Genetics and Molecular Research

Overexpression of *EsMcsu1* from the halophytic plant *Eutrema salsugineum* promotes abscisic acid biosynthesis and increases drought resistance in alfalfa (*Medicago sativa* L.)

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Genet. Mol. Res. 14 (4): 17204-17218 (2015)
Received August 21, 2015
Accepted October 29, 2015
Published December 16, 2015
DOI http://dx.doi.org/10.4238/2015.December.16.20

ABSTRACT. The stress phytohormone abscisic acid (ABA) plays pivotal roles in plants' adaptive responses to adverse environments. Molybdenum cofactor sulfurases influence aldehyde oxidase activity and ABA biosynthesis. In this study, we isolated a novel *EsMcsu1* gene encoding a molybdenum cofactor sulfurase from *Eutrema salsugineum*. *EsMcus1* transcriptional patterns varied between organs, and its expression was significantly upregulated by abiotic stress or ABA treatment. Alfalfa plants that overexpressed *EsMcsu1* had a higher ABA content than wild-type (WT) plants under drought stress conditions. Furthermore, levels of reactive oxygen species (ROS), ion leakage, and malondialdehyde were lower in the transgenic plants than in the WT plants after drought treatment, suggesting that the transgenic plants experienced less ROS-mediated damage. However, the expression of several stress-responsive genes, antioxidant

enzyme activity, and osmolyte (proline and total soluble sugar) levels in the transgenic plants were higher than those in the WT plants after drought treatment. Therefore, *EsMcsu1* overexpression improved drought tolerance in alfalfa plants by activating a series of ABA-mediated stress responses.

Key words: Abscisic acid; Drought tolerance; *Medicago sativa* L.; Molybdenum cofactor sulfurase

INTRODUCTION

Alfalfa (*Medicago sativa* L.) is one of the most important legume forages because it has a high protein content and a variety of vitamins for herbivorous animals, but most cultivars are sensitive to water deficit (Bao et al., 2009). Therefore, drought-tolerant cultivars should be developed to increase crop yields under water-deficit conditions. Conventional breeding methods improve plants' tolerance to abiotic stress, but need long breeding cycles. Recently, genetic engineering has allowed the development of stress-tolerant crops (Park et al., 2008; Yue et al., 2012; Xian et al., 2014).

Drought stress is globally the most destructive factor that affects plant growth and yield (Bohnert et al., 2006). Plants have evolved sophisticated strategies to counteract the damage caused by drought stress via a wide range of morphological, physiological, and biochemical adaptive responses (Shinozaki and Yamaguchi-Shinozaki, 2007). When subjected to drought stress, plants can rapidly accumulate several important metabolites, such as abscisic acid (ABA), ascorbic acid, and osmotically active compounds (Gómez-Cadenas et al., 2002). Among these metabolites, ABA plays a cardinal role in the response to abiotic stress. Previous studies have reported that the application of exogenous ABA increases the tolerance of plants to adverse conditions (Kumar et al., 2012). Conversely, treatment with an ABA synthesis inhibitor significantly impairs abiotic stress tolerance in plants (Kumar et al., 2012). Therefore, ABA content is closely associated with adaptive responses to abiotic stress in plants.

ABA biosynthesis induced by abiotic stress increases *de novo* ABA biosynthesis (Yue et al., 2011). The expression levels of key genes that encode ABA biosynthetic enzymes are increased by various abiotic stressors, such as drought, cold, and high salinity (Barrero et al., 2006; Park et al., 2008; Sun et al., 2014; Xian et al., 2014). ABA biosynthetic pathways have been clearly identified in plants, and mainly involve the following three steps (Seo and Koshiba, 2002): 1) zeaxanthin epoxidase (ZEP) catalyzes the conversion of zeaxanthin to *all-trans*-violaxanthin, and through a series of structural modifications the violaxanthin is converted to 9-*cis*-epoxycarotenoid; 2) 9-*cis*-epoxycarotenoid dioxygenase (NCED) catalyzes the cleavage of 9-*cis*-violaxanthin and 9-*cis*-neoxanthin to generate xanthoxin, which is then converted to abscisic aldehyde (AA) by short-chain dehydrogenase/reductase; 3) AA is oxidized to ABA by AA oxidase (AAO), and molybdenum cofactor (MoCo) sulfurase (MCSU) catalyzes the production of the sulfurylated form of MoCo, which is required for AAO activity.

Recently, several studies have attempted to increase endogenous ABA levels to improve plant drought tolerance. The overexpression of ABA biosynthetic genes, such as *ZEP*, *NCED*, and *AAO*, promotes ABA biosynthesis and increases drought resistance in many plant species, such as *Arabidopsis,* tobacco, and tomato (Yue et al., 2012; Sun et al., 2014; Xian et al., 2014). Therefore, screening key genes involved in ABA biosynthesis increases abiotic stress tolerance in crop plants.

Eutrema s*alsugineum* is an extremophile model plant with an exceptionally high resistance

to drought, salinity, and freezing (Inan et al., 2004), indicating that it is a good source for identifying candidate genes to engineer stress-tolerant plants (Zhu et al., 2014; Zhou et al., 2015). In this study, we isolated and characterized *EsMcsu1*, which encodes a MCSU, from *E. salsugineum*. *EsMcsu1* is an early stress-responsive gene, and we investigated the effect of its overexpression on drought tolerance in transgenic alfalfa plants.

