Overexpression of *BrTSR53* Gene Improves Tolerance of Rice Plant to Salt Stress

A-Ram Kim[†], Hyemin Lim[†], Jung-Il Cho, Chang-Kug Kim, Seung Uk Ji, Soo-Chul Park, Gang-Seob Lee*

Department of Agricultural Biotechnology, National Academy of Agricultural Science, Jeonju, 54874, Korea

ABSTRACT Plant is frequently exposed to various abiotic stress. Salt stress is particularly an important abiotic stress that seriously affects plant growth and development. *BrTSR53* gene, a putative stress-related gene isolated from *Brassica rapa*, was used to generate overexpression transgenic rice. The over-expression of *BrTSR53* in *BrTSR53*-OX transgenic rice was confirmed by quantitative RT-PCR and western blot analysis. To elucidate the role of *BrTSR53* in stress tolerance, responses of *BrTSR53*-OX transgenic rice plants to salt stress conditions were examined. *BrTSR53*-OX #12, #28, and #32 lines were treated with salt stress on MS medium containing 100 mM or 200 mM of NaCl for 5 and 14 days. Morphological analysis revealed differences between the three transgenic *BrTSR53*-OX rice and the wild-type rice. The germination rates of the three transgenic *BrTSR53*-OX lines of rice were significantly higher than that of the wild type rice, indicating that they were more tolerant to 200 mM NaCl than the wild type rice. In addition, the three transgenic *BrTSR53*-OX rice lines had significantly longer length of root and shoot compared to the wild type rice. These results suggest that the *BrTSR53* gene played an important role in the tolerance of rice to salt stress. Therefore, it might be a potential target for the purpose of improving salt tolerance of rice and other crops.

Keywords *Brassica rapa*, *BrTSR53* gene, Salt stress, Transgenic rice

INTRODUCTION

Abiotic stress in plants is likely to be increased due to climate changes. To increase crop cultivation, it is important and necessary to develop stress resistant crops (Kathuria *et al.* 2007). Salt stress is particularly an important factor that limits the growth and productivity of crops. Recently, there has been an increase in cases of crops suffering salt damage, accounting for approximately 20% of global agricultural production (Zhu 2001). Saline soil can reduce crop productivity by increasing the concentration of Na⁺ and Cl⁻ ions in leaves. These ions also can impair cell growth and photosynthesis. They ultimately act as cytotoxins, promote leaf aging, and interfere with carbon fixation and the formation of reproductive organs (Zhu 2001).

Plants possess numerous mechanisms to adapt to physiological, morphological, and biomolecular changes

caused by stress (Yu *et al.* 2013). Genes induced by stress generate important functional proteins, which in turn can regulate genes involved in cell protection and signal transduction during stress response (Nakashima *et al.* 2007). To study abiotic stress-responsive mechanism in *Brassica* species, a number of *Brassica rapa* samples have been used to analyze the transcriptome of abiotic stress-treated *B. rapa* using oligo microarray (Lee *et al.* 2008). Transcription factors are important regulators of gene expression and abiotic-stress responses. It has been demonstrated that plant stress resistance can be improved by inducing the expression of stress-related target genes (Ravikumar *et al.* 2014). Over-expression of genes in transgenic plants has been shown to be able to enhance tolerance of plants to various abiotic stresses (Dubouzet *et al.* 2003; Nakasima *et al.* 2007; Xiang *et al.* 2008).

Rice is one of the most important food crops in the world. It is very sensitive to salinity and water stress. The growth

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*Corresponding author Gang-Seob Lee, kangslee@korea.kr, Tel: +82-63-238-4714, Fax: +82-63-238-4704

[†]These authors contributed equally to this work.

and productivity of rice are frequently affected by various environmental factors. Such as, although it is well known that rice is very sensitive to salt stress, genes that could improve the salt tolerance of rice remain unclear. Therefore, we report the isolation of a putative stress-related gene *BrTSR53* induced under salt stress condition from *Brassica rapa*. The responses of *35S::BrTSR53* over-expression transgenic rice plant to salt stress condition were examined.

MATERIALS AND METHODS

Plant materials and growth conditions

Rice (*Oryza sativa* L. cv. Dongjin) was used in this study. Rice seeds were sterilized in 70% ethanol and 2% NaClO. Sterilized seeds were allowed to germinate at 28°C in a growth chamber. Two-week-old seedlings were transferred to soil culture in a green house with a dark/light cycle of 8/16 h at 28°C. All tissue samples collected were immediately frozen in liquid nitrogen and stored at -70°C.

