Australian Journal of Crop Science AJCS
AJCS 7(3):368-373 (2013) ISSN:1835-2707

OsMSR2, a novel rice calmodulin-like gene, confers enhanced salt tolerance in rice (*Oryza sativa* L.)

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Abstract

OsMSR2 is a novel calmodulin-like gene in rice. Previous study has been demonstrated that *OsMSR2* was a cold, drought and heat-inducible gene. However, the role of *OsMSR2* in rice stress response is still unclear. To reveal the function of *OsMSR2* involved in stress response, the expression pattern and effects of overexpression of *OsMSR2* on salt stress were analyzed in rice. Quantitative real-time RT-PCR analysis showed that *OsMSR2* was rapidly induced by salt stress. Histochemical *GUS* staining assay revealed that *OsMSR2* was mainly expressed in root, leaf, seedling, lamina joint, base of stem and spikelet. Transgenic rice plants with overexpression of *OsMSR2* showed more tolerant to salt stress, with 18.2% survival rate for the wild type and 51.3% for transgenic plants at the end of the salt treatment. In *OsMSR2*-overexpressing transgenic plants, expression levels of some stress-related genes were also altered compared to wild type plants under salt condition. The more accumulated proline and soluble sugars and decreased electrolyte leakage were also observed in transgenic rice compared to wild type plants under salt stress. These results indicate that *OsMSR2* plays important roles in salt stress tolerance in rice, and is useful in developing transgenic crops with enhanced tolerance to salt stress.

Keywords: Stress; Transgenic rice; Gene expression; Ca^{2+} sensors; Calmodulin-like gene.
Abbreviations: CaM- Calmodulin; CBL- Calcineurin B-like protein; CDPK- Ca^{2+}-dependent protein kinase; CML- Calmodulin-like gene; GUS- β-glucuronidase; DREB- Dehydration-responsive element binding transcription factors; P5CS- Pyrroline-5-carboxylate syntheses; WT- Wild type.

Introduction

Throughout their life cycle, plants often confront abiotic stresses such as high salt, drought and cold. These stresses can lead to dramatic changes in plant growth, development, and productivity. In order to survive in these adverse conditions, plants initiate adaptive mechanisms at multiple levels, including molecular, physiological, developmental and morphological characteristics. During these responses and adaptations, many salt stress-related genes are induced (Garg et al., 2002; Sakamoto et al., 2004). High salinity may cause delayed germination, slow seedling growth, and reduced rate of seed set, leading to decreased rice yield. These disorders are generally due to the combined effects of ion imbalance, hyperosmotic stress and oxidative damage. Ca^{2+} is a crucial second messenger and plays a major role in many aspects of plant growth and development. In addition to its function as an essential nutrient and a structure element, Ca^{2+} also plays an important role in response to abiotic and biotic stimuli, including light, high and low temperature, salt and drought, osmotic stress, plant hormones and fungal elicitors (Sanders et al., 1999). These stimuli induce a distinct spatio-temporal pattern of changes in cytosolic-free Ca^{2+} concentration. The transient elevation of Ca^{2+} concentration is sensed by several Ca^{2+} sensors, which usually contain the EF-hand motif, a helix-loop-helix structure. In addition to several well-characterized Ca^{2+} sensors including of calmodulin (CaM), calcineurin B-like protein (CBL) and Ca^{2+}-dependent protein kinase (CDPK), plants also possess a large family of unique CaM-like proteins (CMLs). CMLs are defined by the presence of two to six predicted EF-hand motifs, by the absence of any other identified domains, and by at least 15% amino acid identity with typical calmodulin (McCormack and Braam, 2003). CMLs extend the range of the potential calcium sensors present in plant cells and exhibit significant structural divergence from the typical CaM. Recent data indicated that CaM and CML proteins differ in their Ca^{2+} affinity and target-binding activities (Popescu et al., 2007). At the genome scale, Arabidopsis and rice harbor 50 and 32 CMLs genes, respectively (McCormack and Braam, 2003; Boonburapong and Buaboocha, 2007). Up to now, only several CML genes have been demonstrated to be involved in plant response to external stimulus. *CML37*, *CML38* and *CML39* transcripts were regulated by biotic and abiotic stress as well as hormone and chemical treatment (Vanderbeld and Snedden, 2007). *CML9* altered plant response to abiotic stress and regulated seed germination by abscisic acid (Magnan et al., 2008), and loss function of *CML42* resulted in aberrant trichomes with increased branching (Dobney et al., 2009). *CML24* was shown to play a role in photoperiod-response, ion-homeostasis, and ABA-mediated inhibition of germination and seedling growth (Delk et al., 2005). Expression of soybean CML genes (*SCaM4*, *SCaM5*) in tobacco enhanced resistance to a broad spectrum of virulent and avirulent pathogens (Heo et al., 1999). In previous

