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ORIGINAL PAPER

Cloning and functional validation of molybdenum cofactor sulfurase gene from *Ammopiptanthus nanus*

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Abstract

Key message **The molybdenum cofactor sulfurase gene (*AnMCSU*) was cloned from xerophytic desert plant *Ammopiptanthus nanus* and validated for its function of tolerance toward abiotic stresses by heterologous expression in *Arabidopsis thaliana*.**

Abstract Molybdenum cofactor sulfurase participates in catalyzing biosynthesis of abscisic acid, which plays a crucial role in the response of plants to abiotic stresses. In this study, we cloned molybdenum cofactor sulfurase gene (*AnMCSU*) from a super-xerophytic desert plant, *Ammopiptanthus nanus*, by using rapid amplification of cDNA ends method. This gene has a total length of 2544 bp, with a 5′- and a 3′-untranslated region of 167 and 88 bp, and an open reading frame of 2289 bp, which encodes an 84.85 kDa protein of 762 amino acids. The putative amino acid sequence shares high homology and conserved amino acid residues crucial for the function of molybdenum cofactor sulfurases with other leguminous species. The encoded protein of the *AnMCSU* gene was located in the cytoplasm by transient expression in *Nicotiana benthamiana*. The result of real-time quantitative PCR showed that the expression of the *AnMCSU* gene was induced by heat, dehydration, high salt stresses, and ABA induction, and inhibited by cold stress. The heterologous expression of the *AnMCSU* gene significantly enhanced the tolerance of *Arabidopsis thaliana* to high salt, cold, osmotic stresses, and abscisic acid induction. All these results suggest that the *AnMCSU* gene might play a crucial role in the adaptation of *A. nanus* to abiotic stress and has potential to be applied to transgenic improvement of commercial crops.

Keywords Abiotic stress · *Ammopiptanthus nanus* · Heterologous expression · Molybdenum cofactor sulfurase

Abbreviations

ABA	Abscisic acid
MoCo	Molybdenum cofactor
MCSU	Molybdenum cofactor sulfurase
cDNA	Complementary DNA
RACE	Rapid amplification of cDNA ends
qRT-PCR	Quantitative real-time PCR
ORF	Open reading frame
UTR	Untranslated region
eGFP	Enhanced green fluorescent protein
CaMV	Cauliflower mosaic virus
OCS	Octopine synthetase

Introduction

Abscisic acid (ABA) is a crucial phytohormone functioning in many developmental processes and in response of plants

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to abiotic stresses and pathogens (Pennisi 2009; Santner and Estelle 2009; Cutler et al. 2010; Leng et al. 2014). ABA-mediated signaling triggers expression of relative candidate genes, resulting in accumulation of osmotic metabolites, and enhancement of tolerance to stress conditions (Shinozaki and Yamaguchi-Shinozaki 2007; Mansouri and Asrar 2012). The last step of the ABA biosynthesis is that aldehyde oxidase catalyzes the oxidation of abscisic aldehyde into ABA. The post-translational activation of aldehyde oxidase relies on binding the sulfur ligand-modified molybdenum cofactor (MoCo), and the sulfuration of MoCo is catalyzed by molybdenum cofactor sulfurase (Bittner et al. 2001; Schwarz and Mendel 2006; Wollers et al. 2008). The expression of the molybdenum cofactor sulfurase gene was found to be induced by salt and other abiotic stress in *Arabidopsis* (Bittner et al. 2001; Xiong et al. 2001), and its mutants were more sensitive to freeze and salinity (Xiong et al. 2001). Recent researches demonstrated that the overexpression of the encoding gene of molybdenum cofactor sulfurase (*LOS5/ABA3*) cloned from *Arabidopsis* significantly enhanced the tolerance of transgenic maize, soybean, cotton, tobacco, and rice to abiotic stresses (Xiao et al. 2009; Yue et al. 2011, 2012; Li et al. 2013; Lu et al. 2013). Except for the *LOS5/ABA3* gene, the *OsMCSU* gene from rice, and the *FLACCA* (MoCo sulfurase) gene from tomato (Sagi et al. 2002; Huang et al. 2009), no more genes have been validated for their function as molybdenum cofactor sulfurase, although several sequences have been registered as *MCSU* genes at Genbank (http://www.ncbi.nlm.nih.gov/).

Ammopiptanthus nanus, one of the two species of the *Ammopiptanthus genus* (*Leguminosae*) (Cheng 1959), is a unique evergreen broadleaf shrub in the Mid-Asia deserts and known as a guard plant to prevent further desertification and keep the ecological stability of its habitat. It is endemic to the environments of arid climate (annual precipitation <200 mm), extreme temperatures (from −30 to 40 °C), dryness (dryness usually more than 4), poor soil quality, and high salinity. The habitats of *A. nanus* are usually stony and/or sandy slopes. To cope with these harsh environments, this tenacious species has evolved remarkable tolerance to drought, salinity, and extreme temperatures (Liu et al. 2013; Pang et al. 2013; Wu et al. 2014; Guo et al. 2014). Our previous researches demonstrated that the antifreeze protein gene and betaine aldehyde dehydrogenase gene cloned from *A. nanus* had more significant function in tolerance toward abiotic stresses than their counterparts from other plants (Deng et al. 2014; Yu et al. 2014).

As a relict species, *A. nanus* survives at the west edge of the Tarim Basin from the disappearance of the ancient Mediterranean in the Tertiary Period (Yan et al. 2000). Significant genetic differentiation with other leguminous plants has evolved during its long history of autophilous evolution in the separated habitat (Ge et al. 2005; Chen et al. 2007). Homologous cloning of genes is not easy from this species. In this study, the encoding gene of molybdenum cofactor sulfurase (*AnMCSU*) was cloned from *A.nanus* by rapid amplification of cDNA ends (RACE), analyzed for its characteristics, investigated for its expression pattern in response to abiotic stresses, and validated for its function by heterologous expression in *Arabidopsis*.

