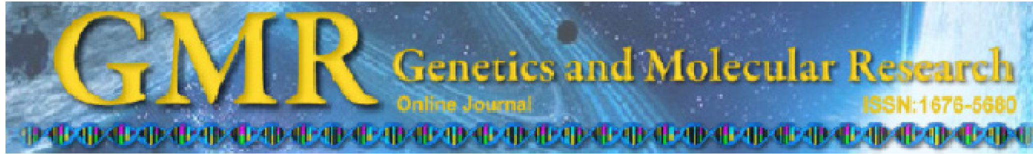

Molecular cloning and functional characterization of a novel CBL-interacting protein kinase NtCIPK2 in the halophyte *Nitraria tangutorum*

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Genet. Mol. Res. 13 (3): 4716-4728 (2014)
Received April 5, 2013
Accepted September 12, 2013
Published July 2, 2014
DOI http://dx.doi.org/10.4238/2014.July.2.1

ABSTRACT. CBL-interacting protein kinases (CIPKs) mediate many plant responses to abiotic stress. However, their functions are poorly understood in halophytes. In this study, we isolated a *CIPK* gene, *NtCIPK2*, from the halophyte *Nitraria tangutorum*. By sequence alignment and the construction of a phylogenetic tree, we found that NtCIPK2 is similar to CIPK2 proteins from other plants, and contains conserved domains and motifs. The promoter of *NtCIPK2* harbors many cis-acting elements that might be recognized and bound by transcription factors that are related to hormones and stress responses. *NtCIPK2* was ubiquitously and robustly expressed in all tested organs, and was induced by salinity, drought, heat, and cold stress. The overexpression of *NtCIPK2* in *Escherichia coli* caused better growth against high salinity, alkalinity, and osmotic conditions, dehydration, and extreme temperatures (i.e., heat and cold) compared to the control.

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Thus, *NtCIPK2* is a candidate gene that might improve the stress tolerance of crops and herbs through genetic manipulation.

Key words: CIPK; Halophyte; *Nitraria tangutorum*; Stress tolerance

INTRODUCTION

Adverse environmental conditions, such as salt, drought, and extreme temperature, are ubiquitous in agricultural areas, negatively affecting the growth and yield of plants. To survive and complete their life cycle, plants have evolved a complex network of signal transduction pathways to cope with such stresses. As a universal second messenger, calcium is critical in the stress-signaling pathways (Knight, 2000). Most stresses elicit temporal and spatial changes in cytosolic free Ca^{2+} concentrations (Sanders et al., 2002). Stress-induced Ca^{2+} signals are decoded by Ca^{2+} sensors, and transduced through interactions with their protein kinases to regulate the expression of stress-responsive genes (Li et al., 2009).

Recently, a novel plant-specific calcineurin B-like protein (CBL)-interacting protein kinase (CIPK) network was dissected. CBL proteins contain 4 elongation factor hand domains for binding Ca^{2+} (Sánchez-Barrena et al., 2005), and associate with a group of serine/threonine kinases, designated as CIPKs (Kim et al., 2000). In general, CIPKs comprise a conserved N-terminal kinase domain with a putative activation loop between the DEF and APE residues (Batistic and Kudla, 2004) and a unique C-terminal-regulatory domain. A highly conserved FISL motif in the C-terminal domain of CIPKs mediates the CBL-CIPK interaction (Albrecht et al., 2001).

A database search revealed 26 *CIPK*s in the *Arabidopsis* genome and 30 *CIPKs* in the rice genome (Kolukisaoglu et al., 2004; Weinl and Kudla, 2009). Subsequent studies have demonstrated the functional conservation of the CBL-CIPK pathway in *Populus* (Yu et al., 2007), grape (Weinl and Kudla, 2009), sorghum (Li et al., 2010), and maize (Chen et al., 2011). Enhanced tolerance to diverse stresses has also been regularly observed in transgenic *Arabidopsis* because of the overexpression of *CIPKs* (Zhao et al., 2009; Chen et al., 2012), rice (Xiang et al., 2007), tomato (Wang et al., 2012; Huertas et al., 2012), tobacco (Tripathi et al., 2009), and apple (Hu et al., 2012).

Remarkable progress has been made in determining the functions of CIPKs in response to abiotic stress in model plants and certain crops (Imamura et al., 2008; Zhao et al., 2009; Tai et al., 2013). For example, in *Arabidopsis*, *CIPK1* and *CIPK3* are crosstalk nodes between the ABA-dependent and ABA-independent pathways under environmental stress (Kim et al., 2003; D'Angelo et al., 2006). *CIPK6* is required for development and salt tolerance (Tripathi et al., 2009), while *CIPK7* is involved in cold responses (Huang et al., 2011). The ectopic expression of *CIPK8* exhibits resistance to high glucose concentrations (Gong et al., 2002). *CIPK9* and *CIPK23* activate K^+ channels or transporters, and regulate K^+ uptake under low K^+ stress (Xu et al., 2006; Pandey et al., 2007; Liu et al., 2013). *SOS2/CIPK24* is critical for salt tolerance, maintaining Na^+ and Ca^{2+} homeostasis (Cheng et al., 2004; Qiu et al., 2004). Yet, there is a paucity of reports about the function of CIPKs in other plants, particularly halophytes (Li et al., 2012).

Nitraria tangutorum Bobr. is a shrub that belongs to the *Nitratia* genus in the Zygophyllaceae family. This species is a typical and native desert halophyte that grows under

extreme conditions in northwest China. *N. tangutorum* seeds were collected from the Eastern Alxa-Western Ordos area (106° 27 E-111° 28'E, 39° 13'N-40° 52'N, elevation 1500-2100 m above sea level), which is a salinized desert in Inner Mongolia. This desert is characterized by a strong continental, weak monsoon, and arid or semiarid climate, with an average annual precipitation of 140.9-302.2 mm and an average annual temperature of 6.0°-9.2°C. The mean soil salinity and pH in this area are 0.7% and 9.0, respectively (Li, 1990).

