CIPK11: a calcineurin B-like protein-interacting protein kinase from *Nitraria tangutorum*, confers tolerance to salt and drought in *Arabidopsis*

Lu Lu
Nanjing Forestry University
Xinying Chen
Nanjing Forestry University
Pengkai Wang
Nanjing Forestry University
Ye Lu
Nanjing Forestry University
Jingbo Zhang
Chinese Academy of Forestry
Xiuyan Yang
Chinese Academy of Forestry
Tielong Cheng
Nanjing Forestry University
Jisen Shi
Nanjing Forestry University
Jinhui Chen (chenjh@njfu.edu.cn)
Nanjing Forestry University https://orcid.org/0000-0002-4994-4681

Research article

Keywords: halophyte, Nitraria tangutorum, CIPK11, salt stress, drought stress

Posted Date: November 24th, 2020

DOI: https://doi.org/10.21203/rs.3.rs-78110/v1

Version of Record: A version of this preprint was published at BMC Plant Biology on March 1st, 2021. See the published version at https://doi.org/10.1186/s12870-021-02878-x.

Abstract

Background

The *ClPKs* are a group of plant-specific Ser/Thr protein kinases acting in response to calcium signaling, which plays an important role in the physiological and developmental adaptation of plants to adverse environments. However, the functions of halophyte-derived *ClPK*s are still poorly understood, that limits a potential application of *ClPKs* from halophytes for improving the tolerance of glycophytes to abiotic stresses.

Results

In this study, we characterized the *NtClPK11* gene from the halophyte *Nitraria tangutorum* and subsequently analyzed its role in salt and drought stress tolerance using transgenic experiments with *Arabidopsis*. *NtClPK11* expression was upregulated in *N. tangutorum* root, stem and blade tissues after salt or drought treatment. Overexpressing *NtClPK11* in *Arabidopsis* improved seed germination on medium containing different levels of NaCl. Moreover, the transgenic plants grew more vigorously under salt stress and developed longer roots under salt or drought conditions than the WT plants. Furthermore, *NtClPK11* overexpression altered transcription of genes encoding key enzymes involved in proline metabolism in *Arabidopsis* exposed to salinity.

Conclusions

We conclude that *NtClPK11* promotes plant growth and mitigates damage associated with salt stress by regulating the expression of genes controlling proline accumulation. These results extend our understanding on the function of halophyte-derived *ClPK* genes and suggest that *NtClPK11* can serve as a candidate gene for improving the salt and drought tolerance of glycophytes through genetic engineering.

1. Background

Soil salinity and drought are critical environmental threats to plant development that limit plant growth by negatively affecting the availability, transportation, and partitioning of nutrients and water. These effects are threatening to decline crop productivity worldwide and increase the pace of soil desertification, further affecting the ecological balance [1]. Therefore, understanding halophyte plant tolerance to salt and drought stress is critical for sustaining agricultural productivity by breeding new stress-tolerant plants that may cope with abiotic stresses [2]. *Nitraria tangutorum* belongs to the family Nitrariaceae *Nitraria* in Sapindales, which is widely distributed in northwestern China [3–6]. *N. tangutorum* is a desert halophyte adapted to severe drought and high salinity, and generally grows in arid or semiarid regions with high salinity [7, 8]. Moreover, this species can efficiently alleviate the degree of soil salinity and fix moving

sand, thus playing an important ecological role in environmental balance [8, 9]. Previous studies have shown that *Nitraria* may adapt to abiotic stress conditions through increased antioxidant enzyme activities, proline accumulation, level of soluble carbohydrates and reduced the intracellular Na^+ / K^+ ratio [7, 10–13]. However, the molecular mechanisms underlying the physiological adaptability of *N. tangutorum* to various stresses need further study [14–16].

To perceive salinity and drought stress, plants have evolved various stress sensors, signaling pathways, transcription factors and promoters to elicit the necessary responses by altering their metabolism, growth and/or development [17, 18]. Ca^{2+} acts as an ubiquitous messenger in various signal transduction networks to induce specific cellular responses, such as responses to signals of abiotic stress [19, 20]. Previous studies have identified proteins able to sense Ca^{2+} levels, including *CaM*, *CDPK* and *CBL*. *CBL*s function through interacting with *CIPK*s to activate specific targets and transduce signals [21, 22]. *CIPK*s contain a highly conserved N-terminal kinase domain with a putative activation loop and a unique C-terminal regulatory region with a conserved NAF amino-acid motif that have been found to promote stress tolerance by regulating various physiological responses [23–25]. Overexpression of *OsCIPK12* improved rice tolerance to cold, drought, and salt stress by inducing the accumulation of proline and soluble sugars [26]. *CaCIPK6* from chickpea has been shown to mediate auxin transport to regulate the salt tolerance of tobacco seedlings [27]. Overexpression of *BrCIPK1* enhanced abiotic stress tolerance by increasing proline biosynthesis in rice [28]. In addition, *CIPKs* may regulate the activity of the ROS scavengers POD, SOD and CAT to reduce the content of H_2O_2 and MDA, and to improve stress tolerance [29, 30] or they may control ion and water homeostasis to improve salt tolerance [31, 32]. These findings have continuously revealed the importance of *CIPK*s in regulating physiological factors that may improve plant stress tolerance.

Here, we identified a novel member of the *CIPK* gene family from *N. tangutorum*, *NtCIPK11*, and described its role in the molecular regulation of salt and drought tolerance. We found that *NtCIPK11* was induced in root, stem and leaf tissues by 500 mM NaCl or 200 mM mannitol, but the transcripts preferentially accumulated in the leaves. To further explore how *NtCIPK11* might function molecularly, we overexpressed it in *Arabidopsis*. The transgenic plants showed a higher germination rate and better growth than the WT plants after NaCl or mannitol treatment. In addition, we found that genes involved in glutamate-derived proline biosynthesis [33–35], were regulated in transgenic plants. Our data show that *NtCIPK11* is able to control the expression of key genes regulating proline metabolism in *Arabidopsis* and thereby is able to increase tolerance to stress.

2. Results

2.1. *N. tangutorum* physiologically responded salt treatment

As a halophyte with adaptability in a salt environment, *N. tangutorum* has been the focus of studies designed and implemented to investigate the mechanism of salt tolerance using biochemical methods [7, 10, 12] and molecular biology techniques [14, 15, 36]. To better understand the salt tolerance, we

observed the growth morphology of *N. tangutorum* upon 400 mM NaCl treatment (Fig. 1). The seedlings watered with tap water showed unchanging growth state for 18 days (Fig. 1A - H). However, the plants treated with 400 mM NaCl exhibited dynamic change in appearance. The bottom leaves gradually withered and turned yellow with treatment extension. After one week, the seedlings under salt stress conditions were significantly different from the untreated seedlings, especially the bottom leaves (Fig. 1A-F). However, plants treated with salt for one week recovered when tap water was used for another 10 days and displayed more vigorous growth than the untreated plants. More new leaves appeared at the top of the salt-treated seedlings (Fig. 1G, H and H'). To further study the physiological mechanism of salt tolerance, the activity of antioxidant enzymes POD, SOD and CAT, was tested in plants after 400 mM NaCl treatment (Fig. 1I-K). The results showed that the activity of these antioxidant enzymes was differentially affected by salinity. POD and SOD activity increased significantly at a 400 mM salinity level on the first day of treatment (Fig. 1I and J). However, CAT did not positively respond to salt treatment in our experiment (Fig. 1K). However, free proline, generally thought to have a positive role in plants responses to environmental stresses, such as drought and salinity [37, 38], was observed to significantly accumulate in *N. tangutorum* after salt treatment (Fig. 1L). In addition, the MDA content, which indicates the integrity of the membrane [39], was slightly changed during the salt treatment (Fig. 1M). Thus, these data taken together suggested that *N. tangutorum* significantly increased the activity of some antioxidant enzymes and increased to proline content to protect the cell membrane from being drastically affected by salinity stress under our experimental conditions.

2.2. *NtCIPK11* identification and bioinformatics analysis

The large number of genes showing responses to stresses have been identified as potential resources for genetic engineering. However, most of these candidate genes were isolated from glycophytes, which possess a relatively poor ability to tolerate environmental stresses [40]. Thus the molecular information from halophytes that can be used to analyze the mechanisms of stress tolerance is limited. As a consequence, *N. tangutorum* was selected for functional gene exploration in our study. We used 5' and 3' RACE to determine the complete cDNA nucleotide sequence of the novel gene and found that it is 1677 bp in length, with a 236 bp 5'UTR and a 127 bp 3'UTR. The coding region is 1314 bp long and encodes a 438 amino acid polypeptide with a calculated molecular mass of 49.4126 kDa. BLASTP searches and multiple alignment analyses showed that the deduced protein sequence of this clone displayed a high identity with *CIPK* orthologs in other species (Fig. 2A). The protein sequence showed 73.48% identity with *Hevea brasiliensis CIPK11* (XP_021639925.1), 72.62% identity with *CIPK11* (XP_006431996.1) of *Citrus clementina* and 67.34% identity with *AtCIPK11* (AAK16686.1) of *Arabidopsis thaliana* (Fig. 2A). Similar to its homologues, this deduced protein possesses an N-terminal serine/threonine protein kinase domain (26–279 aa) with an ATP-binding site, an active site and a C-terminal regulatory domain (310–369 aa) with a *CBL*-interacting NAF/FISL module (Fig. 2A), motifs that are highly conserved in the *CIPK* family. A hydrophobicity blot and transmembrane domain prediction indicated that the most hydrophobic segment of NtCIPK11 is located between amino acid residues 210 and 221 (Fig. 2B and C). In addition, a phylogenetic analysis of the *N. tangutorum* CIPK protein and 26

Arabidopsis thaliana CIPK proteins showed that the novel halophyte CIPK clusters as a sister branch of AtCIPK11 to the intron-free subgroup [41]; hence we referred to it as *N. tangutorum CIPK11* (*NtCIPK11*) (Fig. 3).

2.3. *NtCIPK11* in *N. tangutorum* positively responds to salt treatment

To study whether *NtCIPK11* expression is regulated by salt in *Nitraria*, we treated seedlings with 500 mM NaCl for a duration of two hours. The qPCR expression profiling showed that, untreated *NtCIPK11* was expressed in the roots, stems and leaves, with the latter two tissues expressing 1.4- and 1.8-fold higher levels than the roots (Fig. 4A). After treatment with 500 mM NaCl, we found that the *NtCIPK11* transcript level increased 7-fold in the roots, 17-fold in the stems and up to 118-fold in the leaves compared to the expression level in the untreated roots. This finding shows that *NtCIPK11* transcripts accumulate preferentially in leaf tissues after salt treatment (Fig. 4A).

