTGGAACAAG-3′
CIPK14	F: 5′-CAAAGTCTCCAAGGGCAGGTTCT-3′ R: 5′- CGTCTGCGGATTCGTGTCTAAAA-3′
RD29A	F: 5′-GCGGGAACTGTTGATGAG-3′ R: 5′-ACCAAACCAGCCAGATGA-3′
RD29B	F: 5′-AAGGAGACGCAACAAGGG-3′ R: 5′-ACGGTGGTGCCAAGTGAT-3′
RD22	F: 5′-TTCGGAAAAGCGGAGAT-3′ R: 5′-CTTTGAAGGCCAAGTGGT-3′
RAB18	F: 5′-GAATGCTTCACCGCTCCG-3′ R: 5′-ACGACCGAATGCGACTGC-3′
KIN1	F: 5′-CGCTGGCAAAGCTGAGGA-3′ R: 5′-TTCGGATCGACTTATGTATCGT-3′
KIN2	F: 5′-CTGGCAAAGCTGAGGAGA-3′ R: 5′-CGTAGTACATCTAAAGGGAG-3′
DREB1A	F: 5′-GGCGGGTCGTAAGAAGTT-3′ R: 5′-GATCCGTCGTCGCATCAC-3′
DREB2A	F: 5′-CTGTTGAGACTCCTGGTT-3′ R: 5′-GAGGTATTCCGTAGTTGA-3′
SOS2	F: 5′-CTACAAAAGCAGTGCAGTGT-3′ R: 5′-CTACCTTTTGTGAAGTCCTCC-3′

Note: F, forward primer; R, reverse primer.

1.3 Isolation and PCR analysis of the cipk14 T-DNA insertion mutant

Two T-DNA insertion lines SALK_009699 and SALK_147899 were obtained from a TAIR seed stock. The *R0* primer annealing to the left border of the inserted T-DNA, together with *CIPK14*-specific primers (Table 2), was used for the identification of putative mutant lines by PCR analysis. Plants homozygous for the *cipk14* mutant were used for further germination assay or RNA analysis.

1.4 Hypocotyl and root elongation assay

Seeds, treated with 70% ethanol for 1 min, then with 30% household bleach for 10 min, and washed five times with sterile water, were plated on MS medium solidified with 0.8% agar incubated at 4℃ for 4 d before being placed at 23℃ under continuous white light. The hypocotyl length and root elongation of 6-day old seedling were investigated. For root elongation under ABA treatment assay, seeds were germinated and grown on MS agar medium for 3 d. Seedlings of similar growth rate both from wild type and mutants were transferred to

Table 2 Primers of the *cipk14* T-DNA insertion mutant

Gene name	Primer sequence	Site
R0	5′-TGGTTCACGTAGTGGGCCATCG-3′	
SALK_009699	F: 5′-CAAAGTCTCCAAGGGCAGGTTCT-3′ R: 5′-GTCTGCGGATTCGTGTCTAAAA-3′	176bp－198bp 390bp－370bp
SALK_147899	F: 5′-AGGTCCTCGCCACCAAATCCAA-3′ R: 5′-CCGGCGTACCACAGAGTGTGTG-3′	509bp－531bp 805bp－784bp

Note: F, forward primer; R, reverse primer.

MS containing different concentrations of ABA (0, 20, 30, 40 μmol/L) for 2 weeks, and the root length was measured and recorded. Results were presented as average values ± SE from three experiments.

1.5 Exogenous calcium treatment

Three-week old plants of wild type and *CIPK14* mutant in the MS solution with different calcium concentration (0, 0.1, 1, 10, 100 mmol/L) were treated with the addition of 0.25 μmol/L ABA or 125 mmol/L NaCl in the medium for 30 h. and all samples (Ca+ABA and Ca+ NaCl and only Ca) were collected for RNA analyses. Approximate 100 seeds each from the wild type (col-4) and the *cipk14* mutant were sowed in half on MS medium with different concentrations(0, 0.1, 1, 10, 50 mmol/L) of calcium with or without 0.45 μmol/L ABA, 150 mmol/L NaCl and incubated at 4℃ for 4 d before being placed at 23℃ under long-day conditions. For root elongation assay, seeds were germinated and grown on MS medium containing different concentrations of calcium with 0.15 μmol/L ABA for 1 week before the root length was recorded.

2 Results

2.1 Spatiotemporal expression of *CIPK14* in *Arabidopsis*

CIPK14 mRNA levels in different tissues were determined by using RT-PCR. The highest level of *CIPK14* mRNA was found in flowers and distinct level in roots, whereas lower levels were detected in stems, leaves and siliques (Figure 1(a)). To analyze the expression of *CIPK14* in different development status, we found that the mature seeds accumulated the lowest level of transcripts, while detectable transcripts exhibited in the young seedling (cotyledon and two true leaves seedling) (Figure 1(a)).

Interestingly, high concentrations of salt (300 mmol/L) and ABA (100 μmol/L) strongly induced the expression of the *CIPK14* gene. The expression level continuously increased as the time passed after the treatment (Figure 1(b)).