N. tangutorum has a vital ecological function because of its superior tolerance to salinity, alkalinity, drought, wind, and dust (Zheng et al., 2011). In addition, the berries of *N. tangutorum* are used as a nutritional food source and traditional herb by villagers for the treatment of hypertension, abnormal menstruation, and indigestion. These berries also decrease blood lipid levels and have antioxidative effects (Wang et al., 2007). As an important species that has ecological and economic value in a high-salinity and arid zone, identifying the tolerance-related genes of *N. tangutorum* would be beneficial for crop improvement. However, most research on *N. tangutorum* has been restricted to its physiological responses to stress environments. For instance, enhanced proline accumulation and elevated levels of calcium in *N. tangutorum* under NaCl treatment have been reported by Yang et al. (2010, 2013). In contrast, little is known about the molecular mechanisms underlying the perception and adaptability of *N. tangutorum* to various stressors.

In this study, a novel *CIPK* gene (*NtCIPK2*) was isolated from the halophytic shrub *N. tangutorum* by the rapid amplification of cDNA end (RACE) cloning. In addition, the *NtCIPK2* promoter was isolated and analyzed. The expression of *NtP5CS* was upregulated by various abiotic stresses. Its function in tolerance to high salinity, alkalinity, and osmotic conditions, dehydration (drought), and extreme temperatures (hot and cold) was also examined in *Escherichia coli*.

MATERIAL AND METHODS

Plant growth and stress treatment

Seeds were sown and grown in a growth chamber at 25°C with a 12-h light/dark period and relative air humidity of 50%. After 2 months of culture, the young leaves were used for cloning and various organs (root, shoot and leaves) were used for gene expression assays. In addition, seedlings were treated with 200 mM sodium chloride and 10% polyethylene glycol (PEG), then subject to either 50° or 4°C for 6 h. Subsequently, the leaves were collected to analyze the responses of *NtCIPK2* to abiotic stress.

Isolation of total RNA and synthesis of first-strand cDNA

Total RNA was extracted using the RNAiso Kit (Takara, Dalian, China), following manufacturer protocols, and treated with deoxyribonuclease, before the reverse transcription (RT) reaction. One microgram of total RNA was used for cDNA synthesis. First-strand cDNA was synthesized using the M-MLV Reverse Transcriptase Kit (for semi-quantitative PCR; Promega, USA) or the SMARTer™ *RACE* cDNA Amplification Kit (for RACE; Clontech, USA). The cDNA was diluted with nuclease-free water to obtain a total volume of 20 ng/µL.

Cloning of full-length *NtCIPK2* cDNA

Degenerate primers (DECIPKF and DECIPKR; Table 1) were designed based on the alignment of all known amino acid sequences of *AtCIPK* members. PCR was performed in a 25-µL mixture, containing 20 ng template cDNA, 1.0 µM of each primer, 200 µM dNTPs, and 1.0 U TransStart™ Taq DNA polymerase (TransGen, China). DNA amplification was completed at 94°C for 5 min, 35 cycles at 94°C for 30 s, 50°C for 30 s, and 72°C for 30 s, followed by a final step at 72°C for 10 min. The amplicon was gel-purified, cloned into pMD19-T (Takara), and sequenced (Sangon, China).

Table 1. Primer sequences.

Experiment	Primer name	Primer sequence (5'-3')
NtCIPK2 degenerate PCR	DEF	CACCGTGA(T/C)CTAAA(A/G)CCAG
	DER	TGACCCC(G/C)CAAGACCA(A/G)ATA
5'- and 3'-RACE	GSP5	GGGAGCAACATAGGCTGGGGTTC
	GSP3	GGGTTGAGCGCTCTCTCTGATTCG
	UPM (long)	CTAATACGACTCACTATAGGGCAAGC AGTGGTATCAACGCAGAGT
	UPM (short)	CTAATACGACTCACTATAGGGC
Amplification of the complete coding region of *NtCIPK2*	CDSF	ATGATGGAACACAAAGGGAAAG
	CDSR	CTAATGGTAATGCTGTAGCCGAG
Isolation of the *NtCIPK2* promoter	SFP1	CACGACACGCTACTCAACAC
	SFP2	ACTCAACACACCACCTCGCACAGC
	CIPKP1	CTTGGTCTTGGTGGCCATGACCTC
	CIPKP2	CCTTAACTGCTACACTCTGGCTGG
	CIPKP3	CCTTAGCGAAGGTCCCCTGGCCCA
Semiquantitative PCR	CIPK2F	ACGACACTTCCTGCCTCAAATA
	CIPK2R	CCCAAACAATGTCCTCCAGAGC
	ACTINF	GGAATCCACGAGACCACCTACA
	ACTINR	GATTGATCCTCCGATCCAGACA
Construction of *NtCIPK2* expression vector	NTCMQF	CGCGGATCCATGATGGAACAC
	NTCMQR	ACGCGTCGACCTAATGGTAATG

Sequences underlined indicate restriction enzyme sites.

The sequence was analyzed by BLAST, and gene-specific primers (Table 1) were designed for 5'- and 3'-RACE. RACE was performed using the SMARTer™ *RACE* cDNA Amplification Kit, following manufacturer protocols, in a 25-µL mixture, containing 20 ng 5'- or 3'-RACE cDNA, 1.0 µM GSP5 or GSP3, 0.04 µM long UPM, 0.2 µM short UPM, 200 µM dNTPs, and 1.0 U Taq DNA polymerase. DNA was amplified at 94°C for 30 s, 68°C for 30 s, and 72°C for 3 min for 30 cycles, followed by an extension at 72°C for 10 min.

The 5'- and 3'-RACE products were extracted, inserted into the pMD19-T vector, and analyzed for sequence. After assembly of the fragments, the complete coding region of the cDNA was amplified with the specific primer pairs in Table 1. The PCR program was as follows: an initial denaturation step at 94°C for 5 min, followed by 30 cycles at 94°C for 30 s, 55°C for 30 s, and 72°C for 1.5 min, and a final extension at 72°C for 10 min. This gene was designated as *NtCIPK2*.

