

Expression analysis and characterization of rice oligopeptide transport gene (*OsOPT10*) that contributes to salt stress tolerance

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Abstract Knock-out of a gene by insertional mutagenesis is a direct way to address its function through the mutant phenotype. Among ca. 15,000 gene-trapped *Ds* insertion lines of rice, we identified one line from selected sensitive lines in highly salt stress. We conducted gene tagging by TAIL-PCR, and DNA gel blot analysis from salt sensitive mutant. A gene encoding an oligopeptide transporter (OPT family) homologue was disrupted by the insertion of a *Ds* transposon into the *OsOPT10* gene that was located shot arm of chromosome 8. The *OsOPT10* gene (NP_001062118.) has 6 exons and encodes a protein (752 aa) containing the OPT family domain. RT-PCR analysis showed that the expression of *OsOPT10* gene was rapidly and strongly induced by stresses such as high-salinity (250 mM), osmotic, drought, 100 μ M ABA. The subcellular localization assay indicated that *OsOPT10* was localized specifically in the plasma membrane. Overexpression of *OsOPT10* in *Arabidopsis thaliana* and rice conferred tolerance of transgenic plants to salt stress. Further we found expression levels of some stress related genes were inhibited in *OsOPT10* transgenic plants. These results suggested that *OsOPT10* might play crucial but differential roles in plant responses to various abiotic stresses

Keywords *Arabidopsis*, *Ds* insertion mutant, GFP, OPT family, rice, salt stress, transgenic plant

Introduction

Rice (*Oryza sativa* L.), one of the most important crops, is an ideal model species for the study of crop genes due to its commercial value, relatively small genome size (ca.430 Mb), diploid origin ($2x = 24$) and close relationship to other important cereal crops. Insertional mutagenesis based on T-DNA and maize Activator/Dissociator (*Ac/Ds*) transposons is most suitable for a systematic functional analysis of multifamily plant genes. In the previously studies, we used the Activator/Dissociation (*Ac/Ds*) two-component transposon system (Han 2002) in rice to prepare a large collection of gene-trapped *Ds* insertion lines of rice carrying a single *Ds* insertion (Chin et al. 1999; Kim et al. 2002, 2004). To assessing the potential of reverse genetics to identify insertional mutants in salt stress, we screened salt adaptation mutants from *Ds*-tagged rice lines. One of the most efficient ways to achieve the goal of producing more rice is to develop different varieties that are high seed yielding, biotic and abiotic stress resistant by modern breeding technology.

One of the abiotic stress factor, high salinity influence plant growth and development, and are major limit factor on crop productivity (Teige et al. 2004; Bray 2004; Yun 2005). Salt stress, mostly caused by excess of NaCl, elicits two primary effects on plants such as osmotic stress and ionic toxicity due to high levels of Na^+ and/or Cl^- ions in the cytoplasm (Greenway & Munns 1980; Zhu 2001). One of the early responses of plant cells to many stresses, including salt stress, is the generation of a cytosolic Ca^{2+} increase. In plant cells, Ca^{2+} serves as a second messenger during stress signaling and it activated the signal delivery system against the stress of

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the cell (Sanders, Brownlee & Harper, 1999). It was in control of influx of K^+ ions and relationship of K^+/Na^+ which participate to salt stress adaptation of the cell (Maeda et al. 1994; Yun 2005). However, at high NaCl condition, Na^+ competition at binding sites for K^+ may result in K^+ deficiency (Maathuis & Amtmann 1999).

Knockout of a gene by insertional mutagenesis is a direct way to address its function through the mutant phenotype. Among ca. 15,000 gene-trapped *Ds* insertion lines of rice (data not shown), we identified one line from selected sensitive lines in highly salt stress that were closely linked to insertion sites of the *Ds* element, and termed salt sensitive mutant which has a disrupted oligopeptide transporters (*OsOPT10*) gene. Peptide transport is characterized by the ability of cells to transport peptides or peptide derivatives across membranes in an energy-dependent manner (Lubkowitz et al. 1997; Stacey et al. 2006). Peptide transporters can be classified into three groups, namely the ATP-binding cassette transporters (ABC family), the peptide transporters (PTR family) and the oligopeptide transporters (OPT family) (Stacey et al. 2002, 2006). The OPT family was first characterized in the pathogenic yeast *Candida Albicans* and likely use the proton gradient to energize transport (Lubkowitz et al. 1997; Koh et al. 2002; Stacey et al. 2002, 2006). Database searches for homology with the *C. albicans* CaOpt1p revealed nine putative *Arabidopsis* OPT orthologs that form a distinct subfamily compared with the fungal OPTs. Both of these motifs (i.e. NPG motif, NPG[P/A]F[N/T/S]XKEH[V/T/A][L/I/V][I/V][I/T/S/V][I/V/M][F/M][A/S][N/S/A] and KIPPR motif, K[L/F][G/A][H/M/T]YMK[I/V/L][P/D/S] PR) were found in regions of the protein predicted to be hydrophilic. Members of the PTR family transport dipeptides and tripeptides as well as amino acids and nitrate (reviewed by Williams & Miller 2001; Stacey et al. 2002a). PTR proteins have been identified in yeast (Perry et al. 1994), human (Liang et al. 1995), rabbit (Fei et al. 1994, 2000), mouse (Ganapathy et al. 1995), barley (West et al. 1998), and *Arabidopsis* (Frommer et al. 1994; Steiner et al. 1994).

