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Molecular characterization and subcellular localization of salt-inducible lipid transfer proteins in rice

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Abstract

Rice (*Oryza sativa* L.) is a salt-sensitive species. Salt stress can cause injury to the plant cellular membrane. Plant lipid transfer proteins (LTPs) are abundant lipid binding proteins that are important in membrane vesicle biogenesis and trafficking, however, the biological importance of LTPs on salt-stress response in rice remains unclear. Therefore, salt-responsive rice LTPs were identified and characterized in this study. Microarray analysis showed seven genes positively regulated by salinity, including five *Ltp* genes (*LtpII.3*, *LtpII.5*, *LtpII.6*, *LtpV.1*, and *LtpV.2*) and two *Ltp*-like (*LtpL; LtpL1*, and *LtpL2*) genes. Amino acid alignment revealed that all these *Ltp* and *LtpL* genes contained the N-terminal signal peptide. Apart from *LtpL1*, all salt-inducible *Ltp* genes had the conserved eight cysteine residue motifs backbone. Verification of gene expression to different stimuli in rice seedlings revealed that salt-regulated *Ltp* genes differentially responded to drought, cold, H_2O_2, abscisic acid (ABA) and $CaCl_2$. Furthermore, the expression of *Ltp* and *LtpL* genes was tissue-specifically regulated by ABA-dependent and independent pathway. *In silico* analysis of a 1.5-kb 5'-upstream region of these genes showed regulatory *cis*-elements associated with ABA, calcium, and cold/drought responses. Three *LtpII* subfamily genes, including *LtpII.3*, *LtpII.5*, and *LtpII.6*, were strictly expressed in flowers and seeds, and *LtpIII.1* mRNA strongly accumulated in stem tissue. Subcellular localization analysis of LTP-DsRed fusion proteins revealed that the five LTPs and two LTPLs localized at the endoplasmic reticulum. The results provide new clues to further understanding the biological functions of *Ltp* genes.

Additional key words: abscisic acid, calcium, cold, drought, gene expression, *Oryza sativa*, subcellular localization; salinity.

Introduction

Lipid transfer proteins (LTPs) are characterized by their ability to transfer phospholipids across membranes *in vitro* (Kader 1996). They are small ubiquitous and highly abundant plant proteins (~9 kDa) that have high pI (~9) and contain eight-cysteine motif (8-CM) at conserved positions (Kader 1997). 3-D structure analysis showed that LTPs have a hydrophobic cavity enclosed by four α-helices held together by four disulfide bonds between the eight cysteine residues (Shin *et al.* 1995, Heinemann *et al.* 1996, Charvolin *et al.* 1999). Sequence analysis showed that LTPs contain a signal peptide driving the peptide insertion into the endoplasmic reticulum lumen (Madrid 1991). LTPs have been found in the medium of cell cultures, plasma membrane, cell wall, and glyoxysomes (Liu *et al.* 2015).

In a survey of *Arabidopsis thaliana* genes that may be involved in acyl lipid metabolism, Beisson *et al.* (2003) identified 71 LTP-like sequences on the basis of the conserved 8-CM. However, a more recent analysis of the *Arabidopsis* genome showed that the number of small cysteine-rich peptide such as LTPs may have been underestimated (Silverstein *et al.* 2007). Recent study revealed 52 *Ltp* genes in the rice genome (Boutrot *et al.* 2008, Edstam *et al.* 2011). So far, there are no conserved

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Abbreviations: ABA - abscisic acid; ABRE - ABA-responsive element; 8-CM - eight-cysteine motif; DRE/CRT - dehydration-responsive/C-repeat element; ER - endoplasmic reticulum; LTPs - lipid transfer proteins; LtpL - Ltp-like; MYB - MYB transcription factor recognition sequence; MYC - MYC transcription factor recognition sequence; ROS - reactive oxygen species; ROSE - ROS/oxidative stress-responsive element; RT - reverse transcription; TNG67 - Tainung 67.