In silico analysis of NtCIPK2

The BLAST program (NCBI) was used to verify CIPK2 protein homology. Multiple-sequence alignment was performed using Clustal X. Conserved domains of NtCIPK2 were

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predicted using Prosite (http://au.expasy.org/tools), and a phylogenetic tree of CIPK2 was constructed with the amino acid sequences of NtCIPK2 and *Arabidopsis* CIPK proteins by the neighbor-joining algorithm in MEGA 4.0, with 1000 bootstrap replications. Theoretical isoelectric points and mass values for the proteins were predicted by ExPASy ProtParam (http://web.expasy.org/protparam). A structural search for NtCIPK2 was performed by TMHMM2.0 (http://www.cbs.dtu.dk/services/THHMM-2.0).

Isolation of *NtCIPK2* promoter

Genomic DNA was prepared from *N. tangutorum* seedlings using the Plant Genomic DNA Kit (Tiangen, China). Approximately 0.1 g of the sample was ground to a fine powder in liquid nitrogen, and the *NtCIPK2* promoter was isolated by SiteFinding PCR, as previously described (Tan et al., 2005). PLACE (http://www.dna.affrc.go.jp/PLACE/signalup.html) and PlantCARE (http://bioinformatics.psb.ugent.be/webtools/plantcare/html) were used to analyze cis-regulatory elements.

Analysis of expression patterns by semi-quantitative PCR

Semi-quantitative PCR was performed to analyze the expression profile of *NtCIPK2*. *β-actin* (GenBank accession No. AB617805) was used as the reference gene. The 25-μL PCR product contained 20 ng cDNA, 1.0 μM of each primer, 200 μM dNTPs, and 1.0 U Taq DNA polymerase. PCR was performed at 94°C for 1 min, followed by 28 cycles at 94°C for 20 s, 55°C for 20 s, and 72°C for 20 s. The *NtCIPK2*-specific and *β-actin* primers are listed in Table 1. The experiment was repeated in 3 separate times.

Construction of *NtCIPK2* expression vector and heterologous protein expression

ORF primers with restriction enzyme sites for *Bam*HI and *Sal*I were designed (Table 1) based on the *CIPK2* sequence of *N. tangutorum*. The amplified products were cloned into pGEX 4T1 at the *Bam*HI-*Sal*I site to express the CIPK2 protein that was fused with glutathione S-transferase (GST) at the N-terminus. Recombinant plasmids were transformed into *E. coli Rosetta* (DE3) cells. Transformants that harbored pGEX 4T1-NtCIPK2 were designated as RO/NtCIPK2, while those with pGEX 4T1 were named as RO/pGEX and used as the control.

RO/NtCIPK2 and control cells were cultured overnight in LB (Luria-Bertani) with 100 μg/mL ampicillin on a shaker at 37°C. Fresh LB medium was inoculated with 1% volume of the overnight culture until A_{600} reached 0.6. Isopropyl β-D-thiogalactopyranoside (IPTG) was added to a final concentration of 1 mM to induce the expression of *NtCIPK2*, and all cells were grown at 30°C for another 4 h. Next, the bacteria were pelleted, sonicated, diluted 2-fold in sample buffer, and loaded onto a 12% SDS-PAGE gel for analysis.

Functional validation of *NtCIPK2* in *E. coli* under various abiotic stresses

To evaluate the protective properties of the NtCIPK2 fusion protein, the effects of salt (400 mM NaCl), osmosis (1 M sorbitol), drought (10% PEG), alkali (150 mM $NaHCO_3$, pH 9.0), heat (50°C), and cold (-20°C) on the growth of RO/NtCIPK2 and control cells were

examined. Transformed *E. coli* cells were grown as described in the previous section. The original A_{600} values of the samples were adjusted to 1.0, and 100 µL cells was added to 10 mL LB containing NaCl, sorbitol, PEG, or $NaHCO_3$. Separately, 100 µL cells was incubated at 50°C for 6 h or -20°C for 3 days, and added to 10 mL LB liquid medium.

The bacterial suspension was harvested every 2 h for 12 h, and A_{600} was measured. All experiments were repeated 3 times with 3 replicates each time. The results are reported as means ± standard deviation (SD). Statistical analysis was performed using one-way analysis of variance (ANOVA) with SPSS 19.0. A P value <0.05 indicated a significant difference.

RESULTS

Cloning and characterization of *CIPK2* cDNA from *N. tangutorum*

The *NtCIPK2* cDNA is 2049 nucleotides long with a 5'-UTR of 437 nucleotides, a predicted ORF of 1344 nucleotides, and a 3'-UTR of 268 nucleotides. The predicted ORF encodes a protein of 447 amino acid residues with a theoretical molecular mass of 50.64 kDa. The pI of this protein is 8.80. Our BLAST demonstrated that *NtCIPK2* shared high similarity with plant CIPKs. *AtCIPK2* is its ortholog among the 26 *Arabidopsis CIPK* members, showing 67% sequence identity. Thus, *NtCIPK2* cDNA was deposited into the GenBank (accession No. KC823044) as a novel member of the plant *CIPK* family.

By multiple alignment analysis, the deduced NtCIPK2 sequence contained 2 domains: an N-terminal kinase domain with an activation loop and a C-terminal-regulatory domain with a CBL-interacting FISL motif (Figures 1 and 2). Ser 156, Thr 168, and Tyr 175, which are conserved in AtSOS2 and other CIPKs, were also observed in NtCIPK2 (Figure 1). As in AtCIPK2, there is no transmembrane helix domain in NtCIPK2, according to the prediction by TMHMM 2.0.