In contrast to PTR peptide transporters, the yeast OPT transporters predominantly recognize tetra- and pentapeptides (Lubkowitz et al. 1997; Lubkowitz et al. 1998), including the endogenous opioids Met-enkephalin (YG GFM) and Leu-enkephalin (YGGFL) (Hauser et al. 2000). Database searches for homology with the *C. albicans* CaOpt1p revealed nine putative *Arabidopsis* OPT orthologs that form a distinct subfamily compared with the fungal OPTs (Koh et al. 2002; Stacey et al.

2002). Full-length cDNAs for seven *Arabidopsis* OPT transporters (*AtOPT1* to *AtOPT7*) were cloned and tested for their ability to take up various peptides when expressed in yeast (Koh et al. 2002). More recently, OsGT1 from *Oryza sativa* (Zhang et al. 2004), and AtOPT6 from *Arabidopsis* (Cagnac et al. 2004) have been reported to enable the uptake of glutathione (GSH and GSSG) and glutathione derivatives (GS conjugates) when expressed in yeast. The OPT family of transporters are larger and more diverse than was previously recognized. However, no OPT transporter has yet been identified in animals (Stacey et al. 2006). Until now in plants, unique expression patterns were also found for selected AtOPTs such as expression in pollen tubes, ovules, endosperm, integument of developing seeds and tissue-specific expression pattern (Stacey et al. 2006; Koh et al. 2002). However, the exact role of peptide transport in plants is still undefined. In this study, we carried out physiological analysis on the selected salt sensitive and expression pattern *OsOPT10* gene. Also, we will find expression pattern and gene function of *OsOPT10* in transgenic *Arabidopsis* and rice.

Materials and methods

Plant growth and stress treatment

We used japonica rice cultivar (cv.) Dongjin from Activator/Dissociation (*Ac/Ds*) two-component transposon system (Han 2002) in rice to prepare a large collection of gene-trapped *Ds* insertion lines of rice carrying a single *Ds* insertion (Kim et al. 2002, 2004; Chin et al. 1999). Rice seeds were germinated for 20 days under normal conditions at 28 °C and 14 h light / 10 h darkness in soil in a green house. For high salt stress treatment, 20-day-old (three or four-leaf-old) rice seedlings were grown in nutrient solution containing 250 mM NaCl, and imbibed for 14 days under a normal light/dark cycle at 28 °C. Plants were measured for fresh weight according to Xiang et al. (2007).

Isolation of *Ds*-flanking sequences by TAIL-PCR

Analysis of TAIL-PCR and DNA gel blot was carried out as described by Kim et al. (2004). The sequence of FSTs (flanking sequencing tags) was analyzed online at the following website: <http://www.ncbi.nlm.nih.gov> and

<http://www.gramene.org>. The insertion site of *Ds* element was confirmed by PCR analysis between *Ds* and *OsOPT10* gene region. The *Ac/Ds* PCR analysis and Southern blot were carried out as described by Kim et al. (2004). Total RNA was isolated with easy-spin plant RNA extraction kit (iNtRON Biotechnology, South Korea) according to the manufacturer's manual. The first-strand cDNA was generated by using SuperscriptTM III Reverse Transcriptase according to the manufacturer's instructions (Invitrogen, USA). Based on the flanking sequence of *OsOPT10* gene, the 2.25 kb full-length gene primer set Fw (5'-ATGGA GCAACCACGGCT TGA-3') and Rv (5'-TCACTGCA CCGGGCAGC-3') was designed and synthesized. The amplification program consisted of an initial step at 94°C for 2 min, 30 cycles (94°C for 15 s, 58°C 30 s, 72°C for 1 min), and a final step at 72°C 10 min. The PCR product was ligated into the pGEM-T easy vector (Promega, USA) and sequenced.

Phylogenetic analysis

Identification of nucleotide sequence was established by comparing with the GRAD Rice Genome Annotation Database (Sasaki et al. 2002, <http://rgp.dna.affrc.go.jp/RiceHMM/>). All of the OPT sequences were compared using MegAlign (Version 7.0, DNASTAR Inc.). This led to the identification of two conserved motifs found in all OPT proteins. The bioinformatics tools at the web site <http://www.expasy.org> were used to analyze the deduced protein. A phylogenetic tree was constructed using at the web site <http://www.phylogeny.fr/>.

The *Arabidopsis* orthologs were identified by comparison of the CaOpt1p sequence (Lubkowitz et al. 1997). For phylogenetic analysis, OPT family members in *Arabidopsis* OPT orthologs gene sequences were retrieved from GenBank database (Koh et al. 2002). Using DNASTAR program, overall amino acid sequences were aligned, and then the unrooted phylogenetic tree was generated. This tree was set up with the distance matrix using the neighbor-joining method. Poisson correction with the complete deletion of gaps was used to calculate protein distances. Bootstrap values are based on 100 iterations.

