MsCIPK, a CBL-interacting Protein Kinase in *Medicago sativa* L., Confers Salt and Osmotic Stress Tolerance in Transgenic Tobacco

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Research Article

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Abstract

Alfalfa is an important perennial forage but suffers from salt and osmotic stresses worldwide. Calcineurin B-like proteins (CBLs) and CBL-interacting protein kinases (CIPKs) are reported to play important roles in response to diverse plant stresses, but are largely unvalidated in alfalfa. In this study, we cloned a MsCIPK gene, which contained 1530 bp, coding 509 amino acids, with typical CIPK functional domains. The expression pattern of MsCIPK was measured using qRT-PCR under salt, drought, heat, cold and ABA stresses. Under NaCl, heat and ABA treatment, the expression pattern of MsCIPK was generally similar, with a first steady decrease and then a gradual increase pattern. The highest expression of MsCIPK was all observed at the start point of all treatments, except in cold treatment. Using transgenic tobaccos of MsCIPK, we further measured the content of malondialdehyde (MDA), superoxide dismutase (SOD), soluble protein (SOP), and proline (Pro) under 21 days' salt and 24 hours' cold treatment. Under both salt and cold conditions, the content of MDA, SOP and Pro had a similar overall increase pattern with the time of treatment. These results indicated that the MsCIPK played an important role in improving alfalfa's salt and osmotic tolerance.

Introduction

Medicago sativa L.cv. or, alfalfa, is an important perennial forage worldwide, with a cultivated area exceeds 32 million hectares [1]. It has high protein content and also rich in diverse vitamins, and has been widely applied in improving the soil nutritional conditions in wide pastoral area in the northwest of China. However, the majority of alfalfa cultivars were sensitive to salt and drought stresses [2–4]. In order to survive these stresses, multi-scale diverse regulations and feedbacks are required, from genetic [5] to transcriptional [6,7] to proteomic [8] and to physiological aspects [6–8]. Breeding new alfalfa cultivars with strong salt and osmotic stress resistance remains as a major challenge in the security and long-term sustainability of animal husbandry.

Calcineurin B-like proteins (CBLs) and CBL-interacting protein kinases (CIPKs) were important genes in response to diverse plant stresses, such as salt and osmotic stresses [9]. These enzymes play important roles in phosphorylating downstream components to transduce calcium (Ca2+) signals in response to diverse stresses [10–13]. CBLs and CIPKs in Arabidopsis revealed that these genes responded to various stresses, including drought, salinity and osmotic stresses [9,14,15]. Similar phenomena were also recently observed on diverse species, such as rice [14,16,17], maize [18], wheat [19], soybean [20], tobacco [21], apple [22], pepper [23], eggplant [24], pear [25] and grapewine [26]. However, whether CBL and CIPKs are involved in mediating different stress responses remains largely unknown in alfalfa.

In this report, we cloned an alfalfa CIPK (MsCIPK) and examined its role in salt and osmotic stresses. The main purposes of this study are: 1) clone the full length of MsCIPK; 2) provide detailed bioinformatic prediction of MsCIPK; 3) evaluate the evolutionary position among related species; 4) evaluate the expression patterns of MsCIPK under different stresses; 5) evaluate the impacts of MsCIPK in transgenic

plants on key stress-induced metabolites. These findings will promote the transgenic applications of CBLs and CIPKs in improving the resistance of alfalfa, which could also be useful for other species..

Materials And Methods

Plant Materials and gene clone

Seedings of Medicago sativa L.cv. 'Baoding', after 20 weeks growth in artificial climate growth chamber, were used for the full-length clone of MsCIPK. Total RNA was extracted following the protocol of Takara (Takara, Dalian, China). The total RNA integrity and concentration was qualified using the EasyPure® Quick Gel Extraction Kit (Beijing TransGen Biotech Co., Ltd, Beijing, China) and Thermo Scientific NanoDrop 2000 (Thermo Fisher Scientific Co., Ltd, Waltham, MA, USA). The forward and reverse PCR primer was 5'-ATGGCAGTTGTAGCTGCTCCCAAGC-3' and 5'-TCAGGTATCTAAGTTCAGAGATTC-3', respectively, which were designed by Primmer v5. After transforming recombinant plasmids, positive clones were selected for sequencing verification, which was performed by Yingjun Technology Co., Ltd., China. Transgenic plants were obtained via the tobacco leaf disc transformation approach. Positive transgenic plants were identified via by PCR detection, using the extracted transgenic tobacco DNA.