2.2 Isolating and classifying the T-DNA knock-out *CIPK14* insertion mutant

A 1.8 Kb DNA region was identified as encoding *CIPK14* gene (At5g01820) which had only one extron. To genetically dissect the in vivo function of *CIPK14* gene, T-DNA insertion lines, SALK_009699 and SALK_147899 were obtained from a TAIR seed stock center (Figure 2(a)), PCR was performed with either *CIPK14*-specific primers beside the insertion site or T-DNA-specific primer in the LB. Six of SALK_009699 were verified as homozygote, and three heterozygote of SALK_147899 were classified (Figure 2(b)). RT-PCR, performed with either *CIPK14*-specific primers or actin-specific primers (Table 1) to verify expression of *CIPK14* in the various T-DNA insertion lines, results confirmed that the expression of the selected gene was disrupted in these mutants (Figure 2(c)).

In this study, we performed assays with two independent Salk alleles that behaved in a similar manner. Detailed analysis was performed on Salk-009699 (denominated as *cipk14* mutant in the following). Perceptible delay of seedling growth was observed in *cipk14* mutant plants compared with wild-type plants under normal growth conditions or in the growth chamber or greenhouse (Figure 2(d)). This founding was consistent with high level of expression of *CIPK14* in seedling under normal conditions

2.3 *CIPK14* regulates the transcription of stress-related marker genes in *Arabidopsis*

The plants from *cipk14* mutant and the wild-type under normal conditions during the life cycle were found no significant phenotypic changes except for slower seedling growth in the mutant ones (Figure 2(d)). To determine if stress signaling was modified in the *cipk14* mutant, the expression patterns of various stress related marker genes were analyzed in *cipk14* mutant and the wild type by RT-PCR. As shown in Figure 3, all marker genes were induced by ABA and NaCl in the wild-type

plants, consistent with the results from previous studies[14−17], while the expression levels were different between the *cipk14* mutant and the wild type. Under ABA treatment, *RD29B* transcript level was much lower in the first 4 h of induction in both lines, reaching the maximal induction and showing evident differences between the wild type and the *cipk14* mutant after 6 h of treatment. The induction of *RAB18* in the wild type was twice as much as in the *cipk14* mutant after 6 h of ABA treatment, although they had the same increasing trend. The average induction level of *KIN1* was reduced about 40% in the *cipk14* mutant (Figure 3(b)).