2.4. *NtCIPK11* overexpression led to improved salt resistance in *Arabidopsis*

To investigate how *NtCIPK11* acts molecularly, we cloned and overexpressed the gene in *Arabidopsis*. The seeds of transgenic *Arabidopsis* plants showed a 95.66% germination rate on average, close to that of WT seeds (96.05%) on ½ MS medium without salt. However, the *NtCIPK11*-transformed seeds showed 88% or 57% germination rates, respectively, after 5 days of 100 mM NaCl or 150 mM NaCl treatment, approximately twice as high as the WT germination rates of 45% and 25% respectively under the same salt conditions (Fig. 4B and C). After 20 days, the *NtCIPK11*-overexpressing plants showed longer roots and a higher number of leaves and roots than the WT plants, with the difference particularly large between the plants treated with 150 mM NaCl-treated medium (Fig. 5). Therefore, we concluded that *NtCIPK11* overexpression significantly promoted the seed germination and induced the salt tolerance of *Arabidopsis*.

2.5. Overexpression of *NtCIPK11* altered the transcription patterns of genes involved in proline metabolism

In plants, proline has been reported to accumulate after exposure to various stresses including salt, drought and cold [42]. As shown in previous research, *CIPK* overexpression promoted proline accumulation and improved the tolerance of plants exposed to cold and drought stress [43]. To determine the potential mechanism of how ectopic expression of *NtCIPK11* increases salt tolerance, four key genes of proline metabolism, *P5CS1*, *P5CS2*, *P5CR* [34] and *ProDH1* [35], in WT and transgenic plants were measured via qPCR. As shown in Fig. 6, the genes related to proline synthesis had significantly higher expression levels in the *NtCIPK11*-overexpressing plants than they did in the WT plants under the salt stress conditions (Fig. 6A-C). However, *ProDH1*, which regulates proline catabolism, had a lower

expression level in the transgenic plants than in the WT plants (Fig. 6D). These results showed that *NtCIPK11* overexpression affected the expression of proline-related genes.

2.6. *NtCIPK11* positively responded to drought treatment in *N. tangutorum*

To investigate the function of *NtCIPK11* in drought tolerance, we simulated drought stress by treating plants with 200 mM mannitol for 2 hours and observed how *NtCIPK11* expression changed. We found that *NtCIPK11* transcript levels increased dramatically after mannitol treatment, but to a slightly lesser extent than they did upon salt treatment, increasing 15-, 20- and 38-fold in root, stem and leaf tissues, respectively (Fig. 7A). Taken together, these results show that in response to at least two kinds of abiotic stresses, salt and drought stress, *NtCIPK11* expression is increased.

2.7. Overexpression of *NtCIPK11* enhanced the development of *Arabidopsis* seedlings under drought stress

To study how *NtCIPK11* affects the drought stress response, seeds of transgenic *Arabidopsis* and those of WT plants were sown on ½ MS-agar plates containing various concentrations of mannitol. Compared to the seedlings exposed to the salt treatment, the seed germination of both the WT and transgenic plants was not affected by the mannitol treatment (Fig. 7C). However, we found that WT seedlings developed more slowly than those of the transgenic plants, as indicated by the percentage of seedlings that formed two cotyledons 4 days post-germination (Fig. 7B and C). Adding mannitol to the ½ MS medium caused a high number of WT seeds to undergo arrested development, with 31%, 20% and 5% of the seedlings reaching the two-cotyledon stage at concentrations of 100 mM, 150 mM and 200 mM mannitol respectively (Fig. 7B). In contrast, as many as 91%, 80% and 70% of the *NtCIPK11*-transformed seeds developed two cotyledons (Fig. 7B). Therefore, these results showed that *NtCIPK11* can promote seedling development under drought stress conditions at an early stage of plant growth.

2.8. Overexpression of *NtCIPK11* promoted *Arabidopsis* root growth under drought stress

To further study the function of *NtCIPK11* during drought treatment, we observed plant growth on medium containing different concentrations of mannitol for 20 days. The *NtCIPK11*-overexpressing plants showed better growth than the WT plants after mannitol treatment (Fig. 8A). The transgenic lines developed a longer primary root than the WT line, especially after treatment with 150 mM or 200 mM mannitol (Fig. 8A and B). To determine whether *NtCIPK11* functions like its orthologs to regulate the expression of genes related to proline-mediated drought tolerance, the transcripts of four genes, *ProDH1*, *P5CS1*, *P5CS2*, and *P5CR* were measured by qPCR, and the results were compared to the transcription patterns of the WT and *NtCIPK11*-overexpressing plants. We found that *ProDH1* transcription in the transgenic plants was lower than it was in the WT plants after mannitol treatment, which indicates a positive effect on proline accumulation. Nevertheless, the proline synthesis genes exhibited a different

expression pattern compared to the genes in *Arabidopsis* under salt treatment in *Arabidopsis* (Fig. 9B-D). These results suggest that *NtCIPK11* is involved in drought and salt stress signaling by influencing the expression of proline metabolism regulators but to different degrees.

3. Discussion

Salt and drought stress are major environmental factors that threaten agricultural productivity and ecological balance. As a result of natural selection and adaptation to a stressful environment, halophytes have evolved specific and diverse regulatory mechanisms for high stress tolerance, that lead to a significant plasticity in environmental adaptation [44, 45]. Thus, the basic machinery of halophytes for adaptation to harsh stresses deserves further research. In addition, understanding the genetics of halophyte responses to a variety of stress conditions is critical for developing transgenic treatment strategies [46–48].

In our study, we reached two major conclusions on how the *NtCIPK11* gene isolated from *N. tangutorum* increases the salt tolerance of *Arabidopsis*. First, the overexpression of *NtCIPK11* in *Arabidopsis* resulted in a significantly higher seed germination rate after NaCl treatment (Fig. 4B and C). Second, the transgenic plants grew better than the WT plants during salt treatment (Fig. 5). These results revealed the function of this novel gene from the halophyte on salt tolerance were very consistent with the findings of previous studies [49]. The reasons for salt tolerance induction by *CIPK*s have been identified: *CIPK*s mediate the expression of genes encoding various transporters important for ion homeostasis [49–51], increase the amount of antioxidant metabolites [52], or promote the accumulation of compatible osmolyte such as soluble sugars and proline [53, 54] under salt stress conditions. Nevertheless, the previous report discussed the active response of *N. tangutorum P5CS* to salt stress [55], which helped to explain the proline accumulation in *N. tangutorum* observed in our study. In addition, we hypothesized that *NtCIPK11* functions through proline accumulation to protect plants under high salinity conditions. As a result, the key genes regulating proline levels were found to be differentially expressed in *NtCIPK11*-overexpressing plants and WT plants under salt stress. The genes modulating proline synthesis were upregulated (Fig. 6A-C); in contrast, gene regulating proline catabolism was downregulated (Fig. 6D), which would improve proline content in theory. The importance of this study is shown by the finding that the transcription levels of these candidate genes in transgenic plants were significantly different from those in WT plants when NaCl was applied. Moreover, our investigation shared partial points with the research of *CIPK*s from rice [43]. Ectopic expression of rice *OsCIPK03* and *OsCIPK12* led to a significant accumulation of proline, but the results were obtained under cold and drought stress conditions [43]. Thus, we suggest that our halophyte-derived *NtCIPK11* enhances salt tolerance by inducing gene expression to enhance the proline accumulation in plants exposed to salt stress.

Proline has been proposed to act as a compatible osmolyte [56, 57], a reactive oxygen species scavenger [58], and a protectant of macromolecules such as enzymes and cellular structures [42, 59], thus affecting plant adaptability to stress. Salt-induced *NtCIPK11* regulation of genes associated with proline accumulation led to our speculation about the capacity of this gene to cope with drought stress.

Surprisingly, the drought conditions established by 100 mM, 150 mM, and 200 mM mannitol application did not affect seed germination but limited seedling development (Fig. 7C). Correspondingly, proline synthetases were not upregulated in transgenic plants (Fig. 9B-D). However, the transcript level of the enzyme leading to proline degradation was higher in the WT seedlings than in the *NtCIPK11*-overexpressing seedlings (Fig. 9A). These results seem to indicate that *NtCIPK11* has no dramatic effect on the proline level under drought stress. The possible reason for this outcome might be attributed to the different strategies of plants in adapting to salinity and drought. Although stress regulators have multiple functions, they have a low probability of showing the same capacity in response to different stresses. For example, *Arabidopsis CIPK11* has been reported as a negative regulator of the drought stress response by controlling the expression of the transcription factor Di19-3, a gene reported to be involved in the abiotic stress response [60]. *NtCIPK11* overexpression led to the advanced development of seedlings after germination under drought stress caused by mannitol treatment (Fig. 8). Thus, we suggest that *NtCIPK11* can promote drought tolerance but the mechanism may not involve proline accumulation under drought conditions.

4. Conclusion

In summary, we identified a stress-responsive gene *NtCIPK11* from halophyte *N. tangutorum*. Ectopic expression of *NtCIPK11* promoted the seed germination and seedling growth of *Arabidopsis* upon salt treatment. Moreover, the overexpression of *NtCIPK11* caused a different transcription of genes related to proline accumulation in the seedlings treated with NaCl. Although the transcription patterns of the proline synthesis genes were differentially regulated under mannitol treatment compared to the genes in seedlings under salt stress, *NtCIPK11* overexpression still induced a higher tolerance of the seedlings to drought stress. Hence, it is concluded that *NtCIPK11* is a novel halophyte gene that plays positive roles in plant responses to salt and drought. The novel halophyte-derived gene identified may be used as a candidate gene in molecular breeding of commercial plants to obtain better stress tolerance.

5. Methods

5.1. Plant culture and treatment

N. tangutorum

Seeds of *N. tangutorum* from the Experimental Center for Desert Forestry of Chinese Academy of Forestry at Dengkou Inner Mongolia in China were harvested by the author, Jingbo Zhang, a professional researcher on study of *Nitraria*, who made the formal identification of the samples used in our study. The research institution provided the seeds of *N. tangutorum* has a cooperative relationship with Nanjing Forestry University. However, we did not find a voucher specimen of *N. tangutorum* deposited in any publicly available herbarium. For successful germination, *N. tangutorum* seeds were kept in sand with a relative water content of 7% at 4 °C for eight weeks. The seeds germinated in pots containing a mixture of soil and sand (1:1) in a chamber with 55–60% relative humidity, 26 °C ~ 28 °C, and a 16-hour light/8-hour

dark light regime. Two-month-old seedlings were irrigated with 500 mM NaCl and 200 mM mannitol. The roots, stems and blades were sampled after 2 hours, immediately frozen in liquid nitrogen and stored at -80 °C for RNA extraction. Seedlings of the same age were watered with 400 mM NaCl for morphological observation and biochemical parameter assays. The analyses of enzyme activity, proline content and MDA content were conducted following the methods of Janmohammadi et al. (2012) [61] and Zhou et al. (2014) [62].