Bioinformatic analyses

The basic physical and chemical properties of MsCIPK protein were predicted in ProtParam [27]. The cDNA sequence was then blasted with NCBI blastx in order to identify CIPK-like genes. Protein domains were predicted using SMART [28]. The secondary and three-dimensional structure of MsCIPK protein was predicted using the online GOR IV method (http://npsa-pbil.ibcp.fr/cgi-bin/npsa_automat.pl?page=npsa_gor4.html) [29]. The neighbor-joining (NJ) tree was constructed with 1000 replicates using MEGA v7.0 [30]. The NCBI ID for the 26 Arabidopsis CIPK-like genes were AtCIPK1 (AAG28776.1), AtCIPK2 (AAF86506.1), AtCIPK3 (AAF86507.1), AtCIPK4 (AAG01367.1), AtCIPK5 (AAF86504.2), AtCIPK6 (AAF86505.1), AtCIPK7 (AAK16682.1), AtCIPK8 (AAK16683.2), AtCIPK9 (AAK16684.1), AtCIPK10 (AAK16686.1), AtCIPK11 (AAK16686.1), AtCIPK12 (AAK166877.1), AtCIPK13 (AAK16688.1), AtCIPK14 (AAK16689.1), AtCIPK15 (AAK16692.1), AtCIPK16 (AAK50348.1), AtCIPK17 (AAK64513.1), AtCIPK18 (AAK59695.1), AtCIPK19 (AAK50347.1), AtCIPK20 (AAK61493.1), AtCIPK21 (AAK59696.1), AtCIPK22 (AAL47845.1), AtCIPK23 (AAK61494.1), AtCIPK24 (AAK72257.1), AtCIPK25 (AAL41008.1) and AtCIPK26 (NP_850861.2).

Gene expression analysis

Seedings of Medicago sativa L.cv. Baoding, after 4 weeks growth in artificial climate growth chamber, were then treated with different treatments, including 200 mm/L NaCl, drought, 4 ℃ cold and 42 ℃ heat and 10 μM ABA, in order to investigate MsCIPK's role in response to various stresses. After 2h, 4h, 8h, 12h and 24h treatment, above-ground and root tissues were immediately frozen in liquid nitrogen (-80 ℃) before RNA extraction. The PCR amplification verification was carried out with hygromycin Hyg specific primers. Gene expression analysis was performed using quantitative real-time polymerase chain reaction

(qRT-PCR) by SYBR Premix Ex Taq (Takara, Dalian, China). The main PCR reaction parameters were: 95 ℃ pre-denaturation for 1 min; 40 cycles of 95 ℃ for 30 s, 60 ℃ for 30 s, and 72 ℃ for 30 s; 72 ℃ for 10 min and terminated and saved at 4 ℃. β-actin was used an the internal gene and the forward and reverse primer was 5'-TTTGAGACTTTCAATGTGCCCGCC-3' and 5'-TAGCATGTGGGAGTGCATAACCCT-3, respectively. The forward and reverse primer for MsCIPK was 5'- TCAGCCCTACAGTCCTACCACGG-3' and 5'- GTTCCACCTCTCTGCAGCTCATC-3', respectively. The signal of the qRT-PCR was monitored by the CFX96 real-time system (Bio-Rad, CA, USA). The relative expression level of MsCIPK was determined by the 2-ΔΔCt method [31]. Three biological and technical replicates were used to calculate the standard error (SE). The ANOVA significant test was performed in SAS software.

Functional analysis of transgenic tobacco with MsCIPK

In order to validate the functional role of MsCIPK, we treated T1 transgenic tobaccos (Line 3 and Line 7) and non-transgenic tobacco (WT) under salt, drought and cold stresses, and then evaluated the impacts on the contents of four compounds including malondialdehyde (MDA), superoxide dismutase (SOD), soluble protein (SOP), and proline (Pro). ild type tobaccos (WT) were treated with normal Hoagland nutrient solution. For salt treatment, transgenic plants were treated with 1/2 Hoagland nutrient solution and 200 mM NaCl. The salt treatment was conducted for a period of 21 days and samples were collected at every 7 days (0day, 7day, 14day and 21day) and immediately frozen in liquid nitrogen (-80 ℃) before use. For low temperature treatment, plants were treated with 4 ℃ and collected at 0h, 6h, 12h and 24h and frozen in liquid nitrogen for a total of 24 h treatment. The content of MDA was measured by the thiobarbituric acid method [32]. The content of SOD was measured by the NBT method [33]. The content of SOP was measured by the Coomassie Blue Staining method: SOP content (Mg/g) = C × VTV1 × FW × 1000, where C was the standard curve value (μg); VT was the total extract volume (mL); FW was the fresh weight and V1 was the volume added during measurement (mL). The content of Pro was measured by the methods proposed by Bates [34]. All the measurements were performed with nine replicates. Significant tests were performed in SAS software.

Results

Cloning and Sequence Analysis of MsCIPK

The full length of the opening read frame of *MsCIPK* contains 1530 bp, coding 509 amino acids (Fig. 1). The relative molecular weight was predicted at 57175.7 and the protein formula was $C_{2559}H_{4090}N_{700}O_{752}S_{15}$. The stability coefficient was calculated at 37.06, indicating that this protein was a stable protein. BLASTx showed that *MsCIPK* had the typical conserved N-terminal serine/threonine kinase domain (S_TKs, between 26 bp to 281 bp) and the less conserved C-terminal NAF domain (between 350 bp to 406 bp) of *CIPK* genes (Fig. 2A). The predicted secondary structure of *MsCIPK* were mainly composed with random coil, alpha helix and extended strand (46.56%, 37.33% and 16.11%, respectively) (Fig. 2B). The predicted three-dimentional structure of *MsCIPK* was provided in Fig. 2C.