MATERIAL AND METHODS

Plant material and stress treatments

Alfalfa seeds were grown in soil in a growth chamber under a 16 h light/8 h dark cycle at 23°C. *E. salsugineum* seeds were surface-sterilized with 0.1% $HgCl_2$ (w/v) for 10 min, and then rinsed five times with sterile water. The sterilized seeds were cultured on Murashige and Skoog (MS) agar medium under a 16 h light/8 h dark cycle at 23°C. To investigate *EsMcsu1* expression profiles in response to abiotic stress or ABA treatment, 14-day-old seedlings were treated with PEG 6000 (30%), NaCl (300 mM), cold (4°C), or ABA (100 µM) for different periods (0, 6, 12, 24, or 48 h). To perform drought stress assays, wild-type (WT) and transgenic plants were initially grown under well-watered conditions for five weeks. Thereafter, these plants were subjected to drought stress by withholding water irrigation for eight or sixteen days.

Isolation and sequence analysis

Total RNA was extracted from *E. salsugineum* plants using TRIzol reagent (Takara, Japan). The RNA was then reverse-transcripted into cDNA as a template for polymerase chain reaction (PCR) analysis. The *EsMcsu1* coding sequence was found in the NCBI database based on the amino acid sequences of conserved MoCo domains from different plant species using the Blastx program (http://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blastx&PAGE_TYPE=BlastSearch&LINK_LOC=blasthome). *EsMcsu1* was amplified using a pair of gene-specific primers (5'-ATGGAAGAATTTCTTAAGGA-3' and 5'-TTATTCAGTATTTGGATTTAC-3'). The PCR cycling conditions were as follows: 95°C for 10 min, 40 cycles at 95°C for 40 s, 58°C for 40 s, 72°C for 3 min, and a final extension at 72°C for 10 min. In addition, multiple sequence alignments were conducted using the ClustalW2 program. A phylogenetic tree was constructed using MEGA 4.0 software, based on the neighbor-joining method. The theoretical molecular weight (Mw) and isoelectric point (pI) were calculated using the ExPASy online tool (http://web.expasy.org/compute_pi/). Protein structural analysis was performed by searching for conserved motifs (http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi).

Gene expression analysis

Total RNA was isolated from *E. salsugineum* plants using TRIzol reagent (Takara), and 1 µg of total RNA was then reverse-transcribed into cDNA as a template for real-time quantitative reverse transcription PCR (qRT-PCR) to analyze the transcription levels of *EsMcsu1*, *MsP5CS1*, and *MsRD22*. To monitor the expression patterns of *EsMcsu1* in response to stress treatment, qRT-PCR was conducted to analyze its transcriptions in *E. salsugineum* using the primers 5'-GACATGGAAACGGGACTGG-3' and 5'-AGGTAGCCAAACCAAGAGCC-3'; *EsActin2* was used as an internal control (Zhu et al., 2014). To analyze the expression levels

of stress-related genes, primers for *MsP5CS1* (5'-GAGGGGGAATGACAGCCAAA-3′ and 5'-TGAAGCCGTCTGGAACAGTC-3') and *MsRD22* (5'-ACGGCATCAAAGGAGAGGAA-3' and 5'-ACCTTCCAAAGGCACAGAGT-3') were designed to detect their transcription by qRT-PCR. *MsGPDH* was selected as an internal control to normalize targeted gene expression (Bao et al., 2009). The qRT-PCR was performed in an ABI PRISM® 7500 Real-Time PCR System, according to the method described by Zhu et al. (2014).

Generation of transgenic plants

Recombinant plasmids (pBI121-*EsMcsu1*) were transferred into the *Agrobacterium tumefaciens* strain GV301. Transgenic alfalfa plants were generated by the *Agrobacterium*-mediated transformation method (Wang et al., 2014), and the transformed plants were selected on MS agar medium containing 50 mg/L kanamycin. The kanamycin-resistant lines were further verified by PCR amplification of the fragments of *EsMcsu1* and *nptII*, using the primers for *EsMcsu1* (5'-GACATGGAAACGGGACTGG-3' and 5'-AGGTAGCCAAACCAAGAGCC-3') and *nptII* (5'-ATTCGGCTATGACTGGGCAC-3' and 5'-TTCAGTGACAACGTCGAGCA-3'). *EsMcsu1* expression in independent T2 lines was detected by qRT-PCR; *MsGPDH* was used as an internal reference.

Analysis of biochemical and physiological parameters

The ABA content was measured using an indirect enzyme-linked immunosorbent assay, according to a method described by Yang et al. (2001). Leaf water loss and water potential was measured as described by Li et al. (2013). Levels of ion leakage (IL) and malondialdehyde (MDA) were detected according to the method described by Zhou et al. (2012). *In vivo* localization and quantification of reactive oxygen species (ROS), including H_2O_2 and O_2^-, was determined according to the methods reported by Yadav et al. (2012). Antioxidant enzyme activity was measured as described by Xie et al. (2008).

Statistical analysis

Each experiment was performed in triplicate. Means were compared between the treatments using a one-way analysis of variance and a Duncan test at $P < 0.05$.