Arabidopsis thaliana

Arabidopsis thaliana (Columbia ecotype) was used for this study. Transgenic *Arabidopsis* plants were obtained using the floral dip method [63]. To generate seeds for phenotypic analyses, *NtClPK11* overexpressing plants were screened until homozygous seeds were obtained. Each experiment was performed in triplicate, with at least 120 seeds of each genotype. *Arabidopsis* seeds were surface sterilized and sown on ½ MS containing different concentrations of NaCl or mannitol, and then cultured in a growth chamber at 23 °C using a 16-h-light/8-h-dark cycle. Four days post-germination, the germination rate and seedling development of the plants were observed, and then, 20 days post-germination the growth state was analyzed. *Arabidopsis* seedlings at 20 days post germination were immediately frozen in liquid nitrogen and stored at -80 °C for qPCR detection.

5.2. *NtClPK11* gene cloning

Total RNA was extracted from the leaves of *N. tangutorum* seedlings using a total RNA purification kit (Norgen, Thorold, ON, Canada), followed by removal of genomic DNA contaminant using DNase I (TaKaRa, Japan). Ultraviolet spectrophotometry was used to quantify the total RNA concentration and gel electrophoresis was used to evaluate its integrity. Double-stranded cDNA was synthesized by reverse transcriptase according to the manufacturer's instructions (Invitrogen, Carlsbad, USA). Degenerate primers for *NtClPK11* fragment isolation were designed based on the poplar *ClPK* homeodomain. Primers used for *NtClPK11* fragment isolation are listed in supplementary table 1. The full length *NtClPK11* sequence was cloned by 5' and 3' RACE using the primers listed in supplementary table 2, as indicated in the SMARTerTM RACE cDNA amplification kit user manual (BD Bioscience Clontech, USA). The complete coding sequence of *NtClPK11* was obtained from cDNA based on the assembled RACE sequences, using the primers listed in supplementary table 3.

5.3. *NtClPK11* sequence analysis

NtClPK11 orthologues from other species were searched with NCBI BLASTP. The molecular mass of *NtClPK11* was predicted by the online software package ExPASy (https://web.expasy.org/cgi-bin/protparam/). Multiple sequence alignments of *NtClPK11* and its orthologs were performed using DNAMAN 6.0. The feature motifs and domains in *NtClPK11* were predicted using InterProScan online software (http://www.ebi.ac.uk/InterProScan/). The accession numbers of the sequences and species used for the alignment are listed in supplementary table 4. Hydrophobic analysis and transmembrane domain prediction of the NtClPK11 protein were conducted using the ProtScale tool (http://ca.expasy.org/tools/protscale.html) and the TMHMM Server 2.0

(http://www.cbs.dtu.dk/services/TMHMM/). Phylogenetic analysis was performed with amino acid sequences of *NtCIPK11* and 26 *CIPKs* from *Arabidopsis* using Mega 6 by the NJ method with 1000 bootstrap replications and the JTT model. The accession numbers of the sequences used for the phylogenetic tree are listed in supplementary table 5.

5.4. Quantitative real-time PCR analyses

To confirm the response of *NtCIPK11* to salt and drought stress, qPCR was performed using total RNA from *Nitraria* root, stem and leaf tissues treated with 500 mM NaCl or 200 mM mannitol for 2 hours. *NtCIPK11*-overexpressing and WT *Arabidopsis* germinated on medium with 100 mM NaCl and 200 mM mannitol were used for the transcription analysis of proline-related genes. Total RNA was reverse transcribed as mentioned previously. qPCR was carried out using a SYBR-Green PCR Master Mix on a LightCycler®480 real-time PCR detection system (Roche, Basel, Switzerland) according the manufacturer's instructions. The expression levels of the target genes were normalized by the transcription of the housekeeping gene actin in *Nitraria* [64] and *UBQ10* in *Arabidopsis* [65]. Three independent experiments were performed. The primers used for the qPCR analyses were designed with Primer3 http://frodo.wi.mit.edu/). The sequences of the specific primers for each gene are listed in supplementary table 6.

Abbreviations

ROS
Reactive oxygen species
POD
Peroxidase
SOD
Superoxide dismutase
CAT
Catalase
MDA
Malondialdehyde
P5CS
Pyrroline-5-carboxylate synthetase
P5CR
Pyrroline-5-carboxylate reductase gene
ProDH1
Proline dehydrogenase gene
qPCR
Quantitative real-time PCR
JTT
Jones-Taylor-Thornton

UBQ10
Ubiquitin 10
CaM
Calmodulin
CDPK
Calcium-dependent protein kinase
CBL
Calcineurin B-like proteins
CIPK
CBL-interacting protein kinases
NAF
Asn-Ala-Phe
FISL
Phe-Ile-Ser-Leu
RACE
Rapid amplification of cDNA ends
WT
Wild type
OX
Overexpression

Declarations

Ethics approval and consent to participate

Not applicable.

Consent to publish

Not applicable.

Availability of data and materials

NtCIPK11 sequence data has been submitted to NCBI database with accession no. MW014363. All the other data supporting the results of this article are included within the paper and its supplementary file as figures or tables.

Competing interests

The authors declare that they have no competing interest.

Funding

This research was supported by the Nature Science Foundation of China (31770715), Key Research and Development Plan of Jiangsu Province (BE2017376), Foundation of Jiangsu Forestry Bureau (LYKJ [2017]42), Qinglan Project of Jiangsu Province, Natural Science Foundation of Jiangsu Province (BK20181176), Priority Academic Program Development of Jiangsu Higher Education Institutions (PAPD), and Doctorate Fellowship Foundation of Nanjing Forestry University (grant 163010107). The funding organizations did not play any role in the design of the study, data collection and analysis, interpretation of data, or writing the manuscript.

Authors' Contributions

CJH and SJS contributed to the design of this research; WPK, LY, ZJB, YXY, and CTL carried out the statistical analysis; LL and CXY performed the experiments and wrote sections of the manuscript. All authors contributed to manuscript revision and approved the submitted version.

Acknowledgements

The authors thank the support provided by the Experimental Center of Desert Forestry, Chinese Academy of Forestry. The authors appreciate the editor and reviewers for their helpful comments and suggestions.

Authors' Information

Lu Lu and Xinying Chen contributed equally to this work.

References

1. Aarati K, Shital D, Raffaella G, Asaph A, Trijatmiko KR, Nayelli MM, Arjun K, Nataraja KN, Makarla U, Andy P: **Improvement of water use efficiency in rice by expression of HARDY, an Arabidopsis drought and salt tolerance gene**. *Proceedings of the National Academy of Sciences of the United States of America* 2007, **104**(39):15270-15275.
2. Mahajan S, Tuteja N: **Cold, salinity and drought stresses: An overview**. *Archives of Biochemistry & Biophysics* 2005, **444**(2):139-158.
3. Du J, Yan P, Dong Y: **Phenological response of Nitraria tangutorum to climate change in Minqin County, Gansu Province, northwest China**. *International Journal of Biometeorology* 2010, **54**(5):583-593.
4. Chase MW, Reveal JL: **A phylogenetic classification of the land plants to accompany APG III**. *Botanical Journal of the Linnean Society* 2010, **161**(2):122-127.
5. Lu L, Li X, Hao Z, Yang L, Zhang J, Peng Y, Xu H, Lu Y, Zhang J, Shi J: **Phylogenetic studies and comparative chloroplast genome analyses elucidate the basal position of halophyte Nitraria sibirica (Nitrariaceae) in the Sapindales**. *Mitochondrial DNA* 2018, **29**(5):1-11.
6. Bremer B, Bremer K, Chase MWF, Michael F, Reveal JL, Soltis DE, Soltis PS, Stevens PF, Anderberg AA, Moore MJ, Olmstead RG: **An update of the Angiosperm Phylogeny Group classification for the orders**

and families of flowering plants: APG III. *Botanical Journal of the Linnean Society* 2009, **161**(2):105-121.

7. Yang Y, Wei X, Shi R, Fan Q, An L: **Salinity-induced Physiological Modification in the Callus from Halophyte Nitraria tangutorum Bobr**. *Journal of Plant Growth Regulation* 2010, **29**(4):465-476.
8. Zhao K, Hai F, Ungar IA: **Survey of halophyte species in China**. *Plant Science* 2002, **163**(3):491-498.
9. Kang JJ, Yue LJ, Wang SM, Zhao WZ, Bao AK: **Na compound fertilizer stimulates growth and alleviates water deficit in the succulent xerophyte Nitraria tangutorum (Bobr) after breaking seed dormancy**. *Soil Science & Plant Nutrition* 2016, **62**(5-6):11.
10. Yang Y, Shi R, Wei X, Fan Q, An L: **Effect of salinity on antioxidant enzymes in calli of the halophyte Nitraria tangutorum Bobr**. *Plant Cell Tissue & Organ Culture* 2010, **102**(3):387-395.
11. Wang L, Ma YK, Li NN, Zhang WB, Mao HP, Lin XF: **Isolation and characterization of a tonoplast Na+/H+ antiporter from the halophyte Nitraria sibirica**. *Biologia Plantarum* 2016, **60**(1):113-122.
12. Yang F, Ding F, Duan X, Zhang J, Li X, Yang Y: **ROS generation and proline metabolism in calli of halophyte Nitraria tangutorum Bobr. to sodium nitroprusside treatment**. *Protoplasma* 2014, **251**(1):71-80.
13. Yang Y, Yang F, Li X, Shi R, Lu J: **Signal regulation of proline metabolism in callus of the halophyte Nitraria tangutorum Bobr. grown under salinity stress**. *Plant Cell Tissue & Organ Culture* 2013, **112**(1):33-42.
14. Zheng L, Dang Z, Li H, Zhang H, Wu S, Wang Y, Zheng L, Dang Z, Li H, Zhang H: **Isolation and characterization of a Δ1-pyrroline-5-carboxylate synthetase (NtP5CS) from Nitraria tangutorum Bobr. and functional comparison with its Arabidopsis homologue**. *Molecular Biology Reports* 2014, **41**(1):563-572.
15. Zheng LL, Gao Z, ., Wang J, ., Zhang HR, Wang YC: **Molecular cloning and functional characterization of a novel CBL-interacting protein kinase NtCIPK2 in the halophyte Nitraria tangutorum**. *Genetics & Molecular Research Gmr* 2014, **13**(3):4716.
16. Cheng T, Chen J, Zhang J, Shi S, Zhou Y, Lu L, Wang P, Jiang Z, Yang J, Zhang S: **Physiological and proteomic analyses of leaves from the halophyte Tangut Nitraria reveals diverse response pathways critical for high salinity tolerance**. *Frontiers in Plant Science* 2015, **6**:30-30.
17. Shinozaki K, Yamaguchishinozaki K: **Gene networks involved in drought stress response and tolerance**. *Jexpbot* 2007, **58**(2):221.
18. Golldack D, Li C, Mohan H, Probst N: **Tolerance to drought and salt stress in plants: Unraveling the signaling networks**. *Front Plant Sci* 2014, **5**(151):151.
19. Albrecht V, Weinl S, Blazevic D, Dangelo C, Batistic O, Kolukisaoglu U, Bock R, Schulz B, Harter K, Kudla J: **The calcium sensor CBL1 integrates plant responses to abiotic stresses**. *Plant Journal* 2003, **36**(4):457-470.
20. Bertorello AM, Zhu JK: **SIK1/SOS2 networks: decoding sodium signals via calcium-responsive protein kinase pathways**. *Pfluegers Archiv* 2009, **458**(3):613-619.