Gene expression analysis under the abiotic stress treatments

Seeds of rice (*Oryza sativa*) var. Dongjin were grown under controlled conditions having 28°C day / 25°C night temperatures, 14 h light / 10 h darkness in Yosida solution (Yosida et al. 1976), and 75% relative humidity. After 2 weeks of germination, seedlings were exposed to high-salinity (250 mM), osmotic (15% polyethylene glycol (PEG) 6000, 15% PEG6000 + 250 mM NaCl), drought, 100 μM ABA stresses. The plants were harvested after 0, 30', 1, 5, 10, and 24 h of stress treatment, frozen in liquid nitrogen, and stored at -80°C for further analysis (Rabbani et al. 2003). Total RNA from were extracted using Trizol reagent (Invitrogen, USA) according to the manufacturer's protocol. Real-time PCR was conducted using a Bio-RAD I Cycler IQ5 machine as previously described using RT pre-mix (TOYOBO Co., Japan). The threshold cycle (Ct) values of the triplicate PCRs were averaged and the relative quantification of the expression levels was performed using the comparative Ct method (Livak et al., 2001). The fold change in total RNA (target gene) relative to the reference gene (actin gene) was determined by the following formula: fold change = $2^{-\Delta\Delta Ct}$, where $\Delta\Delta Ct = (Ct_{\text{target gene}} - Ct_{\text{actin gene}})_{\text{transgenic plants}} - (Ct_{\text{target gene}} - Ct_{\text{actin gene}})_{\text{wild-type plants}}$. The following primers were used for the gene specific primers, i.e. *OsOPT10*: 5'-ACCGAAGAGGAG GTAGACGA-3' and 5'-GCCACCACATGTAAGGTG AG-3', *Actin2*: 5'-CGCCCCGCATCGTGCGGTTTC-3' and 5'-TCCGCGGGGT CCATGGTCTG-3'. RT-PCR analysis of vegetative tissue (leaf, stem, and root) was conducted using 50 ng of total RNA treated *DNase* I. The amplification program consisted of an initial step at 42°C for 60 min, 94°C for 5 min, 30 cycles (94°C for 1 min, 58°C for 1 min, 72°C for 1 min), and a final step at 72°C for 10 min and the amplified fragments were run on 1.2% agarose gel.

Subcellular localization analysis

The full open reading frame (ORF) of *OsOPT10* was amplified using the cDNA clone mentioned above as template. The PCR product was ligated into the pGWB5-GFP vector, resulting in an in-frame fusion protein of GFP

(green fluorescent protein) gene and the *OsOPT10* ORF. The construct (p35S:GFP-*OsOPT10*) was transformed into onion epidermal cells by particle bombardment using a Biolistic PDS-1000/He gene gun system (BIO-RAD). After 24 h incubation of transformed onion epidermal cells, GFP signal was detected by a confocal fluorescence microscope.

Transformation to *Arabidopsis* and rice

OsOPT10 gene isolated from rice (*Oryza sativa*) var. Dongjin was introduced into the pB2GW7,0 gateway vector for *Agrobacterium*-mediated plant transformation (Karimi et al. 2002). *Arabidopsis thaliana* (ecotype Columbia, Col-O) and rice (Japonica ‘Dongjin’) were transformed with *Agrobacterium* LBA4404. Stable transformation of *Arabidopsis* was transformed by the floral dip of simplified method as described by Clough and Bent (1998). Sixty-four independent transformants (T0) were screened on the media containing 3 mg/L phosphinotricine (PPT) and selfed. T1 transformants showing 3:1 segregation ratio were selected, made for T2 homozygous transformants, and analyzed. For the experiments of germination rate of transgenic *Arabidopsis* homozygote lines (TG-OPT 3), seeds of wild-type and transgenic plants were germinated on ½ Murashige and Skoog (MS) medium (Murashige & Skoog 1962) with or without supplemented NaCl for 2–3 weeks in a culture room maintained at 25°C under a 16 h light/8 h dark cycle. The construct included *OsOPT10* gene was transformed into *Oryza sativa* L. cv. Japonica as described by Toki (2006). Transgenic plants in rice were selected in MS medium containing 3 mg/L phosphinotricine (PPT) and analyzed transgenic rice (T2 generation). Genomic DNA was extracted from leaves with the CTAB (hexadecyltrimethyl ammonium bromide) method (Xu et al. 2000). For 35S::*OsOPT10* transgenic analysis, PCR primer set 35S Fw (5'-CGCACAATCCCA CTATCCTT-3'), *OsOPT10* Rv (5'-GATGAGCACATG CTCCTTGA-3') and bar Fw (5'-GTTCATCAGATTTT CCGTGACG -3'), bar Rv (5'-TT ATTGCGCGTTCAA AAGTC-3') were designed and synthesized. The amplification program consisted of an initial step at 94°C for 2 min, 30 cycles (94°C for 15 s, 53°C 30 s, 72°C for 1 min), and a final step at 72°C 10 min. Total RNA from 14-day-old seedlings was isolated with

easy-spin plant RNA extraction kit, respectively. RT-PCR analysis was performed as mentioned in the previous chapter.

Salt treatments to transgenic plants

*Arabidopsis 35S::*OsOPT10** transgenic T2 seeds germinated for 7 days in MS medium containing 3 mg/L phosphinotricine (PPT) were transferred to pots filled with a mixture of peat/vermiculite (1:1, v/v) under light at 23°C for 3 weeks. Three-week-old plants were exposed to 150 mM NaCl for 6 days and 6 days for recovery. Then, the selected plant (TG-T3) was measured for fresh weight compared with wild type (Fig. 6A and B). The four transgenic rice seeds (T4, T7, T9 and T13) were germinated on MS medium containing 6 mg/L PPT for 5 days and grown in MS medium for 3 days with wild type plants. Then at least 60 seedlings for each family were transferred to pots in a green house and grown for 6 days and used in the subsequent salt stress assay. They were exposed to 250 mM NaCl for 7 days and allowed to grow on water in a green house for recovery for 7 days (Fig. 8C). For performance of high salt tolerance of two transgenic plants (T4 and T9) and wild type plants after 250 mM NaCl treatment at 25°C for 3 weeks and recovery for 2 weeks (Fig. 8B).