RESULTS

Cloning and sequence analysis of *EsMcsu1* from *E. salsugineum*

A full-length cDNA sequence of *EsMcsu1* from *E. salsugineum* was retrieved from the NCBI database (GenBank accession no. XM_006416743). Based on this sequence, the *EsMcsu1*coding sequence was amplified by PCR. Sequencing analysis revealed that the *EsMcsu1*coding sequence was 2460 bp in length, and encoded a predicted polypeptide of 819 amino acids with a calculated Mw of 91.7 kDa and a pI of 6.53. Multiple sequence alignments of MCSU homologs from different organisms were performed using ClustalW2 (Figure 1a). The results showed that the EsMcsu1 protein had 90% amino acid similarity to the *Arabis alpina* homolog, 89% similarity to *ABA3/LOS5* of *Arabidopsis thaliana*, 66% similarity to *MCSU* isoform1 of *Theobroma cacao*, and 48% similarity to the MCSU-like protein of *M. truncatula.*

Analysis of the conserved domains revealed that the entire amino acid sequence of *EsMcsu1* contained three putative domains. The N-terminal region exhibited a high homology with the NifS-like protein belonging to the aspartate aminotransferase superfamily (fold type I) of Class V pyridoxal phosphate (PLP)-dependent enzymes, which consist of a PLP binding domain and a cysteine domain (Kisher et al., 1997). The second domain was a beta-barrel domain that connected the NifS-like domain to the C-terminal domain, but its functions are unknown. The last domain was a MoCo sulfurase C-terminal motif, which is in MCSU and several other proteins, in both prokaryotes and eukaryotes (Amrani et al., 1999; Watanabe et al., 2000). To further analyze the evolutionary relationship of EsMcsu1 and its homologs, a phylogenetic tree was constructed based on amino acid alignments, using MCSU homologs of 12 different plant species. As shown in Figure 1b, EsMcsu1 was clustered in the same group as *A. thaliana*, *A. alpine*, and *Brassica rapa*, indicating that *EsMcsu1* might have similar biological functions to the others.

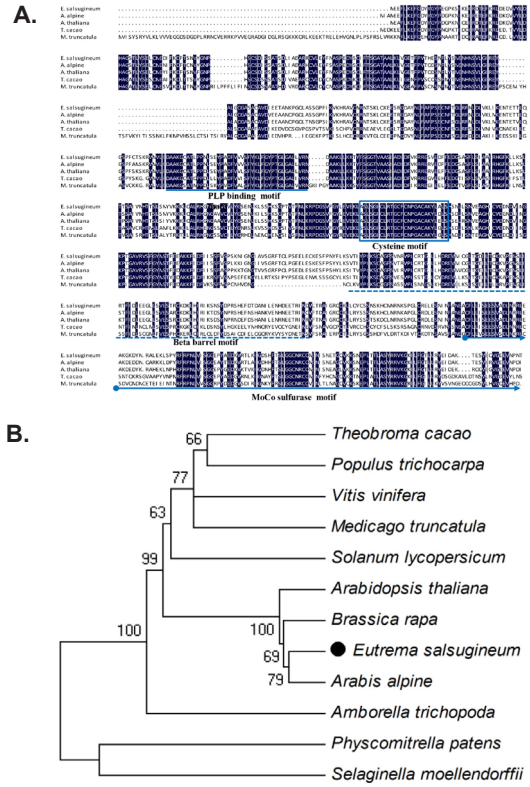

Figure 1. Sequence analysis of the EsMcsu1 protein. **A.** Multiple sequence alignment of EsMcsu1 and its counterparts from several different plant species. The conserved pyridoxal phosphate (PLP) binding region is underlined with a solid line; the cysteine motif is boxed. The unknown C-terminal beta-barrel motif is indicated with a dashed line, and the conserved molybdenum cofactor (MoCo) sulfurase motif is labeled with a straight line and an arrow. **B.** Phylogenetic tree of EsMcsu1 and its homologs, constructed using MEGA 4.0 software. GenBank accession numbers of amino acid sequences used in this study were as follows: *Eutrema salsugineum* (XP_006416806), *Arabis alpine* (KFK43865), *Brassica rapa* (XP_009149088), *Arabidopsis thaliana* (NP_564001), *Theobroma cacao* (XP_007022214), *Vitis vinifera* (XP_003634800), *Populus trichocarpa* (XP_002310102), *Medicago truncatula* (XP_003605400), *Physcomitrella patens* (XP_001756554), *Selaginella moellendorffii* (XP_002983445), *Solanum lycopersicum* (NP_001234144), and *Amborella trichopoda* (XP_006853371).

EsMcsu1 expression patterns in response to abiotic stress or ABA treatment

To examine *EsMcsu1* expression patterns in the roots, leaves, stems, flowers, and siliques of six-month-old *E. salsugineum* plants, its transcription level was analyzed by qRT-PCR. The data showed that *EsMcsu1* transcription was detected in all of the organs tested, while its expression varied between organs (Figure 2a). In addition, we examined *EsMcsu1*expression in response to abiotic stress. As shown in Figure 2b, *EsMcsu1* expression was significantly upregulated after abiotic stress or ABA treatment. *EsMcsu1* expression was quickly induced by cold treatment within 24 h, and then decreased by 48 h. After PEG 6000 treatment, *EsMcsu1* transcription levels had increased substantially at 6 h, subsequently decreasing after 24 h. Upon exposure to 300 mM NaCl, *EsMcsu1* expression peaked at 12 h, thereafter decreasing to 48 h. ABA treatment led to an obvious increase in *EsMcsu1* expression at 12 h, which then gradually decreased until 48 h.