21. Luan S, Lan W, Lee SC: **Potassium nutrition, sodium toxicity, and calcium signaling: connections through the CBL-CIPK network**. *Current Opinion in Plant Biology* 2009, **12**(3):339-346.
22. Tang R-J, Zhao F-G, Garcia VJ, Kleist TJ, Yang L, Zhang H-X, Luan S: **Tonoplast CBL-CIPK calcium signaling network regulates magnesium homeostasis in Arabidopsis**. *Proceedings of the National Academy of Sciences of the United States of America* 2015, **112**(10):3134-3139.
23. Kyung-Nam K, Hwa CY, Grant JJ, Pandey GK, Sheng L: **CIPK3, a calcium sensor-associated protein kinase that regulates abscisic acid and cold signal transduction in Arabidopsis**. *Plant Cell* 2003, **15**(2):411-423.
24. Kudla J, ., Xu Q, ., Harter K, ., Gruissem W, ., Luan S, . **Genes for calcineurin B-like proteins in Arabidopsis are differentially regulated by stress signals**. *Proc Natl Acad Sci U S A* 1999, **96**(8):4718-4723.
25. Albrecht V, ., Ritz O, ., Linder S, ., Harter K, ., Kudla J, . **The NAF domain defines a novel protein-protein interaction module conserved in Ca2+–regulated kinases**. *Embo Journal* 2014, **20**(5):1051-1063.
26. Yong X, Yuemin H, Lizhong X: **Characterization of stress-responsive CIPK genes in rice for stress tolerance improvement**. *Plant Physiology* 2007, **144**(3):1416-1428.
27. Tripathi V, Parasuraman B, Laxmi A, Chattopadhyay D: **CIPK6, a CBL-interacting protein kinase is required for development and salt tolerance in plants**. *The Plant Journal* 2009.
28. Abdula SE, Lee H-J, Ryu H, Kang KK, Nou I, Sorrells ME, Cho Y-G: **Overexpression of BrCIPK1 Gene Enhances Abiotic Stress Tolerance by Increasing Proline Biosynthesis in Rice**. *Plant Molecular Biology Reporter* 2016, **34**(2):501-511.
29. Deng X, Wei HU, Wei S, Zhou S, Zhang F, Han J, Chen L, Yin LI, Feng J, Fang B: **TaCIPK29,a CBL-interacting protein kinase gene from wheat,confers salt stress tolerance in transgenic tobacco**. *Plos One* 2013, **8**(7):e69881.
30. Wang Y, Sun T, Li T, Wang M, Yang G, He G: **A CBL-Interacting Protein Kinase TaCIPK2 Confers Drought Tolerance in Transgenic Tobacco Plants through Regulating the Stomatal Movement**. *PLoS One* 2016, **11**(12):e0167962.
31. Pandey GK, Poonam K, Amarjeet S, Leonie S, Amita P, Yadav AK, Indu T, Sanyal SK, Beom-Gi K, Sung-Chul L: **Calcineurin B-Like Protein-Interacting Protein Kinase CIPK21 Regulates Osmotic and Salt Stress Responses in Arabidopsis**. *Plant Physiology* 2015, **169**(1):780.
32. Chen X, Huang Q, Fan Z, Bo W, Wang J, Zheng J: **ZmCIPK21, A Maize CBL-Interacting Kinase, Enhances Salt Stress Tolerance in Arabidopsis thaliana**. *International Journal of Molecular Sciences* 2014, **15**(8):14819-14834.
33. Strizhov N, Abrahám E, Okrész L, Blickling S, Zilberstein A, Schell J, Koncz C, Szabados L: **Differential expression of two P5CS genes controlling proline accumulation during salt-stress requires ABA and is regulated by ABA1, ABI1 and AXR2 in Arabidopsis**. *The Plant journal : for cell and molecular biology* 1997, **12**(3):557-569.
34. Jung Y, Park J, Choi Y, Yang J, Kim D, Kim B, Roh K, Lee D, Auh C, Lee S: **Expression Analysis of Proline Metabolism-related Genes From Halophyte Arabis stelleri under Osmotic Stress Conditions**.

Journal of Integrative Plant Biology 2010, **52**(10):891-903.

35. Senthil-Kumar M, Mysore KS: **Ornithine-delta-aminotransferase and proline dehydrogenase genes play a role in non-host disease resistance by regulating pyrroline-5-carboxylate metabolism-induced hypersensitive response**. *Plant Cell Environ* 2012, **35**(7):1329-1343.
36. Lu L, Chen X, Zhu L, Li M, Chen J: **NtCIPK9: A Calcineurin B-Like Protein-Interacting Protein Kinase From the Halophyte Nitraria tangutorum, Enhances Arabidopsis Salt Tolerance**. *Frontiers in Plant ence* 2020, **11**:1112.
37. Liu J, ., Zhu JK: **Proline accumulation and salt-stress-induced gene expression in a salt-hypersensitive mutant of Arabidopsis**. *Plant Physiology* 1997, **114**(2):591-596.
38. Verbruggen N, Hermans C: **Proline accumulation in plants: a review**. *Amino Acids* 2008, **35**(4):753-759.
39. Bailly C, Benamar A, Corbineau F, Come D: **Changes in malondialdehyde content and in superoxide dismutase, catalase and glutathione reductase activities in sunflower seeds as related to deterioration during accelerated aging**. *Physiologia Plantarum* 2010, **97**(1):104-110.
40. Jha B, Sharma A, Mishra A: **Expression of SbGSTU (tau class glutathione S-transferase) gene isolated from Salicornia brachiata in tobacco for salt tolerance**. *Molecular Biology Reports* 2011, **38**(7):4823-4832.
41. Yu Y, Xia X, Yin W, Zhang H: **Comparative genomic analysis of CIPK gene family in Arabidopsis and Populus**. *Plant Growth Regulation* 2007, **52**(2):101-110.
42. Verbruggen N, Hermans C: **Proline accumulation in plants: a review**. *Amino Acids* 2008, **35**(4):753.
43. Xiang Y, Huang Y, Xiong L: **Characterization of stress-responsive CIPK genes in rice for stress tolerance improvement**. *Plant Physiol* 2007, **144**(3):1416-1428.
44. Teruaki T, Motoaki S, Masakazu S, Tetsuya S, Masatomo K, Kanako I, Yoshihiro N, Mari N, Jian-Kang Z, Kazuo S: **Comparative genomics in salt tolerance between Arabidopsis and aRabidopsis-related halophyte salt cress using Arabidopsis microarray**. *Plant Physiology* 2004, **135**(3):1697-1709.
45. Zhao KF, Harris PJC: **The Effects of Iso-osmotic Salt and Water Stresses on the Growth of Halophytes and Non-halophytes**. *Journal of Plant Physiology* 1992, **139**(6):761-763.
46. Liu L, Wang Y, Wang N, Dong Y, Fan X, Liu X, Yang J, Li H: **Cloning of a Vacuolar H+-pyrophosphatase Gene from the Halophyte Suaeda corniculata whose Heterologous Overexpression Improves Salt, Saline-alkali and Drought Tolerance in Arabidopsis**. *Journal of Integrative Plant Biology* 2011, **53**(9):731-742.
47. Ben SR, Zouari N, Ben RW, Azaza J, Meynard D, Guiderdoni E, Hassairi A: **Improved drought and salt stress tolerance in transgenic tobacco overexpressing a novel A20/AN1 zinc-finger "AlSAP" gene isolated from the halophyte grass Aeluropus littoralis**. *Plant Molecular Biology* 2010, **72**(1-2):171.
48. Yao M, Zeng Y, Liu L, Huang Y, Zhao E, Zhang F: **Overexpression of the halophyte Kalidium foliatum H⁺-pyrophosphatase gene confers salt and drought tolerance in Arabidopsis thaliana**. *Molecular Biology Reports* 2012, **39**(8):7989-7996.

