Acta Physiol Plant (2009) 31:545–551
DOI 10.1007/s11738-008-0264-1

ORIGINAL PAPER

Tissue-specific regulation of rice molybdenum cofactor sulfurase gene in response to salt stress and ABA

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Received: 17 June 2008 / Revised: 12 November 2008 / Accepted: 5 December 2008 / Published online: 22 January 2009

Abstract Molybdenum-containing aldehyde oxidase is a key enzyme for catalyzing the final step of abscisic acid (ABA) biosynthesis in plants. Sulfuration of the molybdenum cofactor (MoCo) is an essential step for activating aldehyde oxidase. The molybdenum cofactor sulfurase (MCSU) that transfers the sulfur ligand to aldehyde oxidase-bound MoCo is thus considered an important factor in regulating the ABA levels in plant tissues. In this study, we identified the rice *MCSU* cDNA (*OsMCSU*), which is the first *MCSU* gene cloned in monocot species. According to the functional domain analysis of the predicted amino acid sequence, the OsMCSU protein contains a Nifs domain at its N-terminus and a MOSC domain at the C-terminus. Expression of the *OsMCSU* gene was up-regulated by salt stress in root tissues of rice seedlings, but this effect was not observed in leaf tissues. In roots, regulations of *OsMCSU* expressions could be mediated by both ABA-dependent and ABA-independent signaling pathways under salt stress condition.

Keywords Abscisic acid · Molybdenum cofactor sulfurase · Salt stress

Abbreviations

ABA	Abscisic acid
MCSU	Molybdenum cofactor sulfurase
MoCo	Molybdenum cofactor
MOSC	MCSU C-terminal conserved domain
NCED	9-*Cis*-epoxycarotenoid dioxygenase
PLP	Pyridoxal phosphate

Communicated by G. Klobus.

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Introduction

Salinity stress is a serious abiotic stress that results in limited plant growth and development due to the ionic toxin combined with osmotic stress. Plants respond to unfavorable environments mostly through the regulation of stress-related genes, which are usually involved in protection mechanisms, such as osmoprotectant and ion homeostasis production.

Several studies have previously identified abscisic acid (ABA)-mediated signaling as one of the transduction pathways that regulates the salt stress-responsive genes (Narusaka et al. 2003; Zhu et al. 2005). Changes in ABA content in various tissues under salinity conditions have been observed in many plant species (Zhu et al. 2005; Fricke et al. 2006). ABA is a molecule derived from carotenoids, and its biosynthesis involves oxidative cleavage of 9-*cis*-epoxycarotenoid to produce xanthoxin, which is catalyzed by 9-*cis*-epoxycarotenoid dioxygenase (NCED) in plastids (Schwartz et al. 1997). Xanthoxin is further modified to generate abscisic aldehyde in the cytosol via alcohol dehydrogenase (Gonzalez-Guzman et al. 2002); abscisic aldehyde is finally converted to ABA via aldehyde oxidase (Cutler and Krochko 1999). Thus, absence of aldehyde oxidase would result in ABA deficiency in plants (Bittner et al. 2001; Xiong et al. 2001; Sagi et al. 2002; Porch et al. 2006).

In addition to the concentration of available aldehyde oxidase, the activation of aldehyde oxidase is a key regulatory step for controlling the biosynthesis of ABA. The