Construction of *35S::BrTSR53* expression vector

The full-length cDNA of *BrTSR53* (BRAS0001S00024153) gene from *Brassica rapa* was amplified by RT-PCR. Myc-tag was added to primer site of the coding region. The following primers were used: *BrTSR53*-OX F 5'-TCTAGA ATGGAACAAAACTCATCTCAGAAGAAGATCTT ATGACTTCATGGAAGAAAAC-3' containing *Xba*I and myc-tag, and *BrTSR53*-OX R 5'-GGATCC TTAAGAA-GACAGATTGTTTG-3' containing *Bam*HI. *BrTSR53*-OX under the control of CaMV 35S promoter and pinII terminator in pPZP vector was used in PCR to generate expression vector *35S::BrTSR53*.

Generation of transgenic rice plants

To generate transgenic rice plants, *Agrobacterium tumefaciens* LBA4404 was transformed with *35S::BrTSR53* vector using freeze-thaw method. Rice transformation was performed using method described by Hiei *et al.* (1994) and Toki *et al.* (2006). Transgenic rice plants were regenerated from transformed calli using selection medium containing 4 mg/L phosphinothricin and 250 mg/L cefotaxime.

Genomic DNA extraction and TaqMan copy number analysis

Genomic DNAs of transgenic plants were extracted using Inclone™ genomic DNA prep kit (Inclone, Korea). *Bar* gene selection of transgenic plants was performed using PCR with the following primers: *Bar*-F 5'-ATG-AGCCCAGAACGACGCCCCG-3', *Bar*-R 5'-GATCTC-GGTGACGGGCAGGA-3'. PCR reaction conditions included an initial denaturation at 95°C for 5 min, followed by 35 cycles of denaturation for 30 sec at 95°C, annealing for 30 sec at 56°C, extension for 1 min at 72°C, and 7 min extension at 72°C.

Copy number of the transgene is an important standard to determine the utility of transgenic plants. Multi copies of transgene can result in co-suppression and gene silencing (Lowe *et al.* 2009). Copy number assay was performed on Applied Biosystems StepOnePlus™ (Applied Biosystems, Foster City, CA) with TaqMan® Gene Expression Master Mix (Applied Biosystems) kit. As an endogenous control, primers and probes of pre-designed TaqMan® copy number assay for rice *tubulin alpha-1 chain* gene (AK102560) were used. For the transgene, primers and probes were specifically designed for the terminator of *nos* gene. Primers used for the terminator of *nos* gene were: NOS-F (5'-GCATGACGTTATTTATGAGATGGGTTT-3'), NOS-R (5'-TGCGCGCTATATTTTGTCTTCTATCG-3'), and NOS-probe (5'-TAGAGTCCCGCAATTAT-3'). PCR amplifications were performed with the following parameters: enzyme activation at 95°C for 10 min, followed by 40 cycles of denaturation for 15 sec at 95°C, annealing and extension for 1 min at 60°C. To calculate the copy number of gene, relative quantitation analysis of genomic DNA targets was analyzed based on real-time PCR data using Applied Biosystems CopyCaller® Software v2.0 (Applied Biosystems) according to the manufacturer's instructions.

RNA extraction and quantitative real-time PCR

Total RNA was extracted using RNeasy® Plant mini kit (QIAGEN, Germany) according to the manufacturer's protocol. cDNA synthesis was performed using *amfiRivert Platinum* cDNA synthesis master mix (GenDEPOT,

Barker, TX). Quantitative real-time PCR (qRT-PCR) was performed on an Applied Biosystems StepOnePlus™ (Applied Biosystems) with SYBR® Premix Ex Taq™ (TaKaRa, Japan) kit. *BrTSR53* gene expression in transgenic plants was determined by qRT-PCR with the following primers: *BrTSR53*-F 5'-GGTGACGTCATGGCTTGTGGCT-3', *BrTSR53*-R 5'-ACACGTGAAAAACGAG-AGGACAACA-3'. *OsActin1* gene was used as internal control. *OsActin1*-F 5'-ACAGGTATTGTGTTG-GACTCTGG-3' and *OsActin1*-R 5'-AGTAACCACGCTCCGTCAGG-3' were used for qRT-PCR with the following cycling parameters: 95°C for 30 sec followed by 40 cycles at 95°C for 5 sec and 60°C for 30 sec. Relative expression level was calculated using the comparative CT method (Livak and Schmittgen *et al.* 2001).