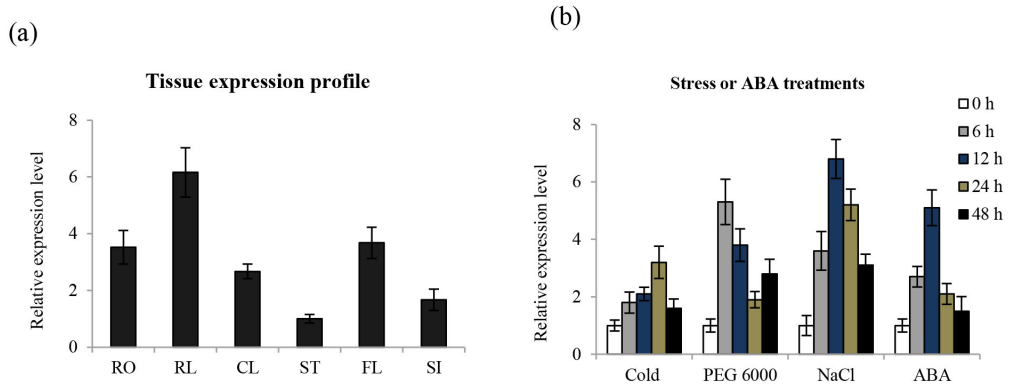

Figure 2. Quantitative reverse transcription polymerase chain reaction analysis of *EsMcsu1* expression profiles in different organs in response to abiotic stress or abscisic acid (ABA) treatment. (**a**) *EsMcsu1* transcription profiles in major organs of six-month-old *Eutrema salsugineum* plants, including roots (RO), rosette leaves (RL), cauline leaves (CL), stems (ST), flowers (FL), and siliques (SI). (**b**) Total RNA isolated from 14-day-old *E. salsugineum* seedlings that were subjected to cold (4°C), PEG 6000 (30%), NaCl (300 mM), or ABA (100 µM) for different periods (0, 6, 12, 24, or 48 h). *EsActin2* was used to normalize gene expression. Bars indicate the means and standard errors of three biological repeats.

Identification of transgenic alfalfa harboring *EsMcsu1*

To study the physiological roles of *EsMcsu1* in response to drought stress, the construct harboring *EsMcsu1* that was driven by a constitutive promoter (CaMV 35S) (Figure 3a) was introduced into alfalfa plants. Plants regenerated from transformed leaf discs were selectively grown on MS agar medium containing 50 mg/L kanamycin. *EsMcsu1* and *nptII* amplification was used to confirm the presence of transgenes in all of the transgenic lines by genomic PCR, and 10 independent transgenic lines were obtained (Figure 3b). Furthermore, 10 independent T2 transgenic lines were selected to detect *EsMcsu1* transcription levels by qRT-PCR. As shown in Figure 3c, *EsMcsu1* was highly transcribed in these transgenic lines, and TG2 and TG7 had more transcript products than the other lines.

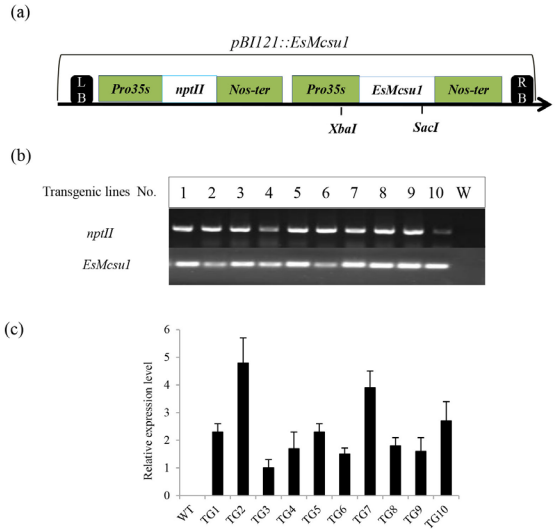

Figure 3. Molecular characterization of alfalfa plants overexpressing EsMcsu1. (**a**) Physical map of EsHdzip1-overexpressing plasmids with a kanamycin-resistance gene (nptII) as a screening marker. (**b**) Molecular identification of the transgenic lines by polymerase chain reaction amplification of nptII and EsMcsu1. TG1-TG10, T2 transgenic lines; WT, wild-type plants. (**c**) EsMcsu1 expression levels in WT and transgenic lines. Bars represent the means and standard errors of three biological repeats.

EsMcsu1-overexpressing lines exhibited increased tolerance to drought stress

We assessed the performance of the *EsMcsu1*-overexpressing lines (TG2 and TG7) under drought stress conditions (Figure 4a). After eight days of water deprivation, the WT and transgenic lines still exhibited similar phenotypic symptoms. However, TG2 and TG7 exhibited better leaf growth that the WT plants, the leaves of which exhibited pronounced yellowness and wilting after 16 days of water deprivation. In order to evaluate whether the activation of *EsMcsu1* expression increased the water capacity of the transgenic plants, the rates of water loss from leaves detached from 35-day-old WT and transgenic plants were measured. As shown in Figure 4b, TG2 and TG7 exhibited 19.6 and 25.7% less water loss, respectively, than WT plants after 10 h of dehydration stress.