Determination of chlorophyll fluorescence

The leaf discs of detached leaves of transgenic *OsOPT10* plants (2 weeks old) and WT plants were floated in 1/2 MS liquid medium containing 250 mM NaCl without sucrose (MSH) for 48 h under light at 23°C to examine the health and chlorophyll contents of various discs. Measurement of chlorophyll fluorescence was performed according to Aono et al. (1993). We repeated experiments on salt stress tolerance three times (Fig. 6C and D).

Results

Mutant selection under salt stress

Altogether, ~15,000 seedlings of T4 seeds from gene-trapped *Ds* insertion lines, in which *Ds* had a promoterless

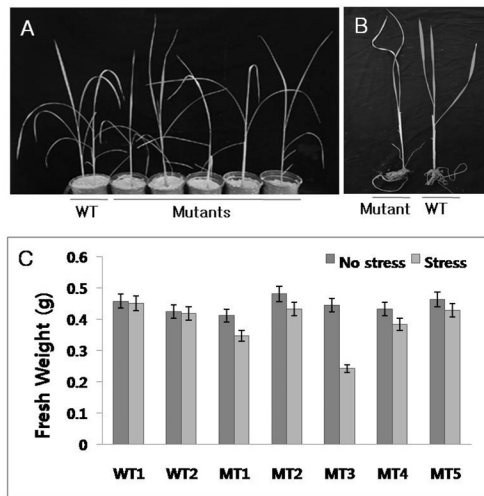


Fig. 1 Appearance of *Ac/Ds* transgenic plants and wild-type plants after high salt stress in rice. A and B, Growth performance of transgenic in soil containing 250 mM NaCl for 3 days and selection of salt-susceptible mutant rice. C, Fresh weight of mutant line and wild type growing in the 250 mM NaCl containing water for 3 days. The average values were calculated from measures of salt treatment at least 3 times

uidA gene encoding β -glucuronidase (GUS) to trap gene (Han, 2002), were screened the one of highly salt-stress sensitive mutants on 250 mM NaCl by using the NaCl responses of Wu et al. (1996). Southern blot analysis showed that the mutant contained single copy of *Ds* element (data not shown). Flanking sequences of the *Ds* element revealed that the *Ds* element was inserted into *OPT* gene by Blast analysis. Fig. 1 shows the phenotype of the salt sensitive mutants under the conditions of 250 mM NaCl stress. *Ds* insertion lines on salt-containing solution had significantly lower fresh weight per plant than the wild type (Fig. 1).

Cloning of *OsOPT10* gene

To identify the locus in which the *Ds* element insertion, DNA fragments adjacent to the 5' and 3' ends of the inserted *Ds* were amplified from salt sensitive mutant seedlings using the thermal asymmetric interlaced (TAIL) polymerase chain reaction (PCR) technique (Liu and Whittier 1995). To further characterize the *Ds* element insertion and fusion transcript from the mutant allele, the junction sequence between the first *OsOPT10* exon and β -glucuronidase (GUS)-encoding region was amplified by RT-PCR using mRNA from mutant leaves. Analysis of

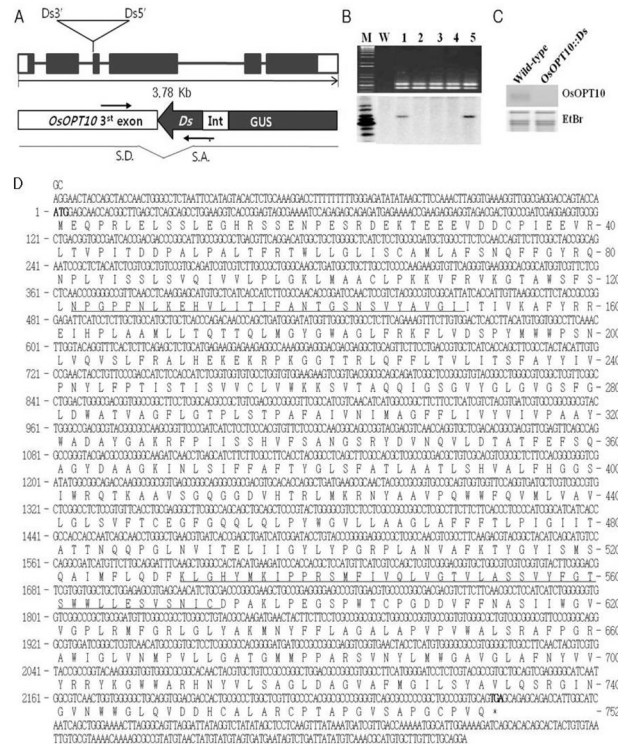


Fig. 2 Transcript analysis of *OsOPT10::Ds*. A, Genomic structure of *OsOPT10* showing the position of the trap *Ds* element. Exons are represented by boxes and introns by lines. The arrows correspond to inserted site. The putative splicing donor (S.D.) and acceptor sites (S.A.) are indicated by the bent arrow. B, Southern hybridization of *EcoRI*-digested DNA from *OsOPT10* plants was performed using probes recognizing a 1.2 kb GUS-encoding region and PCR analysis from *OsOPT10* genomic DNA. C, RT-PCR analysis of *OsOPT10* mRNA. Total RNA was extracted from the leaves of wild-type and *OsOPT10::Ds* plants. D, Nucleotide and deduced amino acid sequence of *OsOPT10*. The OPT family motifs in *OsOPT10* gene are underlined with bold lines