sulfur ligand-modified molybdenum cofactor (MoCo) that was bound on aldehyde oxidase plays an essential role in this activation process (reviewed by Schwarz and Mendel 2006). The sulfuration of MoCo is catalyzed by MoCo sulfurase (MCSU) (reviewed by Schwarz and Mendel 2006). *mcsu* mutants have been found in tomato and *Arabidopsis*, and mutation of the *MCSU* gene results in a decrease of aldehyde oxidase activity and deficiency in ABA (Marin and Marion-Poll 1997; Schwartz et al. 1997; Xiong et al. 2001). The MCSU proteins encoded by the *Arabidopsis ABA3/LOS5* gene and tomato *FLACCA* gene are both composed of two functional domains: a NifS-like sulfurase domain at the N-terminus and a MCSU conserved domain (MOSC) at the C-terminus (Bittner et al. 2001). The NifS domain functions as cysteine sulfurase using pyridoxal phosphate (PLP) as a cofactor, and it transfers the sulfur from L-cysteine to target molecules and releases L-alanine (Zheng et al. 1993; Heidenreich et al. 2005). The conserved C-terminal is considered to function in the recognition of molybdenum enzymes (Bittner et al. 2001). Both the *Arabidopsis los5-1* and *los5-2* mutants contain mutations within the N-terminus of MCSU, while the *aba3-1* and *aba3-2* mutants contain mutation site at the N-terminal region of the MOSC domains (Xiong et al. 2001). The mutation in tomato *flacca* occurs at the C-terminus of the MCSU protein (Sagi et al. 2002). Previous studies of the *mscu* mutants showed that mutation at either the N-terminal or C-terminal portion of the protein disrupted MCSU function and reduced aldehyde oxidase activity and ABA content in plant tissues (Seo et al. 2000, 2004; Xiong et al. 2001; Sagi et al. 2002).

As mentioned above, MCSU is considered the key regulator for controlling ABA levels. In this study, we used RT-PCR to isolate the first *MCSU* gene in monocot species, rice *OsMCSU*. We found that *OsMCSU* expression is positively regulated by salt stress and ABA in the roots of rice seedlings but not in the leaves, indicating that the regulation of *MCSU* genes in response to salt stress is tissue specific. Furthermore, the tissue-specific regulations of *MCSU* gene by salinity are different between rice and *Arabidopsis* plants.

Materials and methods

Plant material and growth conditions

Rice (*Oryza sativa* L. cv. Tainung 67) seeds were sterilized in 1% sodium hypochlorite with Tween 20 for 15 min and subsequently washed three times with distilled H_2O. Seeds were then germinated at 37°C in the dark for 2 days, and then grown in a phytotron with half-strength of Kimura B nutrient solution (Chu and Lee 1989) at 30/25°C under natural daylight. For salt-stress treatments, three-leaf-stage seedlings were transferred to various concentrations of NaCl for 24 h prior to analysis of gene expression in roots and leaves of rice seedlings. To examine the effect of ABA on *OsMCSU* gene expression, rice seedlings were cultured in half-strength Kimura B solution with 100 μM ABA for 24 h prior to gene expression analysis in leaf and root samples. To determine whether salt stress-regulated *OsMCSU* expression was ABA-dependent, rice seedlings were pre-cultured in a nutrient solutions containing 200 μM fluridone (an ABA biosynthesis inhibitor) for 1 h prior to the subsequent 24-h treatment of 200 mM NaCl combined with 200 μM fluridone. ABA content and *OsMCSU* expression in roots and leaves were then analyzed.

RNA extraction

Plant samples (100 mg) were homogenized in 1 mL Trizol reagent (Invitrogen, CA, USA) and centrifuged at 10,000×*g*. The supernatant was treated with 0.2 mL chloroform, shaken for 15 s, and incubated at room temperature for 3 min. After centrifugation at 12,000×*g* for 15 min at 4°C, the upper layer was transferred to a new tube. RNA was precipitated with 0.5 mL isopropanol and incubated for 10 min at room temperature. After centrifugation, the pellet was dissolved in 0.2 mL H_2O.

Cloning and sequence analysis

Total RNA extracted from leaves of rice seedlings was used as template for RT-PCR amplification of *OsMCSU* cDNA. The primers for RT-PCR amplification of the *OsMCSU* coding regions were designed according to the sequences derived from GenBank (AP003635). The primers used for RT-PCR were *OsMCSU*-F 5′-ATGGAGGTGAGCAAGG A-3′ and *OsMCSU*-R 5′-TCATTCTGTGGAAGGGT-3′. The RT-PCR amplified products were cloned into the pGEM-T Easy vector (Promega, WI, USA) and sequenced. Prediction of the amino acid sequence translated from *OsMCSU* cDNA was processed by the Wisconsin Genetics Computer Group (GCG) Software Package version 10.3. The functional domains in OsMCSU were determined by searching the conserved domain database v2.12 in NCBI's Entrez database system, and the specific motifs were derived by aligning the amino acid sequence with those of *Arabidopsis* ABA3 and tomato FLACCA (Xiong et al. 2001; Sagi et al. 2002). The multiple-sequence alignment was performed using the CLUSTALW software (http://www.ch.embnet.org/software/ClustalW.html).