We also determined the extent of the stomatal apertures in the WT and transgenic lines (Figure 4c). Stomatal apertures in TG2 and TG7 were 70.5 and 80.3%, respectively, of those in WT plants after eight days of drought treatment. After 16 days of drought treatment, stomatal apertures in TG2 and TG7 were 37.8 and 45.1% smaller than those in WT plants. However, there was no significant difference in stomatal apertures between the WT and transgenic lines under well-watered conditions. To further assess the water-holding capacity of the transgenic lines, the leaf water potential (LWP) and relative water content (RWC) of leaves from the WT and transgenic lines were examined (Figure 4 d and 4e). The results showed that the LWP in TG2 and TG7 were not obviously different to those in WT plants under well-watered conditions. However, the LWP of both the WT and transgenic lines decreased sharply under drought stress conditions. After 8 and 16 days of drought treatment, the LWP was significantly lower in the WT plants than in TG2 and TG7. Similar results were obtained for the RWC in the WT and transgenic lines. These results indicate that the transgenic lines efficiently increased their capacity to conserve water under drought stress conditions.

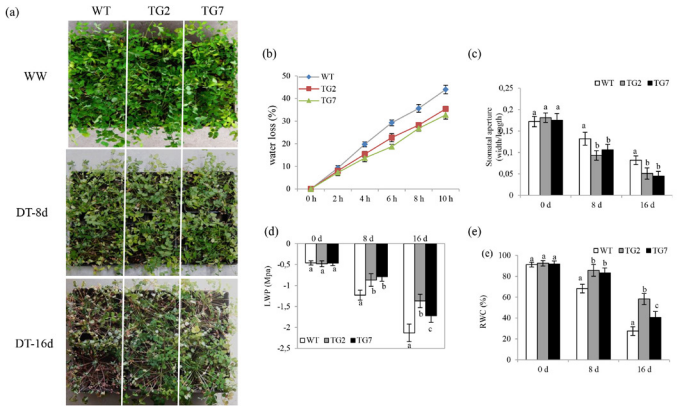

Figure 4. Improved drought tolerance in transgenic alfalfa plants. (**a**) Phenotype of 35-day-old wild-type (WT) and EsMcsu1-overexpressing lines (TG2 and TG7). 35-day-old WT and transgenic alfalfa plants were first grown under well-watered conditions; the plants were then subjected to drought conditions for 8 or 16 days. WW, well-watered conditions; DT-8d, after eight days of drought treatment; DT-16d, after 16 days of drought treatment. (**b**) Water loss from leaves of WT and transgenic lines (TG2 and TG7) at 2-h intervals during 10 h of dehydration stress. (**c**) Stomatal apertures, (**d**) leaf water potential (LWP), and (**e**) relative water content (RWC) in leaves from WT and transgenic lines grown under well-watered and drought stress conditions. Each bar represents the mean of three independent experiments. Error bars are standard deviations of the means, with different letters (a, b, or c) indicating significant differences between WT and transgenic lines.

EsMcsu1 overexpression promoted ABA biosynthesis and upregulated the transcription of stress-responsive genes

To determine whether *EsMcsu1* positively regulates the accumulation of ABA in transgenic alfalfa, the ABA content was measured. As shown in Figure 5a, there was no significant difference in ABA content between the WT and transgenic lines under well-watered conditions, whereas the transgenic lines exhibited a much higher ABA content than the WT plants under drought stress conditions. After eight days of drought treatment, TG2 and TG7 had a 25.9 and 39.5% higher ABA content than WT plants, respectively. After 16 days of drought treatment, TG2 and TG7 had a 19.7 and 40.5% higher ABA content than WT plants, respectively. The expression levels of several important stress-responsive genes, including *MsP5CS* and *MsRD22*, were analyzed by qRT-PCR (Figure 5b and 5c). Their transcription levels had increased in the WT and transgenic lines after drought treatment, and were significantly higher in TG2 and TG7 than in the WT plants.

EsMcsu1 overexpression decreased ROS, IL, and MDA levels under drought stress

Abiotic stress can lead to the overproduction of ROS in various plant species, and cause oxidative damage to cellular structures (Suzuki et al., 2012). EsMcsu1overexpression increased drought resistance in transgenic plants, possibly by reducing ROS accumulation. The levels of two major types of ROS (H_2O_2 and O_2^-) were examined in the leaves of the WT and transgenic lines. Firstly, the in vivo localization of H_2O_2 and O_2^- was conducted by histochemical staining with diaminobenzidine tetrahydrochloride and nitroblue tetrazolium, respectively. As shown in Figure 6a and 6b, TG2 and TG7 exhibited fewer staining spots than did WT plants after 8 and 16 days of drought treatment, but there was no significant difference under well-watered conditions. The H_2O_2

and O_2^- content of the WT and transgenic lines was then quantified (Figure 6c and 6d). After eight days of drought treatment, the H2O2 and O2- content of TG2 (33.4 and 37.7%, respectively) and TG7 (24.6 and 40.1%, respectively) was significantly lower than that in WT plants. After 16 days of drought treatment, the H2O2 and O2- content was significantly higher in TG2 (40.6 and 42.9%, respectively) and TG7 (46.5 and 35.2%, respectively) than in the WT plants. However, there was no difference between the WT and transgenic lines under well-watered conditions. Therefore, EsMcsu1overexpression increased the ability of transgenic alfalfa plants to eliminate cellular ROS, indicating that the transgenic lines experienced less oxidative damage than the WT plants.