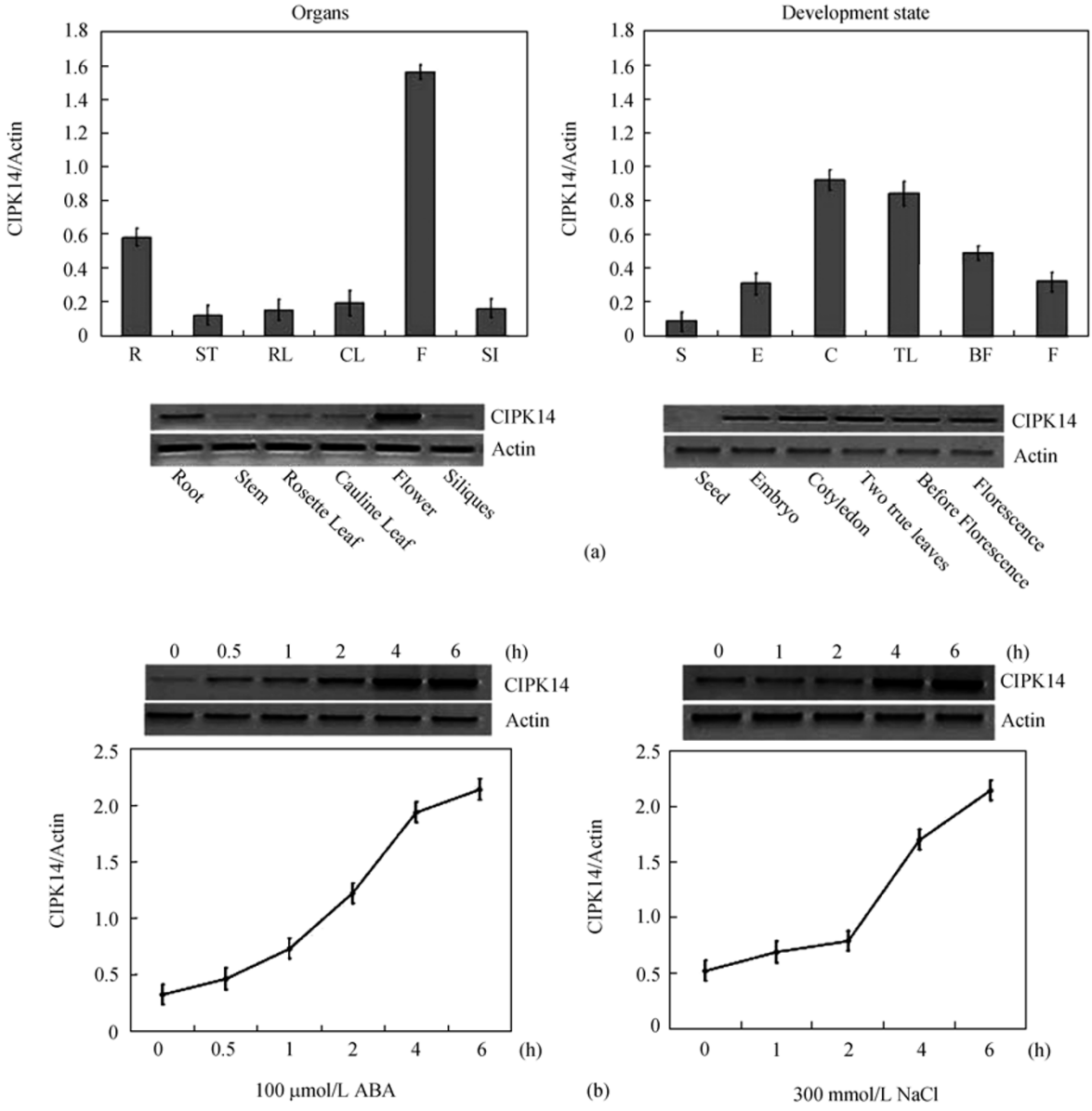

Figure 1 Expression patterns of the *CIPK14* gene. (a) RT-PCR analysis of *CIPK14* transcripts during different development status and in different organs of *Arabidopsis* plants. Total RNA was isolated from root, stem, leaf, flower, and silique of wild-type plants or from seeds, embryo seedling, cotyledon seedling, two true leaves seedling, before florescence state and florescence state plants. RT-PCR was performed with either *CIPK14*-specific primers (top gel) or Actin-specific primers (bottom gel). The corresponding histogram was presented. Levels of mRNA expression were shown as the relative signal intensities (*CIPK14*/*ACTIN*). Abbreviations: R, root; ST, stem; RL, rosette leaf; CL, cauline leaf; F, flower; SI, silique; S, seeds; E, embryo seedling; C, cotyledon seedling; TL, two true leaves seedling; BF, before florescence state; F, florescence state. (b) RT-PCR analysis of the accumulation of *CIPK14* gene transcripts in response to NaCl (300 mmol/L), ABA (100 μmol/L) from 3-week-old *Arabidopsis* plants. The numbers indicated hours after each treatment. Levels of mRNA expression were shown as the relative signal intensities (*CIPK14*/*ACTIN*).