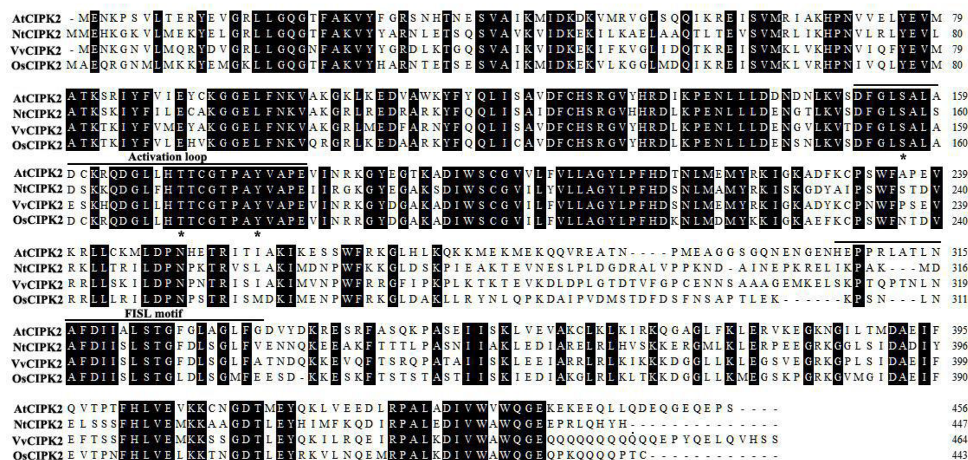

Figure 1. Deduced amino acid sequence alignment of NtCIPK2; peptide alignment of NtCIPK2 with homologs from *Arabidopsis* (AtCIPK2, AAF86506), *Vitis vinifera* (VvCIPK2, XP_002279331), and rice (OsCIPK2, ACD76974). Black boxes indicate identical residues; sequences under the dark line are the activation loop and FISL motif, respectively; three highly conserved residues (Ser, Tyr, and Thr) are marked with an asterisk in the activation loop.

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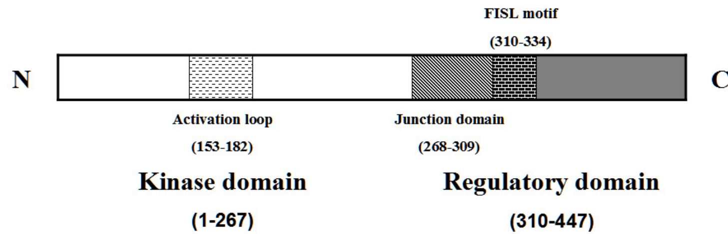

Figure 2. Schematic of the domain structure of NtCIPK2. The positions of the domains were determined based on comparison of the complete sequence of NtCIPK2 with the consensus sequence of all *Arabidopsis* CIPKs.

In the phylogenetic tree of the deduced amino acid sequences of *NtCIPK2* and the *Arabidopsis CIPK* family, NtCIPK2 was closer to AtCIPK2 than AtSOS2, and is clustered into the subgroup without introns (Figure 3). We confirmed that NtCIPK2 did not have any introns in the *N. tangutorum* genome by amplifying it from cDNA and genomic DNA (data not shown).

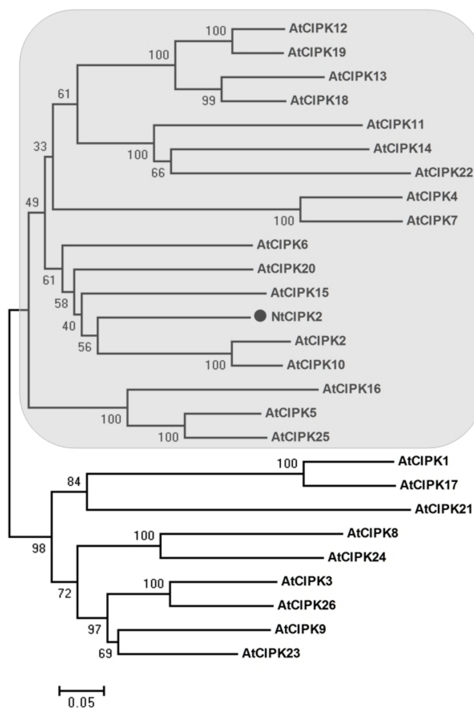

Figure 3. Phylogenetic tree of CIPK proteins. The tree was generated with NtCIPK2 and *Arabidopsis* CIPK proteins using MEGA 4.0, with 1000 bootstrap replications; the subgroup of CIPKs without introns is shownin the gray background; the Genbank accession numbers of the 26 AtCIPK proteins are: AtCIPK1, AAG28776; AtCIPK2, AAF86506; AtCIPK3, AAF86507; AtCIPK4, AAG01367; AtCIPK5, AAF86504; AtCIPK6, AAF86505; AtCIPK7, AAK16682; AtCIPK8, AAK16683; AtCIPK9, AAK16684; AtCIPK10, AAK16685; AtCIPK11, AAK16686; AtCIPK12, AAK16687; AtCIPK13, AAK16688; AtCIPK14, AAK16689; AtCIPK15, AAK16692; AtCIPK16, AAK50348; AtCIPK17, AAK64513; AtCIPK18, AAK59695; AtCIPK19, AAK50347; AtCIPK20, AAK61493; AtCIPK21, AAK59696; AtCIPK22, AAL47845; AtCIPK23, AAK61494; AtCIPK24, AAK72257; AtCIPK25, AAL41008; AtCIPK26, NP_850861.

Promoter analysis

To identify putative cis-regulatory elements, approximately 1600 bp of the upstream sequence from the start codon of *NtCIPK2* was isolated and analyzed. Most elements in the NtCIPK2 promoter were classified into 3 groups by function: light-responsive elements, phytohormone-responsive elements, and stress-related elements (Table 2).

Table 2. Prediction of stress response cis-acting elements in the *NtCIPK2* promoter.