study, transgenic Arabidopsis plants with expression of *OsMSR2* improved the tolerance to drought and salt stresses (Xu et al., 2011). To further explore the mechanism of *OsMSR2* involved in rice stress tolerance, we overexpressed *OsMSR2* in rice 93-11 (*Oryza sativa* L. ssp. *Indica* cv 93-11). In the present study, we demonstrated that the expression of *OsMSR2* was rapidly induced by salt, and transgenic rice shows enhanced tolerance to high-salinity accompanied by altered expression of stress-related genes, suggesting a potential function of *OsMSR2* in rice response to environmental stimuli.

Results and discussion

OsMSR2 was a salt-inducible gene in rice

Microarray data revealed that *OsMSR2* was a cold, drought and heat-inducible gene, and expression of *OsMSR2* conferred enhanced tolerance to drought and salt in Arabidopsis (Xu et al., 2011). To investigate whether *OsMSR2* was a salt-inducible gene in rice, qRT-PCR analysis was performed to monitor the expression levels of *OsMSR2* under high salt stress. The result showed that the transcript of *OsMSR2* rapidly reached the maximum level 1 h after the salt treatment and maintained the moderate level up to 12 h (Fig 1). Strong and rapid induction of *OsMSR2* expression by salt suggests that this gene might be involved in salt tolerance. In response to stimuli, the cytosolic Ca^{2+} concentration in plants is rapidly elevated via an increased Ca^{2+} influx, and then quickly returns to the basal level by Ca^{2+} efflux (Evans et al., 2001). To transducer Ca^{2+} signal to downstream signaling responses, Ca^{2+} sensors such as *OsMSR2* should be expressed to perceive cytosolic Ca^{2+} changes. A larger number of studies have demonstrated that Ca^{2+} sensors are induced by various stresses and play roles in stress tolerance. *CBL1* was induced by cold, drought and wounding at both mRNA and protein levels and overexpression of *CBL1* improved stress tolerance in transgenic Arabidopsis (Kudla et al., 1999; Cheong et al., 2003). Expression of *MCaM-3* could be increased significantly under cold, drought and salt stress (Fang et al., 2011). Previous study showed that *OsMSR2* was a Ca^{2+}-binding protein, and was localized in the cytoplasm (Xu et al., 2011). The expression pattern of *OsMSR2* was consistent with the model of Ca^{2+} changing in cytoplasm under salt stress. Taken together, *OsMSR2* was a salt-inducible gene and may act as a Ca^{2+} sensor to sense the changing concentrations of Ca^{2+} in cytoplasm.

Overexpression of OsMSR2 increased tolerance to salt stress in rice

As shown in Fig 1, the expression of *OsMSR2* was rapidly induced by salt stress. To elucidate the biological function of the *OsMSR2* gene in rice, transgenic rice plants overexpressing *OsMSR2* were generated. Semi quantitative RT-PCR was performed to determine the expression levels of *OsMSR2* in transgenic rice lines (Fig 2), and two independent transgenic lines, L-13 and L-27, were chosen for further study based on their higher expression levels among different transgenic lines. The obtained data showed that seeds of transgenic plants displayed higher germination rate than wild type ones on the ½ MS medium supplemented with 150 mM NaCl (Fig 3a). Transgenic seedlings also showed improved tolerance to high salt stress at post-germination stage, displaying longer roots, longer shoots and heavier fresh weights (Fig 3b, c, d). The sensitivity of wild type and transgenic plants to high salt was also evaluated by measuring plant survival rates after salt treatment. After recovery from the salt treatment, transgenic plants showed greater salt tolerance than the wild type (Fig 3e).