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functional perspective, systematic expression analysis, or clear gene expression profiles of members of LTPs or LTP-like proteins (LTPLs). In addition to LTPs, 8-CM containing proteins are largely distributed in plants and have different functions; examples are 2S-albumins, some protease inhibitors, and hybrid proline-rich proteins (Jose-Estanyol *et al*. 2004).

Several lines of evidence indicate that *Ltp* genes are implicated to modulate abiotic stress tolerance, plant defense, and development. In *Arabidopsis*, *Ltp3* was found to be induced by abscisic acid (ABA) and drought stress and confers plant tolerance to cold, drought, and oxidative stress (Seo *et al*. 2011, Guo *et al*. 2013). Stress-activated *Ltp* genes from wheat increase both biotic and abiotic stress tolerance of transgenic *Arabidopsis*. The enhancement of wheat *TdLtp4* in *Arabidopsis* increased the tolerance to NaCl, H_2O_2, and fungal resistance (Safi *et al*. 2015), whereas overexpression of wheat *TaLTP3* in *Arabidopsis* showed higher thermotolerance than in control plants at the seedling stage (Wang *et al*. 2014). Rice *OsC6* was expressed only in the tapetal cells of anther and was found important for postmeiotic anther development in rice (Zhang *et al*. 2010).

Expression patterns of *Ltps* have been elucidated in many plants. The cultivated potato *StLtpa7* is induced by salicylic acid, methyl jasmonate, ABA and calcium (Gao *et al*. 2009). The strawberry *Fxaltp* responds to ABA, SA, and wounding, and is repressed by cold stress (Yubero-Serrano *et al*. 2003). In wheat, the *TaLtp1* gene was found to be induced by drought and salt stress (Jang *et al*. 2004), and type I *Ltp* genes, *TaLtpIb.1* and *TaLtpIb.5*, were induced under chilling, wounding, and drought (Yu *et al*. 2014). In rice, the expression of rice *Ltp1* was induced in scutellum cells after inoculation with *Magnaporthe grisea* (Guiderdoni *et al*. 2002). *OsLtp2* transcripts were induced by ABA, mannitol, or NaCl (Garcia-Garrido *et al*. 1998). Strong *OsLtp6* promoter activity was found in inflorescence, in particular in anthers, with no expression in germinated seeds, roots, and shoots of rice seedlings (Liu *et al*. 2013). Moreover, rice *Ltp5* expression is prominent in stems and flowers and induced by cutin monomer (Kim *et al*. 2008).

Plant cell membranes are the first targets of many plant stresses. Various kinds of environmental stresses can directly or indirectly perturb the integrity and stability of the cell membrane and cause increased permeability and leakage of ions (Wahid *et al.* 2007). Salt stress has attracted attention recently because of increasing soil salinization and its deleterious effect on global crop production. At least 20 % of total irrigated agricultural lands in the world is affected by salt (Munns and Tester 2008). The negative effects of salinity on plants growth are associated with low osmotic potential of soil solution, nutritional deficiencies or imbalance (Grattan and Grieve 1999), ionic stress (Munns and Tester 2008), and oxidative stress (Mittler 2002) as salt stress induces the generation of reactive oxygen species (ROS), including hydrogen peroxide, superoxide anion, and hydroxyl radical, thereby triggering lipid peroxidation in both cellular and organellar membranes (Mittler 2002, Gill and Tuteja 2010). Membrane damage caused by salt stress-induced ROS was found a major cause of the cellular toxicity in various plant species such as rice, tomato, citrus, pea, and mustard (Gueta-Dahan *et al*. 1997, Dionisio-Sese and Tobita 1998, Mittova *et al*. 2004, Salama *et al*. 2007, Zamani *et al*. 2010, Ahmad *et al*. 2012,). To protect against salt stress-induced damage in membranes, plants have evolved an effective membrane repair system to maintain the membrane integrity.