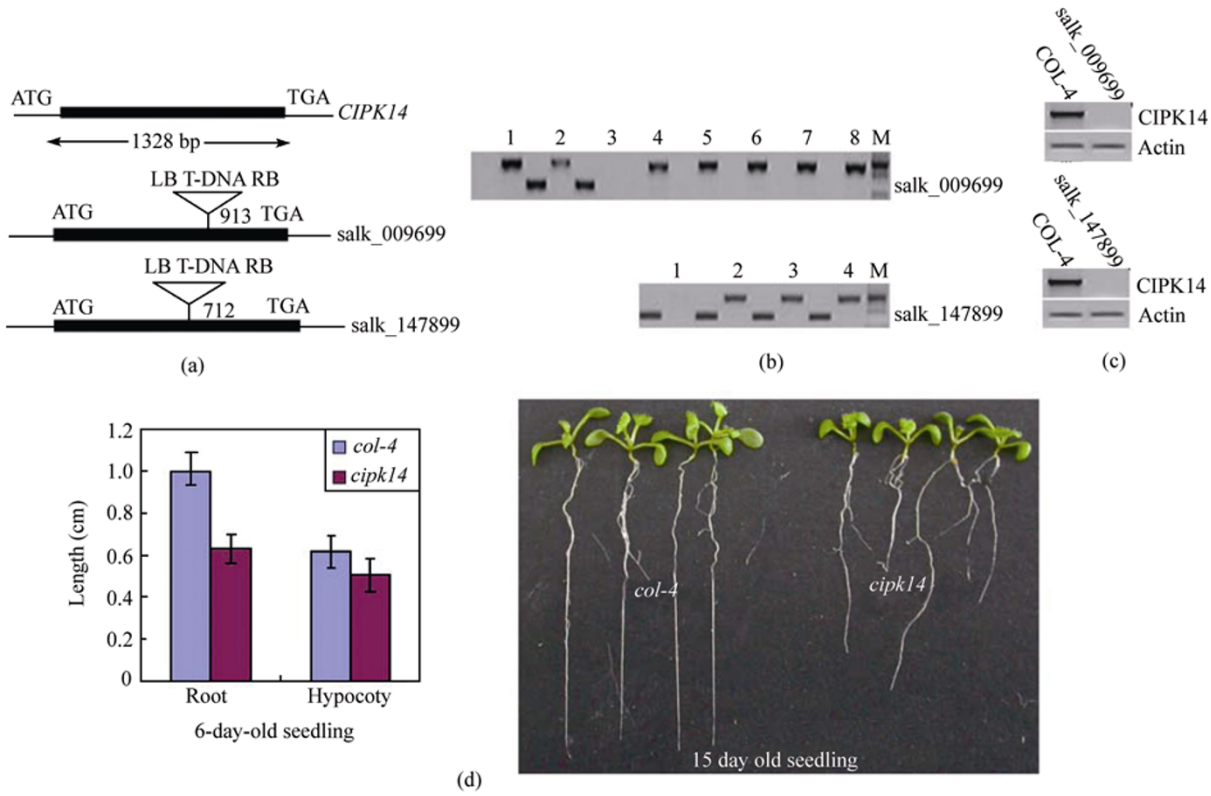

Figure 2 Isolation and identification of the *CIPK14* insertion mutants. (a) Scheme of the *Arabidopsis CIPK14* gene. Exon was indicated solid box. The position and orientation of the T-DNA insertion was shown (not to scale). The numbers represent nucleptides. LB: left border; RB: right border. (b) PCR identification of the *CIPK14* insertion mutants. PCR was performed with either *CIPK14*-specific primers (F, R, left sample) beside the insertion site or T-DNA-specific primers (F, R0, right sample) in the LB respectively. No. 1, 4, 5, 6, 7, 8 alleles of SALK_009699 were verified as homozygote, No. 2 allele was heterozygote, and No. 3 allele is wild type; No. 2, 3, 4 alleles of SALK_147899 were verified as heterozygote, No. 1 allele is wild type. (c) RT-PCR was performed with either *CIPK14*-specific primers (top gel) or Actin-specific primers (bottom gel) to verify expression of *CIPK14* in the T-DNA insertion lines. (d) 6-day-old seedlings of *Arabidopsis thaliana* plants (WT) and *cipk14* mutant grown on MS medium under continuous white light with the fluence rates of 50 μmol $m^{-2} s^{-1}$, hypocotyl and root elongation were measured (left), 15-day-old seedlings of WT and *cipk14* mutant grown on MS medium under normal continuous white light (right). Vertical bars represent the S.D. (n=3).

In both wild type and *cipk14* mutant plants, *RD29B* was induced under high salt conditions with similar kinetics and induction level to ABA treatment. The *RD29B* mRNA level in the wild type plants was five times higher than that in the *cipk14* mutant under salt treatment (Figure 3(d)). The maximal induction of *KIN1/KIN2* occurred two hours earlier in wild type than in *cipk14* mutant. The expression of *RD29A* and *KIN1/KIN2* genes is under the control of *CBFs/DREBs*, transcription factors that bind to the *cis*-acting elements (*CRE/DRE*) in the promoter regions of *RD29A* and *KIN1/KIN2*[17,18]. Genes that encode *CBFs/DREBs* also are stress genes themselves. In the present study, *DREB1A/DREB2A* was induced by salt and ABA stimulus. Their transcripts rapidly reached the peak at 1 h of ABA treatment in both tested plant lines, although the average induction level in *cipk14* mutant was less than 45% of that in wild type (Figure 3(b)). This was consistent with the fact that *DREB* proteins are transcriptional activators for *RD/KIN* genes. Differently, the induction peak of *DREB1A/DREB2A* in *cipk14* mutant delayed more than four hours under high salt conditions (Figure 3(d)).

Interestingly, the induction level of *SOS2* was significantly lower (each 60%) in the *cipk14* mutant compared with wild-type plants on both stress treatments in all the analyzed times. This result suggested that *CIPK14* might be involved in the regulation of stress signal pathway of *SOS2/SOS3* in *Arabidopsis*.

2.4 Sensibility assay of *cipk14* mutants to stresses

To confirm a role of *CIPK14* in stress or ABA signaling processes, we tested the responses of *cipk14* mutant plants to ABA and abiotic stress conditions. The seeds of *cipk14* mutant and wild-type plants were plated on MS medium[13] containing various concentrations of