49. Sanchezbarrena MJ, Martinezripoll M, Zhu J, Albert A: **The Structure of the Arabidopsis Thaliana SOS3: Molecular Mechanism of Sensing Calcium for Salt Stress Response**. *Journal of Molecular Biology* 2005, **345**(5):1253-1264.
50. Li R, Zhang J, Wu G, Wang H, Chen Y, Wei J: **HbCIPK2, a novel CBL-interacting protein kinase from halophyte Hordeum brevisubulatum, confers salt and osmotic stress tolerance**. *Plant, cell & environment* 2012, **35**(9):1582-1600.
51. Miranda RDS, Alvarezpizarro JC, Costa JH, Paula SDO, Prisco JT, Gomesfilho E: **Putative role of glutamine in the activation of CBL/CIPK signalling pathways during salt stress in sorghum**. *Plant signaling & behavior* 2017, **12**(8).
52. Hu D, Ma Q, Sun C, Sun M, You C, Hao Y: **Overexpression of MdSOS2L1, a CIPK protein kinase, increases the antioxidant metabolites to enhance salt tolerance in apple and tomato**. *Physiologia Plantarum* 2016, **156**(2):201-214.
53. Hare PD, Cress WA, Van Staden J: **Dissecting the roles of osmolyte accumulation during stress**. *Plant Cell and Environment* 1998, **21**(6):535-553.
54. Abdula SE, Lee HJ, Ryu H, Kang KK, Nou I, Sorrells ME, Cho Y: **Overexpression of BrCIPK1 Gene Enhances Abiotic Stress Tolerance by Increasing Proline Biosynthesis in Rice**. *Plant Molecular Biology Reporter* 2016, **34**(2):501-511.
55. Zheng L, Dang Z, Li H, Zhang H, Wu S, Wang Y: **Isolation and characterization of a Δ1-pyrroline-5-carboxylate synthetase (NtP5CS) from Nitraria tangutorum Bobr. and functional comparison with its Arabidopsis homologue**. *Molecular Biology Reports* 2014, **41**(1):563-572.
56. Gadallah MAA: **Effects of Proline and Glycinebetaine on Vicia Faba Responses to Salt Stress**. *Biologia Plantarum* 1999, **42**(2):249-257.
57. Hellebusi JA: **Osmoregulation**. *Plant Biology* 1976(27):485-505.
58. Smirnoff N, Cumbes QJ: **Hydroxyl radical scavenging activity of compatible solutes**. *Phytochemistry* 1989, **28**(4):1057-1060.
59. Misra N, Gupta AK: **Effect of salt stress on proline metabolism in two high yielding genotypes of green gram**. *Plant Science* 2005, **169**(2):331-339.
60. Ma Y, Cao J, Chen Q, He J, Liu Z, Wang J, Li X, Yang Y: **The Kinase CIPK11 Functions as a Negative Regulator in Drought Stress Response in Arabidopsis**. *International journal of molecular sciences* 2019, **20**(10):2422.
61. Janmohammadi M, Abbasi A, Sabaghnia N: **Influence of NaCl treatments on growth and biochemical parameters of castor bean (Ricinus communis L.)**. *Acta Agriculturae Slovenica* 2012, **99**(1).
62. Zhou G, Nimir NEA, Lu S, Zhai F, Wang Y: **Gibberellic Acid and Salinity Affected Growth and Antioxidant Enzyme Activities in Castor Bean Plants at Early Growth Stage**. *Agronomy Journal* 2014, **106**(4):1340-1348.
63. Clough SJ, Bent AF: **Floral dip: a simplified method for Agrobacterium-mediated transformation of Arabidopsis thaliana**. *Plant Journal* 2010, **16**(6):735-743.

64. Li W, Fei-Feng LI, Wen-Bo Z, Gui-Lin C, Xiao-Fei L: **Isolation and characterization of Nitraria sibirica actin gene**. *Acta Prataculturae Sinica* 2012, **21**(4):151-158.
65. Norris SR, Meyer SE, Callis J: **The intron of Arabidopsis thaliana polyubiquitin genes is conserved in location and is a quantitative determinant of chimeric gene expression**. *Plant Molecular Biology* 1993, **21**(5):895-906.
66. Grobeta-Hardt, R.: **WUSCHEL signaling functions in interregional communication during Arabidopsis ovule development**. *Genes & Development* 2002, **16**(9):1129-1138.
67. Li-Li L, Hui-Min R, Li-Qing C, Yi W, Wei-Hua W: **A protein kinase, calcineurin B-like protein-interacting protein Kinase9, interacts with calcium sensor calcineurin B-like Protein3 and regulates potassium homeostasis under low-potassium stress in Arabidopsis**. *Plant Physiology* 2013, **161**(1):266-277.
68. Heringer AS, Vale EM, Barroso T, Santa-Catarina C, Silveira V: **Polyethylene glycol effects on somatic embryogenesis of papaya hybrid UENF/CALIMAN 01 seeds**. *Theoretical & Experimental Plant Physiology* 2012, **25**(2):116-124.

Figures

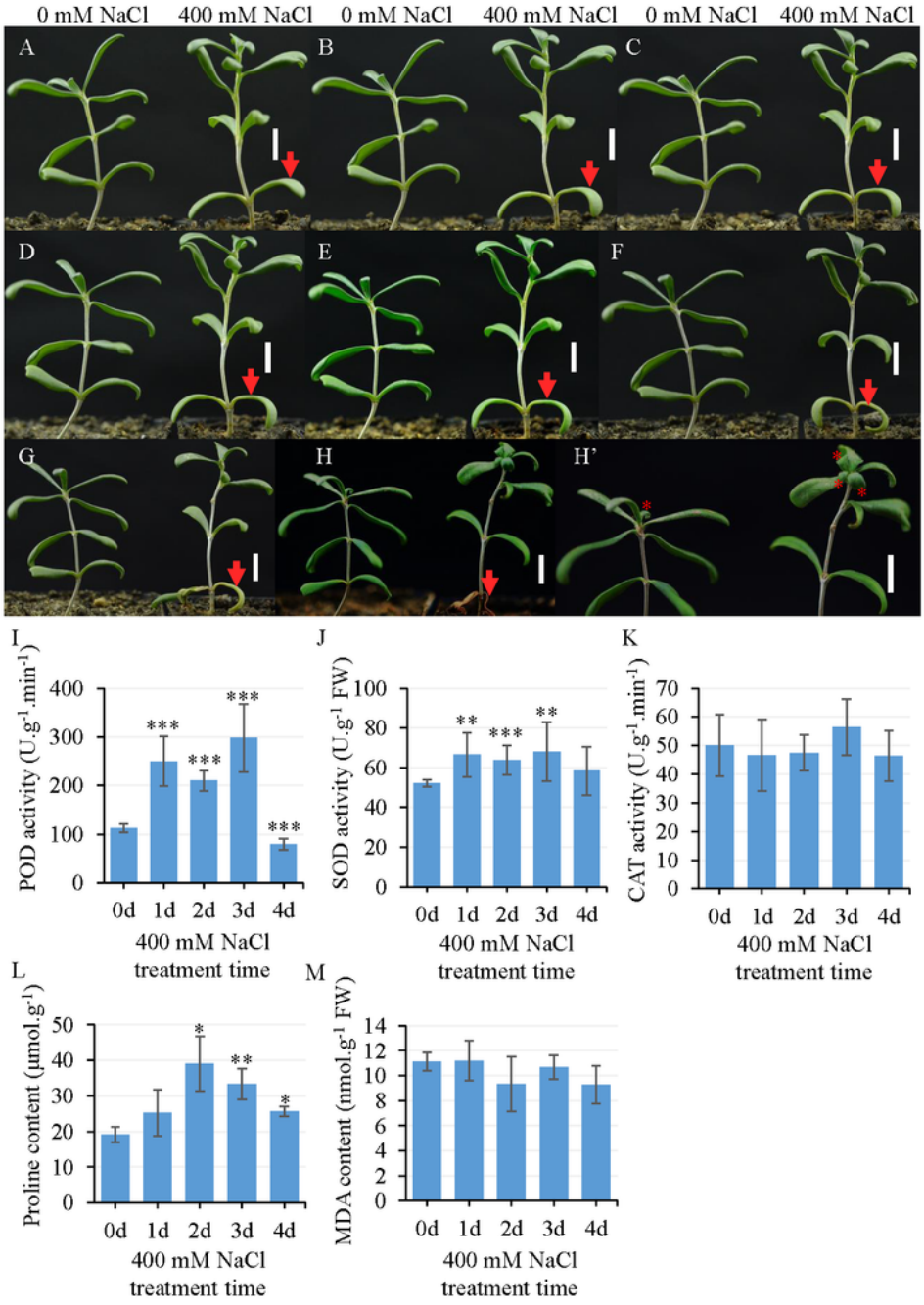

Figure 1

N. tangutorum morphologically and biochemically responded to NaCl stress. (A-H) Morphology of N. tangutorum during salt treatment; red arrowheads indicate withering leaves; red stars indicate new leaves; scale bar: 1 cm. (I-M) Effect of NaCl stress on biochemical parameters: activities of POD (I), SOD (J), and CAT (K), proline content (L), and MDA content (M) in the N. tangutorum leaves. The values and standard

errors of three replications are shown; statistical analyses were performed with ANOVA, '*' $p < 0.05$, '**' $p < 0.01$, '***' $p < 0.001$.