Salt stress can cause injury to the cellular membrane and limit rice production. Lipid transfer proteins were reported to have a function in repairing stress-induced damage in membranes by facilitating the transfer of phospholipids, glycolipids, fatty acids, and steroids between membranes (Torres-Schumann *et al*. 1992, Holmberg and Bulow 1998). Although the roles of a few rice LTPs have been elucidated, how *Ltp* genes are regulated by salinity at the molecular level during plant development is not well understood. Here, we aimed to identify and characterize potential *Ltp* genes that might be important for recovery after salt stress-induced damage in rice. To efficiently identify salt responsive *Ltp* genes, we used microarray assay. We also investigated the abiotic stress-responsive expression patterns and subcellular localization of salt-inducible LTPs/LTPLs to provide new clues about the possible roles of rice salt-inducible *Ltps/LtpL*s in plant development and stress response.

Materials and methods

Plant materials, growth conditions, and stress treatment: Seeds of rice (*Oryza sativa* L.) *japonica* type cv. Tainung 67 (TNG67) were surface-sterilized with 2.5 % (m/v) NaOCl for 15 min, washed extensively with sterile water, and germinated in the dark on wet filter paper in Petri dishes at 37 °C for 48 h. After incubation, uniformly germinated seeds were selected and cultivated in a 150 cm^3 beaker containing half-strength Kimura B (Yoshida *et al.* 1972) solution replaced every 3 d. The hydroponically cultivated seedlings were grown in a *Phytotron* (Agricultural Experimental Station, National Taiwan University, Taipei) under natural irradiance and day/night temperatures of 30/25 °C and a 90 % relative humidity. Two-week-old seedlings underwent different treatments by replacing the medium with fresh medium supplemented with 200 mM NaCl, 200 mM mannitol, 10 mM H_2O_2, 20 μM ABA, or 10 mM $CaCl_2$. For cold stress, the seedlings were transferred to a growth chamber and maintained at 4 °C. Samples were collected after 0, 1, 3, 6, and 12 h, frozen in liquid nitrogen, and stored at -80 °C.

Microarray analysis: For microarray analysis, 2-week-old seedlings were treated in hydroponic solution with or without 200 mM NaCl for 6 h. RNA samples were prepared from shoot tissue by use of *TRIzol* reagent (*Invitrogen*, Carlsbad, CA, USA). Transcriptome analysis involved the use of the *Rice 4 × 44K Microarray RAP-DB* (G2519F#15241; *Agilent Technologies*, Santa Clara, CA, USA) for three biological replicates and color swaps for each replicate. Fluorescent probe labeling and hybridization were performed according to the manufacturer's instructions. Slides were scanned on an *Agilent G2505C* DNA microarray scanner. Scanned images were analyzed with *Feature Extraction Software v. 10.5.1.1* (*Agilent Technologies*), with default parameters, to obtain background-subtracted and spatially detrended processed Cy3 signal intensities. For statistical analysis, genes with signal intensities less than 100 in all experiments were excluded after correction of the dye effect by averaging the two color swaps. Statistical analysis involved unpaired Student *t*-test with the use of *GeneSpring GX11* (*Agilent Technologies*). In the *t*-test, *P* values were adjusted for multiple testing by the Benjamine and Hochberg's method to correct the false discovery rate for multiple testing. The fold change for each probe after salt treatment was calculated by the mean of three biological replicates. Expressions of lipid transfer proteins with greater than two-fold change regulated by NaCl were selected for further analysis.