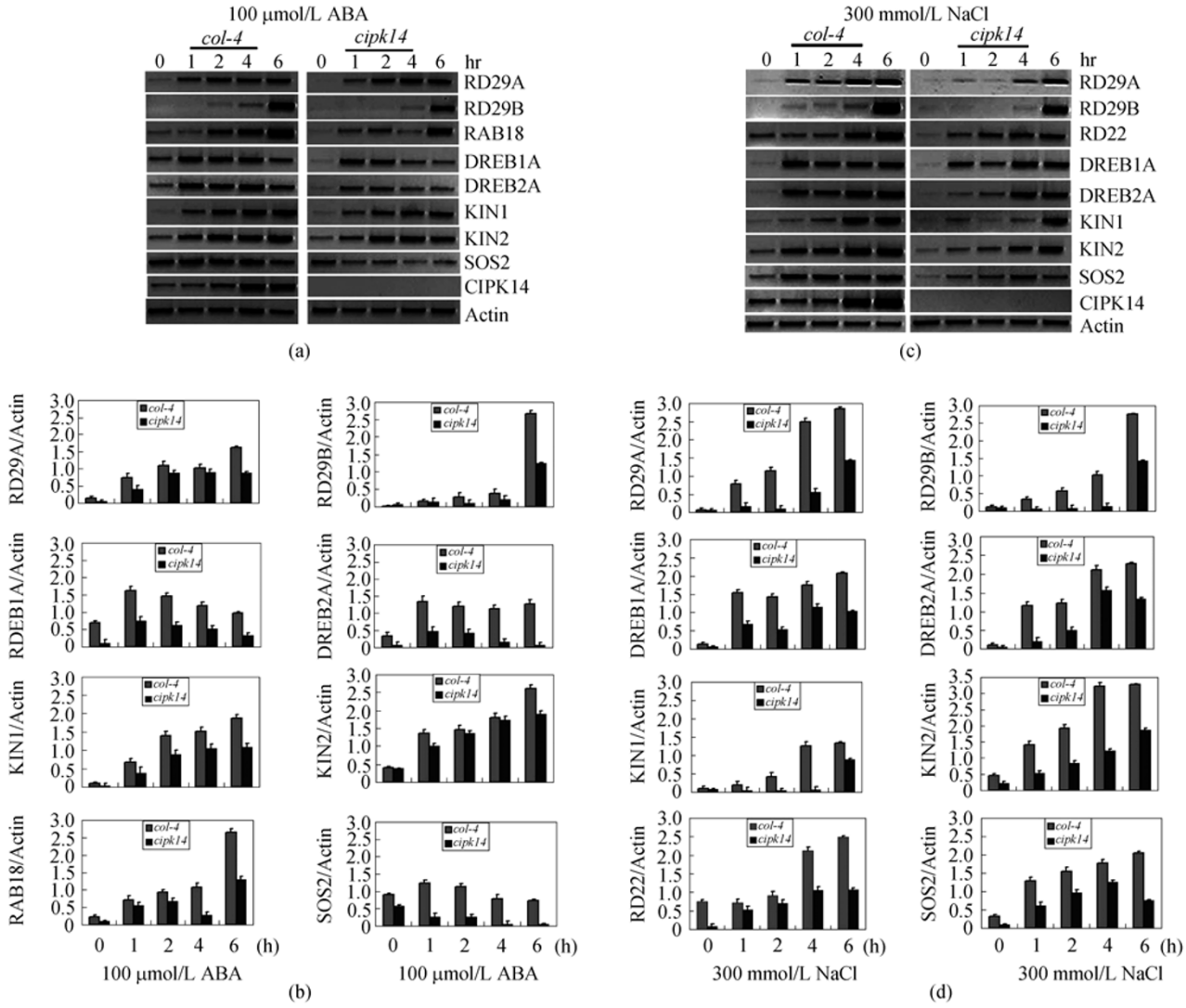

Figure 3 Expression of ABA and salt response related genes in wild type and *cipk14* mutant. (a) Expression of stress-related marker genes under 100 μmol/L ABA treatment by RT-PCR. The numbers indicated hours after each treatment. (b) The corresponding histogram was presented. Levels of mRNA expression were shown as the relative signal intensities (*GENE* /*ACTIN*). Vertical bars represent the S.D. (n=3). (c) Expression of stress-related marker genes under 300 mmol/L NaCl treatment by RT-PCR. The numbers indicated hours after each treatment. (d) The corresponding histogram was presented. Levels of mRNA expression were shown as the relative signal intensities (*GENE*/*ACTIN*). Vertical bars represent the S.D. (n=3).

ABA, NaCl, Glucose and mannitol. Seed germination of *cipk14* mutant under these stresses was generally delayed about 2—3 d showing evident inhibition in compare with the wild-type (Figure 4(b)). On medium containing 0.35 μmol/L ABA, the *cipk14* mutant seeds had, at the 6th day after seed sowing, the germination of 45%, much lower than that of wild-type (93%). Under the treatment with 150 mmol/L NaCl, 35% of wild-type seeds germinated after 2 d, 4 d earlier than that of the *cipk14* mutant seeds (Figure 4(b)). At the 6th day, the germination rate for the wild type ascended to 89%. It was also found that the germination of both wild-type and *cipk14* seeds was inhibited significantly in 6% glucose. And the germination of *cipk14* mutant was almost completely inhibited in 0.25 mol/L mannitol in our study (Figure 4).

To analyze the possible relationship between the inhibition of seed germination by salt, glucose and mannitol and ABA accumulation, the ABA biosynthesis inhibitor norflurazon[12,19] was employed in the assay. Norflurazon in the normal MS medium did not affect the germination of wild-type and *cipk14* seeds. However, when norflurazon was on the salt-containing MS medium, *cipk14* mutant had the germination rate four times higher than that on the same salt-containing medium without norflurazon. In contrast, norflurazon could not