Materials and methods

Abiotic stress treatments and total RNA extraction

The seeds of *A. nanus* were soaked in 60 °C water for 5 min and sown in sterilized nutrient soil. The seedlings were grown in a growth chamber under 25/20 °C day/night, relative humidity of 60–70 %, and illumination of 200 μmol m^{-2} s^{-1} for a photoperiod of 14 h light/10 h dark for 5 weeks. At the five-leaf stage, the plants were subjected to heat (45 °C), cold (–4 °C), dehydration (as described by Wei et al. 2012 and Wang et al. 2014), high salt (250 mM NaCl), and ABA (100 μM) treatments, respectively, with three replicates. At the 0 (control), 1, 3, 6, and 9 h of these treatments, the leaves were collected, ground in liquid nitrogen, and used to extract total RNA by using RNAiso plus kit (TaKaRa, Japan). The possible genomic DNA was removed by using RNase-free DNase (TaKaRa, Japan).

Cloning of full-length cDNA

A pair of degenerate primers [5′-GTCGGAGAGR(A/G)CK(G/T)TTTCCR(A/G)TGG-3′/5′-TGCACAW(A/T)GCD(A/G/T)CCW(A/G/T)GGATTR(A/G)-3′)] was designed for amplifying the core cDNA sequence of *MCSU* ortholog from *A.nanus*, based on multiple alignment among the full-length cDNA sequences of *Arabidopsis* and other leguminous plants. As described by Yu et al. (2014), four primers [3RS-outer (5′-GCATGGCGATGGATCTAGCATGTGCAT-3′), 3R-outer (5′-TACCGTCGTTCCACTAGTGATTT-3′), 3RS-inner (5′-GGCTCTTGGTATGGATACCGTGAAGTGG-3′), and 3R-inner (5′-CGCGGATCCTCCACTAGTGATTTCACTATAGG-3′)] were used to perform 3′-RACE using 3′-Full RACE Core Set Ver.2.0 (TaKaRa, Kapan). Four primers [5RS-outer (5′-TTCACCAAGTCAAGGCCAAATCTC-3′), 5R-outer (5′-GCTGATGGCGATGAATGAACACTG-3′), 5RS-inner (5′-GCTGATGGCGATGAATGAACACTG-3′), and 5R-inner (5′-CGCGGATCCGAACACTGCGTTTGCTGGCTTTGATG-3′)] were used to perform 5′-RACE using First Choice® RLM-

RACE Kit (Ambion, USA). After sequencing of the core sequence, the 3′-RACE, and the 5′-RACE amplifications, the full-length cDNA sequence of the *AnMCSU* gene was assembled.

Bioinformatics analysis

The open reading frame (ORF) of the *AnMCSU* gene was identified from the full-length cDNA sequence by online software ORF finder (http://www.ncbi.nlm.nih.gov/gorf/gorf.html). The putative amino acid sequence was deduced online (http://web.expasy.org/translate/) from the full-length cDNA sequence and used for phylogenetic analysis using MEGA5.1 software (http://mega.software.informer.com/5.1b/), multiple alignment using ClustalX2.1 software (http://mac.softpedia.com/get/Math-Scientific/ClustalX.shtml), and conserved domain analysis using Conserved Domains Search Tool (http://www.ncbi.nlm.nih.gov/cdd/). The physical and chemical properties, secondary structure, transmembrane regions and orientation, subcellular localization, and signal peptide were predicted using Expasy (http://www.expasy.org/), GOR IV (http://npsa-pbil.ibcp.fr/cgi-bin/npsa_automat.pl?page=npsa_gor4.html), TMHMM Server v. 2.0 (http://www.cbs.dtu.dk/services/TMHMM-2.0/), PSORT Prediction (http://psort.hgc.jp/form2.html), and SignalP 4.1 (http://www.cbs.dtu.dk/services/SignalP/), respectively.

Quantitative real-time PCR amplification

A pair of specific primers (5′-GCATGGCGATGGATCTAGCA-3′/5′-GCCAGCTTTTCCACTTCACG-3′) was designed to amplify the 144 bp fragment of the *AnMCSU* gene using the Primer-BLAST software (http://www.ncbi.nlm.nih.gov/tools/primer-blast). Each of the retained total RNA samples was reverse transcribed into cDNA separately using a PrimeScript™ reagent kit (TaKaRa, Japan) and used as template for quantitative real-time PCR (qRT-PCR) of the *AnMCSU* gene using SYBR® Premix Ex Taq™ II (Tli RNaseH Plus) (Takara, Japan) in CFX96™ Real-Time System (Bio-Rad, USA). Referring to Wei et al. (2012), the *AnActin2* gene was cloned from *A. nanus* (GenBank accession number: KJ873129) and used as internal reference for qRT-PCR. A pair of specific primers (5′-AGCCTGGATTGCCACATAC-3′/5′-TCAGCAACTGGGACGACAT-3′) was designed to amplify the 185 bp fragment of this gene. The temperature cycle of two-step qRT-PCR was as follows: 95 °C for 30 s; 39 cycles of 95 °C for 5 s, 57 °C for 30 s; then, the temperature was increased by 0.5 °C s^{-1} to 95 °C, so that a melting curve could be calculated and used to differentiate between specific and non-specific amplicons. The $2^{-\Delta\Delta CT}$ method of the CFXManger™ software version 2.0 (Bio-Rad, USA) was used to normalize the expression differentiation between the internal reference and the *AnMCSU* gene (Livak and Schmittgen 2001). The statistical significance among the three biological replicates was tested by the method of Student's *t* test with IBM-SPSS software (http://www-01.ibm.com/software/analytics/spss/).

Subcellular localization

The ORF of the *AnMCSU* gene without the stop codon (TAA) was amplified from the *AnMCSU* inserting pMD19-T vector with primers (5′-TACCCGGGATGGACGACGCCAAAAAAG-3′/5′-TCCGTCGACTGCACAATAGCATGAACTTT-3′; the underlined bases were restriction sites of *Sma* I and *Sal* I, respectively) and inserted at the relative cloning sites of the pC2300–*eGFP* vector containing the enhanced green fluorescent protein gene (*eGFP*), to generate an in-frame fusion gene *AnMCSU–eGFP* under the control of the CaMV *35S* promoter and octopine synthetase terminator (*OCS*) (Fig. S. 1a). After identification by double digestion with *Sma* I/*Sal* I and bacterial sequencing, the reconstructed vector pC2300–*35S–AnMCSU–eGFP*, as well as the control vector pC2300–*35S–eGFP*, was transformed into the *Agrobacterium tumefaciens* strain EHA 105 by freeze and thaw method, respectively (Holsters et al. 1978).