Tissue-specific expression analysis and gene expression profiles under abiotic stresses: For tissue-specific gene expression analysis, shoots and roots of 2-week-old rice seedlings and then flag leaves, leaf sheaths, stems, roots, flowers (at booting stage), and seeds (at milky stage) were harvested for reverse transcription (RT)-PCR analysis. Total RNA was isolated from shoot tissues of 2-week-old rice seedlings by the use of *TRIzol* reagent (*Invitrogen*). To prevent DNA contamination, RNA was treated with *Turbo DNase I* (*Ambion*, Austin, TX, USA) at 37 °C for 30 min before real time PCR analysis. Control PCR amplifications involved RNA used as a template after *DNase I* treatment to verify the complete elimination of contaminated DNA. Reverse transcription reactions involved 200 ng total RNA with the *SuperScript III* platinum one-step quantitative RT-PCR system (*Invitrogen*). The gene-specific primers were designed from the 3′-UTR of rice *LtpII.3* (Os03g0111300), *LtpII.5* (Os05g0550600), *LtpII.6* (Os06g0705400), *LtpV.1* (Os01g0849000), *LtpV.2* (Os04g0415800), *LtpL1* (Os06g0686400), and *LtpL2* (Os07g0290500) (Table 1 Suppl.). The RT-PCR program was 50°C for 30 min; 94 °C denaturation for 5 min, followed by 94 °C for 30 s, 22 to 32 cycles of 50 °C for 30 s, and 68 °C for 30 s. All tests were repeated at least three times, and a representative repeat is shown. For all treatments, three replicates of RT-PCR were conducted with 3 batches of total RNA samples isolated independently. The PCR products were resolved by electrophoresis in 2 % (m/v) agarose gel and stained with ethidium bromide. Rice *Actin1* was used as a reference for normalization.

Sequence alignment and phylogenetic analysis: To identify the rice LTPs and LTPLs homologs, sequences were aligned with the use of *Clustal W 2.0* (Larkin *et al.* 2007), followed by manual alignment. Phylogenetic trees were constructed by using conserved regions of protein sequences with the neighbor-joining algorithm (*MEGA 5.0*) with the option of pairwise deletion (Tamura *et al.* 2011). To test inferred phylogeny, we used bootstraps with 1 000 bootstrap replicates. The transit peptide was predicted by *ChloroP 1.1* (Emanuelsson *et al.* 1999). *Weblogo* was generated using *Weblogo 3.4* (http:// weblogo.threeplusone.com/)(Crooks *et al.* 2004).

Construction of plasmids: The coding sequences of *LtpII.3*, *LtpII.5*, *LtpII.6*, *LtpV.1*, *LtpV.2*, *LtpL1*, and *LtpL2* without a stop codon were amplified by using PCR and then fused with the *DsRed* reporter gene (Matz *et al.* 1999). The maize *ubiquitin1* promoter, together with its intron (*Ubi1*), was used to drive the constitutive expression (Christensen and Quail 1996) of *green fluorescent protein* (*GFP*), and *Ltps-DsRed*. Primers used for construction are in Table 1 Suppl. The *pGOM2* plasmid was used as an ER marker (Wu *et al.* 2015).

Analysis of subcellular localization: Rice protoplasts were isolated from *in vitro* culture of 2-week-old rice seedlings as described by Zhang *et al.* (2011). PEG-mediated transient transformation was done according to the method described by Yoo *et al.* (2007). To detect endoplasmic reticulum, GFP-KDEL plasmid was used for PEG transient transformation. Fluorescence signals were visualized under a confocal laser-scanning microscope (*Leica TCS SP5 II*, *Leica*, Wetzlar, Germany). The excitation wavelength for GFP and chlorophyll auto-fluorescence was 488 nm. The emission wavelengths were 500 - 535 nm for GFP, 650 - 750 nm for chlorophyll autofluorescence and 570 - 630 nm for DsRed. All fluorescence experiments were repeated independently at least 3 times.

***In silico* analysis of rice *Ltps* and *LtpLs* promoters:** Search for *cis*-regulatory elements in the 1.5-kb 5' regions of *Ltps* and *LtpLs* involved the signal scan search provided by *PLACE* (http://www.dna.affrc.go.jp/htdocs/PLACE) (Higo *et al.* 1999).