Protein extraction and immunoblot analysis

Total protein was extracted using method of Martinez-Garcia *et al.* (1999). Protein expression was determined through western blot analysis. Total protein was separated by SDS-PAGE (stacking gel at 5% and running gel at 12%) and transferred to membranes. Anti-myc tag (Upstate, Billerica, MA) and anti-mouse IgG (Sigma, St. Louis, Missouri) were used as primary and secondary antibody, respectively. After washing membranes three times, immuno-reactive proteins were detected using Amersham™ ECL™ Prime Western Blotting Detection Reagent solution (Amersham Life Sciences, UK).

Salt stress treatment to transgenic rice

Salt stress treatment to transgenic rice was performed using method of Duan *et al.* (2012). Seeds of transgenic rice were sterilized in 70% ethanol and 2% NaClO. Sterilized rice seeds were transferred into 1/2 Murashige and Skoog (MS) liquid medium, 1/2 MS liquid medium containing 100 mM NaCl, or 1/2 MS liquid medium containing 200 mM NaCl. Seeds were incubated at 28°C for 5 days or 14 days. Shoot length, root length, and seed germination rate were recorded. All data from experiment of three times were analyzed by *t*-test analysis. Significantly different are indicate at $p < 0.05$ (*) and $p < 0.01$ (**).

RESULTS

Production and analysis of *BrTSR53*-OX rice plants

The *BrTSR53* gene (putative Triple Stress Resistance53, BRAS0001S00024153) is categorized as an unknown gene in NCBI database. It has been reported that *BrTSR53* is phylogenetically close to halophyte *Eutrema salsugineum* showing salt resistance in yeast cell (Kim *et al.* 2014). *BrTSR53* gene isolated from *Brassica rapa* cDNA using RT-PCR was cloned into a pPZP vector, which included the CaMV 35S promoter, PinII terminator, and *bar* genes (Fig. 1A). A total of 32 T0 generation lines of *BrTSR53*-OX rice plants were generated. After extracting genomic DNAs from these 32 lines, PCR analysis was used to search for plants that possessed the *bar* gene (Fig. 1B). TaqMan copy number assay was used to select single-copy lines (Fig. 1C). *BrTSR53*-OX rice plant lines with T-DNA inserted in the intergenic region were selected by flanking sequence analysis. Eight lines of *BrTSR53*-OX rice plants with single-copy and an intergenic insertion were selected and separated to produce homozygous T2 generation. These single-copy/intergenic/homozygous *BrTSR53*-OX rice plants were used in subsequent experiments.

Expression of the *BrTSR53* gene in transgenic rice plants

The transcriptional levels of the *BrTSR53* gene in the eight lines of single-copy/intergenic/homozygous *BrTSR53*-OX rice plants were determined using qRT-PCR. Compared to *BrTSR53*-OX #32, *BrTSR53*-OX #17 showed higher level of expression. *BrTSR53*-OX #10 showed a relatively low level of expression compared to other transgenic rice lines. However, it was 6-fold higher compared to *BrTSR53*-OX #32 (Fig. 2A). In addition, the protein expression levels of *BrTSR53* were determined by western blot analysis. *BrTSR53* protein was detected in *BrTSR53*-OX lines at molecular weight of 10 to 15 kDa. However, this protein band was not detected in the wild-type rice plant. *BrTSR53*-OX #12, #28, and #32 lines showed the highest expression of *BrTSR53* protein (Fig. 2B). Therefore, these three lines were used in salt stress experiment.

Fig. 1. *BrTSR53*-OX vector and molecular identification of transgenic rice. (A) Vector construction of *35S::BrTSR53-myc*. RB: right border; LB: left border; CaMV 35S: cauliflower mosaic virus 35S promoter; TPInII: PinII terminator; TNOS: NOS terminator; *Bar*: selection marker. (B) DNA analysis by PCR. M: 1 kb DNA ladder; P.C: positive control; N.C: negative control. (C) Copy number assay by quantitative RT-PCR.

Fig. 2. Expression level of *BrTSR53* gene or protein in *BrTSR53*-OX transgenic rice plants. (A) Expression of the *BrTSR53* gene in transgenic rice by quantitative real time-PCR. *OsActin* was used as internal control. (B) *BrTSR53* protein levels in transgenic rice plants based on western blot analysis.