Tobacco (*Nicotiana benthamiana*) seedlings were grown in a growth chamber at 25 °C under a light/dark cycle of 16/8 h for 6 weeks and then used for agroinfiltration experiment with the transformed EHA 105 strains as described by Huang et al. (2013). After growth for 36 h under the same conditions, the tobacco leaves were collected and used to examine the transient expression of the *eGFP* gene by image acquisition on a Nikon A1 confocal laser scanning microscope (Nikon, Japan).

Arabidopsis transformation

A pair of specific primers (5′-GAGGACAGGGTACCCGGGATGGACGACGCCAAAAAAG-3′/5′- CCTGGCATGCCTGCAGGAAGGGCCATCTAACTACAACAGCAT-3′; the underlined bases were homologous sequence to vector ends) were designed and used to amplify the ORF of the *AnMCSU* gene from the *AnMCSU* inserting pMD19-T vector. The product was recombined into expression vector pC2300 by using In-Fusion® HD Cloning Kit (TaKaRa, Japan). The *AnMCSU* gene was under the control of CaMV *35S* promoter and octopine synthetase terminator (*OCS*). The selection marker gene *npt II* was under the control of CaMV *35S* promoter and poly A terminator (Fig. S. 1b). The recombinant vector pC2300–*35S–AnMCSU* was transformed into the *Agrobacterium tumefaciens* strain EHA 105 by freeze and thaw method (Holsters et al. 1978).

Arabidopsis (ecotype Col-0) were grown in 7 cm × 7 cm pots filled with a mixture of soil and vermiculite (4:1, V/V) in a greenhouse at 25 °C and 60–70 % humidity under a 10 h-light/14 h-dark photoperiod. About 6 weeks later, the plants were transformed by the transformed *A. tumefaciens* strain by the flower-dipping method (Clough and Bent 1998).

Screening and identification

The T_0 seeds were harvested and sown on 1/2 MS medium (Murashige and Skoog 1962) supplemented with 50 mg/L kanamycin (Sigma, USA). The surviving T_1 plants were selfed to produce T_2 seeds. The T_2 lines were screened on the same selection medium, and the surviving T_2 lines with a 3:1 segregation ratio (resistant:sensitive) were selfed to produce T_3 seeds. The homozygous T_3 lines without segregation of resistance were selected on the same selection medium and identified by specific PCR amplification of the *AnMCSU* and the *35S* promoter.

The plants of the homozygous T_3 lines, along with the wild-type control cultured on 1/2 MS medium, were transferred into nutrient soil, grown for 3 weeks, and subjected to high salt (300 mM NaCl, twice with an interval of 3 days) and cold (−12 °C for 5 h) treatments, respectively. The resistant lines were selected according to their phenotypes after recovering for 10 days.

Reverse transcription PCR

Total RNA was extracted from T_3 plants and wild type using RNAiso plus kit (TaKaRa, Japan) and used as template to synthesize the first strand of cDNA using PrimeScript™ II 1st Strand cDNA Synthesis kit (TaKaRa, Japan). A pair of specific primers (5′-TTTACCTCTGG GGCAACAGC-3′/5′-CAGGTGGCATGGTAGCAGA-3′) was designed to amplify the 451 bp fragment of the *AnMCSU* ORF. Another pair of specific primers (5′-CCG AGGCTCCTCTTAACCCA-3′/5′-CCAACAGAGCACCT AAGGTCG-3′) was designed to amplify the 518 bp fragment of the *AtActin2* gene of *Arabidopsis* and used as control. The amplified product was separated by 2 % agarose gel electrophoresis and used to identify the heterologous expression of the transformed *AnMCSU* gene.

Measurement of ABA and proline content

The seeds of the T_3 lines and the wild-type control were sown in nutrient soil, grown for 3 weeks, and subjected to high salt (300 mM NaCl) for 3 days and cold (4 °C) for 3 h, respectively, as described by (Gu and Cheng 2014). The leaves were sampled. Half of each sample was ground in 0.1 M phosphate-buffered saline (pH 7.4) and centrifuged at 3000*g* for 20 min. The supernatants were collected and used to measure the content of endogenous ABA by the method of enzyme-linked immunosorbent assay as described by Yang et al. (2001). The other half of each sample was homogenized in 1 ml with 3 % sulfosalicylic acid on ice and centrifuged at 5000*g* for 10 min. The supernatants were treated with acetic acid and ninhydrin, boiled in a water bath for 30 min, and used to measure the absorbance at 520 nm in a UV–visible spectrophotometer model UV-8000 (Thermo Scientific, USA) with L-proline as the standard and double-distilled water as the blank control. The content of free proline was calculated as described by Bates et al. (1973) with minor modification.

Germination assay and root length measurement

Referring to Ying et al. (2012) and Wang et al. (2014) with minor modification, the seeds of the T_3 lines and the wild-type control were surface sterilized with 75 % (V/V) ethanol for 1 min and 10 % NaClO for 10 min, washed three times with sterile distilled water, and suspended by adding 300 µl 0.1 % sterilized agar. According to our preliminary screening, approximately100 seeds with three replicates were plated in a dish containing 1/2 MS agar medium, supplemented without (control) and with 200 mM NaCl, 800 mM mannitol, and 8 µM ABA, respectively, incubated at 4 °C in the dark for 2 days, and transferred to a greenhouse under 16 h-light/8 h-dark cycle. The germination rate was investigated on the seventh day.

According to our preliminary screening, approximately 30 sterilized seeds with three replicates were plated in a dish containing 1/2 MS agar medium, supplemented without (control) and with 100 mM NaCl, 150 mM mannitol, and 0.25 µM ABA, respectively, incubated at 4 °C in dark for 2 days, and vertically cultured in a greenhouse under 16 h-light/8 h-dark cycle. After 2 weeks, the average length of the primary roots was investigated.

Results

Full-length cDNA sequence

From the cDNA template reversely transcribed from the mixed total RNA sample, a core sequence of 960 bp was amplified with the degenerate primers (Fig. S. 2a). A 1170 bp and a753 bp fragment were amplified with the inner primers of the 3′-RACE and 5′-RACE (Fig. S. 2b, c), respectively. These three fragments perfectly matched with each other during their overlapping sequences and were assembled into a full-length cDNA 2544 bp long, with a 5′-untranslated region of 167 bp, an ORF of 2289 bp, and a 3′-untranslated region of 88 bp (Fig. S. 3).

