J. Korean Soc. Appl. Biol. Chem. **53**(1), 15-21 (2010)

Article

Response of Calcineurin B-like Protein Kinase Gene in Tomato to Various Abiotic Stresses

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Received December 9, 2009; Accepted December 31, 2009

This study describes the expression patterns of the CBL interacting protein kinase *(SlCIPK)* gene in *Solanum lycopersicum* after treatment with various abiotic stresses, such as salt, dehydration and mechanical wounding. The cDNA of the *SlCIPK* gene from the tomato leaf encodes 446 amino acids and shows significant homology with other plant CIPK proteins. *SlCIPK* is a single copy gene in the tomato genome. The organ-specific expression pattern of the *SlCIPK* gene revealed that the transcript of this gene was present in all tissues examined (roots, stems, leaves and flowers), with strong expression being observed in the roots. Additionally, the *SlCIPK* gene was upregulated in response to salt, dehydration and wounding. Moreover, *SlCIPK* was regulated by abscisic acid (ABA) and calcium. Taken together, these results indicate that *SlCIPK* gene may play a role in the response to abiotic stresses via ABA-mediated calcium signaling.

Key words: abiotic stress, calcium, CIPK, expression analysis, *Solanum lycopersicum*

Excessive salt, drought, cold and high temperature are common adverse environmental factors that can induce considerable changes in plants at the cellular and molecular level [Mittler *et al.*, 2006; Xiang *et al.*, 2007], thereby affecting plant growth and productivity [Mahajan and Tuteja, 2005; Gao *et al.*, 2008]. Extreme abiotic stress can disrupt the life cycles of plants, leading to plant death [Cho *et al.*, 2006; Zeba *et al.*, 2006]. High or low temperature, drought and salinity affect plant metabolism, enhance the generation of reactive oxygen species (ROS) and reduce the photosynthetic activity of the plant [Hasegawa *et al.*, 2000]. Plant adaption to stress occurs through a complex network of signaling molecules [Zhu, 2001]. To cope with adverse environmental situations, plants employ various protective mechanisms [Yi *et al.*, 2004] and activate some stress-responsive genes to ensure their continued existence [Xiang *et al.*, 2007]. However, free Ca^{2+} ion is involved in the stress signal transduction pathway and helps to activate the stress gene in the pathway; therefore, it may contribute to the differential developmental process and signaling [Cheong *et al.*, 2003; Xiang *et al.*, 2007]. Indeed, the cytosolic free Ca^{2+} ion level has been reported to increase inside the cell in response to stress, leading to the regulation of plant growth and productivity [Rudd and Franklin-Tong, 2001].

Various Ca^{2+} ion sensing proteins and protein kinases are involved in the biotic and abiotic stress responsive pathway, including calmodulin (CaM), calcium-dependent protein kinase (CDPK), CBL and CIPK [Hamada *et al.*, 2009]. CIPK is a Ser/Thr protein kinase that contains a N-terminal catalytic domain, C-terminal regulatory domain and FISL/NAF motif [Xiang *et al.*, 2007]. The N-terminal and C-terminal domain of the *CIPK* gene contribute to salt tolerant calcium signaling [Albrecht *et al.*, 2001]. CBL interacts with CIPK and controls the activity of kinase enzyme [Batistic and Kudla, 2009]. Kinase activity remains inactive under normal conditions due to the activity of the FISL motif, which functions as an autoinhibitory domain [Xiang *et al.*, 2007]. SOS2 (Salt Overlay Sensitive 2) type protein kinase is closely related to the SOS3 type Ca^{2+} sensors [Gong *et al.*, 2004]. During SOS3-SOS2 interaction via FISL motif, SOS2 is released from inactive state to active state in a calcium-dependent manner [Halfter *et al.*, 2000].

Solanum lycopersicum is an important crop that is sensitive to unfavorable growth conditions. It has been reported that calcium sensors play an important role in the stress signaling pathway, but no studies have been conducted to evaluate the molecular response of *SlCIPK* gene to

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doi:10.3839/jksabc.2010.003

abiotic stresses. Therefore, in this study, we isolated the *Solanum lycopersicum* calcineurin B-like protein kinase gene (*SlCIPK)* and analyzed its mRNA expression pattern following various abiotic stresses and wounding. The functional characterization of *SlCIPK* under salt, dehydration, ABA and wound stress is reported here.