Quantitative real-time reverse transcriptase PCR

Total RNA (200 ng) was used as template for quantitative real-time RT-PCR analyses with the Brilliant SYBR Green QRT-PCR Master Mix (Stratagene, La Jolla, CA), and the PCR reactions were performed using a Multiplex 3000P Real-Time PCR System (Stratagene, La Jolla, CA). The gene-specific RT-PCR primers for *OsMCSU* were 5′-AC ATAGTCAGAGTGATTCAAGC-3′ and 5′-TAACTTCC ATTGTCTTTTGC-3′. RT-PCR was carried out as follows: 50°C for 30 min and 95°C for 10 min, followed by 40 cycles of 95°C for 1 min, 50°C for 1 min, and 72°C for 1 min. To accurately quantify relative expression levels of the genes, the C_T value of *OsMCSU* was normalized to the C_T value of *Actin*. For all real-time RT-PCR analyses, three independent experiments were carried out, and the data are presented as mean ± SD.

ABA content analysis

After NaCl or fluridone treatment, the tissue samples were frozen with liquid nitrogen and stored at −80°C. The samples (15 mg) were then extracted with 1.5 mL of 80% (v/v) methanol combined with 2% glacial acetic acid, and the ABA content was determined with the Phytodetek ABA test kit (Agdia Incorporated, IN, USA) using the competitive antibody binding method according to the manufacturer's protocol. The samples were loaded along with alkaline phosphatase-labeled ABA-tracers (ABA competitor) into wells that were coated with anti-ABA monoclonal antibodies. The levels of yellow color presented after the paranitrophenolphosphate (PNP) substrate and alkaline phosphatase reaction were inversely proportional to the ABA dosages in the samples. The intensity of the yellow color was correlated to the ABA concentration using a standard curve. Three replicates of experiments were processed and the final data were presented as mean ± SD.

Results and discussion

Analysis of the cDNA of *OsMCSU*

A sequence search of the National Center for Biotechnology Information (NCBI) Nucleotide database revealed the presence of several genes encoding MOSC domain-containing proteins in the rice genome. However, only one *MCSU* gene (*OsMCSU*) was found. *MCSU* is also a single copy gene in both *Arabidopsis* and tomato (Xiong et al. 2001; Sagi et al. 2002). In this study, the cDNA fragment encoding the rice MCSU protein was amplified by RT-PCR (*OsMCSU* cDNA accession number: DQ855409), and this amplified cDNA encoded a protein of 824 amino acids. The predicted amino acid sequence of OsMCSU was 58.9 and 53% identical with that of the *Arabidopsis* and tomato MCSU proteins, the products of the *ABA3* and *FLACCA* genes, respectively, and OsMCSU, ABA3, and FLACCA all share the same functional motifs (Fig. 1). The OsMCSU protein is composed of two major domains (Fig. 2). The N-terminal domain shows homology to bacterial Nifs proteins that could function as pyridoxal phosphate (PLP)-dependent sulfurtransferases for the desulfuration of cysteine to alanine. In addition to the PLP-binding domain, the N-terminal region also contains a conserved cysteine motif (Fig. 2). The C-terminus of OsMCSU contains a MOSC domain, and the N-terminal portion of this domain (MOSC_N) forms a beta barrel structure (Fig. 2). The function of the MOSC domain is still unclear, but is present in the C-terminal region of all MCSU proteins that have been identified thus far in humans, animals, and plants (Sagi et al. 2002). A previous research suggested that the MOSC domain could function to recognize the target molecular (Bittner et al. 2001).