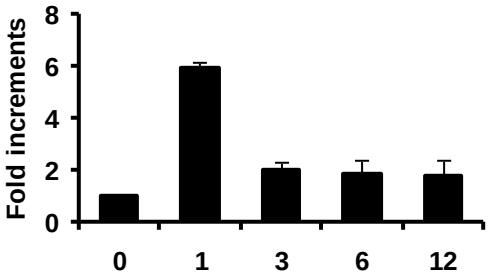

Fig 1. Fold increments of *OsMSR2* under salt stress. Four-week-old 93-11 seedlings were treated with 200 mM NaCl, plant materials were harvested at different time points (0, 1, 3, 6, 12 h), and qRT-PCR was performed. Error bar represents SD for three independent experiments.

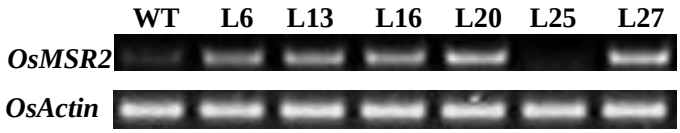

Fig 2. Semi-quantitative RT-PCR analysis of *OsMSR2* expression levels in transgenic lines and wild type plants. WT, wild type; L-6, L-13, L-16, L-20, L-25 and L-27, transgenic lines 6, 13, 16, 20, 25 and 27.

At the end of salt treatment, the survival rate was only 18.2% for the wild type and an average rate of 51.3% for transgenic plants (Fig 3f).

Changes of proline and soluble sugar contents and electrolyte leakage

It is known that proline and soluble sugar contents and the electrolyte leakage are indicator of salt response. Changes of these parameters in wild type and transgenic plants were examined under normal and salt conditions. Under normal conditions, proline and soluble sugar contents were comparable among wild type and transgenic plants. Although the proline contents were increased both in wild type and transgenic plants under salt-stress conditions, the proline contents detected in transgenic plants were higher than that in the wild type plant (Fig 4a, b). A similar situation was observed for the soluble sugar content (Fig 4b). The electrolyte leakage was significantly lower in transgenic plants than that in the wild type under NaCl treatment (Fig 4c). In response to abiotic stress, many plants can accumulate compatible osmolytes, such as proline and soluble sugars, to protect their subcellular structures from damage by adjusting the intracellular osmotic potential (Garg et al., 2002; Armengaud et al., 2004). The obtained result indicated that *OsMSR2* may function as a salt tolerance gene by regulating the accumulation of proline and soluble sugar and the electrolyte leakage in rice under salt stress.

OsMSR2-overexpressing altered expressions of stress-related genes

To elucidate the molecular mechanism of *OsMSR2* action during the salt stress, qRT-PCR analysis was performed to monitor the expression levels of several stress-related genes in transgenic lines and wild type plants, including *OsP5CS2* encoding pyrroline-5-carboxylate syntheses (Hur et al., 2004), *OsDREB2A* encoding a DREB-type transcription factor