Phylogenetic analysis of MsCIPK

Phylogenetic analysis revealed that *MsCIPK* had the closest relationship with *Medicago truncatula*, with a total of 98% similarity. Maximum-likelihood gene tree topology analysis showed that these *CIPK* genes could be mainly divided into two classes. Class I were those CIPKs with introns, and Class II were without introns. *MsCIPK* had 13 introns and were clearly grouped in Class I (Fig. 3A). We further analyzed the phylogenetic relationship between *Medicago sativa* and *Arabidopsis thaliana* (*AtCIPK1* to *AtCIPK26*), and found that *MsCIPK* had the highest similarity with *AtCIPK12* (Fig. 3B).

Expression patterns of MsCIPK under different stresses

In this study, the expression patterns of *MsCIPK* were evaluated under various stresses, including salt stress, drought, low and high temperature and ABA stresses (Fig. 4). Under all treatment conditions, the relative expression of *MsCIPK* in root was significantly higher than that in shoot. The overall expression pattern of *MsCIPK* in shoot and root was also not always consistent. Under NaCl, heat and ABA treatment, the expression pattern of *MsCIPK* was generally similar, with a first steady decrease and then a gradual increase pattern. The highest expression of *MsCIPK* was all observed at the start point of all treatments, except in cold treatment (the expression of *MsCIPK* was increased in overall), while the lowest expression was observed at different time points in different treatments. In sum, the expression patterns under different treatments were different both in shoot and root, which indicated that it's a quite complex regulatory mechanism of *MsCIPK* in responding to stresses.

Impacts of transgenic MsCIPK on malondialdehyde (MDA), superoxide dismutase (SOD), soluble protein (SOP) and proline (Pro) under stresses

In order to demonstrate the biological role of *MsCIPK* under stresses, we measured the contents of four important compounds, including MDA, SOD, SOP and Pro, using salt and cold stress treatment as an example. Under both salt and cold stress conditions, the content of MDA was increased with the time of treatment and peaked at 14 days, where significant difference ($P < 0.05$) was observed between the control and the two trangenic plants (Fig. 5a, 6a). Similar increasing trends were also observed for SOP (Fig. 5c, 6c) and Pro contents till at the end of treatment (Fig. 5d, 6d). In contrast, the relationship between the time of treatment and the content of SOD was different compared with MDA, SOP and Pro (Fig. 5b, 6b). In additon, the content of these four compounds at different treatment time points both in salt and cold condition was not always higher or lower than the control. It's suggested that *MsCIPK* might have complex feedback mechanisms in regulating the content of important stress-related metabolites.

Discussion

Though the role of cellular Ca^{2+} in transducing stress-related signals is well established in *Arabidopsis thaliana* and other plant species, many of the CIPKs are still functionally unknown [12,13, 17–21,24,26,35,36]. This Ca^{2+}-induced signal is a quite complex network, which enables different plant species to adapt to different stresses timely and efficiently. The number of CIPKs in different species also differed a lot, which complexified the Ca^{2+} signal network even more [22,23,25,26,35,36]. In this study, we found that the *MsCIPK* gene shared a similar secondary and three-dimensional structure compared to other CIPKs, providing evidence of the common function of CIPKs in different plant species. In addition, the targeted subcellular locations of CIPKs might also differed from cytoplasm to the nucleus and others, which were interacted with corresponding CBL partners, which also greatly enriched the network complexity[9,37,38].

Transcriptional analysis revealed that *MsCIPK* had a significantly higher expression level under stresses conditions when compared to normal control conditions. It revealed that *MsCIPK* might enhance alfalfa resistance performance to stress conditions by increasing its expressions. Similar results were also observed in other species [17,38,39]. The continuously increase of stress-induced metabolites, such as MDA, SOD, SOP and Pro, under consistent stress conditions, demonstrated that *MsCIPK* might had a long-term effect on protecting alfalfa from stresses.

A well-balanced mineral elements in the soil is crucial for the growth and development of plants, including alfalfa. However, plants could suffer from morphological to physiological harms and even to death, when the balance between different elements were broken or insufficient [40,41]. The accumulation of Na^+ will influence K + and the ratio of K^+/Na^+ is crucial for plant growth [42,43]. The inter-connected network of CIPKs and CBLs in plants could export Na^+ to vacuoles or transport to elder leaves in order to enhance salt tolerance [13,44].

However, the difference between different transgenic lines of *MsCIPK* demonstrated the complexity and difficulty in introducing CIPKs to high-salt/osmotic stress resistant alfalfa breeding. Natural alfalfa genetic resources could serve as a useful gene pool for diverse resistance gene discovery [2, 5–7,45]. Molecular markers, structural variants in the genomic level and functional validations of novel candidate genes at the transgenic level will jointly promote the new breeding era of alfalfa with fast biotechnological advancements[1,46,47].

Conclusion

This study cloned a MsCIPK gene, which contained 1530 bp, coding 509 amino acids, with typical CIPK functional domains,and play important roles in response to diverse plant stresses. Under NaCl, heat and ABA treatment, the expression pattern of MsCIPK was generally similar, with a first steady decrease and then a gradual increase pattern. The highest expression of MsCIPK was all observed at the start point of all treatments, except in cold treatment. The overexpression of MsCIPK in tobacco also exhibit abiotic stress tolerance. These results indicated that the MsCIPK played an important role in improving alfalfa's salt and osmotic tolerance.