Materials and Methods

Plant growing. Seeds of tomato (*Solanm lycopersicum* L. cv Mircro-tom) were surface sterilized according to Cortina and Culianez-Macia [2005], immersed in 70% (v/v) ethanol for 3 min, followed by soaking the seeds in 20%(v/v) sodium hypochlorite (NaClO) solution for 10 min, and then three times washing with distilled water. The seeds were placed on Murashige-Skoog (MS) medium (Duchefa Biochemie, Haarlem, The Netherlands) containing 3%(w/v) sucrose and 0.8%(w/v) agar at 25°C for 16 h under light and 8 h under dark. After germination, two-week-old seedlings were transferred to pot soil and then were kept in green house.

Stress and sample collection. Five-week-old healthy plants were uprooted from the pot soil, washed their roots, and then the plants were soaked in a solution containing 250 mM NaCl for salt treatment and 100 μM ABA for ABA treatment, respectively for 0, 4, 8, 12, 24, and 48 h. Dehydration treatment was performed as previously explained by Yamaguchi-Shinozaki and Shinozaki [1994], kept the seedlings on filter paper for designated time periods as mentioned above. For mechanical wounding treatment, about 80% leaves of each seedling were punctured with hemostat [Kudla *et al.*, 1999], and subsequently were soaked in water for the required times (0, 1, 3, 6, and 12 h). Similarly, to know the sensitivity of the gene to exogenous calcium, the plants were grown on different concentration of $CaCl_2$ (50, 100, and 200 mM) by dipping their roots in the solution for 24 h. Control (0 mM) seedlings were treated with water for the same period of time. The treated leaves were harvested, frozen in liquid nitrogen and stored at −80°C until RNA extraction. In order to study the organ specificity of the *SlCIPK* gene, tomato organs such as roots, stems, mature leaves, young leaves and flowers were collected, frozen and stored at −80°C.

Isolation of *SlCIPK* gene from tomato cDNA. Total RNA was isolated from tomato leaf tissue (200 mg) using TRIzol reagent (Invitrogen, Carlsbad, CA). DNase treated two microgram RNA was used for cDNA synthesis. The first-strand cDNA was synthesized using the AccuPower PCR PreMix (Bioneer, Daejeon, Korea), including oligo-(dT) primers and Moloney Murine Leukemia Virus Reverse Transcriptase (M-MLV RT, Invitrogen). cDNA was diluted 10 times, and used as the template for detection of *SlCIPK* transcript by the amplification of the genome region, using the primer pair: forward primer (5'-ATGAAGAAAGTGAAGAGAAAGCTT-3') and reverse primer (5'-TCAGCGAGTCCTTGTCCTAAGCAG-3'). The template was denatured at 94°C for 5 min, followed by 30 cycles of 94°C for 1 min, 60°C for 1 min and 72°C for 1.5 min, with a final extension of 7 min at 72°C. The PCR product was extracted from the gel, purified using Power Gel Extraction Kit (Takara, Seongnam-si, Korea), ligated into pGEM-T Easy Vector (Promega, Madison, WI), amplified by T7 and SP6 primers, and then sequenced for sequence confirmation.

Alignment and construction of phylogenetic tree. Amino acids sequence of SlCIPK was aligned by using ClustalW program (http://www.ebi.ac.uk, proteomics server of the European Bioinformatics Institute, EBI.) A phylogenetic tree was constructed using TreeTop software (http://www.genebee.msu.su/services/phtree_full.htm, GeneBee-Molecular Biology Server).

Southern blot analysis. Genomic DNA was isolated from tomato leaf tissues according to Dellaporta *et al.* [1983]. Fifteen microgram DNA were digested with restriction enzymes, such as *Eco* RI, *Xba* I and *Bam* HI, electrophoresed on 1.0%(w/v) agarose gel, transferred to a nylon membrane (Hybond ^{TM}-NX, Amersham, GE Healthcare, Buckinghamshire, UK). Transferred DNA was immobilized by heating the membrane for 2 h at 80°C. The blot was hybridized using a *SlCIPK* cDNA probe labeled with [α-^{32}p] dCTP. Washing of blots and subsequent activities was performed according to Seong *et al.* [2007].