Results

To examine the transcriptomic changes of lipid-transfer-protein genes in rice seedlings under salt stress, the whole genome expression profile of 2-week-old rice seedlings treated with 200 mM NaCl for 6 h was generated by rice 4 X 44K oligonucleotide microarray. Five *Ltp* genes (*LtpII.3*, *LtpII.5*, *LtpII.6*, *LtpV.1*, and *LtpV.2*) and two *Ltp*-like (*LtpL*) genes (*LtpL1* and *LtpL2*) were up-regulated at least two-fold by salt stress in rice seedlings (Table 1). Under salt stress, *LtpII.3*, *LtpII.5*, and *LtpV.2* were most highly up-regulated and *LtpII.6* and *LtpV.1* slightly up-regulated. Among the *LtpL* genes, *LtpL1* and *LtpL2* exhibited 9.1- and 2-fold up-regulation, respectively, with salt stress.

Table 1. Lipid transfer protein genes in rice seedlings significantly up-regulated by 200 mM NaCl for 6 h.

Name	Probe Name	MSU ID	Annotation	Fold change	*P* value
LtpII.3	CUST_33540_Rice4x44kGene	LOC_Os03g02050	nonspecific LTP 2	419.0	0.000
LtpII.5	CUST_33819_Rice4x44kGene	LOC_Os05g47730	nonspecific LTP	97.0	0.000
LtpII.6	CUST_18812_Rice4x44kGene	LOC_Os06g49190	nonspecific LTP 2P	6.3	0.001
LtpV.1	CUST_1754_Rice4x44kGene	LOC_Os01g62980	LTP101	2.4	0.000
LtpV.2	CUST_11592_Rice4x44kGene	LOC_Os04g33920	plant LTP	582.0	0.000
LtpL1	CUST_21527_Rice4x44kGene	LOC_Os07g19000	plant LTP	9.1	0.001
LtpL2	CUST_26263_Rice4x44kGene	LOC_Os10g40510	plant LTP	2.0	0.046