Motif name	Sequences	Sites	Function
ABRE	ACGTG	150(+); 1160(+); 1212(+); 1249(+); 1159(-)	Abscisic acid responsiveness
GARE	TCTGTTG	282(+)	Gibberellin-responsive element
GATA Box	GATA	1477(+); 164(-); 1182(-)	Light-regulated and tissue-specific expression
G-Box	SACGTB	1159(+); 1159(-)	Light responsiveness
GT1	GRWAAW	190(+); 194(-); 202(-); 168(-); 234(-)	Salt- and pathogen-induced gene expression
HSE	AAAAAATTTC	1060(+); 1062(-)	Heat stress responsiveness
LTRE	CCGAAA	186(-)	Low-temperature responsiveness
MYB	CNGTTR	284(+); 110(-); 122(-); 1039(-)	Dehydration responsiveness
MYC	CANNTG	481(+); 720(+); 840(+); 933(+); 1096(+); 1125(+); 1145(+); 1159(+); 1227(+); 1438(+); 481(-); 720(-); 840(-); 933(-); 1096(-); 1125(-); 1145(-); 1159(-); 1227(-); 1438(-)	Multiple stresses (cold, dehydration, freezing, ABA, etc.) responsiveness
W-Box	TCAC	742(+); 1487(+); 848(-)	Salicylic acid, wound, and pathogen responsiveness

Expression analysis

Expression patterns of NtCIPK2 in vivo

NtCIPK2 transcripts were identified in all of the organs that we tested, including the root, shoot, and leaves, but varied between organs. For example, the shoot generated the least *NtCIPK2* transcripts. *NtCIPK2* was upregulated by salinity, drought, heat, and cold stress, with the highest induction being observed for salt and drought stress compared to the other treatments (Figure 4). Our data demonstrated that *NtCIPK2* is involved in multiple stress-responsive pathways.

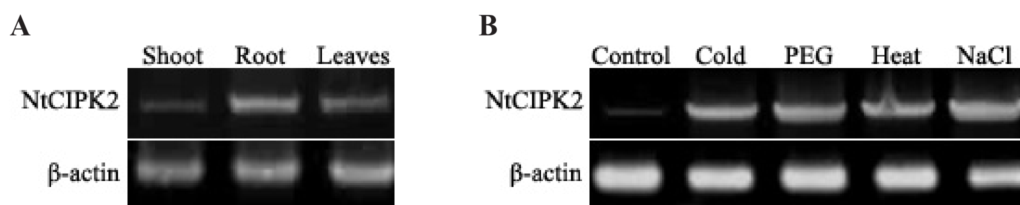

Figure 4. Expression patterns of NtCIPK2 in *Nitraria tangutorum*. **A.** RT-PCR analysis in various organs. **B.** expression level of NtCIPK2 under abiotic stress. *β-actin* was used as an internal control.

Overexpression of NtCIPK2 in E. coli

Vectors were confirmed to have been transformed into *Rosetta* cells by restriction

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enzyme digestion and PCR analysis (data not shown). The supernatants of sonicated cell lysates from induced cells were examined by SDS-PAGE. The deduced size of the proteins from RO/pGEX and RO/NtCIPK2 cells was approximately 26.0 and 76.6 kDa, respectively. We detected GST at -26.0 kDa in RO/pGEX cells, and the GST-NtCIPK2 fusion protein at -76.6 kDa in RO/NtCIPK2 cells (Figure 5, lanes 2 and 4, arrowheads). These results indicate that the *E. coli* overexpression system worked well, and that the fusion proteins were expressed effectively and were soluble.

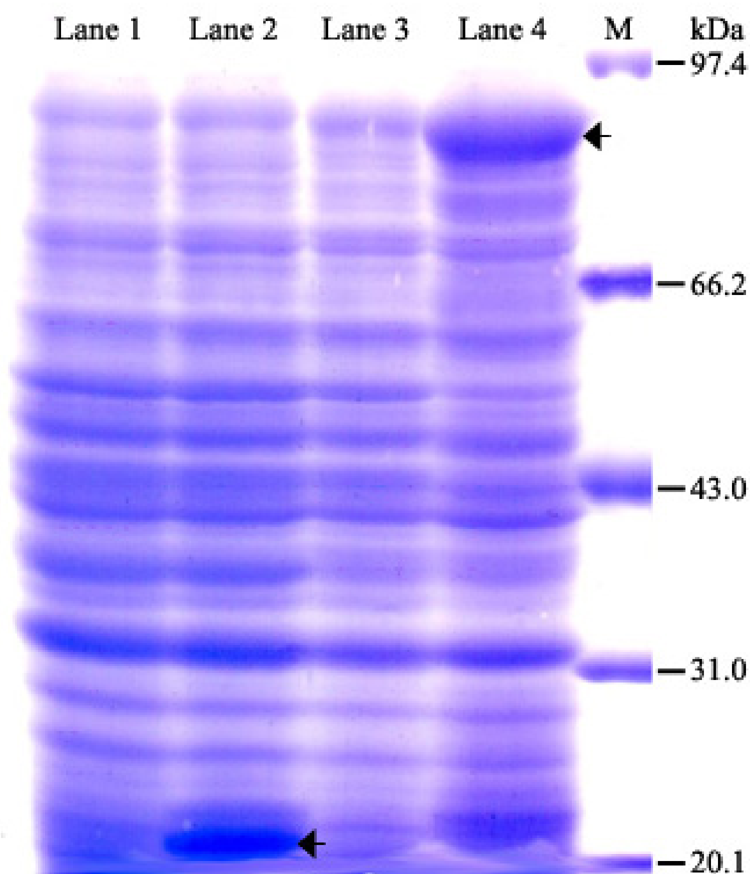

Figure 5. SDS-PAGE (12%) analysis of fusion protein in *Escherichia coli*. *Lane M* = protein marker; *lane 1* = supernatant of RO/pGEX cells without IPTG induction; *lane 2* = supernatant of RO/pGEX cells with IPTG induction; *lane 3* = supernatant of RO/NtCIPK2 cells without IPTG induction; *lane 4* = supernatant of RO/NtCIPK2 cells with IPTG induction.