RNA isolation and Northern blot analysis. Tomato leaf tissues (200 mg) from stress treated and unstressed control (0 h) leaves were ground to fine powder with liquid N_2, and total RNA was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA) using manufacturers guideline. RNA from different organs such as roots, leaves, stems and flowers were extracted to study the organ distribution pattern of the *SlCIPK* transcript. Extracted RNA concentration was checked using a UV spectrophotometer (Optizen 2120UV, Mecasys Co. Ltd., Daejeon, Korea), and the purity was checked by staining RNA with ethidium bromide, visualized on 1%(w/v) formaldehyde agarose gel. Twenty microgram of total RNA were loaded onto a 1%(w/v) agarose gel, visualized and transferred onto nylon a membrane (Hybond ^{TM}-NX, Amersham, GE Healthcare, Buckinghamshire, UK) overnight with 20×SSC (saline-sodium citrate) solutions. RNA was immobilized by heating the membrane at 80°C for 2 h. The blot was hybridized using a *SlCIPK* cDNA probe labeled with [α-^{32}p] dCTP. Hybridization and

subsequent washing was performed according to Seong *et al*. [2007].

RT-PCR analysis. The first-strand cDNA was synthesized using the AccuPower PCR PreMix (Bioneer, Daejeon, Korea) containing Moloney Murine Leukemia Virus Reverse Transcriptase (M-MLV RT, Invitrogen). Two microgram of DNase-treated total RNA was used for cDNA synthesis. The PCR was carried out by denaturing the DNA at 94°C for 5 min, followed by 25 cycles of 94°C for 1 min, 60°C for 1 min and 72°C for 1.5 min, with a final extension at 72°C for 7 min, in which cDNA from each sample was used as template. The reaction products (10 μL) were stained with ethidium bromide and visualized on 1%(w/v) agarose gel.

Results

Isolation and evolutionary analysis of *SlCIPK* gene. The *SlCIPK* gene (GenBank Accession No. AJ717348) was isolated from tomato cDNA by RT-PCR. The sequence harbors an open reading frame (ORF) of 1,341 bp encoding 446 putative amino acids. To determine the stress responsive characteristics of the *SlCIPK* gene, its amino acid sequence was compared with other previously characterized CIPK proteins from different crops (Fig. 1A). CIPK protein contains an N-terminal catalytic domain and a C-terminal regulatory domain, which are essential for stress-tolerance [Xiang *et al.*, 2007]. SlCIPK protein was found to have significant sequence homology with other CIPK proteins from different plants with the same domain compositions (Fig. 1A). Specifically, the SlCIPK sequence was 71% homologous with AtCIPK24 (GenBank Accession No. Q9LD13), 68% homologous with OsCIPK24 (B3GMO7), 65% homologous with OsCIPK8 (Q5JLD8), 60% homologous with AtCIPK23 (Q93VD3) and 59% homologous with OsCIPK23 (B3GMO5).

To assess the evolutionary relationships of *SlCIPK* gene involved in various stress responses, a phylogenetic tree was constructed from the full length amino acid sequences of SlCIPK protein using the 'neighbor-joining' method in the phylip package (Fig. 1B). The SlCIPK protein was clustered with the AtCIPK24, OsCIPK 24 and OsCIPK8 into one branch and other proteins were clustered with another branch. The SlCIPK was found to be most similar to AtCIPK24 (Fig. 1B).

Copy number determination of *SlCIPK* gene. To determine the number of copies of the *SlCIPK* transcript in the tomato genome, Southern blotting was performed using *SlCIPK* cDNA as a probe. Fifteen (15) microgram of genomic DNA was cut by *Eco*RI, *Xba*I and *Bam*HI. Hybridization band in each lane revealed that there was only one copy of the *SlCIPK* gene in the genome (Fig. 2).

Expression of *SlCIPK* gene in different organs of tomato. We examined the mRNA expression levels of *SlCIPK* gene in tomato roots, stems, mature leaves, young leaves and flowers under normal growth conditions. The RT-PCR approach was applied to analyze the expression levels. As shown in Fig. 3A, the *SlCIPK* gene homologue was functional in almost all organs assessed, with the highest induction being observed in the roots. These variations in the expression levels may be indicative of differential functions of *SlCIPK* gene in different organs of tomato.

Response of *SlCIPK* gene to exogenously applied calcium. We also found that SlCIPK is a calcium binding related protein. Specifically, we examined the transcript level of *SlCIPK* gene in response to various concentrations of $CaCl_2$ and observed the response of the gene to exogenously applied calcium (Fig. 3B). The gradual upregulation of the *SlCIPK* transcript was noted in response to various calcium levels.