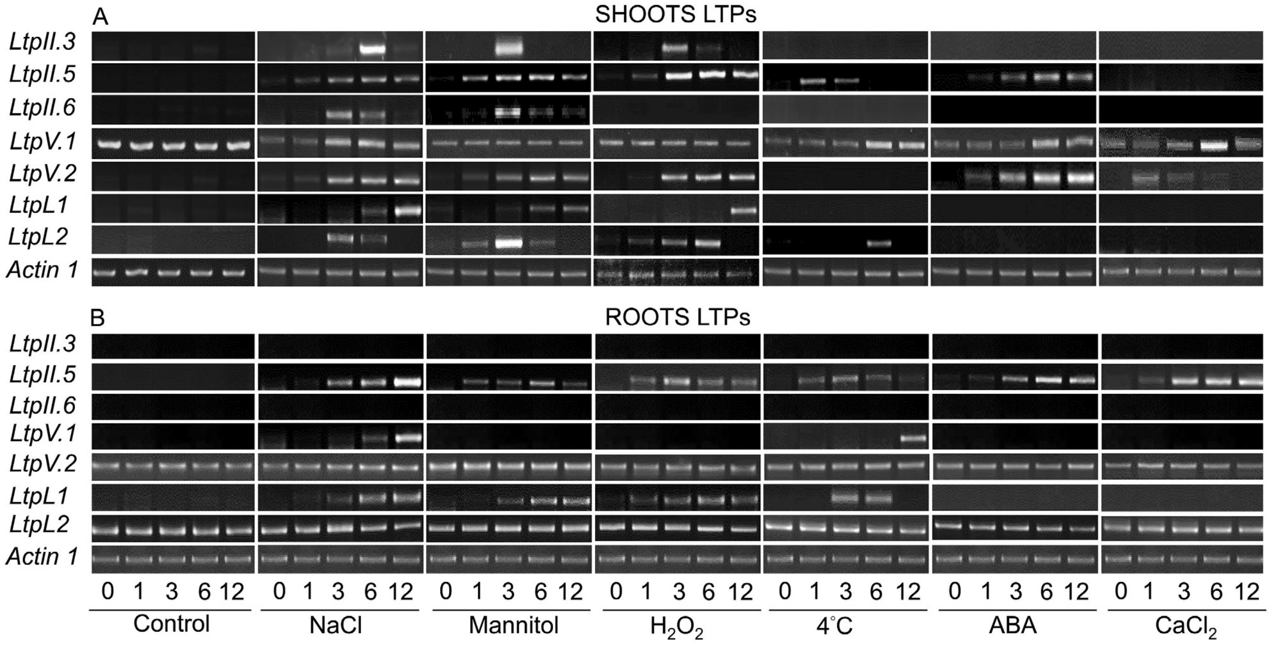

Fig. 1. Expression profiles of the seven salt-inducible *Ltps* in rice shoots (*A*) or roots (*B*) in response to external stimuli. Total RNA was isolated from roots or shoots of hydroponically cultivated 2-week-old seedlings exposed to 200 mM NaCl, 200 mM mannitol, 10 mM H_2O_2, 4 °C, 20 µM abscisic acid (ABA), 10 mM $CaCl_2$ for 0, 1, 3, 6, or 12 h. The rice *Actin1* gene was an internal control.

Phylogenetic analysis revealed that five *Ltp* and two *LtpL* genes formed a clade with high bootstrap value, which indicates high sequence homology (Fig. 1 Suppl.). Comparison of amino acid sequences of the five LTPs and two LTPLs revealed a signal peptide sequence at the N terminus of all LTP-related proteins (Fig. 2 Suppl.). In addition to LTPL1, all LTPs showed eight conserved cysteine residues in the peptide sequences. The LTPL1 peptide sequence showed only five conserved cysteine residues, with the second, seventh, and eighth conserved cysteine residues missing (Fig. 2 Suppl.).

To determine the response of *Ltp*-related genes under other abiotic stresses, we analyzed the temporal gene expression pattern of the five *Ltp* genes and the two *LtpL* genes in shoots of rice seedlings treated with NaCl, mannitol, H_2O_2, 4 °C, ABA, and $CaCl_2$ (Fig. 1*A*). *LtpV.1* was expressed in untreated rice seedlings but not *LtpII.3*, *LtpII.5*, *LtpII.6*, *LtpV.2*, *LtpL1*, and *LtpL2*. With NaCl treatment, all *Ltp* and *LtpL* genes were up-regulated. Both mannitol and H_2O_2 treatments increased the expression of