Functional analysis of *NtCIPK2*

The effects of various stresses on the growth of RO/NtCIPK2 and the control strain were examined. The growth curve assay indicated no apparent distinction between the 2 strains in the absence of stress (data not shown). In contrast, RO/NtCIPK2 cells grew better in all

treatments compared to the control cells (Figure 6). Under heat stress, the A_{600} value of RO/NtCIPK2 cells was elevated ($P < 0.01$) after 6 h compared to the control cells. For all other treatments, the bacterial growth was similar to 4 h in both cell types, after which growth improved significantly ($P < 0.01$) in RO/NtCIPK2 cells versus the control cells. The data indicate that the overexpression of *NtCIPK2* improves the degree of tolerance of *E. coli* to abiotic stress.

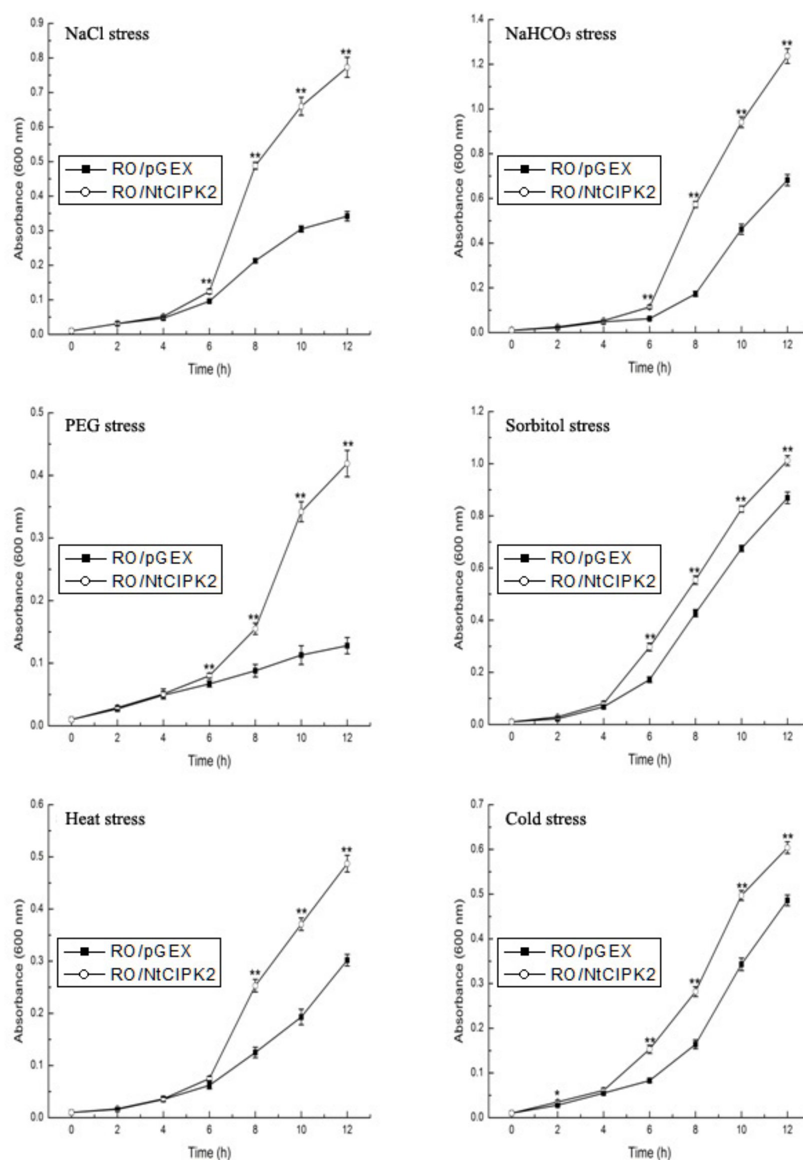

Figure 6. Growth curves of RO/pGEX (control) cells and RO/NtCIPK2 cells in liquid medium under various treatments. The average A_{600} and standard deviations were based on 3 independent experiments; statistical analysis was performed using ANOVA; $*P < 0.05$ and $**P < 0.01$ indicate significant differences.

DISCUSSION

Despite increasing evidence about the critical functions of *CIPKs* in mediating the stress responses of various plant species, there is a paucity of data about *CIPKs* from halophytes. In this study, we examined *NtCIPK2*, which is a member of the CIPK family in the halophyte shrub *N. tangutorum*. Like other CIPKs, NtCIPK2 bears a 2-domain structure that contains an N-terminal kinase domain and a C-terminal-regulatory domain. By mutational analysis, constitutively active forms of CIPKs might be generated by the substitution of 1 of 3 conserved residues in the N-terminal domain (Ser, Thr, and Tyr) to Asp (Guo et al., 2001; Gao et al., 2012). These residues are also found in NtCIPK2, indicating that the activity of NtCIPK2 may be improved by site-directed mutagenesis.

Several recent studies have demonstrated that *CIPK2* is involved in various stress-signaling processes. *Zea mays CIPK2* is upregulated by salt, PEG, cold, and heat; however, its expression differs between the leaves and the root (Chen et al., 2011). In the halophyte *Hordeum brevisubulatum*, *HbCIPK2* is strongly induced in the root and shoot by high salinity, drought, and ABA treatment. Furthermore, the overexpression of *HbCIPK2* in *Arabidopsis* improves its tolerance to salt and osmotic stress (Li et al., 2012). In this study, to better understand the mechanism by which *NtCIPK2* is regulated, an upstream fragment of the start codon was amplified. *In silico* analysis identified the presence of many stress-related cis-regulatory elements, such as MYB, MYC, W-BOX, LTR, and HSE, which were located in the promoter region of *NtCIPK2*. These elements are binding sites of transcription factors that might mediate responses to heat, cold, salinity, injury, and dehydration. We also identified ABRE in the *NtCIPK2* promoter, which indicates that the expression of *NtCIPK2* is ABA-dependent. Based on our promoter analysis, we selected 4 treatments (NaCl, PEG, 50°C, and 4°C) to examine the expression patterns of *NtCIPK2* under stress conditions. Like *ZmCIPK2* and *HbCIPK2*, *NtCIPK2* was ubiquitously expressed in all tested organs, and was induced by salinity, drought, heat, and cold stress.