Expression of *OsMCSU* is regulated by salt stress and ABA in roots of rice seedlings

Roots are usually the primary plant organs that suffer in response to high salt problems in soil. To determine the effect of high salt concentrations on *OsMCSU* expression in seedling roots, three-leaf-stage seedlings were treated with various concentrations of NaCl. Although the effect of 50 mM NaCl on *OsMCSU* gene expression was not significant, the *OsMCSU* mRNA levels were found to increase approximately fourfold and 3.7-fold in roots of seedlings treated with 100 and 200 mM NaCl, respectively (Fig. 3a). However, no significant difference in the transcript levels of *OsMCSU* in leaf tissues between unstressed and stressed seedlings (Fig. 3b). In contrast, the effect of NaCl on *Arabidopsis MCSU* gene (*ABA3*) expression in leaves was more significant than in roots when *Arabidopsis* seedlings were placed on filter paper saturated with NaCl (Barrero et al. 2006). These results implied that the effect of NaCl on *MCSU* gene expression could differ among various plant species. Putative *cis*-elements on *OsMCSU* and *AtABA3* promoter sequences have been characterized with the Database of Plant *Cis*-acting Regulatory DNA Elements (PLACE) (Higo et al. 1999), and the difference has been observed between the two promoters (data not shown). Therefore, the molecular mechanisms of *MCSU* gene transcription could be different between rice and *Arabidopsis*. However, we could not rule out the possibility that the various sensitivities of *MCSU* gene expressions in response to NaCl between rice and *Arabidopsis* tissues