Putative protein

The putative protein of the ORF contains 762 amino acids with a molecular weight of about 84.85 kDa, isoelectric point pH 6.02, instability index (II) 37.95(<40), and grand average of hydropathicity −0.255 (<0). The percentages of α-helices, β-sheets, and random coils in the secondary structure were predicted to be 33.99, 17.72, and 42.39 %, respectively. Its subcellular localization was predicted to be in the cytoplasm. These properties suggest that the putative protein is a stable hydrophilic protein, neither transmembrane nor secretive. In the phylogenetic analysis, this putative protein was clustered into the branch of dicotyledonous leguminous plants (Fig. S. 4).

Homology and conserved domains

Multiple alignment showed that the putative protein shared 85, 83, 64, 64, 52, and 51 % identity with its counterparts in soybean (*Glycine max*, XP_006586740.1), chickpea (*Cicer arietinum*, XP_004506463.1), *Arabidopsis* (NP_564001.1), tomato (*Solanum lycopersicum*, NP_001234144.1), rice (*Oryza sativa*, BAD45451.1), and *Aegilops tauschii* (EMT21536.1), respectively (Fig. 1). The conserved domain analysis showed that the putative protein had the same functional motifs and active sites with its counterparts in other plants (Fig. 1). For example, the four strictly conserved cysteine residues (Cys206, Cys428, Cys430, and Cys435, marked by black triangles) in the NifS-like domain (AAT_I) were perfectly conserved among all the aligned species (Lehrke et al. 2012). The binding and active sites (Ala 110, Thr 111, Leu114, His136, Asp 245, Ala 247, Lys 248, Ser 268, Tyr 270, and Lys 271, marked by black dots) for the pyridoxal phosphate, which is an essential prosthetic group of pyridoxal phosphate-dependent sulfurtransferase, were also conserved among the aligned species except for the two monocotyledonous species (rice and *Aegilops tauschii*). The C-terminus of the putative protein contains a β-barrel domain from 521 to 640 aa (MOSC_N) and a β-strand-rich domain from 671 to 744 aa (MOSC), which were also highly conserved and present in the C-terminal region of all MCSU proteins that have been identified in animals and plants.

All the above evidences substantiate that the putative protein is a molybdenum cofactor sulfurase. Therefore, we registered its encoding sequence as the *AnMUSU* gene at GenBank with accession number KM269293.

Abiotic-inducible expression

The result of qRT-PCR showed that the expression of the *AnMCSU* gene was induced by heat, dehydration, high salt stress, and ABA induction, and inhibited by cold stress (Fig. 2). The expression peaks appeared at the 6th h of the heat and dehydration stress, the 3rd h of the high salt stress, and the 1st h of ABA induction, while the expression valley appeared at the 6th h of the cold stress. All these differences were significant in variance analysis.

Subcellular localization

By laser scanning confocal microscope, the fluorescence signal was observed only in the cytoplasm of the tobacco leaves transformed by the fusion gene vector pC2300–*35S*–*AnMCSU*–*eGFP*, but both in the nucleus and cytoplasm of the tobacco leaves transformed by the control vector pC2300–*35S*–*eGFP* (Fig. 3). This result substantiated the prediction for cytoplasm localization of the AnMCSU protein by the online tool PSORT Prediction.

Transgenic lines

Forty-six T_0 plants survived on the kanamycin selection medium and were considered to represent 46 independent transformation events. After screening of their T_1 and T_2 lines, eight T_3 lines (*L4*, *L7*, *L13*, *L14*, *L19*, *L21*, *L24*, and *L25*) were identified to be homozygous transgenic lines without segregation by the kanamycin screening and specific PCR amplification of the *AnMCSU* and the *35S* promoter (Fig. S.5). From these eight homozygous T_3 lines, three (*L13*, *L19*, and *L25*) were evaluated to be resistant to the high salt (300 mM NaCl) and cold (−12 °C for 5 h) stresses (Fig. 4). Heterologous expression was identified from all the three T_3 lines by reverse transcription PCR (Fig. 5).

Content of endogenous ABA and free proline

Under the high salt and cold stresses, the content of endogenous ABA and free proline of the three T_3 lines and wild-type control was significantly higher than that under the blank control, and the content of endogenous ABA and free proline of the three T_3 lines was significantly higher than that of the wild-type control (Figs. 6, 7). This result confirmed the ABA-dependent response of the transgenic lines to abiotic stresses and implied the association between the content of endogenous ABA and free proline.

Abiotic tolerance

Under the control condition, all the three T_3 lines and the wild-type control germinated normally and their germination rate was almost 100 %. Under a stress of 200 mM NaCl and 800 mM mannitol and the induction of 8 μM ABA, the germination rates of the T_3 lines were