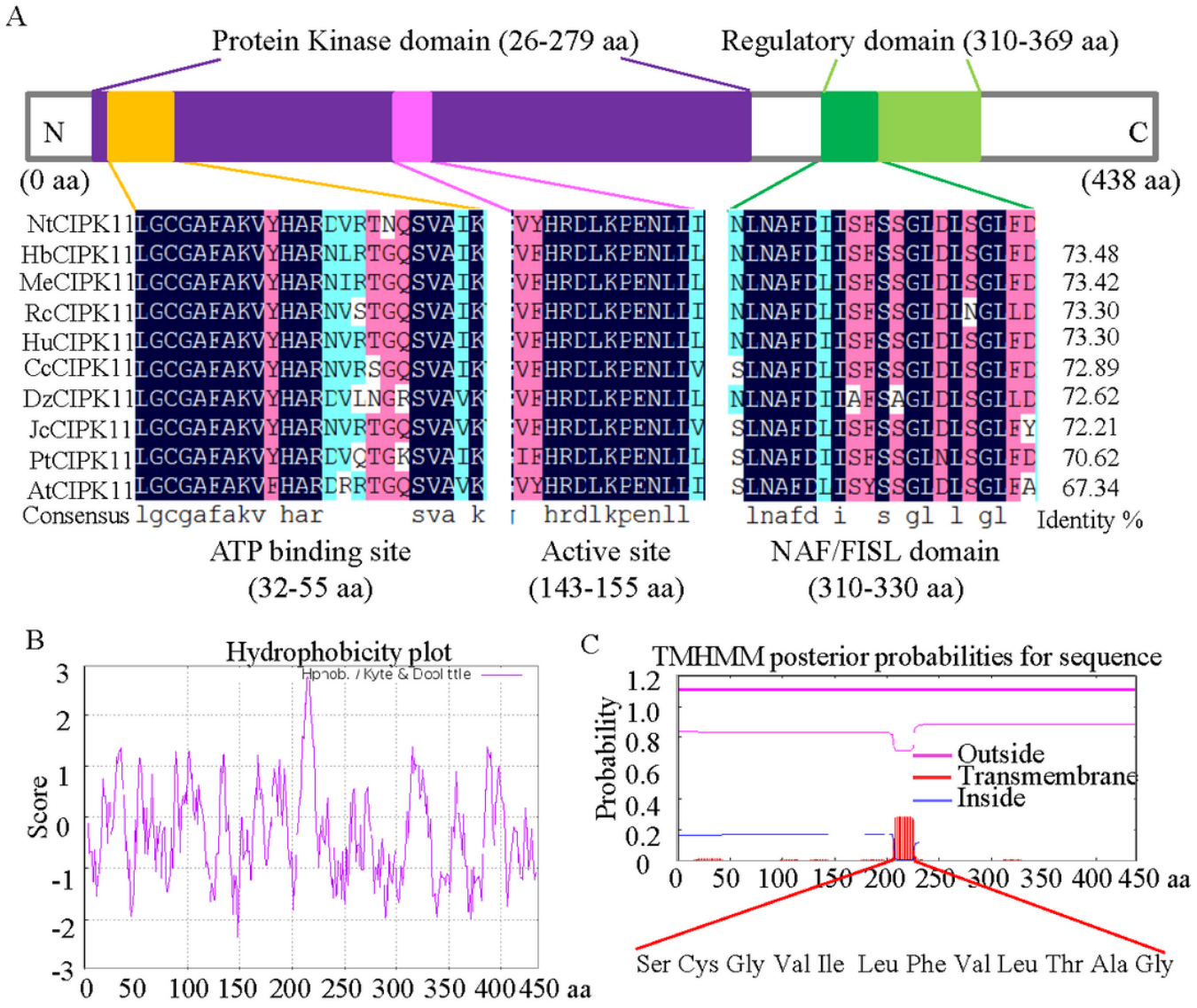

Figure 2

Multiple alignment and domain prediction of NtCIPK11. (A) Multiple alignments for the conserved domains of CIPK11 orthologs from N. tangutorum and other species; the borders of the protein domain were predicted by InterProScan online software. (B) Hydrophobicity plot of NtCIPK11. (C) Predicted transmembrane domain of NtCIPK11.

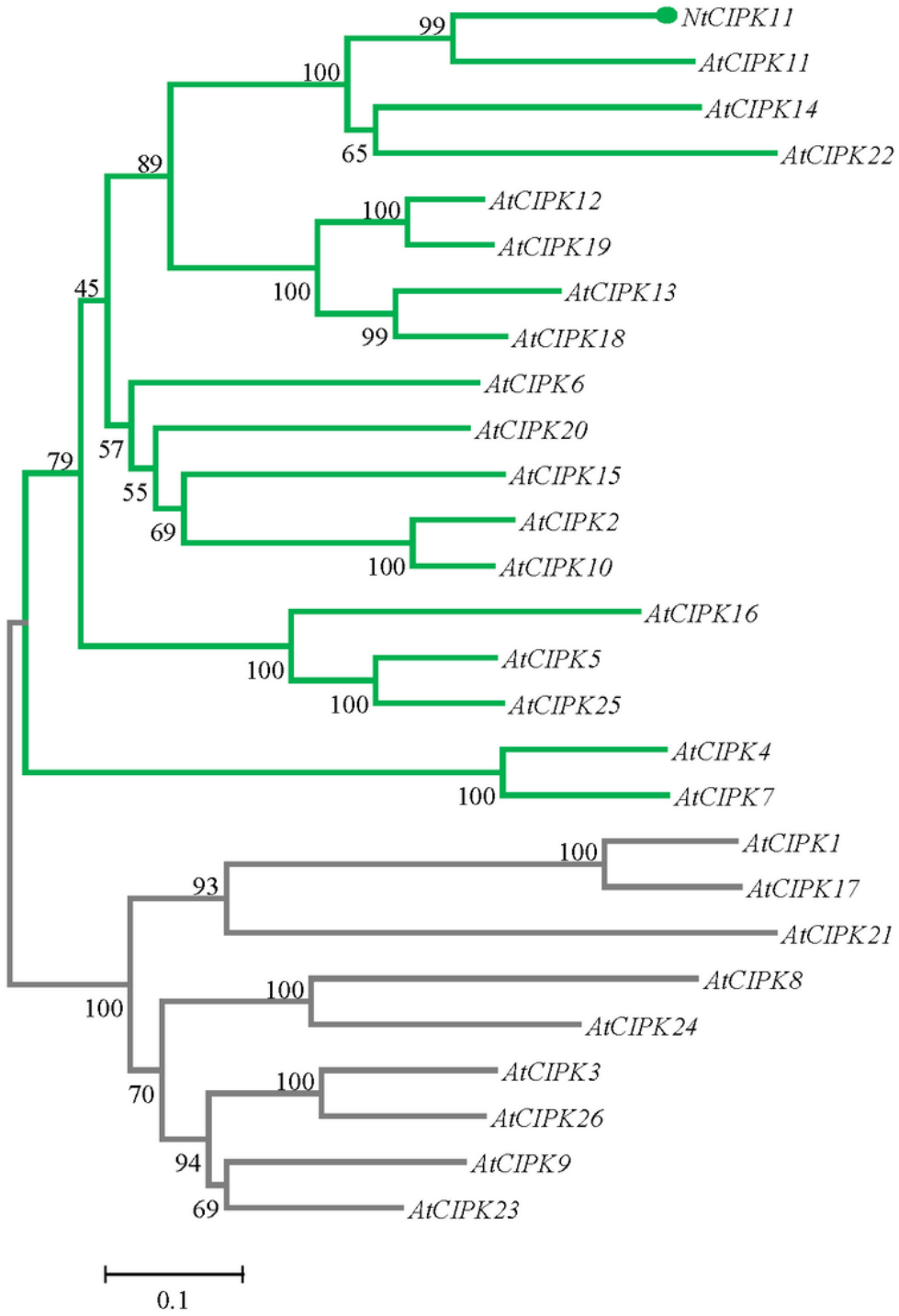

Figure 3

Phylogenetic analysis of NtCIPK11 with Arabidopsis CIPKs. The grey branch represents the subgroup of CIPKs with introns. The green branch represents the clusters without introns.

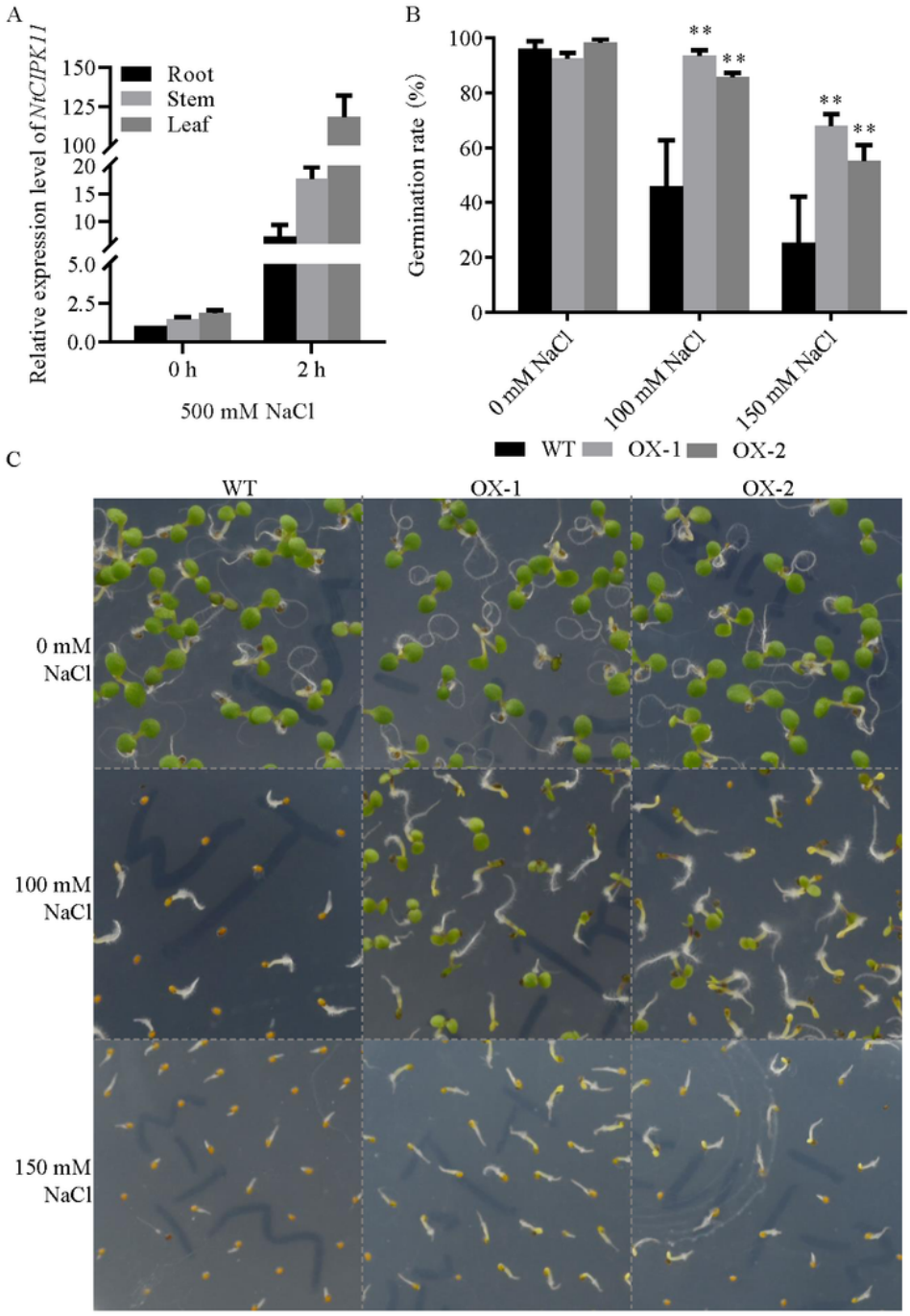

Figure 4

NtCIPK11 responded to salt stress in N. tangutorum and Arabidopsis. (A) NtCIPK11 transcription increased after 500 mM NaCl treatment of N. tangutorum. (B) Germination rate of WT and NtCIPK11 overexpressing seeds. (C) Growth of the seeds germinated on medium with different salt contents. Three biological replicates and three technical replicates were conducted. The data represent the means ± SD from three biological replicates. '**' $p < 0.01$.