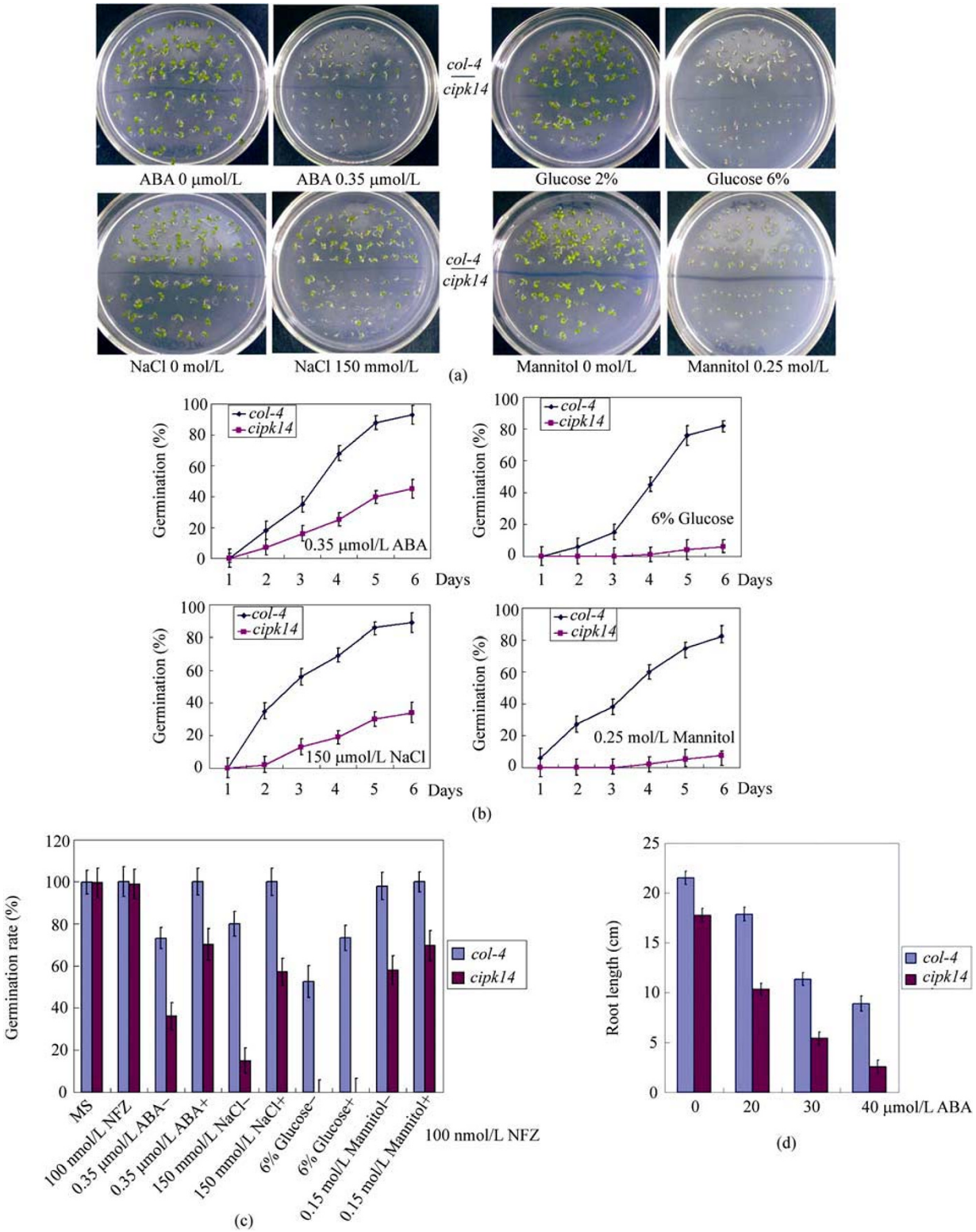

Figure 4 *CIPK14* mutants showed more sensitive to ABA and osmotic stress conditions. (a) Seed germination of *cipk14* mutant and wild type on MS with the treatments of 0.35 μmol/L ABA, 150 mmol/L salt, 6% glucose and 0.25 mol/L mannitol. The photograph was taken on day 6 after transfer to 23℃. (b) Germination rate of wild-type and *cipk14* mutant seeds from 1day to 6 d after transfer to 23℃ in the presence of different concentrations of ABA, salt, glucose and mannitol. (c) ABA biosynthesis inhibitor norflurazon rescued the hypersensitive phenotype of *cipk14* mutant seeds. 6-day-old WT, *cipk14* seedlings were analyzed. (d) Effects of ABA treatments on root elongation of *cipk14* mutants. Seeds of wild type and mutants were germinated and grown on MS medium for 2 d and seedlings were transferred to MS agar containing different concentrations of ABA for 2 weeks before the root length was measured. Vertical bars represent the S.D. (n=3).

recover the seed germination of *cipk14* mutant plants in the 6% glucose and 0.25 mol/L mannitol (Figure 4(c)). The results indicated that *CIPK14* participated in another ABA independent stress pathway simultaneously.

Based on the results in the germination assay, we further tested the effects of ABA treatment on the *cipk14* mutant. The 2-day-old seedlings grown on normal medium were transferred to stress media (0, 20, 30, 40 μmol/L ABA) for 2 weeks before the root length was measured. Obvious inhibition of root growth was observed at all the concentrations of ABA treatments (Figure 4(d)).

2.5 Exogenous calcium concentration regulated CIPK14 transcriptional levels

Ca^{2+} serves as an intracellular messenger in many phyto-hormone signaling processes. Increasing evidence suggests that Ca^{2+} serves as a second messenger in stress and ABA responses. The elevation of Ca^{2+} during the ABA response of guard cells frequently follows a distinct pattern of reiterated phases of increase and decrease, the so-called Ca^{2+}-oscillations that constitute a primary regulator of the output response[20]. To determine whether the *CIPK14* is also calcium-dependent in stress response, different concentrations of exogenous calcium were applied to the media containing ABA and salts and then *CIPK14* transcripts level was analyzed. Three-week old wild type and *cipk14* mutant plants were put in the solution with different calcium concentrations only or in addition with 0.25 μmol/L ABA or 125 mmol/L NaCl, respectively for 30 h. As shown in Figure 5(a), *CIPK14* was induced under the extremely exogenous calcium concentrations ($C \leqslant 0.1$ or $C>10$ mmol/L) without stress treatments in the wild type plants, suggesting the calcium-dependent property of *CIPK14* as expected. When ABA or NaCl was added to the Ca-containing media, similar expression pattern of *CIPK14* was observed, but the transcription level was significantly less in the *cipk14* mutant. Interestingly, the expression of *CIPK14* enhanced in parallel with the increase of Ca^{2+} concentration in the presence of ABA, while more sensitively at lower calcium concentration under salt stress.

The expression of stress marker gene *RD29A* was analyzed in order to see if exogenous calcium could alter stress signaling pathways in plants under ABA and salt treatments. As shown in Figure 5(b), the *RD29A* gene transcript was barely detectable in *cipk14* mutant plants at all the tested exogenous calcium concentrations under ABA and salt treatments; by contrast, the induction pattern of *RD29A* in wild-type was similar to that of *CIPK14*.