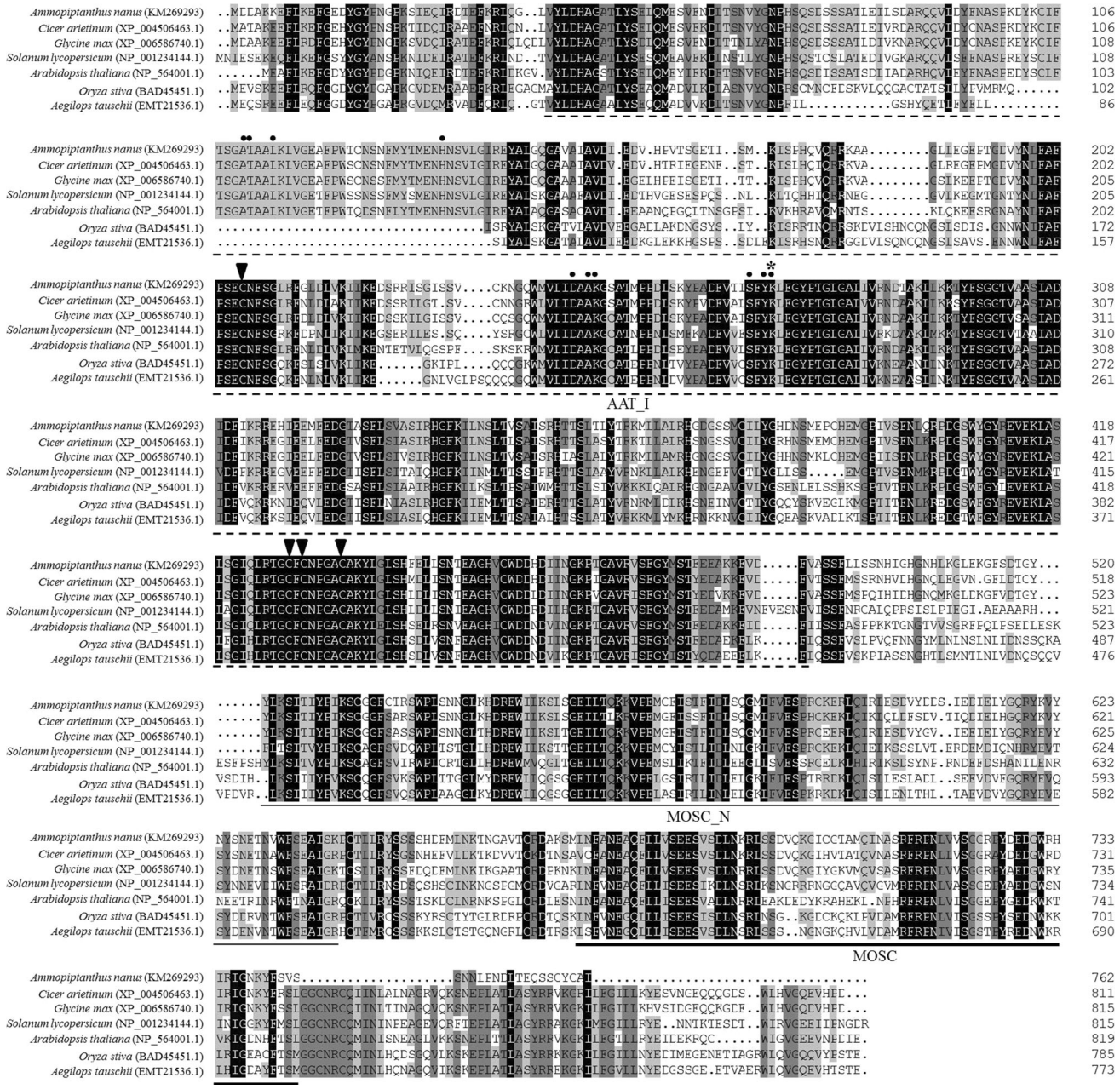

Fig. 1 Multiple alignment among putative proteins of *AnMCSU* gene and *MCSU* genes in soybean (*Glycine max*, XP_006586740.1), chickpea (*Cicer arietinum*, XP_004506463.1), *Arabidopsis* (NP_564001.1), tomato (*Solanum lycopersicum*, NP_001234144.1), rice (*Oryza sativa*, BAD45451.1) and *Aegilops tauschii* (EMT21536.1). The *black*, *dark gray*, *light gray*, and *white* backgrounds indicate perfect (100 %), high (75 %), intermediate (50 %), and low (33 %) conservation, respectively. The cysteine residues (Cys206, Cys428, Cys430, and Cys435) in the NifS-like domain (AAT_I) marked by *black triangles* are strictly conserved among all the aligned species. The amino acids (Ala 110, Thr 111, Leu114, His136, Asp 245, Ala 247, Lys 248, Ser 268, Tyr 270, and Lys 271) marked by the *black dots* are the conserved residues involved in the binding pyridoxal phosphate of pyridoxal phosphate-dependent sulfur transferases belonging to aspartate aminotransferase superfamily type I (AAT_I). The *asterisk* indicates the active site (Lys 271). MOSC_N and MOSC indicate the β-barrel domain from 521 to 640 aa and the β-strand-rich domain from 671 to 744 aa, respectively

significantly ($p \leq 0.01$ or $p \leq 0.05$) higher than that of the wild-type control (Fig. 8).

The result of root length investigation was similar to that of the germination assay. Under the control condition, no significant difference was investigated between the three T_3 lines and the wild-type control. Under a stress of 100 mM NaCl and 150 mM mannitol and induction of 0.25 μM ABA, the average length of the primary roots of the T_3 lines was significantly ($p \leq 0.01$ or $p \leq 0.05$) longer than that of the wild-type control (Fig. 9).

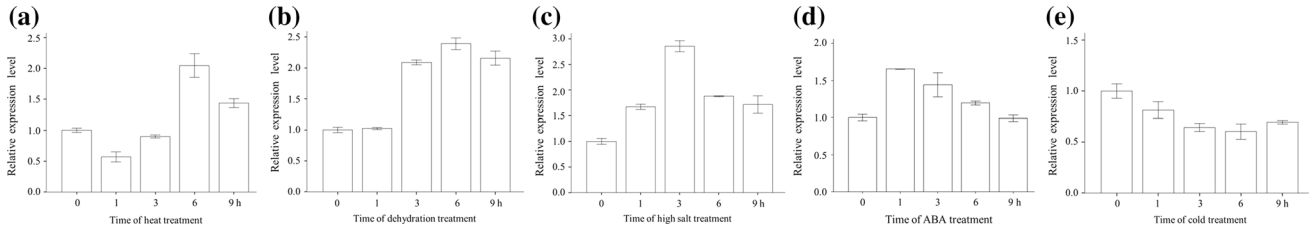

Fig. 2 Relative expression level *AnMCSU* gene abiotic stress and ABA induction detected by qRT-PCR. **a** Under heat (45 °C) stress. **b** Under dehydration. **c** Under high salt (250 mM NaCl) stress. **d** Under ABA (100 μM) induction. **e** Under cold (−4 °C) stress. Data represent mean ± SD ($n = 3$)