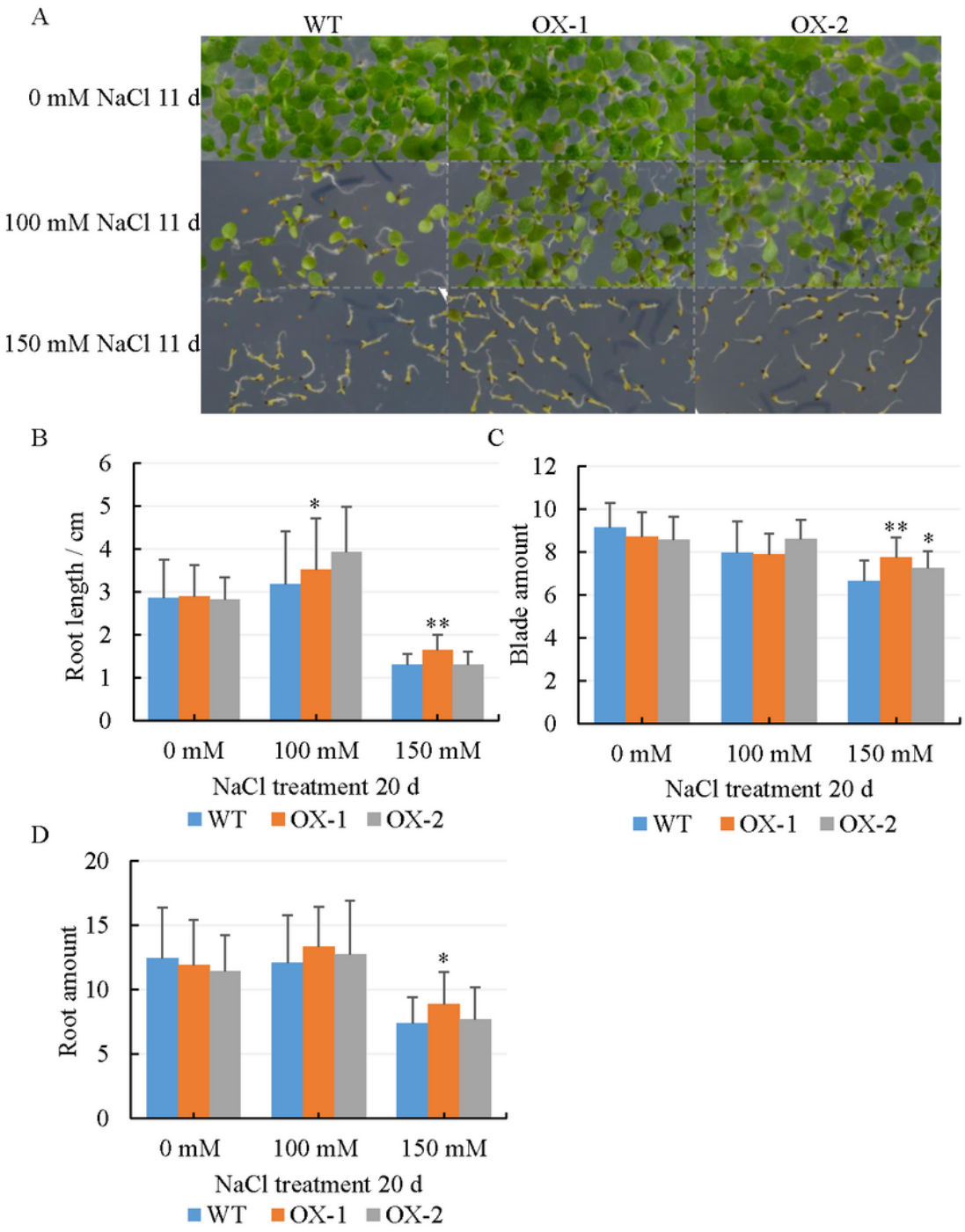

Figure 5

NtCIPK11 overexpression promoted the growth of Arabidopsis under salt conditions. (A) Phenotype of the WT and NtCIPK11-overexpressing plants under different salt conditions for 11 days; (B) root length; (C) blades and (D) roots of WT and transgenic plants 20 days post-germination on medium containing different levels of salt. The data represent means ± SD from three biological replicates, and the statistics analyses were performed with ANOVA, '*' $p < 0.05$, '**' $p < 0.01$.

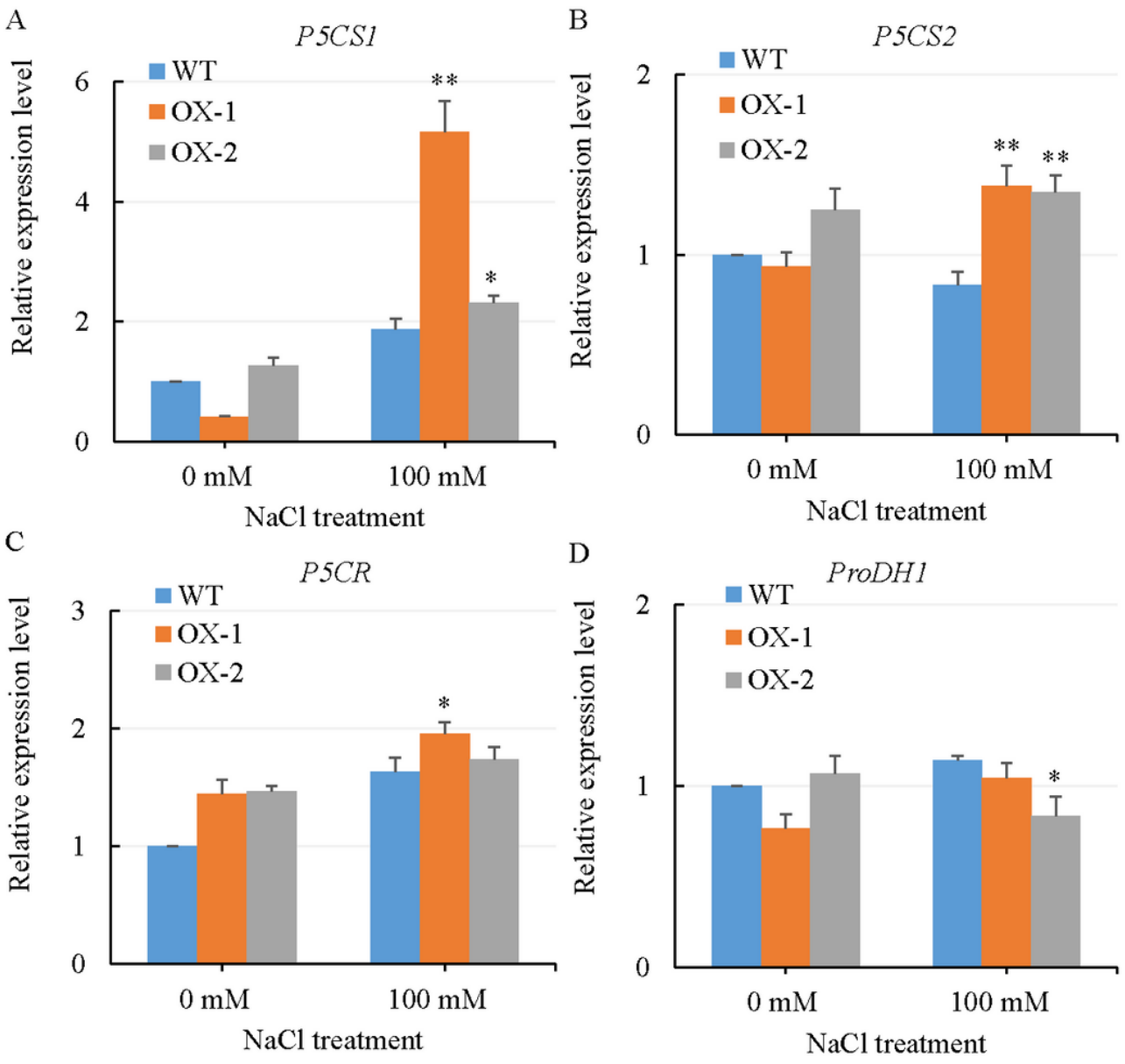

Figure 6

NtCIPK11 induced the transcription of genes involved in proline metabolism under salt treatment. (A-C) Expression levels of proline synthetase genes P5CS1 (A), P5CS2 (B), and P5CR (C). (D) Expression level of the proline catabolism gene ProDH1. The data represents means ± SD of three replicates and the statistical analyses were performed with ANOVA, '*' $p < 0.05$, '**' $p < 0.01$.