2.6 Regulation of the responses to ABA and salt treatments by exogenous calcium

To address whether *CIPK14* genetic disruption could alter the calcium-dependent stress responses, effects of exogenous calcium on seed germination and root elongation, under ABA and salt treatments, were further assayed with a *cipk14* mutant Salk-009699. In a germination assay, seeds were sowed on the media containing different exogenous calcium concentrations, and no effect was found on *cipk14* mutant plants (Figure 6(a)). For the wild type, in the first 2—3 d, the germination

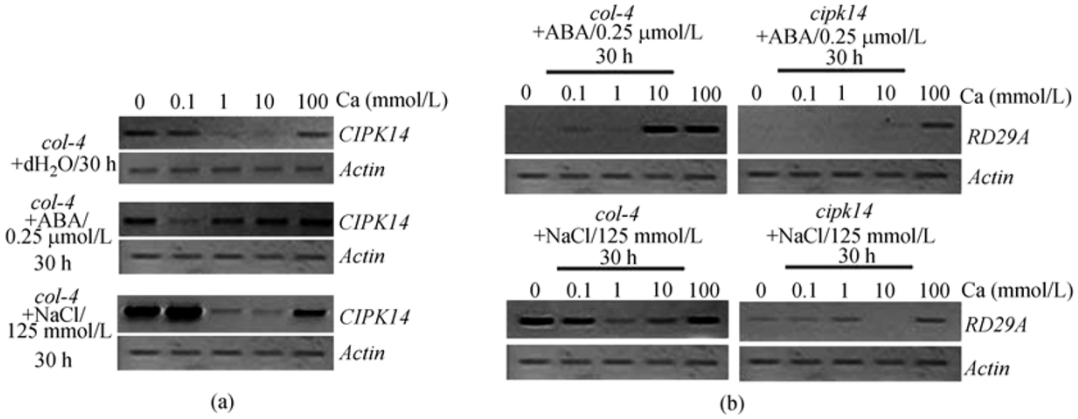

Figure 5 Regulation of *CIPK14* and stress-related gene transcriptional level by different exogenous calcium concentrations under ABA and salt treatment. (a) Regulation of the *CIPK14* gene transcriptional level by different exogenous calcium (0, 0.1, 1, 10, 100 mmol/L) under 0.25 μmol/L ABA and 125 mmol/L NaCl treatment. (b) Regulation of stress related gene transcription level by different exogenous calcium under 0.25 μmol/L ABA and 125 mmol/L NaCl treatment.

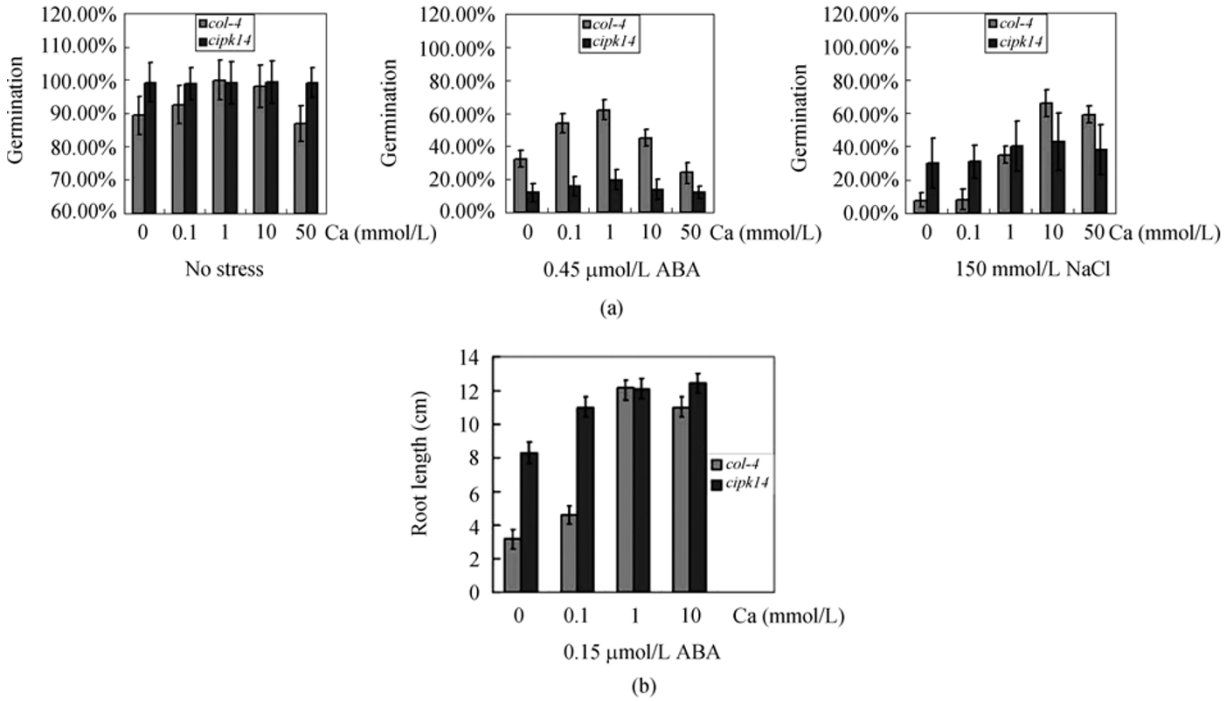

Figure 6 Effects of exogenous calcium on seed germination and root elongation of wild type and *cipk14* mutant under stress. (a) Percentage of seed germination in MS media was measured with exogenous calcium with or without 150 mmol/L NaCl or 0.45 μmol/L ABA. The corresponding histogram corresponded to the germination rate at the 6 th day. (b) Seeds were germinated and grown on MS agar medium containing different concentrations of calcium with 0.15 μmol/L ABA for 6 d before the root length was recorded. Vertical bars represent the S.D. (n±3).