Regulation of *SlCIPK* transcripts in response to abiotic stress, wounding, and a stress hormone (ABA). To reveal the response of the *SlCIPK* gene to stress, Northern blot analysis was conducted following salt, dehydration, mechanical wounding and ABA treatments. RNA from the leaves of plants that were not subjected to stress (0 h) was used as control, and 1341 bp *SlCIPK* cDNA was used as a probe. As shown in Fig. 4, the mRNA level was upregulated gradually until 48 h in response to salt stress (Fig. 4A), whereas the transcript was upregulated at 8 h after dehydration treatment, then increased gradually until strong expression was observed at 24 h, after which it began to decline (Fig. 4B).

The *SlCIPK* gene showed the earliest induction in response to mechanical wounding, with the highest expression being observed at 1 h, followed by a gradual decline (Fig. 5A). To evaluate the ABA sensitivity of the gene, we analyzed the *SlCIPK* transcript after treating the plants with 100 μM ABA. A significant change in the transcription level of the *S1CIPK* gene was noted in response to ABA treatment, with increased expression occurring until 24 h followed by a rapid reduction (Fig. 5B). Overall, our results suggest that expression of the *SlCIPK* gene differs in response to various stress conditions.

Discussion

The SlCIPK protein showed significant sequence identity with a variety of CIPK proteins from different plants (Fig. 1A), and can contribute to stress and other developmental signaling processes. The activation