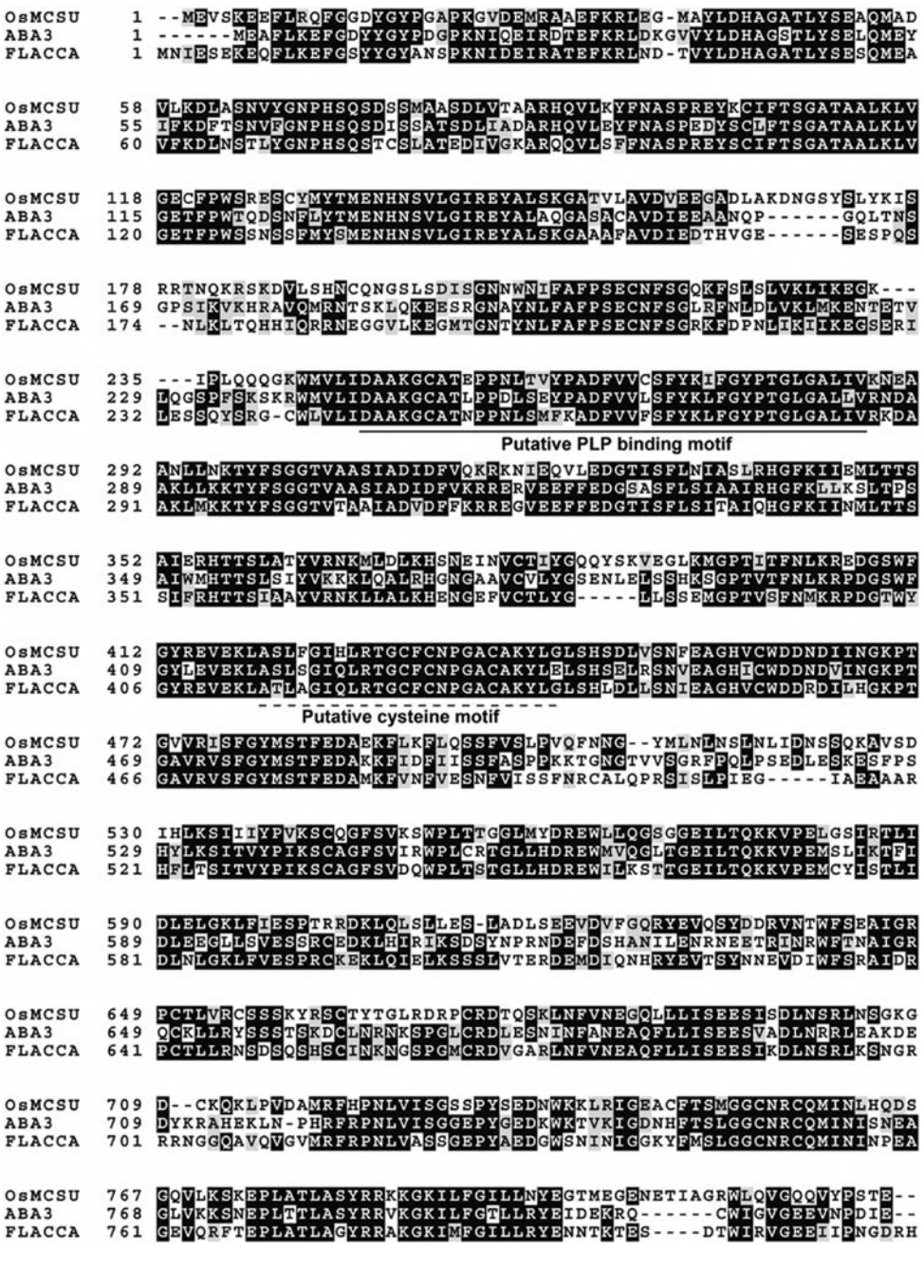

Fig. 1 Sequence alignment of rice *OsMCSU* with the *Arabidopsis* and tomato homologs. The amino acids depicted in *black box* indicate fully identical residues; the residues *shaded in grey* indicate similarity. The putative PLP-binding and cysteine motifs are indicated with *solid and dash underline*, respectively. Sequence accession numbers for OsMCSU, ABA3 and FLACCA are DQ855409, AY034895 and AY074788, respectively

Fig. 2 Functional domains of the *OsMCSU* protein product. The *black box* on Nifs domain indicates the putative PLP-binding motif, and the *dotted box* indicates the putative cysteine motif

was due to the different NaCl-supplied processes within experiments.

Following, we observed the level of ABA in salt-stressed roots was increased as predicted (Fig. 4). On the other hand, although NaCl-induced *OsMCSU* expression was not observed in leaves of 24-h NaCl-treated seedlings (Fig. 3b), we previously observed an increase of ABA in leaf tissues of salt-stressed seedlings (Chen et al. 2007).

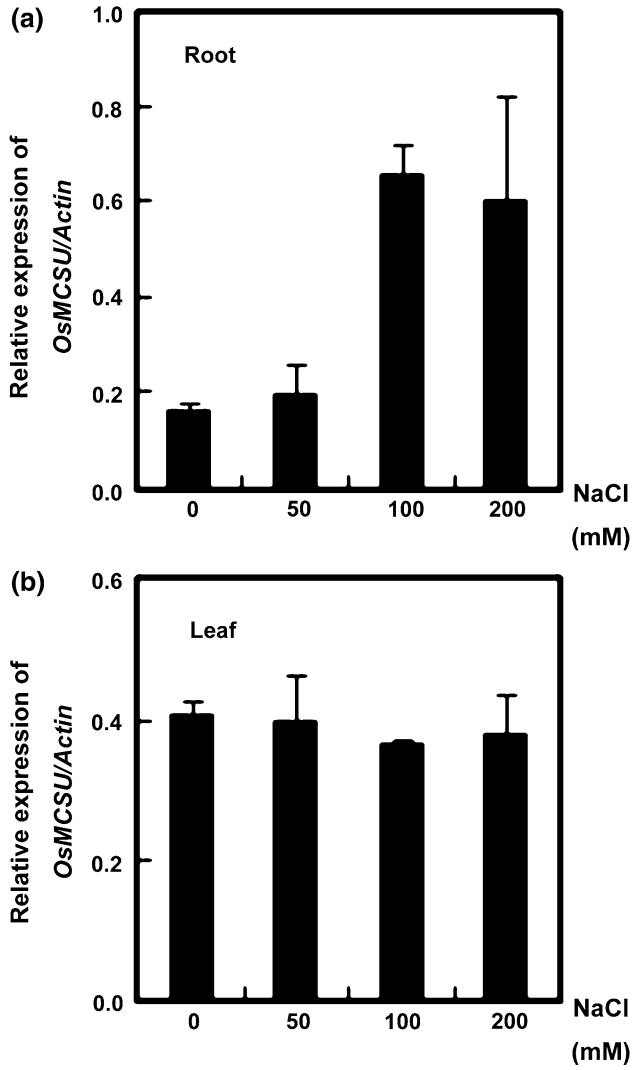

Fig. 3 Expression of *OsMCSU* in salt-stressed rice seedlings. Transcript levels of *OsMCSU* were measured in roots (**a**) and leaves (**b**) of 3-leaf-stage rice seedlings treated with 50, 100, or 200 mM NaCl. Standard deviation is indicated with *vertical bars*