Declarations

Author Contributions:

Dr.Xia Zhao and Pro. Tianming Hu conceived the study. Pro. Tianming Hu designed the experiments. Dr.Xia Zhao, Dr.Yushi liu and Pro.Peizhi Yang conducted the experiments and the data analysis. Dr.Xia Zhao and Pro.Lin Ye wrote and revised the manuscript. All authors read and approved the final manuscript.

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Conflicts of Interest:

The authors declare no conflict of interest.

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Figures

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1    atggca gtt gta gct gct cccaagaagaac aac tca atc aacaagaag gat aat cca aat ctt cta ttg gga cgt ttt gaa tta ggaaaa
1    M  A  V  V  A  A  P  K  K  N  N  S  I  N  K  K  D  N  P  N  L  L  L  G  R  F  E  L  G  K
91   ctc ctt ggc cat ggaacc ttt gcaaaa gtc cac ctt gct aagaac ctc aaaaca ggt gaa tcc gta gct ata aag atc ata agcaaagac
31   L  L  G  H  G  T  F  A  K  V  H  L  A  K  N  L  K  T  G  E  S  V  A  I  K  I  I  S  K  D
181  aaa atc ctt aaa agt ggt tta gtt tca cat atc aaacgagaa att tcc att ctc cgt cgt gtt cgccaccccaac atc gtt caa ctc ttt
61   K  I  L  K  S  G  L  V  S  H  I  K  R  E  I  S  I  L  R  R  V  R  H  P  N  I  V  Q  L  F