was delayed distinctly under the extremely exogenous calcium concentration (C≤0.1 or C>10 mmol/L) and in the 6th day, similar germination to the *cipk14* mutant in all calcium concentrations was found. Interestingly, on medium containing 0.45 μmol/L ABA added with 1 or 10 mmol/L Ca, the wild type had 62% and 45% germination, respectively; whereas only 32.3%, 54%, 24% of germination rates were obtained when the Ca concentrations became 0, 0.1 and 50 mmol/L (Figure 6(a)). Under the salt stress (150 mmol/L NaCl), the seed germination of the wild type was extremely inhibited when the Ca^{2+} concentration was ⩽1 mmol/L (Figure 6(a)). Both *cipk14* mutant and wild type seedlings hardly survived in such conditions. This result was consistent with the previously report of that externally supplied Ca^{2+} reduces the toxic Vects of NaCl[21,22]. We also examined the effects of exogenous calcium with 0.15 μmol/L ABA on root elongation rate in wild type and *cipk14* mutants (Figure 6(b)). The results were similar to those from seed germination assays.

3 Discussions

As reported previously, many extra-cellular signals, including light, biotic, and abiotic stress factors, elicit changes in cellular Ca^{2+} concentration in plants[23−25]. Genetic analysis indicates that ABA-dependent and ABA-independent pathways may cross-talk (or even converge) through components in the signaling pathways[26,27]. A widely accepted candidate that may mediate such cross-talk is calcium. Events that occur before the elevation of Ca^{2+} and post- Ca^{2+} signal transduction are considered when stress signaling is analyzed. A signal component downstream of Ca^{2+}could affect the release of Ca^{2+}. The elevation of Ca^{2+} during the ABA response of guard cells frequently follows a distinct pattern of reiterated phases of increase and decrease, the so-called Ca^{2+}-oscillations that constitute a primary regulator of the output response[20,28].

CIPK14 was highly expressed in roots and young seedlings thus suggested that this gene may be required for the early development and root elongation of *Arabidopsis*. Highest levels of *CIPK14* mRNA in flower organs indicated that *CIPK14* might play a role in florescence which remained to be further investigated. Our results here showed that disruption of *CIPK14* function altered the pattern of stress gene induction by high salt and ABA. Especially, the expression levels of *DREB1A/*

DREB2A, transcription factors for COR/RD/KIN, was declined under stress conditions in the *cipk14* mutant plants. Admittedly, *RD29A* has one copy of ABRE (ABA-responsive element), *RD29B* has two copies of ABRE in the promoter, and *RAB18* contains only the ABRE[29−31]. The induction of *RD29A, RD29B* and *RAB18* was significantly inhibited in the *cipk14* mutant in the present study, suggesting that the transcription factors that bind to ABREs may be controlled by *CIPK14*. In addition, *RD22* with a different *cis*-acting element also was affected in the *cipk14* mutant by salt treatment. The transcription factors for the *RD22* activation may include MYC/MYB-type proteins[32,33]. The fact that *CIPK14* regulated the expression of *RD22, RAB18* and *RD29A*/*RD29B* again supported that *CIPK14* was located upstream of transcription factors in stress-induced gene expression. Although the retardation growth of *cipk14* mutant seedlings was found in normal conditions, the germination of *cipk14* mutant seeds was quite similar to that of the wild type. However, *cipk14* mutant was hypersensitive to stress treatments, including ABA, high salt, mannitol, and glucose, in which the germination and root elongation were all distinctly inhibited. Interestingly, norflurazon could recover the seed germination of *cipk14* mutant plants in the ABA and high salt treatment but 6% glucose and 0.25 mol/L mannitol. The results indicated that *CIPK14* participated in another ABA independent stress pathway simultaneously.

The transcription level of *CIPK14* was influenced by exogenous calcium in our study. Similarly, When different concentrations of exogenous calcium were applied to the *cipk14* mutants under same ABA or salt treatments, the transcription level of stress related genes was invariable compared with that of wild type, implying that *CIPK14* might mediate the calcium-regulated stress gene expression. It was also found that seed germination rate was regulated by exogenous calcium under ABA and salt treatments in the wild type but not in the *cipk14* mutant which NAF domain was destroyed, in which, we found that externally supplied Ca^{2+} reduced the toxic effects of NaCl. Corresponding, root elongation of the wild type was more sensitive to exogenous calcium than that of *cipk14* mutant under ABA treatment. Here we proposed that *CIPK14* was located upstream of transcription factors and downstream of the Ca^{2+} signal. This putative location for *CIPK14* in the signaling pathways was further supported by our results. The transcripts of *CIPK14* fluctuated with modification of exogenous calcium concentrations, while *RD29A* showed the same diversification in wild type and only minor changes in the *cipk14* mutant under the ABA and salt induction.

Our special thanks to Professor Chentao Lin (University of California, Los Angeles, USA) for his guidance in the work. We thank Dr Yuehui He *(National University of Singapore, Singapore) and Dr Ziniu Deng (Agriculture University of Hunan, Changsha, China) for linguistic editing of the manuscript.*

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