flanking sequences revealed that the *Ds* were inserted into near the end of the third exon of *OsOPT10*, which gene locus of chromosome 8 (Fig. 2A). The linkage between the phenotype of salt sensitive mutant plants and the *Ds* insertion was confirmed by DNA gel blot and PCR analysis using genomic DNA isolated from wild-type control, *Ds*-inserted normal plants, *Ds*-inserted salt sensitive mutant plants (Fig. 2B). *OsOPT10* expression in salt sensitive mutant plants was examined by RT-PCR analysis. Total RNA was extracted from the leaves of wild-type and *OsOPT10::Ds* plants. RT-PCR analysis indicated that the *OsOPT10* gene was not expressed in mutant plants. Therefore these results indicate that disruption of the *OsOPT10* gene causes a salt sensitive phenotype (Fig. 2C). Based on the sequence of flanking

region, we obtained the cDNA of *OsOPT10* contains a 2259-bp open reading frame encoding a protein of 752 amino acids with a calculated molecular mass of 82.8 kDa, tentatively designated as *OsOPT10* (GenBank accession no. NP_001062118.) (Fig. 2D).

Molecular characterization of *OsOPT10* protein

An alignment of the predicted amino acid sequence of *OsOPT10* with the complete sequence of the *C. albicans* OPT, CaOpt1p (Lubkowitz et al. 1997) was conducted using the DNASTAR program. This analysis led to the identification of nine possible *Arabidopsis* OPT orthologs and two possible *O. sativa* OPT paralogs (Fig. 3A and C) that exhibited 49% to 53% sequence similarity to CaOpt1p. The OPTs of *Arabidopsis* and rice formed a distinct subgroup when compared with the yeast OPT members and showed 61% to 85% sequence similarity when compared with each other (Fig. 3A). The size of proteins predicted for each of the AtOPTs were similar

in size to CaOpt1p (783 amino acids) and Opt1p (799 amino acids) according to Koh et al. 2002. Interestingly, sequence comparisons revealed two domains that were strongly conserved among all of the nine OPT family members in *Arabidopsis* and the two OPT family members in rice (Vasconcelos et al. 2008). Both of these motifs (i.e. NPG motif and KIPPR motif) were found in regions of the protein predicted to be hydrophilic (Fig. 3B). As reported previously by Lubkowitz et al. (1998), none of the OPT family members showed any significant sequence similarity to the known ABC or PTR peptide transporters (data not shown).

Expression of *OsOPT10* gene with abiotic stresses

The results from RT-PCR analysis showed that the *OsOPT10* gene was detected in various organs including vegetative tissue (leaf, stem, and root), respectively, of rice of 2 weeks old. The gene expression was much higher in one of the leaves than other tissues (Fig. 4A).

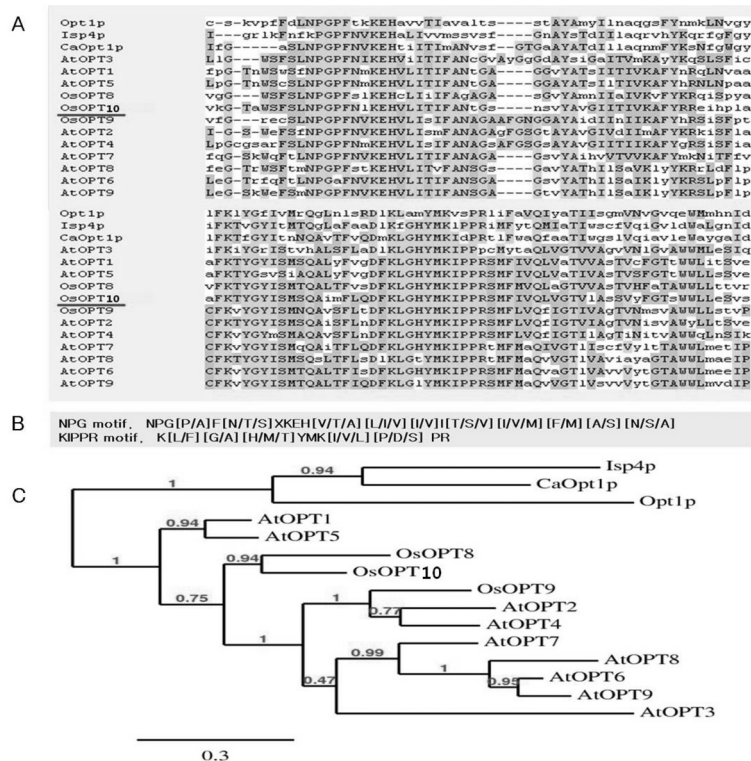


Fig. 3 Sequence alignment of NPG and KIPPR motifs from different proteins. A, Multiple sequence alignment of the known members of the OPT family. Accession numbers are as follows: AtOPT1, AB026659 GI:9758213; AtOPT2, AAB60748 GI:2160185; AtOPT3, Z97341 GI:2244994; AtOPT4, AB008268 GI:9759417; AtOPT5, AL078465 GI:4938497; AtOPT6, AL035602.1 GI:4469024; AtOPT7, AF080119 GI:3600039; AtOPT8, BAB09728.1 GI:9759191; AtOPT9, AB015476 GI:9759190; OsOPT8, AP005859; OsOPT9, AK100814; Opt1p, Z49487; CaOpt1p, U60973 and Isp4p, P40900. B, The residues in motif forming the OPT family protein sites. C, Phylogenetic relationship between *OsOPT10* protein and other plant OPT-domain proteins