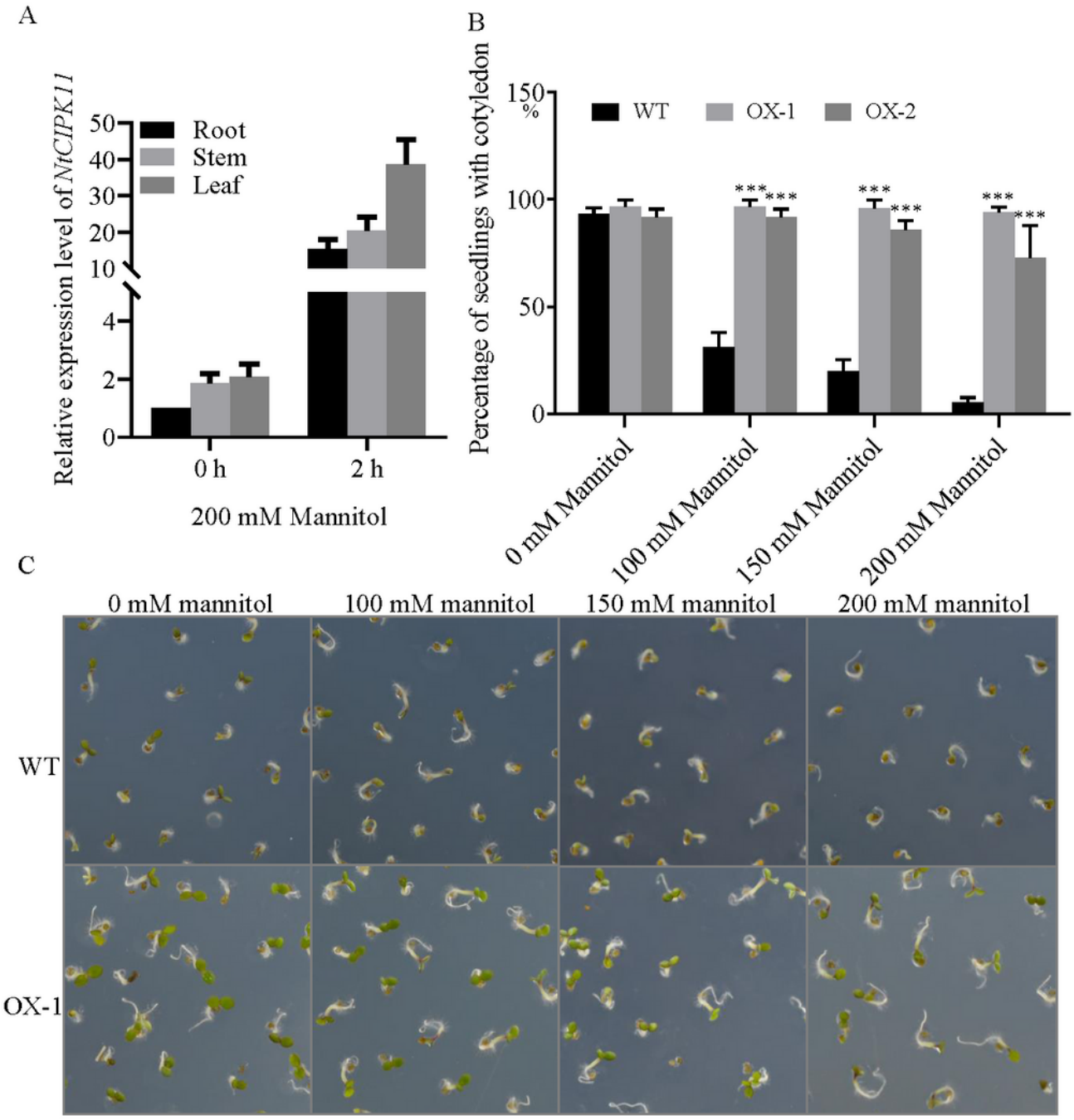

Figure 7

NtCIPK11 responded to drought stress in Nitraria and Arabidopsis. (A) Transcription analysis of NtCIPK11 in N. tangutorum after salt treatment. (B) The percentage of Arabidopsis seedlings with two cotyledons. (C) Morphology of seedling germination of WT and transgenic Arabidopsis plants under increasing mannitol treatment. The data represent means ± SD, three biological replicates, with ANOVA used for the statistical analyses, '*' $p < 0.05$, '**' $p < 0.01$, '***' $p < 0.001$.

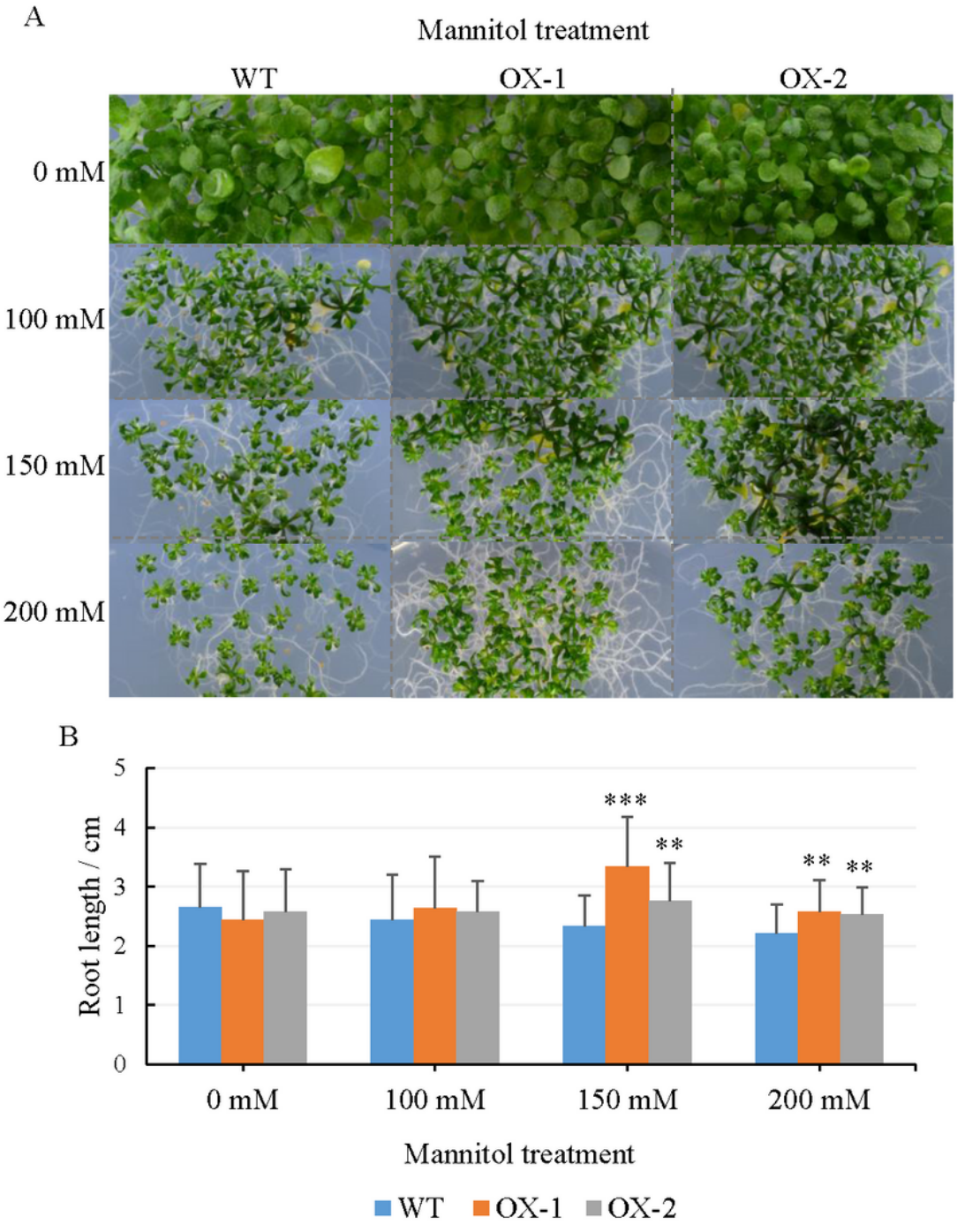

Figure 8

NtCIPK11-overexpressing Arabidopsis plants developed a longer root. (A) WT and transgenic plants on medium with different concentrations of mannitol. (B) The length of the primary root in the transgenic plants and WT. The data represent the means ± SD and three biological replicates, with ANOVA used for the statistical analyses, '*' $p < 0.05$, '**' $p < 0.01$.

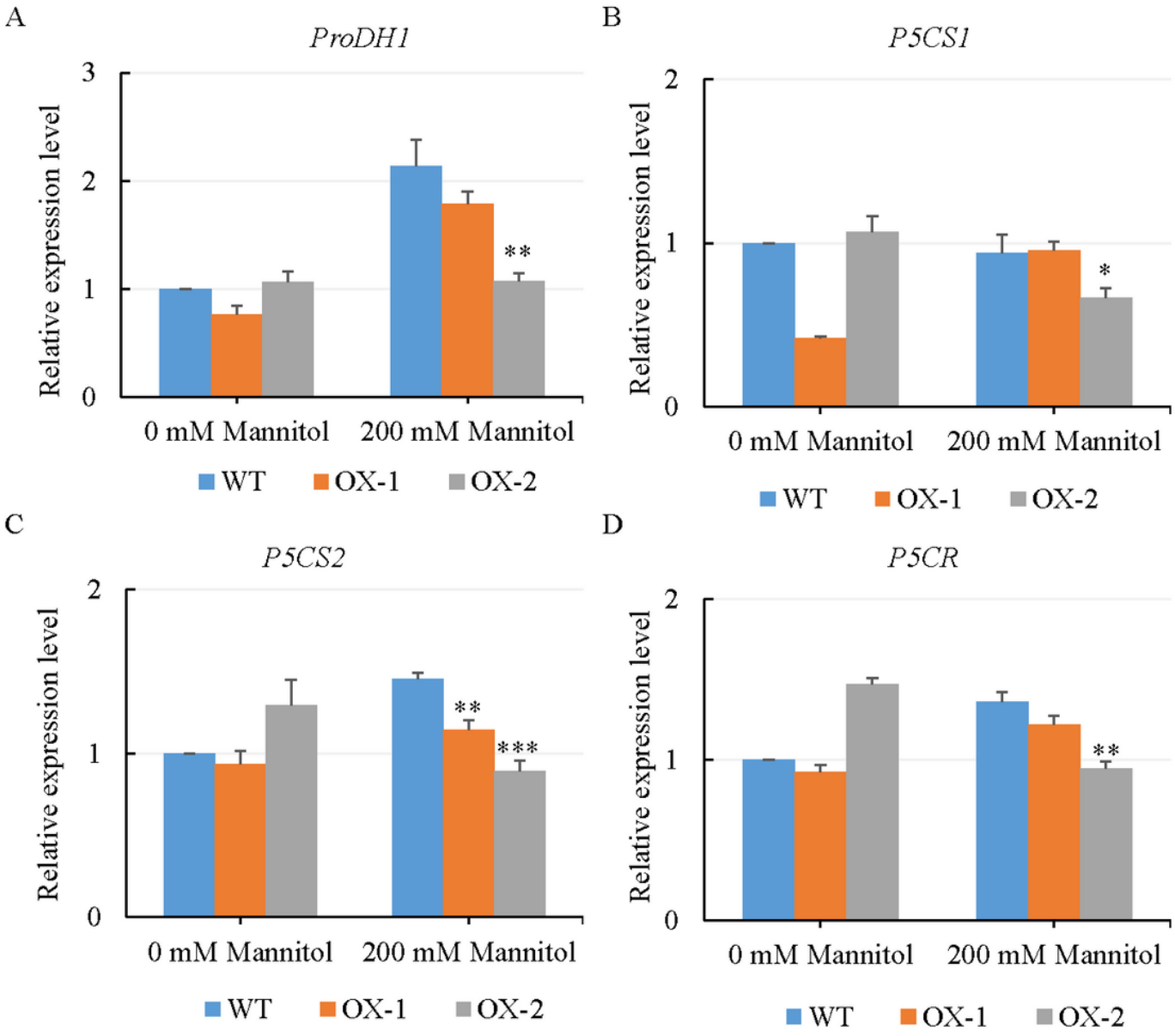

Figure 9

NtCIPK11 influenced the expression of genes controlling proline metabolism under drought stress. (A) Transcription of ProDH1, (B) P5CS1, (C) P5CS2 and (D) P5CR after 200 mM mannitol treatment. The data represent the means ± SD, three biological replicates, and ANOVA was used for the p-value calculations, '*' $p < 0.05$, '**' $p < 0.01$, '***' $p < 0.001$.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- Supplementaryfile1.xlsx