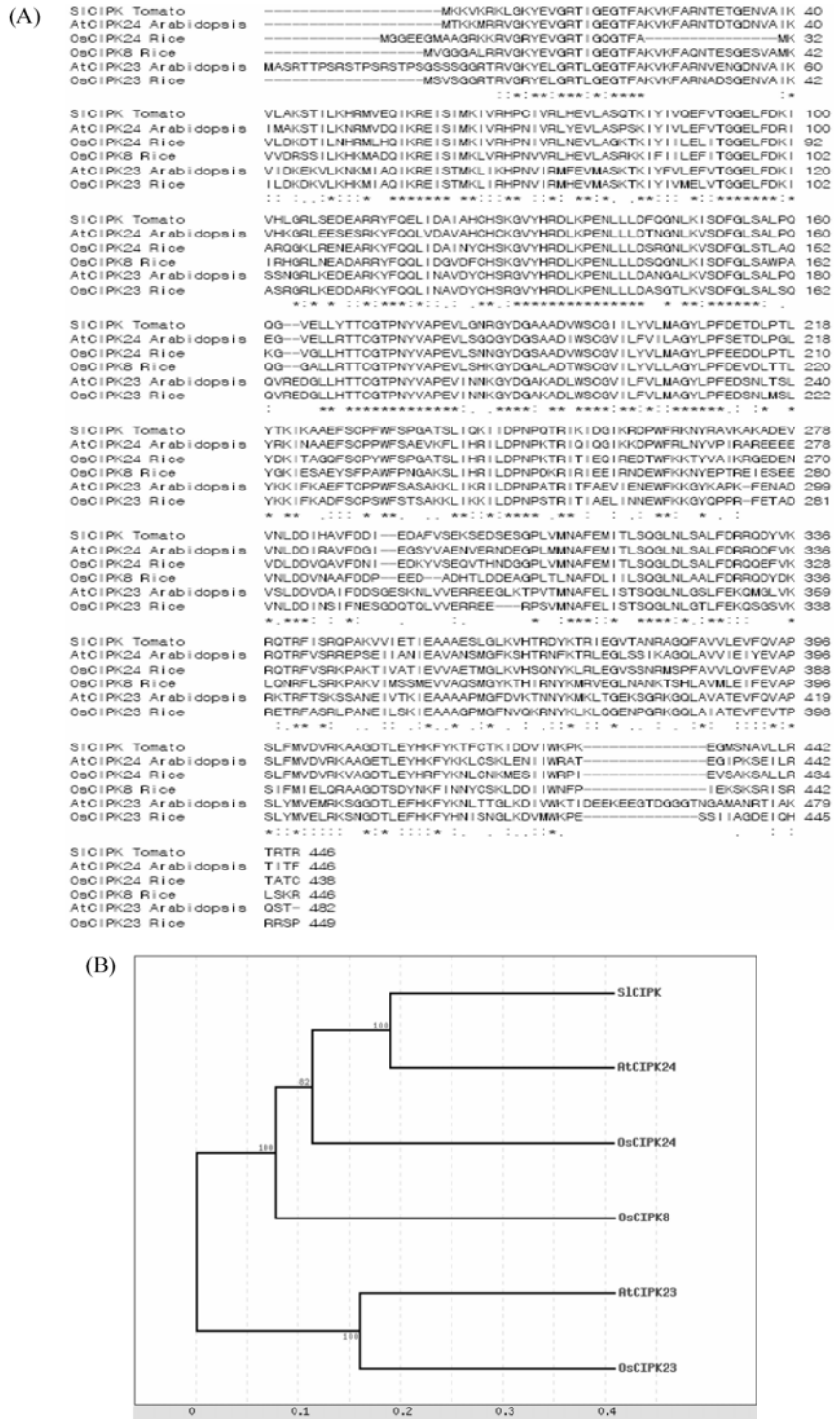

Fig. 1. Alignment of SlCIPK with other CIPK proteins and phylogenetic relationship analysis. (A) Sequence comparison of SlCIPK protein (GenBank Accession No. CAG30526) with CIPK proteins from other plants such as *Arabidopsis thaliana* (AtCIPK24; Accession No. Q9LD13); *Oriza sativa* (OsCIPK24; B3GMO7), *O. sativa* (OsCIPK8; Q5JLD8), *Arabidopsis thaliana* (AtCIPK23; Q93VD3) and *O. sativa* (OsCIPK23; B3GM05). Dashes (-) indicate to maximize alignment. Asterisks (*) represents identical amino acid residues in the sequences, (:) indicate strong conserved residues and (.) indicate weak conserved residues. Amino acid sequences lengths are indicated in the right as number. ClustalW was used to create an alignment. (B) Phylogenetic relationship of SlCIPK (446 aa). Tree was constructed using protein sequences of CIPK from other plants: *Arabidopsis thaliana* (AtCIPK24; 446 aa), *Oriza sativa* (OsCIPK24; 438 aa), *O. sativa* (OsCIPK8; 446 aa), *A. thaliana* (AtCIPK23; 482 aa) and *O. sativa* (OsCIPK23; 449 aa). A PHYLIP package from Genebee-Molecular Biology Server was used to make the final tree. Bootstrap analysis results represent the value as the number of times (as %) in the branch of tree.

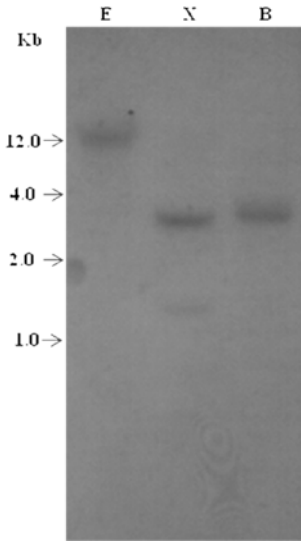

Fig. 2. Southern blot analysis of *SlCIPK*. Genomic DNA (15ig) was completely digested with restriction enzymes such as *Eco* RI (E), *Xba* I (X) and *Bam* HI (B), blotted and hybridized with *LeCIPK* cDNA probe labelled with [α-^{32}p] dCTP. DNA size was indicated in Kb at the left.