271  gaa gtc atg gct acaaagacaaag att tac ttt gtg atggaa tat gta cga ggt ggt gag ctt ttc aacaag gtt gct aaa ggt agg ttg
91   E  V  M  A  T  K  T  K  I  Y  F  V  M  E  Y  V  R  G  G  E  L  F  N  K  V  A  K  G  R  L
361  aaagaagaa gtt gcaagaaaa tat ttt cagcag tta ata tgt gct gtt gga ttt tgt cat gct aga ggt gtt ttt cataga gat cta aag
121  K  E  E  V  A  R  K  Y  F  Q  Q  L  I  C  A  V  G  F  C  H  A  R  G  V  F  H  R  D  L  K
451  cct gaa aat ttg ttg ctt gat gaaaaa ggt aac ctt aaa gtt tca gat ttt ggt ctt agt gct gtg tca gat gaa att aagcaa gat ggg
151  P  E  N  L  L  L  D  E  K  G  N  L  K  V  S  D  F  G  L  S  A  V  S  D  E  I  K  Q  D  G
541  ttg ttt cat act ttt tgt ggt aca cct gct tat gtt gct cct gag gtt ttg tct aggaaa ggt tat gat ggt gct aag gtt gat att tgg
181  L  F  H  T  F  C  G  T  P  A  Y  V  A  P  E  V  L  S  R  K  G  Y  D  G  A  K  V  D  I  W
631  tct tgt ggg gtt gtt ttg ttt gtt ttg atg gct ggt tat tta cct ttt cat gat cct aat aat gtt atg gct atg tat aagaag att tat
211  S  C  G  V  V  L  F  V  L  M  A  G  Y  L  P  F  H  D  P  N  N  V  M  A  M  Y  K  K  I  Y
721  aaa ggt gaa ttt agg tgt cct aga tgg ttt tca cct gag ctt gtt agt ctt ctt act agg ctt ctt gat att aaa cct caa act aga att