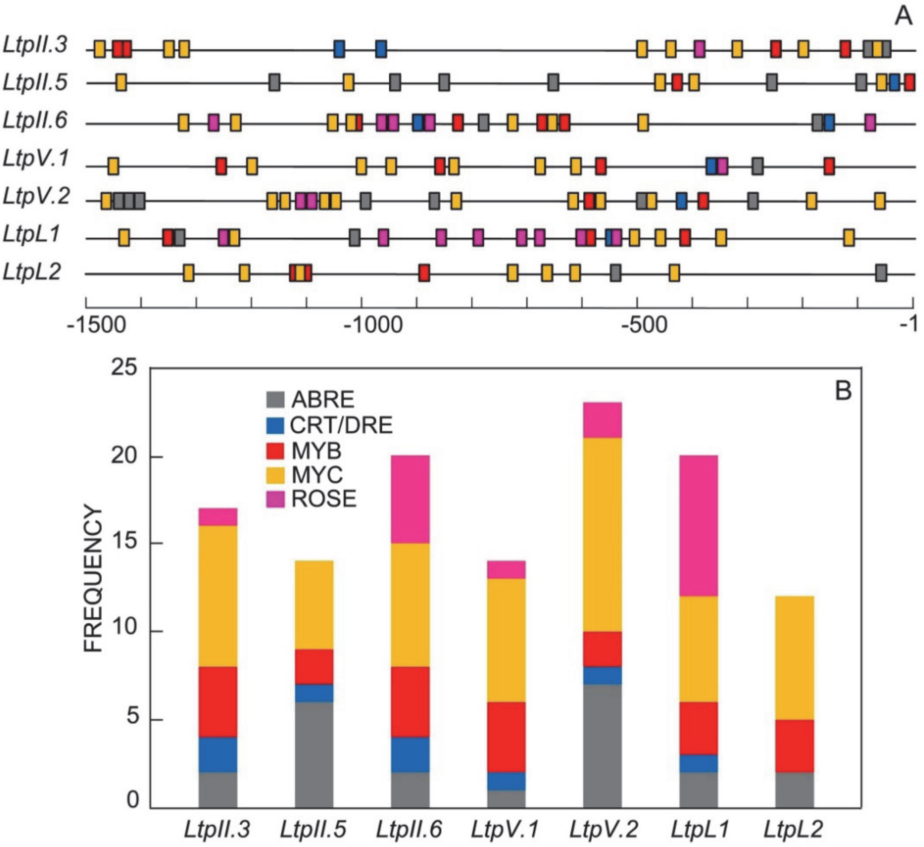

Fig. 2. *In silico* analysis of putative *cis*-acting elements in 1.5-kb 5' regulatory sequences of rice *Ltp* and *LtpL* genes. *A* - Positions are with respect to the first base of the translation start site (ATG). The putative *cis*-elements are indicated in boxes. *B* - Frequency of the various abiotic stress responses-related *cis*-acting elements in LTP promoter regions. ABRE - ABA response element; CRT/DRE - C-repeat element/ dehydration response element; MYB - MYB transcription factor binding site; MYC - MYC transcription factor binding site; ROSE - ROS/oxidative stress-responsive element.

LtpII.3, *LtpII.5*, *LtpII.6*, *LtpV.2*, *LtpL1*, *and LtpL2*, but no increased expression was in *LtpII.6* with H_2O_2 treatment. With cold treatment, *LtpII.5*, *LtpV.1*, and *LtpL2* were induced, but no expression of *LtpII.3, LtpII.6*, *LtpV.2*, *and LtpL1* was detected. With ABA treatment, *LtpII.5, LtpV.1*, and *LtpV.2* were up-regulated, but no expression of *LtpII.3*, *LtpII.6*, *LtpL1*, and *LtpL2* was detected. With $CaCl_2$ treatment, *LtpV.1* and *LtpV.2* were up-regulated, but no expression of *LtpII.3*, *LtpII.5*, *LtpII.6*, *LtpL1* and *LtpL2* was detected. In roots, *LtpV.2* and *LtpL2* were expressed under the normal conditions. With NaCl treatment, *LtpII.5*, *LtpV.1*, and *LtpL1* were up-regulated, but no expression of *LtpII.3* and *LtpII.6* was detected. With mannitol and H_2O_2 treatments, *LtpII.5* and *LtpL1* were up-regulated. With cold treatment, *LtpII.5*, *LtpV.1*, and *LtpL1* were up-regulated. With ABA and $CaCl_2$ treatment, *LtpII.5* was up-regulated. *LtpV.2* and *LtpL2* were constitutively expressed, with no *LtpII.3* and *LtpII.6* expression in root tissues (Fig. 1*B*).