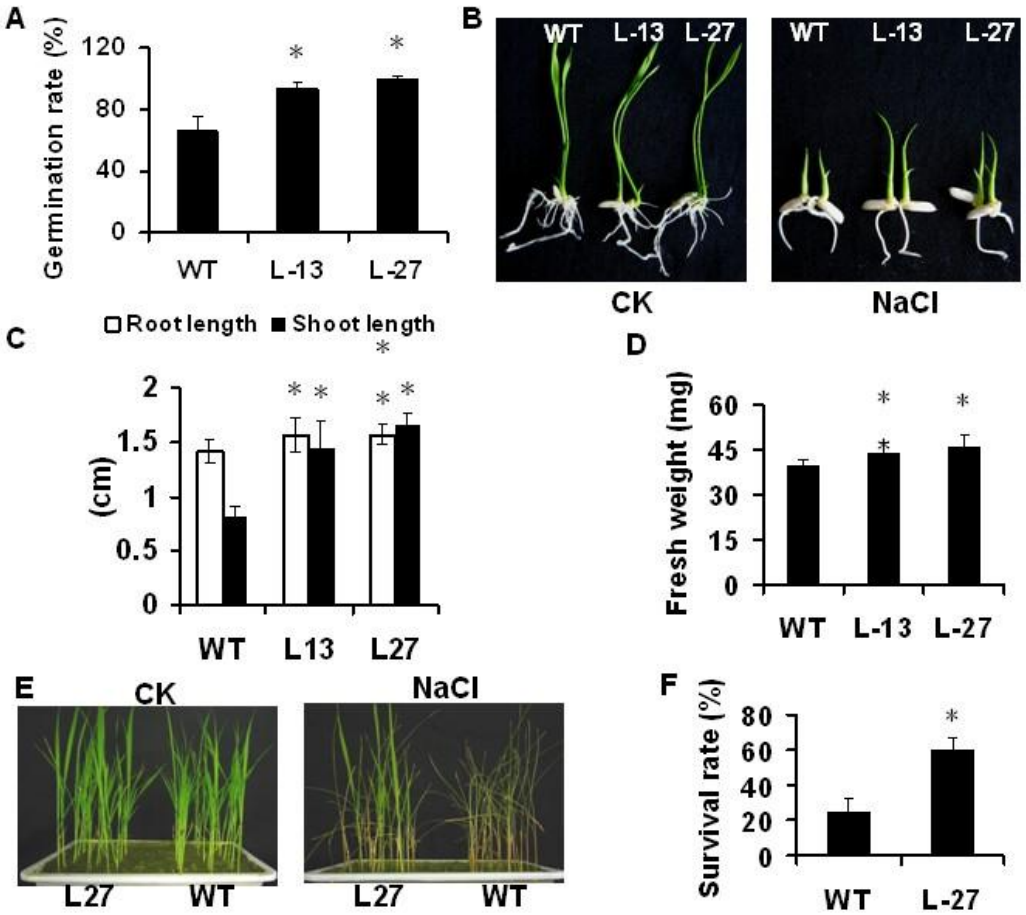

Fig 3. *OsMSR2*-overexpressing transgenic rice showed enhanced tolerance to salt stress. A Germination rates of wild type and transgenic lines under 150 mM NaCl condition. B Transgenic rice showed more salt tolerance at post-germination stage. C, D Root lengths, shoot lengths and fresh weights of rice seedlings from experiment B. E Transgenic rice survived better than wild type plants under high salt condition. Plants were exposed to 200 mM NaCl for 10 days and recovered for another week. F Survival rates of wild type and transgenic plants after salt treatment. Error bars represent SD for three independent replicates. Asterisks indicate statistically significant differences, * $P < 0.05$, ** $P < 0.01$ (by Student's *t* test). WT, wild type; L-13, L-27, transgenic lines 13 and 27; CK, under normal condition; NaCl, under 150 mM NaCl condition.

(Mallikarjuna et al., 2011) and *OsCatA* encoding a catalase protein (Iwamoto et al., 2000). The results showed that the expression levels of *P5CS2* and *DREB2A* were increased more in transgenic plants than those in wild type ones under salt condition (Fig 5), while the abundance of *OsCatA* was comparable between the wild type and transgenic plants (Fig S2). Previous studies showed that *DREB2A* and *P5CS* played positive roles in plant stress tolerance. Gene *AtDREB2A* has been shown to regulate some stress-response genes in plants (Liu et al., 1998). Expression of *OsDREB2A* enhanced the salt and drought tolerance in transgenic rice plants (Mallikarjuna et al., 2011). Gene *P5CS* has been isolated from various plant species. In rice, two alleles of *P5CS* were identified (Hien et al., 2003). The *OsP5CS1* gene was induced by salt, dehydration, cold and ABA (Igarashi et al., 1997), whereas *OsP5CS2* is also induced by NaCl and mannitol (Hien et al., 2003). Hur et al. (2004) revealed that *OsP5CS2* was essential for salt and cold tolerance in rice. In this study, expression levels of *OsDREB2A* and *OsP5CS2* were increased more in transgenic plants overexpressing *OsMSR2* than those in the wild type, indicating that *OsMSR2* may play a role in the enhanced salt tolerance of transgenic plants.

OsMSR2 was expressed in different tissues of rice

To determine the expression pattern of *OsMSR2* in rice, about 1.5-kb promoter region of gene *OsMSR2* was cloned and used to drive expression of the β-glucuronidase (*GUS*) reporter gene in the expression vector pCMBIA1301. The construct was used to transform Pei'ai 64S, and tissues of transgenic plants were subjected to a histochemical *GUS* staining assay. As shown in Fig 6, the *GUS* gene driven by the *OsMSR2* promoter was expressed in root, stem base, leaf blade, seedling, lamina joint and spikelet base. The obtained results suggested that *OsMSR2* was expressed widely in many different tissues, mostly in rapidly dividing and elongation tissues and organs.

Materials and methods

Plant materials and growth conditions

Seeds of 93-11 and Pei'ai 64S (*Oryza sativa* L. ssp. *Indica* cv Pei'ai 64S) were used in this study. All plants were grown under white fluorescent light (600 μmol m^{-2} s^{-1}, 12 h d^{-1} photoperiod) at 28 °C and 75 % relative humidity in a greenhouse.