***BrTSR53*-OX transgenic rice plants have enhanced tolerance to salt stress**

To examine the role of *BrTSR53* in stress response of rice, seeds of *BrTSR53*-OX, *BrTSR53*-OX #12, #28, #32, and wild-type rice plants were sown on MS agar plates containing different concentrations (100 and 200 mM) of NaCl. Their germination rates and seedling growth were monitored. There was no significant ($p > 0.05$) difference between the wild-type rice plant and *BrTSR53*-OX rice

plant cultured in MS medium without NaCl (Fig. 3). However, when they were cultured in MS medium containing 100 mM NaCl for 5 days, the *BrTSR53*-OX #28 and #32 rice lines showed different phenotype from that of the wild-type. Treatment with 200 mM NaCl inhibited the germination of wild-type rice plant. However, the germination of the three *BrTSR53*-OX lines of rice plants were not inhibited by the 200 mM NaCl. With the treatment of 200 mM NaCl, the germination of both *BrTSR53*-OX and wild-type seeds was inhibited the most. After 14 days of

Fig. 3. Growth phenotype of *BrTSR53*-OX and wild-type rice plants under salt stress conditions. (A) Root and height of rice in MS medium containing 0 mM, 100 mM, or 200 mM NaCl for 5 days. Bar: 1cm. (B) Root and height of rice in MS medium containing 0 mM, 100 mM, or 200 mM NaCl for 14 days. Bar: 1cm.

culture in MS medium containing 100 mM NaCl, the germination rates of the wild-type and the *BrTSR53*-OX lines of rice seeds were 90% and 100%, respectively. In MS medium containing 200 mM NaCl, the germination rate of the wild-type rice seed was 30%, while that of the *BrTSR53*-OX #12, #28, and #32 was 70%, 60%, and 90%, respectively (Fig. 4A). These results indicated that these lines were resistant to salt stress.

After 5 days of culture in MS medium, the shoot lengths of *BrTSR53*-OX lines plants were significantly ($p < 0.05$) longer than that of the wild-type (Fig. 4B). In MS medium containing 100 mM NaCl, the shoot lengths of both *BrTSR53*-OX #28 and #32 were significantly ($p < 0.01$) longer than that of the wild-type. After 14 days of culturing in the MS medium, the shoot lengths and the root lengths of the three *BrTSR53*-OX lines were significantly ($p < 0.05$)

longer than that of the wild-type. In MS media containing 200 mM NaCl, the shoot and root lengths of *BrTSR53*-OX #32 were significantly ($p < 0.05$) longer than that of the wild-type (Fig. 4C, D).

DISCUSSION

Plants have developed mechanisms to allow them rapidly respond to stresses in the environment. Expression of stress-related genes is primarily controlled by specific transcription factors such as *MYB*, *bZIP*, *WRKY*, *AP2/EREBP*, *LEA*, and *NAC* (Zhong *et al.* 2012). According to Duan *et al.* (2012), the functions of LEA proteins include maintaining protein, sequestering ions, and functioning as membrane structure or molecular chaperones to help prevent

Fig. 4. Response of *BrTSR53*-OX and wild-type rice to salt stress. (A) Germination rate of *BrTSR53*-OX transgenic rice after culturing for 14 days. (B) Shoot length of *BrTSR53*-OX after culturing for 5 days. (C) Shoot length of *BrTSR53*-OX after culturing for 14 days. (D) Root length of *BrTSR53*-OX after culturing for 14 days. Error bars represents SD of three replicates. Significant differences depending on *t*-test are indicated by asterisks. * $p < 0.05$; ** $p < 0.01$.

the formation of protein aggregates. Over-expressed *LEA* rice has been generated under various abiotic stresses and its phenotype after being subjected to salt stress for 5 days has been analyzed. In MS media containing 100 mM or 200 mM NaCl, transgenic lines of *LEA* rice plants have been found to be more resistant to salt stress than the wild-type rice (Duan *et al.* 2012). Similar trends were observed for *BrTSR53*-OX rice plants in this study.

NAC proteins are involved in the regulation of a number of plant growth processes, such as embryo growth, lateral root formation and growth, flower growth, leaf senescence, and secondary wall thickenings (Hu *et al.* 2008, Zheng *et al.* 2009 and Song *et al.* 2011). Over-expressing NAC transcription factor gene can enhance the tolerance of rice to salt stress compared to the wild-type in rice (Hu *et al.* 2008). These studies have demonstrated that transgenic rice with overexpressed stress-related transcription factor genes are more resistant to salt stress, similar to the findings of this study.

In summary, *BrTSR53*-OX rice plants were found to be more tolerant to salt stress. Whether *BrTSR53*-OX confers resistance to other abiotic stresses such as drought and cold stresses merits future study.

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