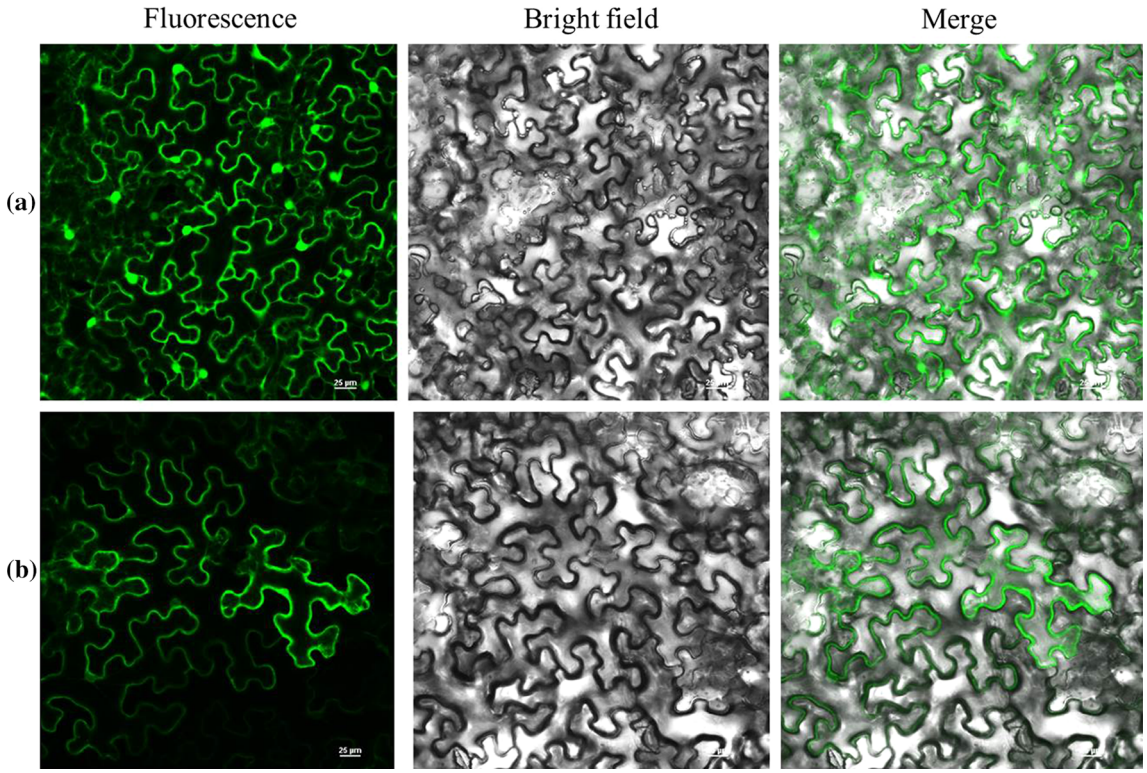

Fig. 3 Subcellular localization of AnMCSU protein. *eGFP* and *AnMCSU–eGFP* fusion gene were driven under the control of the CaMV 35S promoter. **a** Tobacco leaf transformed by pC2300–*35S–eGFP*. **b** Tobacco leaf transformed by pC2300–*35S–AnMCSU–eGFP*

Discussion

Multiple alignment showed that there was a deletion of about 50 amino acids at the C-terminus of the putative protein of the *AnMCSU* gene compared to its counterparts in other plants (Fig. 1). The available literatures suggest that all the conserved cysteine residues (Cys206, Cys428, Cys430, and Cys435), the binding and active sites (Ala 110, Thr 111, Leu114, His136, Asp 245, Ala 247, Lys 248, Ser 268, Tyr 270, and Lys 271) crucial for the sulfuration of MoCo are involved in the N-terminal domain (AAT_I) of the MCSU proteins (Fig. 1, Bittner et al. 2001; Xiong et al. 2001; Lehrke et al. 2012). In contrast, the function of the C-terminal domain has not been identified in detail, although the C-terminal domain was demonstrated to bind a sulfurated Moco (Wollers et al. 2008), which is likely to be incorporated into the target enzymes aldehyde oxidase and xanthine dehydrogenase after reductive cleavage of an Mo–S–S-cysteine/protein bond (Giles et al. 2014). Remarkably, the tomato flacca mutant carrying a mutation exactly in this part of the C-terminal domain, which is lacking in the MOSC domain of AnMCSU, showed impaired sulfuration activity of the resulting MCSU protein, although it retained measureable activity of the aldehyde oxidase in the ABA synthetic pathway and detectable accumulation of ABA (Sagi et al. 1999, 2002). In the present study, the *AnMCSU* gene was cloned from *A. nanus*, which is a relict species from the disappearance of the ancient

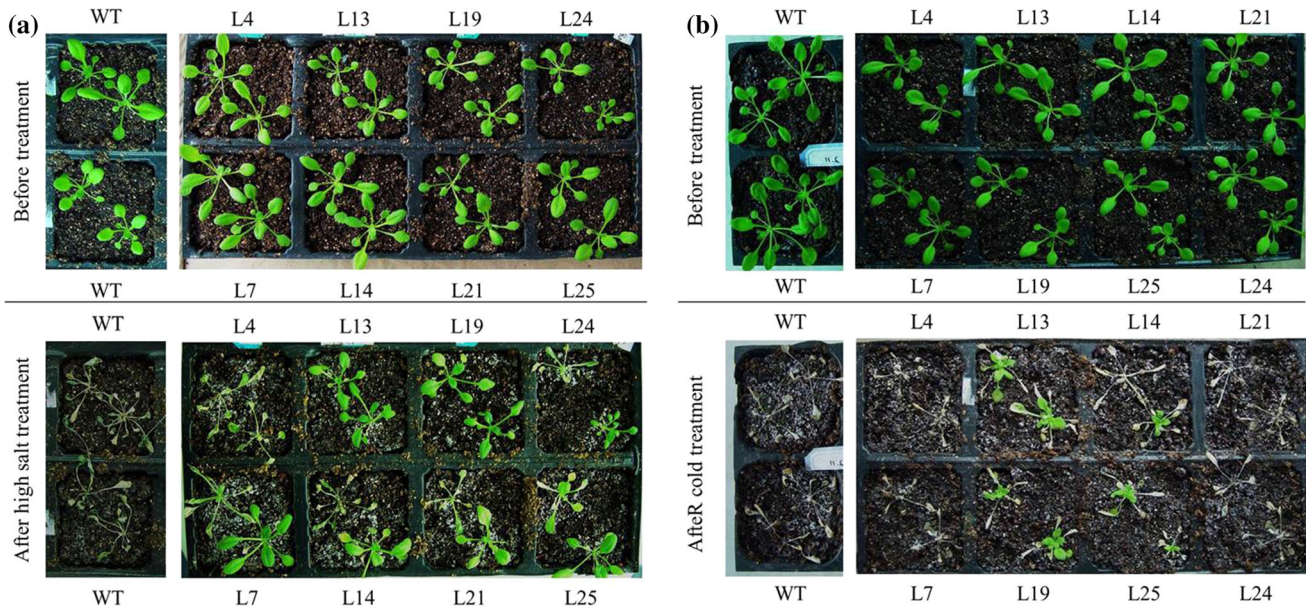

Fig. 4 Resistant phenotype of eight homozygous T_3 lines. **a** High salt (300 mM NaCl, twice with an interval of 3 days) stress. **b** Cold (−12 °C for 5 h) stress