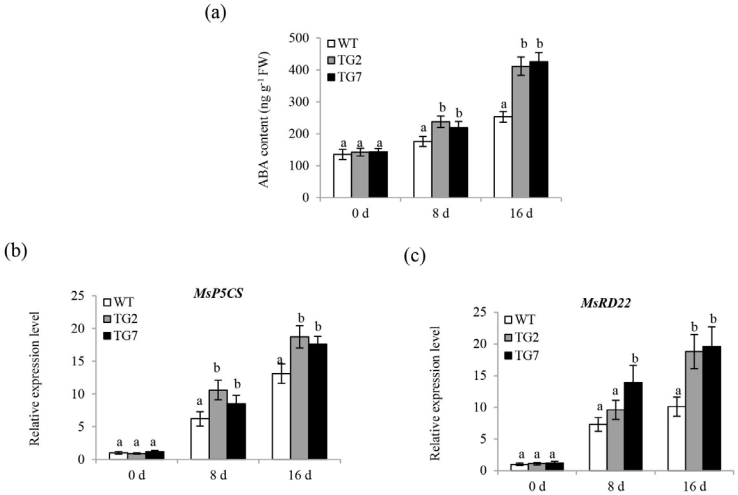

Figure 5. Analysis of abscisic acid (ABA) content and expression levels of stress-related genes in wild-type (WT) and EsMcsu1-overexpressing lines (TG2 and TG7) grown under well-watered conditions or drought stress for 8 or 16 days. (**a**) ABA content. (**b**) Expression levels of MsP5CS and MsRD22. Each bar represents the mean of three independent experiments. Error bars are standard deviations of the means, with different letters (a, b, or c) indicating significant differences between WT and transgenic lines.

IL, which is an index of physical damage to cell membranes (Zhou et al., 2012), was significantly lower in the leaves of TG2 and TG7 than in those of the WT plants, while no difference was observed before drought treatment (Figure 7a). In addition, MDA, which is a key indicator of ROS-mediated damage to plant cells (Moore and Roberts, 1998), exhibited a similar tendency to IL (Figure 7b). These changes in physiological parameters demonstrate that the transgenic lines had a greater ability to minimize oxidative damage caused by drought stress than the WT plants.

EsMcsu1 overexpression increased antioxidant enzyme activity under drought stress

Elevated ABA levels in plants significantly increase abiotic stress tolerance because of increased antioxidant enzyme activity (Sun et al., 2014; Xian et al., 2014); therefore, we investigated superoxide dismutase (SOD), ascorbate peroxidase (APX), catalase (CAT), and peroxidase (POD) activity in leaves of the WT and transgenic lines (Figure 8). Under well-watered conditions, antioxidant enzyme activity did not differ between the WT and transgenic lines. After 8 and 16 days of drought treatment, SOD, CAT, and APX activity was significantly higher in the leaves of TG2

and TG7 than in those of the WT plants, but there was no significant difference in POD activity (Figure 8a-d). These results indicate that *EsMcsu1* overexpression decreases ROS by increasing antioxidant enzyme activity under drought stress conditions.

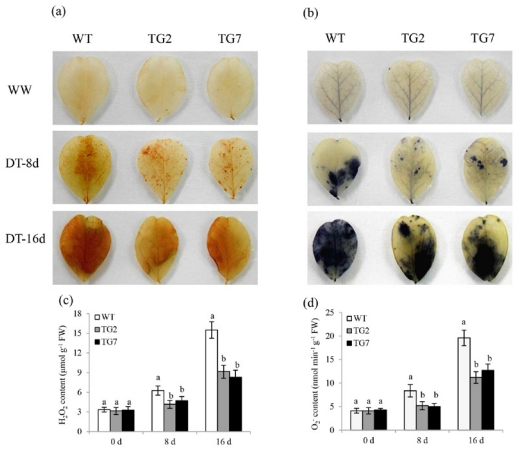

Figure 6. Levels of H_2O_2 and O_2^- in leaves from wild-type (WT) and transgenic lines (TG2 and TG7) under well-watered conditions or drought stress for 8 or 16 days. Histochemical localization of (**a**) H_2O and (**b**) O_2^- was conducted by diaminobenzidine tetrahydrochloride and nitroblue tetrazolium staining, respectively. WW, well-watered conditions; DT-8d, after eight days of drought treatment; DT-16d, after 16 days of drought treatment. Quantification of the (**c**) H_2O_2 content and (**d**) O_2^- content. Each bar represents the mean of three independent experiments. Error bars are standard deviations of the means, with different letters (a, b, or c) indicating significant differences between WT and transgenic lines.

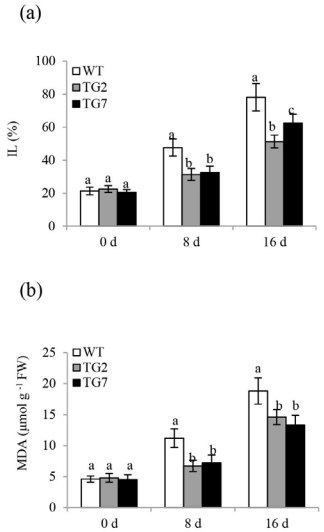

Figure 7. Levels of (**a**) ion leakage (IL) and (**b**) malondialdehyde (MDA) in wild-type (WT) and transgenic lines (TG2 and TG7) grown under well-watered conditions or drought stress for 8 or 16 days. Each bar represents the mean of three independent experiments. Error bars are standard deviations of the means, with different letters (a, b, or c) indicating significant differences between WT and transgenic lines.