RNA extraction and quantitative real-time RT-PCR analysis

Trizol reagent (Invitrogen, Carlsbad, CA, USA) was used for the RNA extraction, and RNA samples were further treated

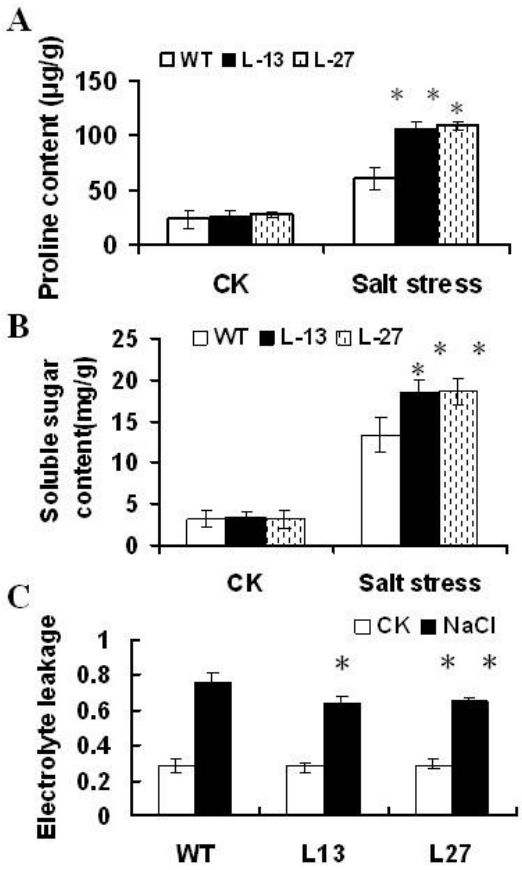

Fig 4. The contents of proline and soluble sugars and electrolyte leakage in wild type and transgenic rice plants under salt stress. Three-week-old seedlings of wild type and transgenic rice plants were treated with 100 mM NaCl for 4 days, and contents of proline (A) and soluble sugars (B) and electrolyte leakage (C) were measured. Asterisks indicated statistically significant differences, * $P < 0.05$, ** $P < 0.01$ (by Student's *t* test). CK, under normal condition; Salt stress, under 100 mM NaCl condition; WT, wild type; L-13, L-27, transgenic lines 13 and 27.

with DNase (Promega, Madison, WI, USA) to eliminate DNA contamination. qRT-PCR analysis was carried out using one-step QuantiTect SYBR Green RT-PCR Kit (Qiagen, Shanghai, China). The gene for *18S* rRNA was used as the internal control. qRT-PCR reactions were performed according to the manufacturer's instructions. The reaction was performed in an ABI 7900HT (Applied Biosystems, Foster City, CA, USA) at 48 °C for 30 min and then 95 °C for 10 min followed by 40 cycles at 95 °C for 15 s and 58 °C for 40 s. The data was analyzed using the comparative *Ct* method. The gene specific primers used in qRT-PCR are shown in Supplementary Table 1 (Table S1).

Construction of the plant expression vector and generation of transgenic plants

For generating construct used for rice transformation, the DNA fragment with the whole open reading frame of *OsMSR2* was cloned into the binary vector pCosAc, generating pCosAc-OsMSR2, as described previously (Fig S1; Xu et al., 2011). The construct was introduced into *Agrobacterium tumefaciens* stain EHA105 and then transformed into 93-11. Expression level of *OsMSR2* in transgenic lines was

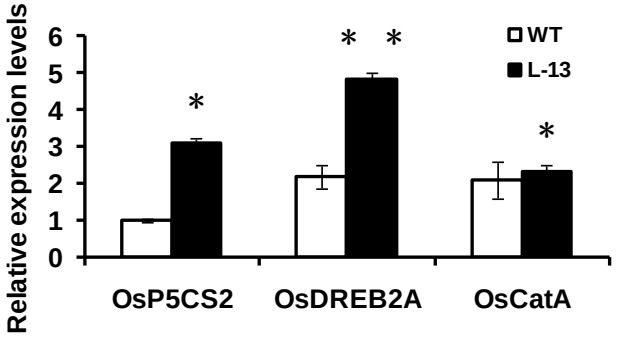

Fig 5. Relative expression levels of salt-related genes in response to salt treatments. Three-week-old wild type and transgenic seedlings were treated with 200 mM NaCl, for 5 h. Total RNAs were extracted from wild type (WT) and transgenic line 13 (L-13), and qRT-PCR analyses were performed. Asterisks indicated statistically significant differences, * $P < 0.05$, ** $P < 0.01$ (by Student's *t* test).