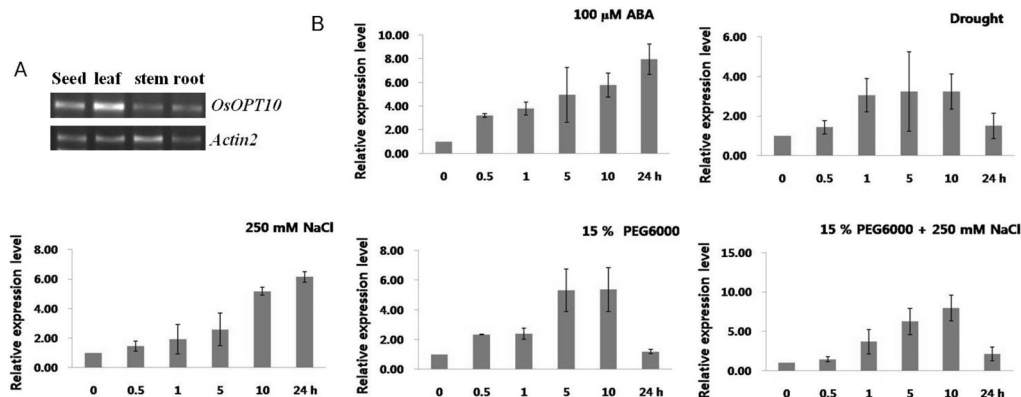


Fig. 4 A, Expression profiles of *OsOPT10* in organs collected from rice. The *actin2* gene was expressed as a positive control and was detected in all organs B, Real-time PCR analysis of *OsOPT10* gene in response to 250 mM NaCl, 15% PEG6000, osmotic (PEG+NaCl), drought and 100 μ M ABA for the indicated amount of time in leaves of 14 days old seedlings, respectively. Data presented are means and standard deviations of 3 independent replicated experiments

Under conditions of salt stress (250 mM NaCl), the expression levels peaked at 24 h and these levels increased to a very high value within 30 min and decreased after 24 h were exposed to 15% PEG6000 and 15% PEG6000 + 250 mM NaCl conditions. In the case of 100 μ M ABA treatment, the *OsOPT10* gene was induced strongly within 30 min and continued to be expressed until 24 h after treatment. However, under drought stress, induction of *OsOPT10* expression was relatively low, though the levels increased to 30 min until 10 h (Fig. 4B). Therefore, it can be concluded that *OsOPT10* gene expression may lead to the tolerance of transgenic plants to salt and osmotic stress.

OsOPT10 gene was localized in the plasma membrane

We analyzed the subcellular localization of *OsOPT10* by *Agrobacterium*-mediated transient expression analysis of a *OsOPT10*-GFP fusion gene in onion epidermal cells. As shown in Figure 5A, cells transformed with *OsOPT10*-GFP displayed fluorescence specifically in the plasma membrane, suggesting that *OsOPT10* is a membrane-localized protein.

Increases of salt stress tolerance in transgenic plants

To study the physiological functions of *OsOPT10*, we generated transgenic *Arabidopsis* and rice plants that overexpressing *OsOPT10* by fusing its full-length cDNA sequence to the CaMV 35S promoter in the pB2GW 7.0 gateway vector (data not shown). The positive trans-

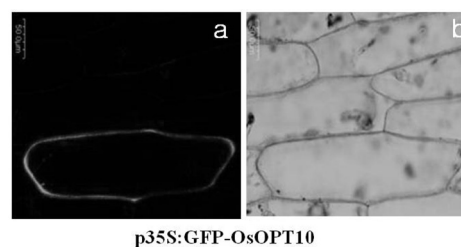


Fig. 5 Plasma membrane localization of *OsOPT10* gene. p35S:GFP-*OsOPT10* was transiently expressed in onion epidermal cells. The photograph was taken in the dark field for green fluorescence (a), under bright light for the morphology of the cell (b)

genic plants were confirmed by genomic PCR, Southern blot, and RT-PCR (data not shown). We measured the fresh weight of transgenic *Arabidopsis* plants used for salt tolerance after 6 days of recovery and transgenic lines that showed no apparent signs of chlorosis (Fig. 6). Figs. 6A and B show absolute variation in the experiment such as the stressed transgenic seedlings decreased 0.17 ~0.21 g/plant of fresh weight, whereas WT plants decreased 1.22~1.31 g/plant of fresh weight as compared to unstressed seedling. When the leaf discs of WT and transgenic plants were floated in 250 mM NaCl solution for 48 h, the discs of the transgenic leaves were fresher than those of WT leaves, which agreed with the measurement of their chlorophyll content (Fig. 6C, D). Salt stress tolerance revealed that the percentage of germination of WT plants was lower than the transgenic plants over 2-3 weeks period under the treatment of 50 mM and 150 mM NaCl (Fig. 7A, B). Also, the percentage of green seedlings between WT and transgenic plants showed a