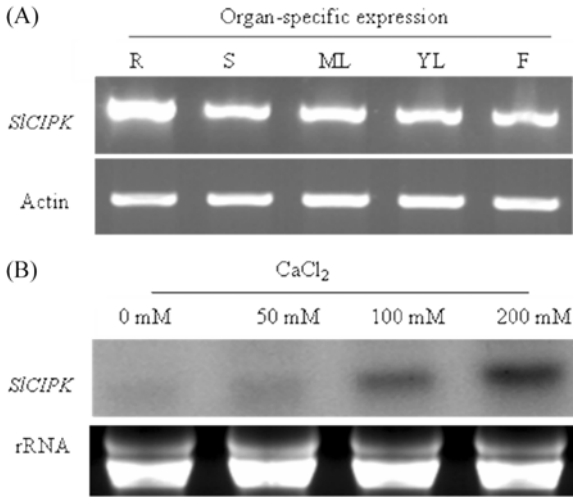

Fig. 3. Tissue distribution and exogenous calcium response of *SlCIPK* gene. (A) RT-PCR based mRNA level was observed in different tomato organs (R, roots; S, stems; YL, young leaves; ML, mature leaves and F, flowers). Two micrograms of total RNA was used for cDNA preparation for RT-PCR analysis. Tomato actin was used as loading control. (B) The *SlCIPK* response in different concentration of calcium. 0 mM to 200 mM $CaCl_2$ was used to treat tomato leaves. Each lane contains twenty micrograms of total RNA as per different treatment, blotted and hybridized with *SlCIPK* cDNA, probe labelled with [α-^{32}p] dCTP. rRNA bands stained with ethidium bromide indicate as loading control.

domain in the N-terminal region and the NAF domain in the C-terminal region of the CIPK protein may create an environment that enables its interaction with CBL to activate the kinase enzyme in a calcium-dependent manner [Xiang *et al.*, 2007]. Plants may harbor single, double or multiple copies of a gene in their genome, but we detected only a single copy of the *SlCIPK* cDNA in the tomato genome. *PsCIPK* in the pea genome has also been identified as a single copy gene [Mahajan *et al.*, 2006].

The *CIPK* gene is expressed differently in different organs of the plant under normal growth conditions [Kim *et al.*, 2003]. Variations in the expression of a gene in different organs may be due to functional diversity of the gene during plant development and signaling [Kudla *et al.*, 1999]. Our study revealed that the highest expression of *SlCIPK* gene occurred in the roots of the tomato plants (Fig 3A). Abiotic stress has been found to be closely linked to the production of cellular Ca^{2+} ions [Batistic and Kudla, 2009]. Calcium may act as a signal transduction molecule in various pathways and works as second messenger in abiotic stress signaling. The Ca^{2+} ion binds to the CBL calcium sensor in the CBL-CIPK regulatory pathway, activates kinase enzymes, and stimulates the expression of the relevant genes [Mahajan *et al.*, 2006]. In our investigation, the *SlCIPK* gene was found to be stimulated by exogenous calcium in a concentration dependant manner (Fig. 3B). Similar results have also been reported in pea *CIPK* [Mahajan *et al.*, 2006].

Plants have developed specific mechanisms to cope with adverse environments and ensure their continued survival. The CBL-CIPK pathway is considered to be an emerging pathway that is regulated by calcium concentrations in the cell and enables plants to acclimate to various stress conditions [Mahajan *et al.*, 2006]. The results of the present study demonstrated that *SlCIPK* gene was induced in response to salt, dehydration, mechanical wounding and ABA (Fig. 4 and 5). The Ca^{2+} ion level has also been reported to increase in plants under abiotic stresses [Gong *et al.*, 2004], thereby helping plants to survive adverse conditions by activating calcium sensors inside the cell. In the CBL-CIPK pathway, the Ca^{2+} ion regulates the activity of the calcium sensor, thus modulating expression of the gene. The cellular free Ca^{2+} ion level has been reported to be higher under abiotic stresses. Indeed, it has been shown that even small changes in the Ca^{2+} ion level can modify the enzyme activity, leading to a stress response [Gong *et al.*, 2004]. In our experiment, Northern blot analysis revealed that the *SlCIPK* gene was upregulated by salt and dehydration treatment, with the highest expression being observed after 48 and 24 h, respectively (Fig. 4A, B). Similarly, *CIPK3* in *Arabidopsis* was upregulated after treatment with salt and drought [Kim *et al.*, 2003]. Gradual upregulation after treatment with salt was also observed