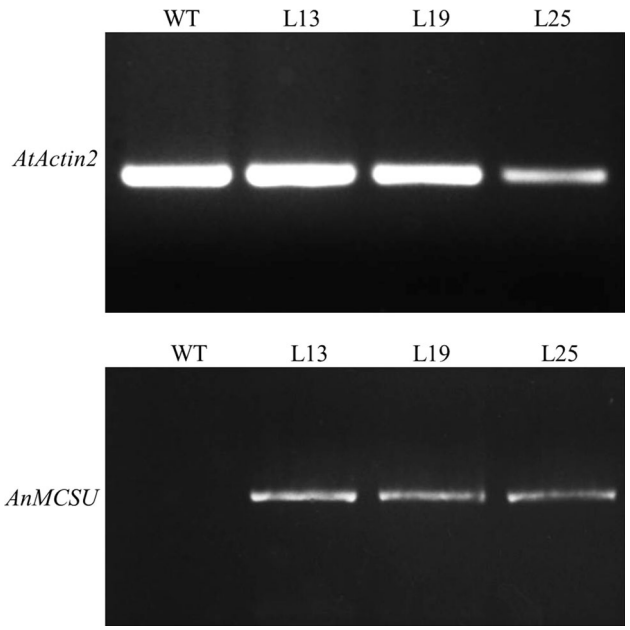

Fig. 5 Reverse transcription PCR amplification of T_3 plant lines. *WT* untransformed wild-type control; *L13*, *L19*, and *L25* T_3 plant lines

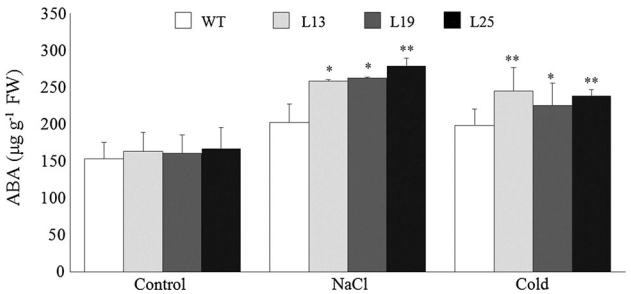

Fig. 6 Content of endogenous ABA in T_3 lines and wild-type control under stress of high salt (300 mM NaCl) for 3 days and cold (4 °C) for 3 h. Data are the mean ± SE of three replicates. * and ** indicate $p \leq 0.05$ and $p \leq 0.01$, respectively

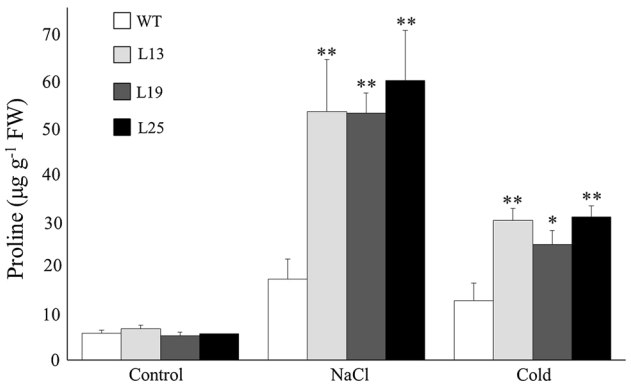

Fig. 7 Content of free proline in T_3 lines and wild-type control under stress of high salt (300 mM NaCl) for 3 days and cold (4 °C) for 3 h. Data are the mean ± SE of three replicates. * and ** indicate $p \leq 0.05$ and $p \leq 0.01$, respectively

Mediterranean in the Tertiary Period. Significant genetic diversity with other leguminous plants has evolved during its long history of the autophilous evolution in the separated habitat (Yan et al. 2000; Ge et al. 2005; Chen et al. 2007). The heterologous expression of the *AnMCSU* gene in *Arabidopsis* significantly increased the content of endogenous ABA (Fig. 6) and enhanced the tolerance of

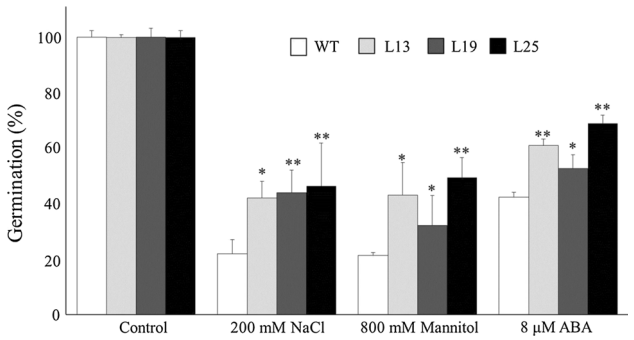

Fig. 8 Germination rates of T_3 plant lines and wild-type control under stress of 200 mM NaCl and 800 mM mannitol, and induction of 8 μM ABA. Data represent mean ± SD ($n = 3$), $*p \leq 0.05$, $**p \leq 0.01$

Arabidopsis to high salt, cold, osmotic stresses, and abscisic acid induction (Figs. 4, 8, 9). Based on these results, the motif affected in the tomato flacca mutant does not appear to be essentially required for mediating tolerance toward the tested abiotic stresses, which is confirmed by the finding that flacca roots still exhibit detectable aldehyde oxidase and xanthine dehydrogenase activities (Sagi et al. 1999). More detailed mechanism remains to be elucidated by structural molecular biology.

In the present study, the endogenous expression of the *AnMCSU* gene was found to be inhibited by cold stress (Fig. 2e). However, the *Arabidopsis* lines transformed by the *AnMCSU* gene showed obvious improvement of cold tolerance (Fig. 4b). The possible explanation was that the heterologous expression of the *AnMCSU* gene in the transgenic lines was driven by the constitutive super promoter *35S*, whereas the endogenous expression of the *AnMCSU* gene in *A.nanus* was driven by its own endogenous promoter. This phenomenon was likewise observed in *Arabidopsis*. The endogenous expression of the *LOS5/ABA3* gene, a homologous counterpart of the *AnMCSU* gene, was cold inhibited, but the mutant of this gene was sensitive to cold stress (Xiong et al. 2001).