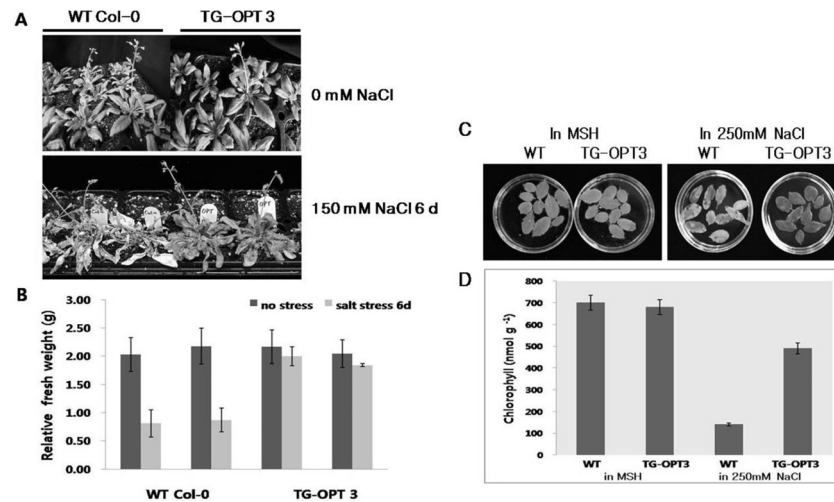


Fig. 6 Phenotype of the 35S::*OsOPT10* transgenic plants and wild-type plant exposed to high-salt stress in *Arabidopsis*. A, Growth performance of transgenic in soil containing 150 mM NaCl for 6 days and 6 days for recovery and selection of salt-resistance mutant in *Arabidopsis*. B, Fresh weight of mutant line and wild type growing in the 150 mM NaCl containing water for 6 days. The average values were calculated from measures of salt treatment at least 3 times. C, For high-salt treatment, leaves from 3-week-old T2 transgenic plants and controls were cut and floated in $\frac{1}{2}$ MS medium containing 250 mM NaCl without sucrose (MSH) for 48 h under light at 23°C. B and C, Phenotype differences were observed and photos were taken at 48 h and chlorophyll contents (nmol g⁻¹ fresh weight) were measured at 48 h for leaves in 250 mM NaCl

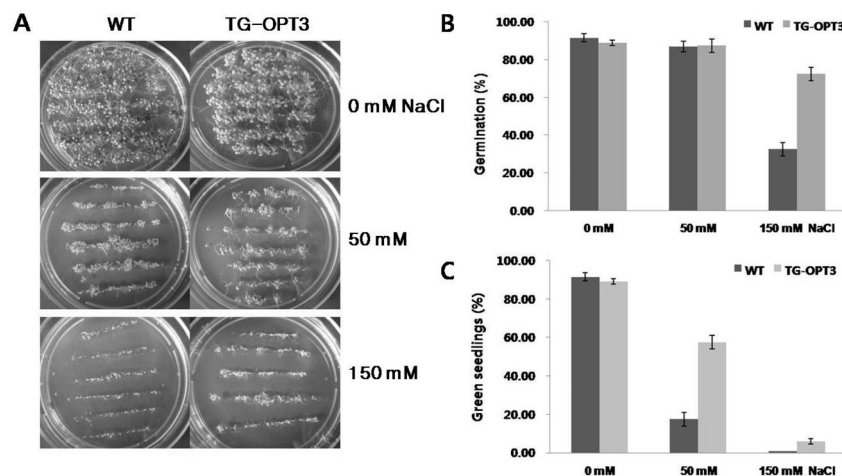


Fig. 7 Effect of salt stress on *Arabidopsis* seedlings from wild type (WT) and *OsOPT10* transgenic homozygote lines (TG-OPT 3). A, Seeds were germinated and grown for 2–3 weeks on agar plates containing $\frac{1}{2}$ MS medium with or without supplemented NaCl. B and C, Salt stress tolerance revealed that the percentage of germination and green seedling of WT and transgenic plants. The percentage is shown relative to germination and green seedling of unstressed seedlings. Error bars are based on three replicates

marked difference (Fig. 7C). To analyze whether over-expression of *OsOPT10* in rice can improve salt tolerance, we generated transgenic rice plants that were confirmed by transcript analysis (Fig. 8A). Four transgenic plants (T4, T7, T9 and T13) were exposed to 250 mM NaCl for 7 days and allowed to grow on water in a green house for recovery for 7 days. In salt stress treatment, we clearly observed transgenic rice plants grew better than wild type plants as reflected by survival rate com-

parisons (Fig. 8C). Also, selected transgenic plant lines (T4 and T9) were significantly higher than the survival rate of the wild type (Fig. 8B). These results suggested that overexpression of *OsOPT10* could enhance the tolerance of transgenic plants to salt stress. Drought tolerance of *OsOPT10* transgenic plants were also tested, but no significant effect was detected for this stress (data not shown).