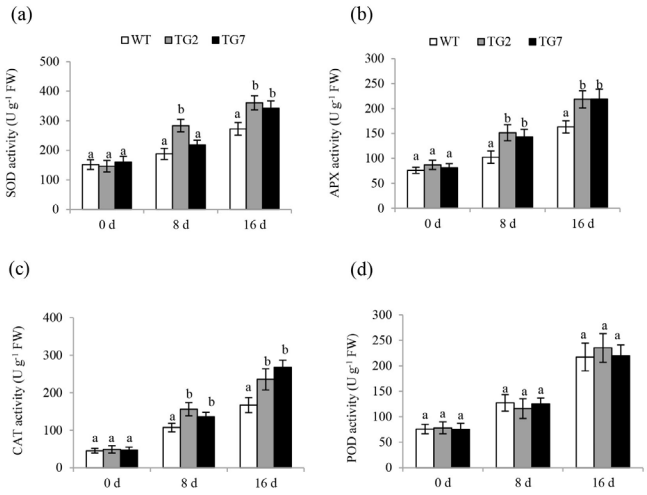

Figure 8. Antioxidant enzyme activity. (**a**) SOD, (**b**) APX, (**c**) CAT, and (**d**) POD in leaves from wild-type (WT) and transgenic lines (TG2 and TG7) grown under well-watered conditions or drought stress for 8 or 16 days. Each bar represents the mean of three independent experiments. Error bars are standard deviations of the means, with different letters (a, b, or c) indicating significant differences between WT and transgenic lines.

EsMcsu1 overexpression increased osmolyte levels under drought stress

The accumulation of proline and total soluble sugar plays an important role in protecting plants from adverse environmental conditions (Yadav et al., 2012), suggesting that proline and soluble sugar levels are closely related to abiotic stress tolerance. As shown in Figure 9a, there was no significant difference in proline levels between the WT and transgenic lines under well-watered conditions. However, drought stress increased proline levels in both the WT and transgenic lines, and they were significantly higher in TG2 and TG7 than in WT plants after 8 and 16 days of drought treatment. In addition, drought stress increased total soluble sugar levels in both the WT and transgenic lines, and they were significantly higher in TG2 and TG7 than in WT plants after 8 and 16 days of drought treatment (Figure 9b).

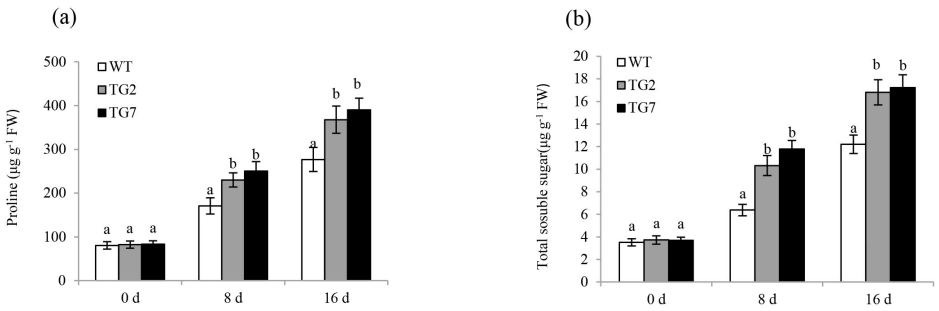

Figure 9. Osmolyte levels in leaves from wild-type (WT) and transgenic lines (TG2 and TG7) grown under well-watered conditions or drought stress for 8 or 16 days. (**a**) Proline, (**b**) Total soluble sugar. Each bar represents the mean of three independent experiments. Error bars are standard deviations of the means, with different letters (a, b, or c) indicating significant differences between WT and transgenic lines.

DISCUSSION

ABA is a plant hormone that plays essential roles in the regulation of water status by modulating stomatal closure and activating the expression of stress-responsive genes (Rock, 2000; Yue et al., 2011). MCSU catalyzes the generation of sulfurylated MoCo that is essential for AAO activity, which is responsible for ABA biosynthesis (Xiong et al., 2001). Therefore, the genetic regulation of ABA biosynthesis has great potential to breed drought-tolerant crops.

EsMcsu1 is a stress-responsive gene that encodes MoCo sulfurase from *E. salsugineum*

Sequence analysis revealed that *EsMcsu1* shares a high sequence identity with other *MCSUs* in several plant species. EsMcsu1 contains a conserved NifS-like domain in its N-terminal region, including a putative PLP binding motif and a conserved cysteine motif, which occur in all other MCSU proteins (Kisher et al., 1997; Amrani et al., 1999). It has previously been found that the PLP and cysteine motifs are essential for MCSU activity in plants (Xiong et al., 2001). In addition, the EsMcsu1 C-terminal MoCo domain is similar to that of MCSUs in a variety of organisms. The MoCo domain functions as a sulfur-carrier, which receives sulfur abstracted by PLP-dependent NifS-like enzymes on its conserved cysteine and then delivers it for the formation of sulfur-metal clusters (Anantharaman and Aravind, 2002). In our study, *EsMcsu1*was ubiquitously expressed in all of the organs tested, suggesting that it plays a variety of roles in plant growth and development. Xiong et al. (2001) reported that knockdown mutants of *Arabidopsis ABA3/LOS5* (that encodes a putative MCSU) exhibited visible alterations in their leaf morphology. Therefore, MCSU plays important roles in a range of plant growth processes.