241  K  G  E  F  R  C  P  R  W  F  S  P  E  L  V  S  L  L  T  R  L  L  D  I  K  P  Q  T  R  I
811  tct att cct gag att atggag aat cgt tgg ttt aag ata ggt ttt aagcaa att aag ttt tat gtt gag gat gat gtt gtt tgt gat ctt
271  S  I  P  E  I  M  E  N  R  W  F  K  I  G  F  K  Q  I  K  F  Y  V  E  D  D  V  V  C  D  L
901  gat tca ctt gat ttt gat ggt gat gat agt aat aaa gta gta aag atc gat gat cat cgc gat gaa gta ctg gaa tca gta tca gaa aat
301  D  S  L  D  F  D  G  D  D  S  N  K  V  V  K  I  D  D  H  R  D  E  V  L  E  S  V  S  E  N
991  gaa tcg gat tct gag gtt gtg aat aga agg atc agg aat cgt ggt tcg ttg cca agg cct gca ggt ttg aat gct ttt gat att ata tcg
331  E  S  D  S  E  V  V  N  R  R  I  R  N  R  G  S  L  P  R  P  A  G  L  N  A  F  D  I  I  S
1081 ttt tcg caa ggt ttt gat ctt tct ggt tta ttt gaggaaaagggc gat gag gct cgg ttt gtg tca agt gca tca gtg cca aag att ata
361  F  S  Q  G  F  D  L  S  G  L  F  E  E  K  G  D  E  A  R  F  V  S  S  A  S  V  P  K  I  I
1171 tca aaa ttg gaa gaa gtt ggt caa atg gtt agg ttc aat gtg aggaagaaa gat tgc aaa gtt cac ttg gag ggt tca aga gaa ggggca
391  S  K  L  E  E  V  G  Q  M  V  R  F  N  V  R  K  K  D  C  K  V  H  L  E  G  S  R  E  G  A