Since *OsMCSU* is a single copy gene, the tissue-specific regulation of *OsMCSU* in response to NaCl in roots compared to leaves of rice seedlings implied that the regulatory mechanisms of NaCl-induced ABA biosynthesis in these tissues are distinct. Even there were many evidences to indicate that ABA could be synthesized in leaves under stress conditions for controlling turgor of guard cells (Pierce and Raschke 1980), it has also been reported that stomatal closure was due to sense the ABA transported from roots when the soil was dried but the leaf water level was remained (Blackman and Davies 1985). Thus, whether the ABA increased in leaves of salt-stressed rice seedlings in our works were transported from root tissues was needed to be further examined in the future. Besides, since the *OsMCSU* transcript level was not changed in salt-stressed seedling leaves, the possibilities caused ABA increase could be (1) changes of *OsMCSU* gene expression in response to NaCl were earlier than 24 h; (2) post-transcriptional regulation of *OsMCSU* could be an important factor to control ABA biosynthesis in leaf tissues.

As shown in Fig. 5, the levels of *OsMCSU* were enhanced by exogenous ABA (100 μM) in roots, but this response was not detectable in leaves. ABA is considered as a stress hormone and a signal molecule involved in regulations of several environmental stresses-controlled gene expressions (Chandler and Robertson 1994). To determine the signal transduction of NaCl-induced *OsMCSU* expression in roots was mediated by ABA-dependent or ABA-independent pathway, the inhibitor of ABA biosynthesis, fluridone, was applied with NaCl in seedling culture solution. As the data showed in Fig. 4, exogenous fluridone could repress ABA biosynthesis that was induced by 200 mM NaCl. However, the level of *OsMCSU* expression in root tissues of NaCl/fluridone co-treated rice seedlings was still similar to that was detected in NaCl only-treated samples (Fig. 6). Therefore, we propose that the ABA-independent signal transduction could be a regulatory pathway for NaCl-induced *OsMCSU* expressions in rice root tissues. Previous studies indicated that the expressions of several ABA biosynthesis-related genes such as *NCED3*, *AAO3,* and *ABA1* were enhanced by NaCl in ABA-deficient mutants (Xiong et al. 2002; Barrero et al. 2006). Together these results support the current concept that the expression of many abiotic stress-responsive genes are regulated by both ABA-dependent and ABA-independent mechanisms, and it also implies that the positive feedback regulation is an important factor in

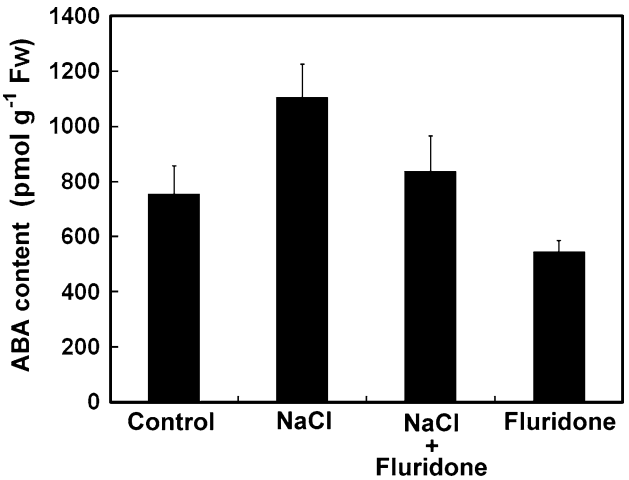

Fig. 4 Changes in ABA levels in root tissues under salt-stress conditions. ABA content in roots was analyzed after 3-leaf-stage rice seedlings were treated with 200 mM NaCl, 200 mM NaCl plus 200 μM fluridone, or 200 μM fluridone only