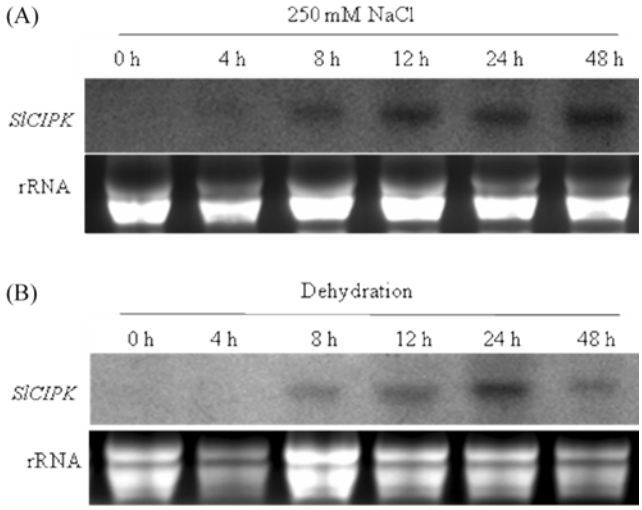

Fig. 4. RNA gel-blot analysis of *SlCIPK* in tomato leaves treated with salt, and dehydration at various time points. The leaf tissues were treated with (A) 250 mM NaCl, and (B) Dehydration. Each lane contains twenty micrograms of total RNA as per different treatment, blotted and hybridized with *SlCIPK* cDNA probe labelled with [α-^{32}p] dCTP. rRNA bands stained with ethidium bromide indicate as loading control.

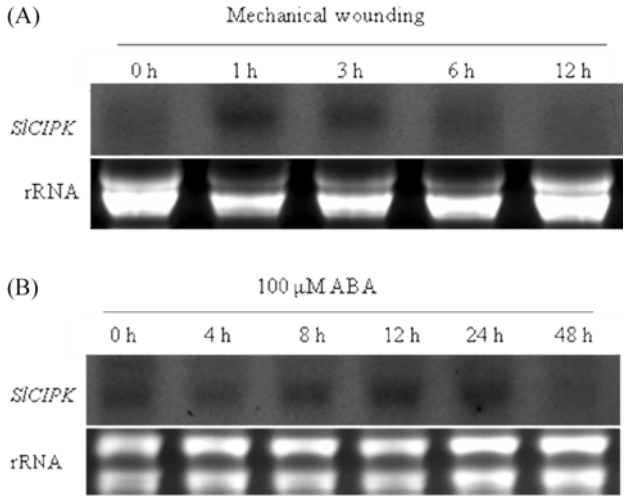

Fig. 5. *SlCIPK* gene induction pattern in response to Mechanical wounding (A) and 100 μM ABA (B). Each lane contains twenty micrograms of total RNA. The RNA was blotted and hybridized with *SlCIPK* cDNA probe labelled with [α-^{32}p] dCTP. rRNA bands stained with ethidium bromide indicate equal loading of RNA.

in *PsCIPK* in pea (Mahajan *et al.*, 2006). Additionally, a rapid increase in free Ca^{2+} ion was observed in plants treated with salt [Knight, 2000]. In *Arabidopsis*, CBL/SOS3-CIPK24/SOS2 controls SOS1 (Na^+/H^+antiporter), thereby enhancing salt detoxification in the cell [Chinnusamy *et al.*, 2004; Luan, 2008]. However, the results of the present study suggest that *SlCIPK* gene functions differentially under different stress conditions. Rice *CIPKs* were also expressed differentially in response to different abiotic stress treatments including salt, drought, ABA, PEG, and cold [Xiang *et al.*, 2007].

Wounding or mechanical damage is a common feature in the field that can be caused by natural calamities, insect attack or many other reasons, and many genes are activated by mechanical wounding in plants [Titarenko *et al.*, 1997]. We noted a time course induction of the *SlCIPK* gene in response to wounding stress, with upregulation being observed after 1 h of wounding treatment followed by a gradual decrease (Fig. 5A) [Mahajan *et al.*, 2006]. The response of *SlCIPK* with wound stress is also suggestive of its response to biotic and abiotic stresses [Sanchez *et al.*, 2000].

To determine the hormonal sensitivity of *SlCIPK* gene, we investigated its transcriptional regulation in response to treatment with 100 μM ABA. The induction of the gene was significantly upregulated in response to the exogenous application of ABA, with its maximum level being observed at 24 h, followed by a decline (Fig. 5B). These findings reflect the involvement of the *SlCIPK* gene in the ABA-mediated signaling pathway. Hormone treatment has been shown to modulate the intracellular Ca^{2+} ion level of cells [Batistic and Kudla, 2009].

This study provides a functional characterization of the *SlCIPK* gene under stress. The transcription levels observed in this study indicate that *SlCIPK* gene may play a role in salt, dehydration, and wound stress responses through ABA-mediated calcium regulatory pathway. Studies on gene silencing activities, and overexpression analysis should help clarify the role of this gene in stress tolerance signaling.

Acknowledgments. This work was supported by the Basic Research Promotion Fund (KRF-2007-313-C00688) in South Korea.

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