1261 aaa gggcga ttg act att gct gcc gag gtc ttc gaa ttg aca cct tct ttg gtg gtg gtt gag gtg aagaaa aaa gga ggt gat aag gtt
421  K  G  R  L  T  I  A  A  E  V  F  E  L  T  P  S  L  V  V  V  E  V  K  K  K  G  G  D  K  V
1351 gag tat gat aaa ttt tta aac act gaa ttg aag cct gct ttg cat agt tta acc aaggaggaa tct gca ggt tct tca tct caa act aca
451  E  Y  D  K  F  L  N  T  E  L  K  P  A  L  H  S  L  T  K  E  E  S  A  G  S  S  S  Q  T  T
1441 cca gat gaa tct ttg caacaa cgt gca ttt tct gat tct gcc att gac ata cat tca gat agt att gaa tct ctg aac tta gat acc tga
481  P  D  E  S  L  Q  Q  R  A  F  S  D  S  A  I  D  I  H  S  D  S  I  E  S  L  N  L  D  T  *
```

Figure 1

Nucleotide and deduced amino acid sequences of MsCIPK.

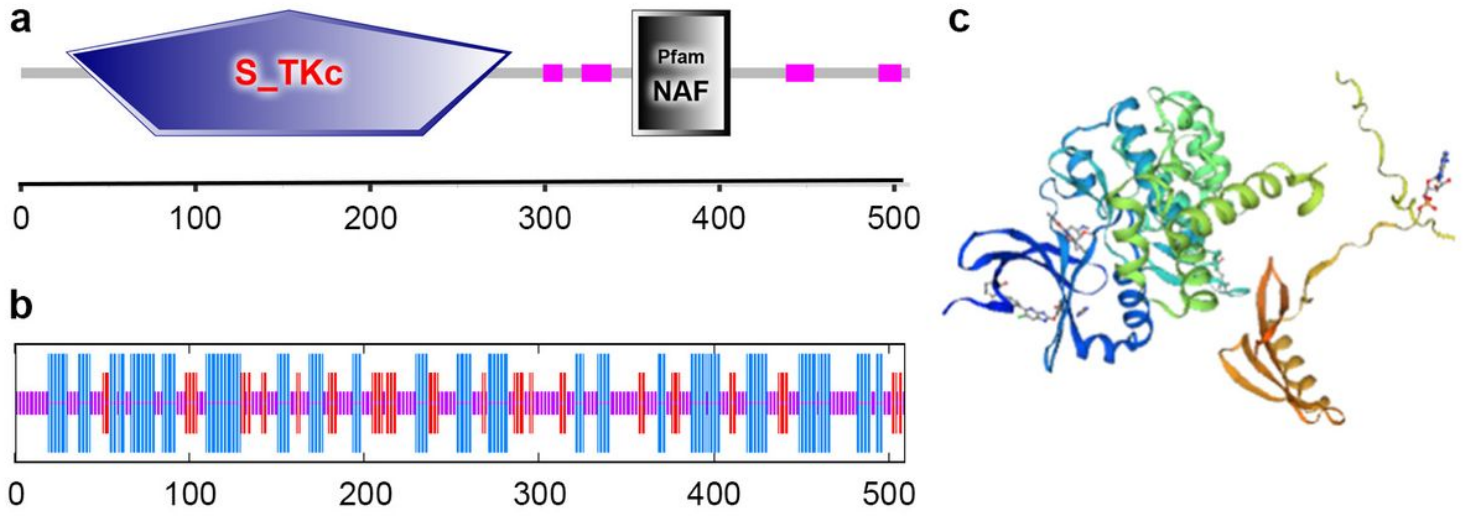

Figure 2

Conserved domain and structural predictions of MsCIPK. (a) Conserved domain of MsCIPK; S_TKs, serine/threonine kinase domain; (b) predicted secondary structure of MsCIPK; alpha helix, extended strand and random coil were represented in blue, red and purple; (c) predicted three-dimensional structure of MsCIPK.

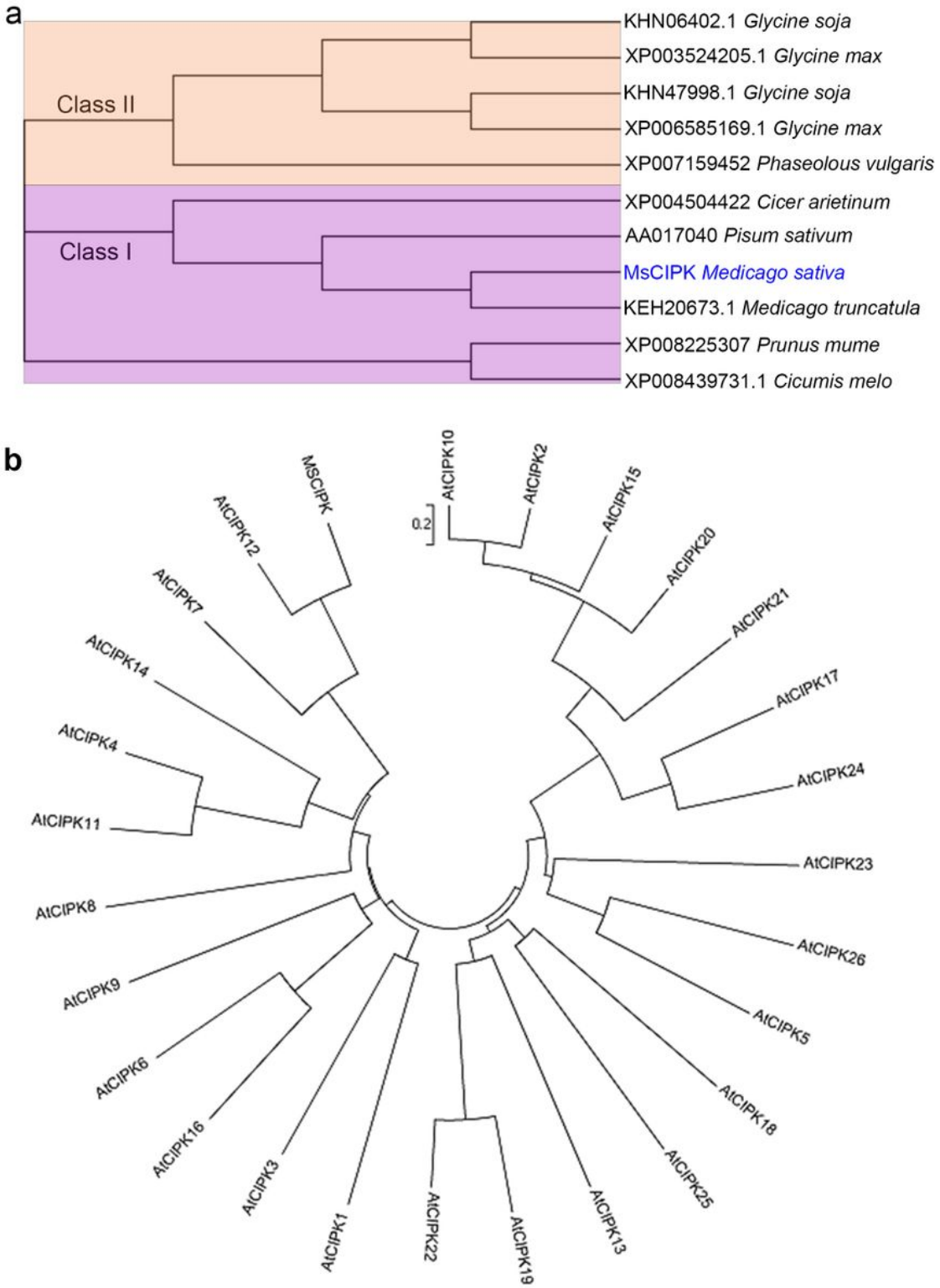

Figure 3