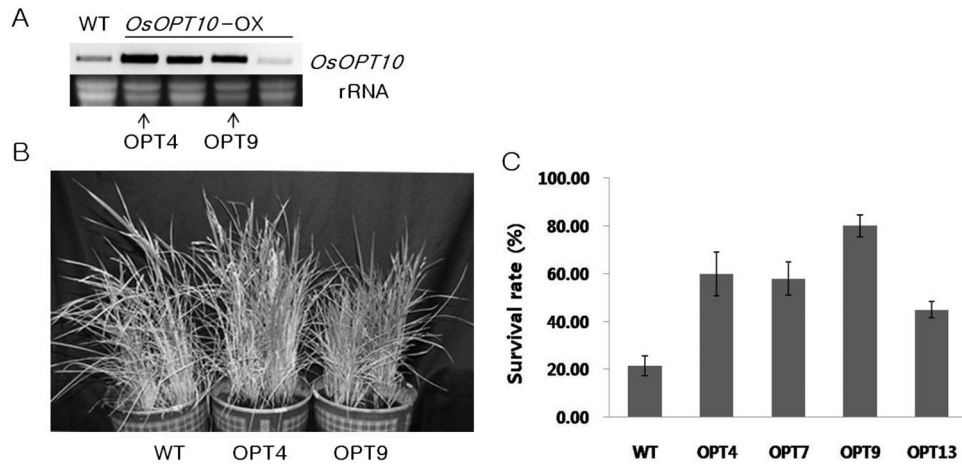


Fig. 8 Identification and salt-tolerance testing of transgenic rice. A, RT-PCR analysis of part of the transgenic plants and the wild type. B, Performance of salt tolerance of two transgenic families and wild type plants after salt treatment (250 mM NaCl for 3 weeks) and recovery (25°C for 2 weeks). C, Survival rate of four transgenic lines and the wild type after salt treatment (250 mM NaCl for 7 days and 25°C for 7 days for recovery). Error bars are based on three replicates

Discussion

In the present study, we showed some lines of evidence that support the role of a rice oligopeptide transporter, *OsOPT10*, as a salt tolerance gene. First, *OsOPT10* overexpressing plants are tolerance to salt stress (Fig. 6–8), and *OsOPT10* *Ac/Ds* knockout mutants are sensitive to salinity (Fig. 1). Second, *OsOPT10* overexpressing plants germinate much better in sodium-containing 1/2 MS agar plates than wild-type plants (Fig. 7). Third, chlorophyll contents and fresh weight of *OsOPT10* overexpressing plants are higher than those of wildtype plants (Fig. 6, 8). Fourth, *OsOPT10* gene is expressed in a cell membrane (Fig. 5). These data suggest that *OsOPT10* contributes to salt tolerance of *Arabidopsis* and rice in cell membrane by reducing sodium contents, at least in part. Searching of the available *Arabidopsis* sequences using the CaOpt1p sequence identified nine (AtOPT1 ~ AtOPT9) possible OPT orthologs (Koh et al. 2002). The presence of several OPT transporters in *Arabidopsis* suggests that these proteins are important and, so small peptides might play a crucial physiological role in plants.

All OPT family members (plant and fungus) appear to be integral membrane proteins with 12 to 14 predicted transmembrane (TM) domains but the rice OPT proteins appear to have 15 to 18 TM domains (Vasconcelos et al., 2008). Furthermore, the availability of these OsOPT sequences in rice allowed us to identify two highly conserved sequence motifs found in all OPT members

such as *Arabidopsis*. The OsOPTs (OsOPT1~ OsOPT9) contain the two signature motifs characteristic of the OPTs of PT clade (Koh et al. 2002) (Fig. 3A and B). The same was true for OsOPT1 and OsOPT2, which showed different expression profiles despite having 81% sequence identity (Vasconcelos et al. 2008). *OsOPT10* gene was predicted to subfamily with OsOPT1 and OsOPT8 (Fig. 3C). It has been shown in *Arabidopsis* and rice that AtOPT3 (Stacey et al. 2002), AtOPT6 (Cagnac et al. 2004) and OsOPT1 (Vasconcelos et al. 2008) are expressed in developing embryos and in seed maternal tissue. In all plants, including rice, the transport of ions across the cell plasma membrane and across intracellular membranes of different organelles must be done with the help of specific ions transporters. It is known that sodium has a toxic effect on many enzymes if present at high concentrations. In addition, high salt conditions in the environment cause low water availability, making it difficult for the plant to acquire water and nutrients.

Therefore, salt stress results in a water-deficient condition in the plant, which resembles a physiological drought. Conversely, drought decreases water contents in the cell and there by increases the salt concentration (Kim et al. 2010). Therefore, high salt, drought and osmotic stresses, which commonly result in dehydration of the cell and osmotic imbalance (Blumwald et al. 2000 Mahajan et al. 2005), can lead to the same defense mechanism involving *OsOPT10* gene. In the present work, we showed that

OsOPT10 confers salt tolerance and relate to osmotic stress (Fig. 4B). Interestingly, some genes induced transcriptionally by osmotic stress also participate in plant defense responses to wounding and pathogen attack. They include peroxidase, PR-1, PR-10 and osmotin (PR-5) genes (Zhu et al. 2000). Pretreatment of plants with SA, an important regulator of systemic acquired resistance and inducer of many pathogenesis-related (PR) genes, induces antioxidant enzymes and leads to increased salinity, drought or chilling tolerance (Kang and Saltveit 2002, Jacob et al. 2005). However, drought stresses induced very low the expression of *OsOPT10* (Fig. 4B). In conclusion, our results show that *OsOPT10* is important for osmotic and salt stress tolerance in *Arabidopsis* and rice, either directly or indirectly by reducing sodium contents in plants. For application, it might be useful to either search for crop varieties with constitutively high expression of the *OsOPT10* gene or to develop crops with enhanced tolerance to salt stress.

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