As in model plant systems and several yeast mutants, *E. coli* may be used to validate the function of plant stress tolerance genes (Gupta et al., 2010; Song and Ahn, 2011; Chaturvedi et al., 2012). The overexpression of *Lycoris radiata S*-adenosylmethionine synthetase confers growth advantages to an engineered *E. coli* strain at high salt concentrations (Li et al., 2013). *SbSI-1* is a novel salt-inducible gene in the halophyte *Salicornia brachiata* that increases tolerance to salt and desiccation in *E. coli* (Yadav et al., 2012). In our study, the growth rate of RO/NtCIPK2 cells was better than control cells under salinity, drought, alkali, heat, cold, and high osmolyte stress. *N. tangutorum* primarily grows in deserts and salinity deserts, such as the Eastern Alxa-Western Ordos area, which is characterized by high soil salinity, hyperdrought, and extreme temperatures. Our results indicate that *NtCIPK2* mediates stress tolerance in relation to the functioning and adaptability of *N. tangutorum* to hostile environments.

In conclusion, we cloned a novel *CIPK2* gene in *N. tangutorum*, and analyzed its function. *NtCIPK2* was upregulated under abiotic stress, and conferred stress tolerance in *E. coli*. Thus, *NtCIPK2* could potentially be used to improve the stress tolerance of crops and herbs through genetic transformation.

ACKNOWLEDGMENTS

Research supported by the National Natural Science Foundation of China (#31140020)

and the Natural Science Foundation Key Project of Inner Mongolia Autonomous Region (#2012ZD05).

REFERENCES

Albrecht V, Ritz O, Linder S, Harter K, et al. (2001). The NAF domain defines a novel protein-protein interaction module conserved in Ca^{2+}-regulated kinases. *EMBO J.* 20: 1051-1063.

Batistic O and Kudla J (2004). Integration and channeling of calcium signaling through the CBL calcium sensor/CIPK protein kinase network. *Planta* 219: 915-924.

Chaturvedi AK, Mishra A, Tiwari V and Jha B (2012). Cloning and transcript analysis of type 2 metallothionein gene (SbMT-2) from extreme halophyte *Salicornia brachiata* and its heterologous expression in *E. coli*. *Gene* 499: 280-287.

Chen L, Ren F, Zhou L, Wang QQ, et al. (2012). The *Brassica napus* calcineurin B-like 1/CBL-interacting protein kinase 6 (CBL1/CIPK6) component is involved in the plant response to abiotic stress and ABA signalling. *J. Exp. Bot.* 63: 6211-6222.

Chen X, Gu Z, Xin D, Hao L, et al. (2011). Identification and characterization of putative CIPK genes in maize. *J. Genet. Genomics* 38: 77-87.

Cheng NH, Pittman JK, Zhu JK and Hirschi KD (2004). The protein kinase SOS2 activates the *Arabidopsis* H^+/Ca^{2+} antiporter CAX1 to integrate calcium transport and salt tolerance. *J. Biol. Chem.* 279: 2922-2926.

D'Angelo C, Weinl S, Batistic O, Pandey GK, et al. (2006). Alternative complex formation of the Ca-regulated protein kinase CIPK1 controls abscisic acid-dependent and independent stress responses in *Arabidopsis*. *Plant J.* 48: 857-872.

Gao P, Kolenovsky A, Cui YH, Cutler AJ, et al. (2012). Expression, purification and analysis of an *Arabidopsis* recombinant CBL-interacting protein kinase 3 (CIPK3) and its constitutively active form. *Protein Expres. Purif.* 86: 45-52.

Gong D, Gong Z, Guo Y, Chen X, et al. (2002). Biochemical and functional characterization of PKS11, a novel *Arabidopsis* protein kinase. *J. Biol. Chem.* 277: 28340-28350.

Guo Y, Halfter U, Ishitani M and Zhu JK (2001). Molecular characterization of functional domains in the protein kinase SOS2 that is required for plant salt tolerance. *Plant Cell* 13: 1383-1400.

Gupta K, Agarwal PK, Reddy MK and Jha B (2010). SbDREB2A, an A-2 type DREB transcription factor from extreme halophyte *Salicornia brachiata* confers abiotic stress tolerance in *Escherichia coli*. *Plant Cell Rep.* 29: 1131-1137.

Hu DG, Li M, Luo H, Dong QL, et al. (2012). Molecular cloning and functional characterization of MdSOS2 reveals its involvement in salt tolerance in apple callus and *Arabidopsis*. *Plant Cell Rep.* 31: 713-722.

Huang C, Ding S, Zhang H, Du H, et al. (2011). CIPK7 is involved in cold response by interacting with CBL1 in *Arabidopsis thaliana*. *Plant Sci.* 181: 57-64.

Huertas R, Olias R, Eljakaoui Z, Galvez FJ, et al. (2012). Overexpression of SlSOS2 (SlCIPK24) confers salt tolerance to transgenic tomato. *Plant Cell Environ.* 35: 1467-1482.

Imamura M, Yuasa T, Takahashi T, Nakamura N, et al. (2008). Isolation and characterization of a cDNA coding cowpea [*Vigna unguiculata* (L.) Walp.] calcineurin B-like protein-interacting protein kinase, VuCIPK1. *Plant Biotechnol.* 25: 437-445.

Kim KN, Cheong YH, Gupta R and Luan S (2000). Interaction specificity of *Arabidopsis* calcineurine B-like calcium sensor and their target kinases. *Plant Physiol.* 124: 1844-1853.

Kim KN, Cheong YH, Grant JJ, Pandey GK, et al. (2003). CIPK3, a calcium sensor-associated protein kinase that regulates abscisic acid and cold signal transduction in *Arabidopsis*. *Plant Cell* 15: 411-423.

Knight H (2000). Calcium signaling during abiotic stress in plants. *Int. Rev. Cytol.* 195: 269-324.