Abiotic stress significantly increases ABA biosynthesis (Xiong and Zhu, 2003), due to the increased transcription of key ABA biosynthetic genes. The expression of some ABA-related biosynthetic genes, such as *AtZEP*, *EsABA1*, and *AtABA3/AtLOS5*, is induced by several abiotic stressors, including high salinity, drought, and cold (Xiong et al., 2001; Park et al., 2008; Sun et al., 2014). In our experiments, *EsMcsu1* transcripts quickly accumulated after treatment with PEG 6000, salt, or cold, suggesting that *EsMcsu1* expression is regulated by abiotic stress. In addition, its expression was also induced by ABA treatment. ABA itself can upregulate the transcription of genes involved in ABA biosynthetic pathways, thereby promoting its synthesis (Yue et al., 2011). Many studies have shown that *ZEP*, *AAO3*, and *LOS5/ABA3* are induced by ABA treatment, but *NCED* is not (Seo et al., 2000; Xiong et al., 2001; Barrero et al., 2006; Park et al., 2008). *Therefore, EsMcsu1 might be involved in ABA-mediated abiotic stress responses.*

EsMcsu1 regulates ABA synthesis and participates in drought responses

The loss of MCSU enzymatic activity decreases ABA biosynthesis in *Arabidopsis* (Xiong et al., 2001), and *MCSU* overexpression markedly increases AAO activity and ABA levels in transgenic plants under drought stress conditions (Yue et al., 2012). In the present study, the *EsMcsu1*coding region was placed under the control of the constitutive promoter CaMV 35S, and the transgenic lines that ectopically expressed this gene exhibited a high level of *EsMcsu1* expression. Intriguingly, our transgenic lines had relatively similar ABA levels to the WT plants, but the ABA content was significantly higher in the transgenic lines than in the WT plants after drought treatment. The coordination of *EsMcsu1* and other stress-inducible ABA biosynthetic

genes may regulate ABA biosynthesis in transgenic alfalfa lines under drought stress conditions. Subsequently, newly produced ABA upregulated the expression of ABA biosynthetic genes such as *ZEP* and *AAO*, and led to a significant increase in endogenous ABA.

The *EsMcsu1*-overexpressing lines exhibited a lower water loss rate and higher LWP and RWC than WT plants under drought stress conditions. A greater water-holding capacity may decrease stomatal apertures in the leaves of transgenic plants; increased ABA levels increase the tolerance of transgenic plants to drought stress by decreasing stomatal closure and water loss (Li et al., 2013; Lu et al., 2013). Furthermore, elevated ABA levels upregulate the expression of several stress-responsive genes (Sun et al., 2014). In our experiments, the transcription of *MsP5CS* and *MsRD22*, which are homologs of *Arabidopsis P5CS* and *RD22*, rapidly increased in the drought-treated WT and transgenic lines, and their expression levels were higher in the transgenic lines than in the WT plants. Collectively, these results suggest that the *EsMcsu1*-overexpressing lines had more effective strategies than the WT plants to conserve water under water-deficit conditions.

EsMcsu1 increases the ability of transgenic alfalfa plants to scavenge cellular ROS

When plants experience drought stress, the overaccumulation of cellular ROS is a common phenomenon (Seo and Koshiba, 2002). ROS are signaling molecules that are involved in modulating adaptive responses to drought stress. However, ROS overaccumulation causes oxidative damage to lipids, nucleic acids, and proteins, and ultimately disrupts normal cell metabolism (Gill and Tuteja, 2010). In this study, the levels of two major types of ROS (H_2O_2 and O_2^-) were significantly lower in the transgenic lines than in the WT plants under drought stress conditions, suggesting that the *EsMcsu1*-overexpressing transgenic lines might have experienced less oxidative damage than the WT plants. The transgenic lines had lower IL and MDA values than the WT plants after drought treatment. Therefore, *EsMcsu1*overexpression effectively minimized oxidative damage to membranes in the transgenic lines.

The lower ROS levels in the transgenic plants than in the WT plants suggests that *EsMcsu1* expression increased the ability of the transgenic plants to effectively eliminate ROS. In plants, detoxified ROS can be acquired by non-enzymatic (e.g., ascorbic acid and glutathione) and enzymatic systems, including SOD, CAT, POD, and APX, to cope with oxidation stress by *eliminating ROS* production (Asada, 1999). In the present study, antioxidant enzyme activity was much higher in the transgenic lines than in the WT plants under drought stress conditions, and a negative correlation existed between the level of cellular ROS and antioxidant enzyme activity in the transgenic lines. This may be because high antioxidant enzyme activity was closely associated with the ABA content of the drought-treated transgenic lines. Previous studies have found that increased ABA levels significantly increase antioxidant enzyme activity in transgenic plants (Yue et al., 2011; Li et al., 2013; Lu et al., 2013). Taken together, our data indicate that *EsMcsu1*overexpression increased the ability of transgenic alfalfa plants to scavenge ROS.

In conclusion, *EsMcsu1* was isolated and characterized from *E. salsugineum*. Its transcription levels were significantly upregulated by various abiotic stressors and ABA treatment. *EsMcsu1* overexpression in alfalfa improved drought tolerance and increased levels of endogenous ABA. The transgenic lines exhibited a lower water loss rate and higher LPW and RWC than WT plants. Furthermore, lower levels of ROS, IL, and MDA were observed in the transgenic lines, probably because of the increased antioxidant enzyme activity that reduced the oxidative damage caused by drought stress. These finding demonstrate that *EsMcsu1* can be exploited to breed drought-resistant crops by genetic engineering.

Conflicts of interest

The authors declare no conflicts of interest.

ACKNOWLEDGMENTS

Research supported by the Anhui Science and Technology University (Grant # ZRC2014403) and the Key Research Projects in Anhui Science and Technology Committee (Grant #1301032151).

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