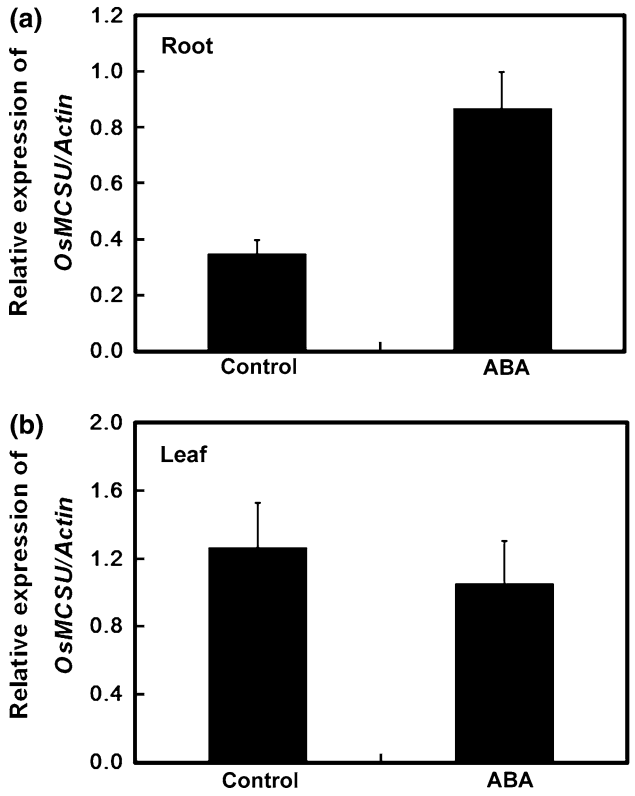

Fig. 5 Expression of *OsMCSU* in ABA-treated rice seedlings. Transcript levels of *OsMCSU* were determined in roots (**a**) and leaves (**b**) of 3-leaf-stage rice seedlings treated with 100 μM ABA

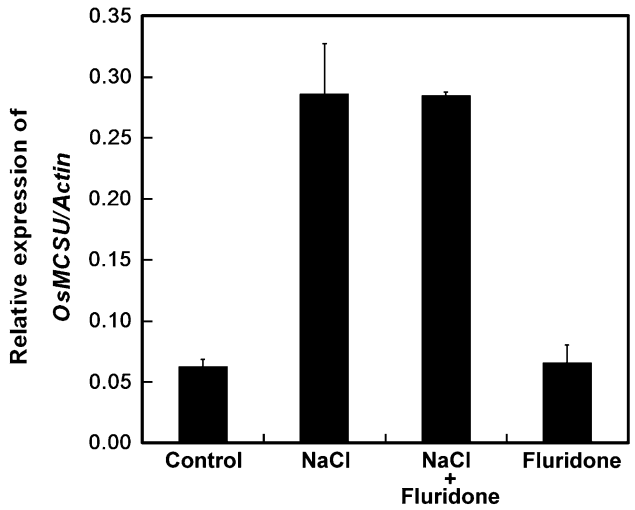

Fig. 6 ABA effect on the NaCl-induced *OsMCSU* expression in roots of rice seedlings. Transcript levels of *OsMCSU* were detected in roots of 3-leaf-stage rice seedlings treated with 200 mM NaCl, 200 mM NaCl plus 200 μM fluridone, or 200 μM fluridone only

controlling ABA biosynthesis under unfavorable conditions (reviewed by Shinozaki et al. 2003).

In summary, our work showed that the rice *OsMCSU* gene was significantly up-regulated by NaCl in roots of seedlings, but was not responsive to salt stress in leaf tissues. The differential salt-stress response of *OsMCSU* between roots and leaves is in contrast to the response observed in dicotyledonous plant species such as *Arabidopsis*. Furthermore, the induction of *OsMCSU* expression in response to NaCl could be mediated by both ABA-independent pathway and ABA-mediated regulatory mechanism in roots of rice seedlings.

Acknowledgments We thank Dr. Chih-Wen Wu for providing rice seeds, and Tzu-Hsuan Kuo for assistance with ABA measurements. This work was financially supported by a research grant from the Council of Agriculture, Executive Yuan, Taiwan.

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