Kolukisaoglu U, Weinl S, Blazevic D, Batistic O, et al. (2004). Calcium sensors and their interacting protein kinases: genomics of the *Arabidopsis* and rice CBL-CIPK signaling networks. *Plant Physiol.* 134: 43-58.

Li B (1990). Natural Resources and Environment Study of Inner Mongolia Ordos Plateau. Science Press, Beijing.

Li LB, Zhang YR, Liu KC, Ni ZF, et al. (2010). Identification and bioinformatics analysis of SnRK2 and CIPK family genes in sorghum. *Agr. Sci. China* 9: 19-30.

Li RF, Zhang JW, Wei JH, Wang HZ, et al. (2009). Functions and mechanisms of the CBL-CIPK signaling system in plant response to abiotic stress. *Prog. Nat. Sci.* 19: 667-676.

Li RF, Zhang JW, Wu GY, Wang HZ, et al. (2012). HbCIPK2, a novel CBL-interacting protein kinase from halophyte *Hordeum brevisubulatum*, confers salt and osmotic stress tolerance. *Plant Cell Environ.* 35: 1582-1600.

Li XD, Xia B, Wang R, Xu S, et al. (2013). Molecular cloning and characterization of S-adenosylmethionine synthetase gene from *Lycoris radiata*. *Mol. Biol. Rep.* 40: 1255-1263.

Liu LL, Ren HM, Chen LQ, Wang Y, et al. (2013). A protein kinase, calcineurin B-like protein-interacting protein kinase 9, interacts with calcium sensor calcineurin B-like protein 3 and regulates potassium homeostasis under low-potassium stress in *Arabidopsis*. *Plant Physiol.* 161: 266-277.

Pandey GK, Cheong YH, Kim BG, Grant JJ, et al. (2007). CIPK9: a calcium sensor-interacting protein kinase required for low-potassium tolerance in *Arabidopsis*. *Cell Res.* 17: 411-421.

Qiu QS, Guo Y, Quintero FJ, Pardo JM, et al. (2004). Regulation of vacuolar Na^+/H^+ exchange in *Arabidopsis thaliana* by the salt-overly-sensitive (SOS) pathway. *J. Biol. Chem.* 279: 207-215.

Sánchez-Barrena MJ, Martinez-Ripoll M, Zhu JK and Albert A (2005). The structure of the *Arabidopsis thaliana* SOS3: molecular mechanism of sensing calcium for salt stress response. *J. Mol. Biol.* 345: 1253-1264.

Sanders D, Pelloux J, Brownlee C and Harper JF (2002). Calcium at the crossroads of signaling. *Plant Cell* (Suppl 14): S401-S417.

Song NH and Ahn YJ (2011). DcHsp17.7, a small heat shock protein in carrot, is tissue-specifically expressed under salt stress and confers tolerance to salinity. *New Biotechnol.* 28: 698-704.

Tai FJ, Wang Q, Yuan ZL, Yuan ZH, et al. (2013). Characterization of five CIPK genes expressions in maize under water stress. *Acta Physiol. Plant* 35: Doi: 10.1007/s11738-012-1197-2.

Tan GH, Gao Y, Shi M, Zhang XY, et al. (2005). Site Finding-PCR: a simple and efficient PCR method for chromosome walking. *Nucleic Acids Res.* 33: e122.

Tripathi V, Parasuraman B, Laxmi A and Chattopadhyay D (2009). CIPK6, a CBL-interacting protein kinase is required for development and salt tolerance in plants. *Plant J*. 144: 1416-1428.

Wang HL, Suo YR, Wang XY, Li YL, et al. (2007). Extraction of *Nitraria tangutorum* seed oil by supercritical carbon dioxide and determination of free fatty acids by HPLC/APCI/MS with fluorescence detection. *Sep. Purif. Technol*. 56: 371-377.

Wang RK, Li LL, Cao ZH, Zhao Q, et al. (2012). Molecular cloning and functional characterization of a novel apple MdCIPK6L gene reveals its involvement in multiple abiotic stress tolerance in transgenic plants. *Plant Mol. Biol.* 79: 123-135.

Weinl S and Kudla J (2009). The CBL-CIPK Ca^{2+}-decoding signaling network: function and perspectives. *New Phytol.* 184: 517-528.

Xiang Y, Huang YM and Xiong LZ (2007). Characterization of stress-responsive CIPK genes in rice for stress tolerance improvement. *Plant Physiol.* 144: 1416-1428.

Xu J, Li HD, Chen LQ, Wang Y, et al. (2006). A protein kinase, interacting with two calcineurin B-like proteins, regulates K^+ transporter AKT1 in *Arabidopsis*. *Cell* 125: 1347-1360.

Yadav NS, Rashmi D, Singh D, Agarwal PK, et al. (2012). A novel salt-inducible gene SbSI-1 from *Salicornia brachiata* confers salt and desiccation tolerance in *E. coli*. *Mol. Biol. Rep*. 39: 1943-1948.

Yang YL, Wei XL, Shi RX, Fan Q, et al. (2010). Salinity-induced physiological modification in the callus from halophyte *Nitraria tangutorum* Bobr. *J. Plant Growth Regul.* 29: 465-476.

Yang YL, Yang F, Li XN, Shi RX, et al. (2013). Signal regulation of proline metabolism in callus of the halophyte *Nitraria tangutorum* Bobr. grown under salinity stress. *Plant Cell Tiss. Org.* 112: 33-42.

Yu YH, Xia XL, Yin WL and Zhang HC (2007). Comparative genomic analysis of CIPK gene family in *Arabidopsis* and *Populus*. *Plant Growth Regul*. 52: 101-110.

Zhao J, Sun Z, Zheng J, Guo X, et al. (2009). Cloning and characterization of a novel CBL-interacting protein kinase from maize. *Plant Mol. Biol.* 69: 661-674.

Zheng J, Li H, Ding CX, Suo YR, et al. (2011). Anthocyanins composition and antioxidant activity of two major wild *Nitraria tangutorum* Bobr. variations from Qinghai-Tibet Plateau. *Food Res Int*. 44: 2041-2046.

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