Proline is a type of compatible osmolyte (Delauney and Verma 1993). The content of free proline was found to vary relative to the degree of plant tolerance to high salt, cold, and other abiotic stresses (Naser et al. 2010; Tang et al. 2013; Gu and Cheng 2014). In the present study, the content of free proline in the transgenic *Arabidopsis* lines was significantly higher than that of the wild-type control under high salt and cold stresses (Fig. 7), while the content of endogenous ABA in the transgenic lines was also higher than the wild-type control under the same stress conditions (Fig. 6). These two results imply the association between the content of endogenous ABA and free proline. The possible explanation should be that the transformation of the *AnMCSU* gene increased the accumulation of endogenous ABA. As a consequence, ABA might promote the expression of the *P5CS* gene. This gene encodes Δ^1-pyrroline-5-carboxylate synthase, a key enzyme in proline biosynthesis (Strizhov et al. 1997; Yue et al. 2012; Lu et al. 2013; Li et al. 2013).

In other plants, the expression of the *MCSU* genes was found to be responsive to abiotic stresses such as drought, high salt, and ABA induction without tissue or organ specificity (Xiong et al. 2001; Huang et al. 2009; Lu et al. 2013). In the present study, the expression of the *AnMCSU* gene was also strongly induced by heat, dehydration, high salt stresses, and ABA induction, and inhibited by cold stress (Fig. 2). However, the content of endogenous ABA was not increased in the transgenic lines, in which the *AnMCSU* gene was driven by the constitutive super promoter *35S*, compared with the wild-type control under the blank control (Fig. 6), although this result was consistent with previous reports (Shi et al. 2003; Wei et al. 2011;

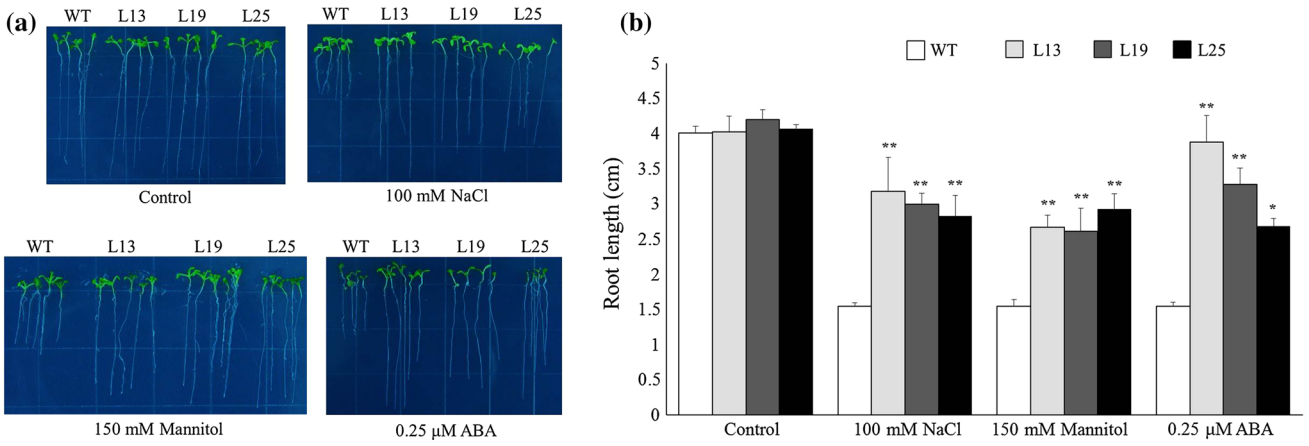

Fig. 9 Average length of primary root under stress of 100 mM NaCl and 150 mM mannitol, and induction of 0.25 μM ABA. **a** The phenotypes. **b** The statistic data. Data represent mean ± SD ($n = 30$), $*p \leq 0.05$, $**p \leq 0.01$

Baisakh et al. 2012; Li et al. 2013; Lu et al. 2013). The possible explanation should be that the stresses could cause a post-transcriptional stablilization of the transcript. Post-transcriptional regulation of transcript accumulation is a major mechanism of gene expression in response to abiotic stresses (Cohen et al. 1999; Shi et al. 2003).

ABA, known as an environmental hormone, plays a crucial role in the response of plants to abiotic stresses (Pennisi 2009; Santner and Estelle 2009; Cutler et al. 2010; Leng et al. 2014). Several candidate genes encoding proteins involved in the pathway of ABA biosynthesis, such as *NCED* (9-*cis*-epoxycarotenoid dioxygenase) from tomato, crowtoe (*Lotus tenuis*), and orange (*Citrus reshni*), *ZEP* (zeaxanthin epoxidase) from *Arabidopsis*, *FLACCA* (MoCo sulfurase) gene from tomato, *OsMCSU*, and *LOS5/ABA3* have been cloned and validated for their function in adaption to abiotic stresses (Agrawal et al. 2001; Xiong et al. 2001; Sagi et al. 2002; Park et al. 2008; Huang et al. 2009; Ji et al. 2014; Espasandin et al. 2014; Xian et al. 2014). In the *MCSU* gene family, however, only the *LOS5/ABA3* gene cloned from *Arabidopsis* has been applied to transgenic operation for improving abiotic tolerance of commercial crops (Xiao et al. 2009; Yue et al. 2011, 2012; Li et al. 2013; Lu et al. 2013). In the present study, the molybdenum cofactor sulfurase gene *AnMCSU* was cloned from the super-xerophytic desert plant *A. nanus* and validated for its function in abiotic tolerance by heterologous expression in *Arabidopsis* under stresses of high salt, cold, and high osmosis, and ABA induction. This result provides one more choice for the transgenic improvement of commercial crops for abiotic tolerance.

Conclusion

The full-length cDNA of the molybdenum cofactor sulfurase gene *AnMCSU* from *A. nanus* is 2544 bp long and contains an open reading frame of 2289 bp, encoding an 84.85 kDa protein of 762 amino acids. Expression of the *AnMCSU* gene is strongly induced by heat, dehydration, high salt stresses, and ABA induction, and inhibited by cold stress. Its encoded protein is located in the cytoplasm. The heterologous expression of the *AnMCSU* gene confers high salt, cold, and dehydration tolerance to *Arabidopsis*.

Author contribution statement HQ Yu performed the experiment and drafted the manuscript; YY Zhang and TM Yong participated in the experiments; YP Liu sampled the material in the field; SF Zhou provided technical support; FL Fu and WC Li provided ideas, designed the research, and edited the manuscript; all authors read and approved the final manuscript.

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Conflict of interest The authors declare that they have no conflict of interest.

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