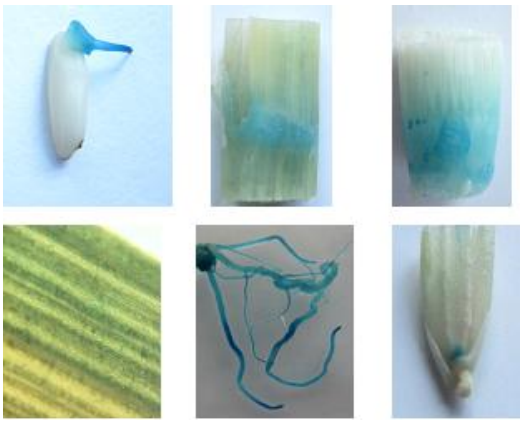

Fig 6. Expression pattern of *OsMSR2* in rice from *GUS* staining assay. Pictures in the upper panels show seedling, lamina joint and stem base, from left to right. Lower panels show leaf blade (left), root (middle) and spikelet base (right).

determined by semi-quantitative RT-PCR, The gene for *OsActin* was used as the internal control. The *OsMSR2* promoter (1,556-bp DNA fragment upstream of the translation start site) was cloned into the expression vector pCAMBIA1301. The construct was introduced into *Agrobacterium tumefaciens* stain EHA105 and then delivered into Pei'ai 64S. Histochemical *GUS* staining assay was performed as described below. Transgenic plant samples were incubated with GUS staining solution (50 mM $NaPO_4$ buffer pH 7.2, 0.2 % Triton X-100, 2 mM potassium ferrocyanide and potassium ferricyanide, 2 mM X-Gluc) overnight at 37 °C. To remove chlorophyll, the X-Gluc staining buffer was replaced with different concentrations of ethanol.

Stress treatments

For qRT-PCR analysis of the expression level of *OsMSR2* under salt stress, four-week-old 93-11 seedlings were treated with 200 mM NaCl, and plant materials were harvested at regular intervals. For the germination assays under salt condition, seeds form wild type and transgenic plants were sown on ½ MS medium supplemented with 150 mM NaCl, and germination rate was recorded at the third day. After germination, seedlings were kept growing, and root and shoot

lengths and fresh weights were measured at the sixth day. For salt stress, the seedlings at the three-leaf stage grown in soil in the greenhouse were water with 200 mM NaCl solution for 10 days. Survival rate was estimated after a recovery period of a week. For qRT-PCR analysis of known salt-related genes, 3-week-old rice was treated with water (control) and 200 mM NaCl. After 5 h treatment, plant materials were harvested and qRT-PCR analysis was performed as described above.

Measurement of proline, soluble sugar contents and electrolyte leakage

Three-week-old seedlings of wild type and transgenic rice plants were treated with 100 mM NaCl for 4 days, and the contents of proline and soluble sugars were determined by sulphosalicylic acid method (Troll and Lindsley, 1955) and the anthrone method (Morris, 1948), respectively. Electrolyte leakage was measured as described by Cao et al. (2007). Percentage electrolyte leakage was calculated as the ratio of the conductivity before autoclaving to that after autoclaving.

Statistical analysis

All of the data from three replicates of experiments were subjected to Student's *t*-test analysis by using SPSS version 13.0 (SPSS, Chicago, USA).

Conclusions

OsMSR2 was a salt-inducible gene, it was expressed mostly in rapidly dividing or elongation tissues and organs. Transgenic plants overexpressing *OsMSR2* showed enhanced salt tolerance, displaying longer roots and shoots, heavier fresh weights and higher survival rates. Proline and soluble sugar contents were increased and electrolyte leakage was decreased in transgenic plants under salt conditions, compared with wild type plants. Overexpression of *OsMSR2* also altered the expression levels of some stress-related genes. Taken together, the enhanced salt tolerance in transgenic rice may be resulted from the increased accumulation of osmoregulation substances and altered expression of stress-related genes.

Acknowledgements

This research was supported by National Natural Science Foundation of China (31171536).

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