Phylogenetic analysis of MsCIPK. (a) Phylogenetic relationship of MsCIPK in 11 species. Class I and Class II were those CIPKs with or without introns, respectively. MsCIPK was highlighted in blue. (b) Maximum-likelihood gene tree topology for the MsCIPK gene and 26 AtCIPK gene family.

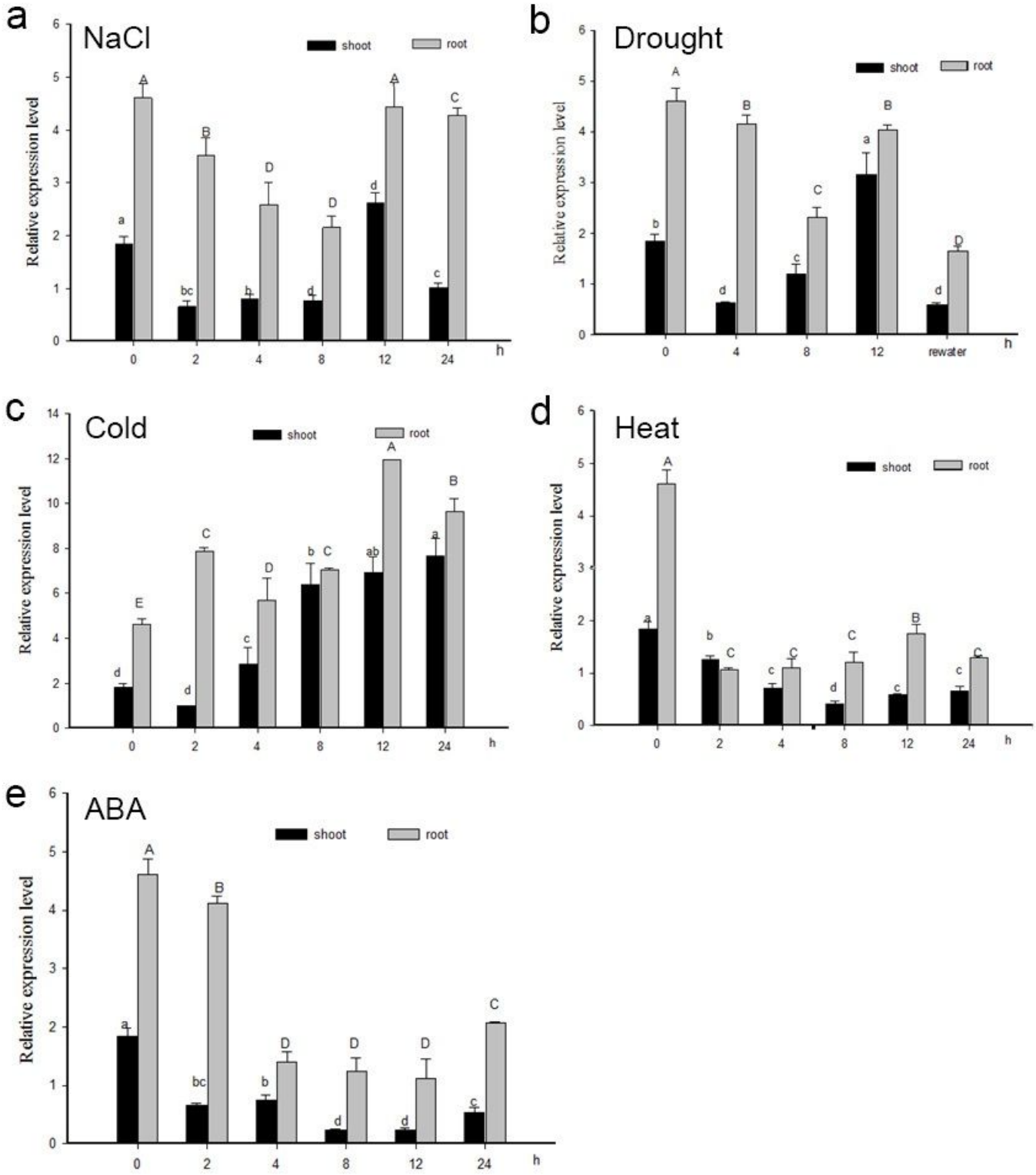

Figure 4

Relative expression patterns of MsCIPK gene under different treatment. (a) Relative expression patterns of MsCIPK gene under NaCl treatment; (b) relative expression patterns of MsCIPK gene under drought

treatment; (c) relative expression patterns of MsCIPK gene under NaCl cold; (d) Relative expression patterns of MsCIPK gene under heat treatment; (e) Relative expression patterns of MsCIPK gene under ABA treatment. Significant (P < 0.05) and high significant (P < 0.01) differences were indicated by lowercase and uppercase letters.

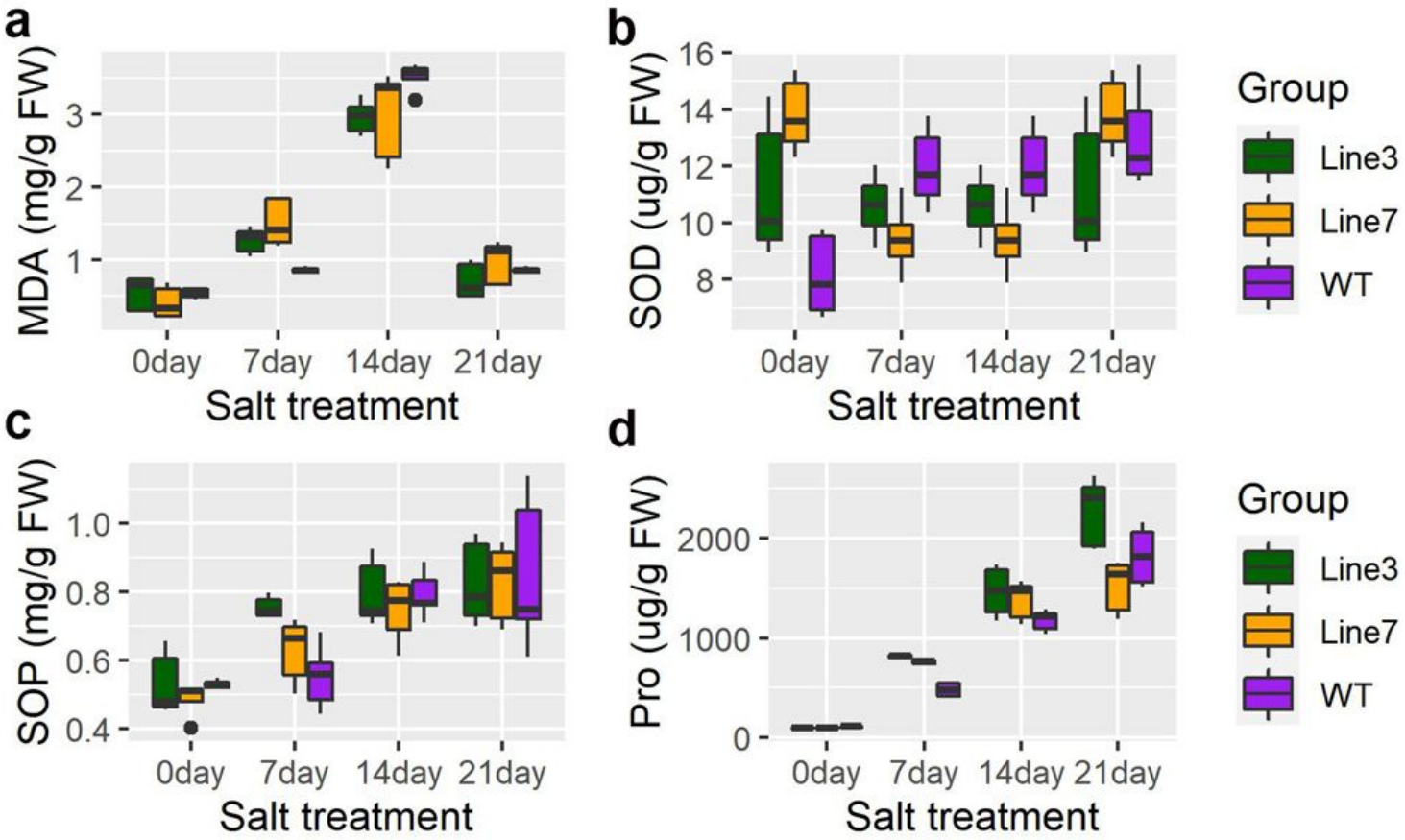

Figure 5

Impacts of MsCIPK on MDA, SOD, SOP and Pro under salt stress treatment. (a) Impacts of MsCIPK on MDA; (b) Impacts of MsCIPK on SOD; (c) Impacts of MsCIPK on SOP; (d) Impacts of MsCIPK on Pro.

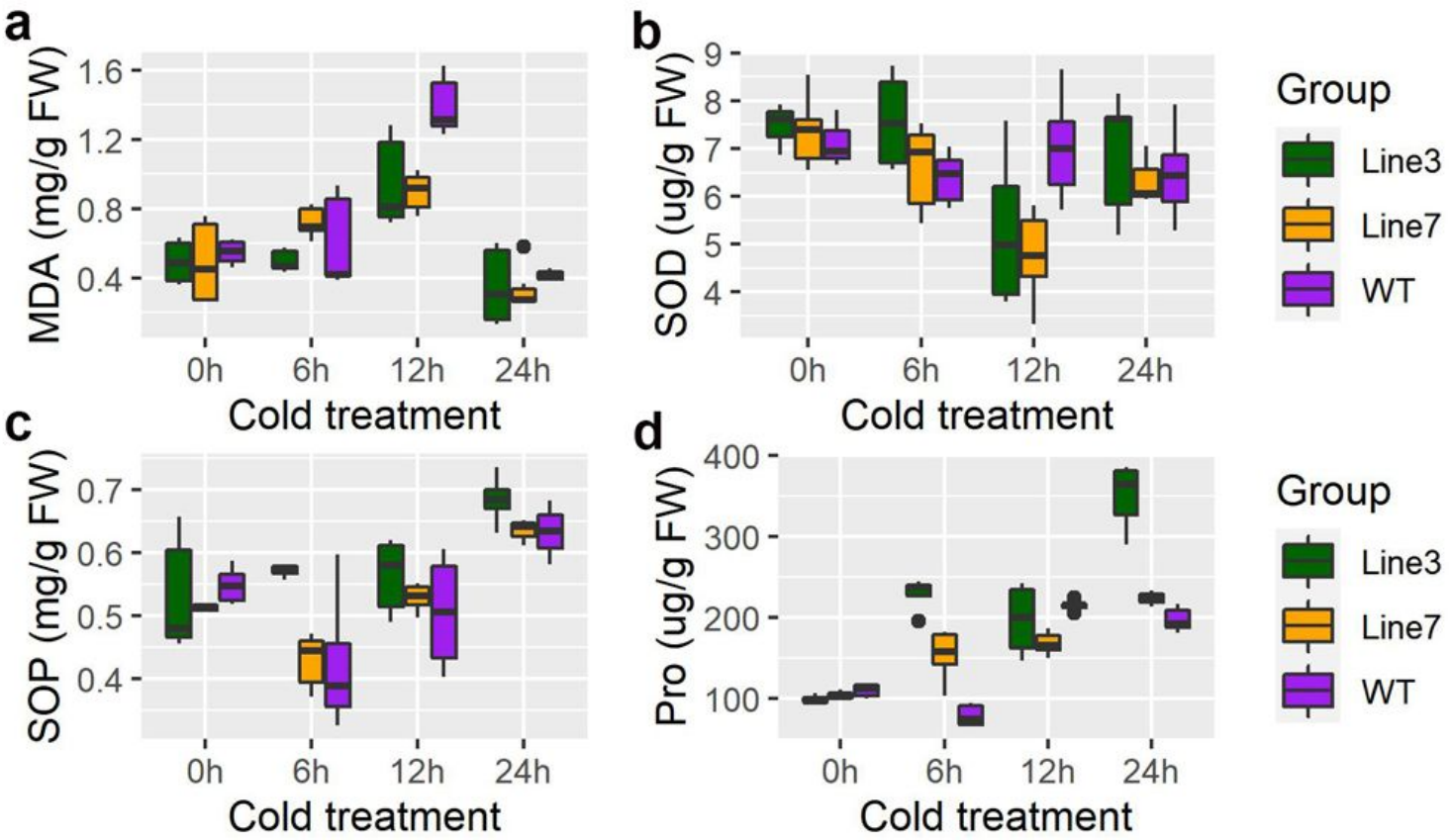

Figure 6

Impacts of MsCIPK on MDA, SOD, SOP and Pro under cold stress treatment. (a) Impacts of MsCIPK on MDA; (b) Impacts of MsCIPK on SOD; (c) Impacts of MsCIPK on SOP; (d) Impacts of MsCIPK on Pro.