An *in silico* analysis of a 1 500-bp upstream region from the translational start site of five *Ltp* and two *LtpL* genes using the *PLACE* database (http://www.dna.affrc.go.jp/PLACE/) revealed the presence of various regulatory elements such as an ABA-responsive element

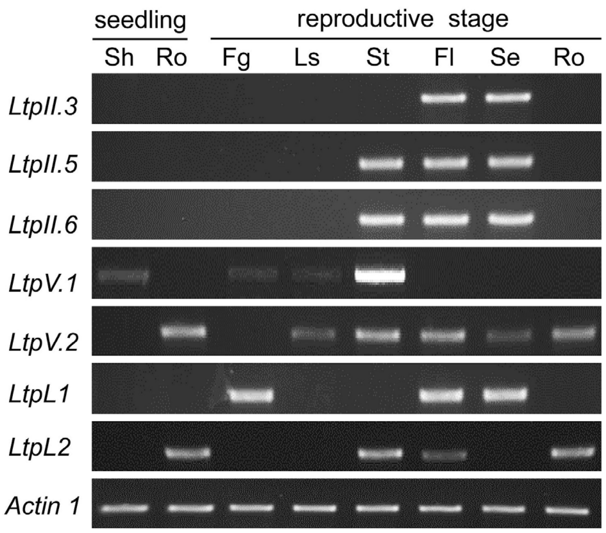

Fig. 3. Developmental and tissue-specific expressions of the salt-inducible *Ltp* and *LtpL* genes in rice. RT-PCR analysis of gene expression in shoots (Sh) or roots (Ro) of 2-week-old rice seedlings, and flag leaf (Fg), leaf sheaths (Ls), stems (St), flower (Fl), seeds (Se), and roots (Ro) at the reproductive stage of rice plants. The rice *Actin1* gene was an internal control.

(ABRE), MYB transcription factor recognition sequence (MYB), MYC transcription factor recognition sequence (MYC), dehydration-responsive/C-repeat element (DRE/CRT), and ROS/oxidative stress-responsive element (ROSE) motif, known to widely regulate various abiotic stress responses. Differences were observed also in the number and position of these *cis*-acting elements among the *Ltp*-related genes. All *Ltp*-related genes possessed most of the above-mentioned motifs except *LtpII.5*, which lacked the ROSE promoter element, and *LtpL2*, which lacked two *cis*-motifs such as DRE/CRT and ROSE. Interestingly, MYB and MYC motifs were present in all the *Ltp*-related genes, accounting for the response of these genes to drought and/or ABA (Fig. 2*A,* 2*B*).

Determination of spatial and temporal expression of *Ltp*-related genes at the seedling stage showed that *LtpV.1* was expressed at low amounts only in shoots, whereas *LtpV.2* and *LtpL2* were expressed only in roots. No RNA transcripts could be detected for *LtpII.3*, *LtpII.5*, *LtpII.6*, and *LtpL1* at the seedling stage. At the reproductive stage, *LtpII.3* was expressed only in flowers and seeds. Expression of *LtpII.5* and *LtpII.6* was restricted to stem, flowers, and seeds. *LtpV.1* was strongly expressed in stem, with lower expression in flag leaves and leaf sheaths and no expression in flowers, seeds, and roots. *LtpL1* expression was restricted to the flag leaf, flowers and seeds. *LtpL2* was expressed in stems, flowers, and roots, with no expression in flag leaves, leaf sheaths, and seeds (Fig. 3).

To observe the subcellular localization of LTP and LTPL proteins in rice cells, we fused the five *Ltp* and two *LtpL* genes to the coding region of DsRed reporter gene and observed their localization by PEG-mediated transient expression in rice protoplasts. The all seven tested genes targeted to the endoplasmic reticulum; the control DsRed protein was localized to the cytosol (Fig. 4).

Discussion

The plant LTPs are a multi-gene family and have been classified into two subfamilies by molecular mass: LTP1 (~ 9 kD) and LTP2 (~7 kD) (Yeats and Rose 2007). In rice, the first *Ltp* gene was described by Vignols *et al.* (1994) and then more genes have been cloned (Vignols *et al.* 1997) and annotated (Boutrot *et al.* 2008). However, their nomenclature may be confusing (Table 2 Suppl.). In this study, we used the accessions of the Rice Genome Annotation Project database (Kawahara *et al.* 2013) and nomenclature proposed by Boutrot *et al.* (2008).

This work reports five *Ltp* and two *Ltp*-like genes up-regulated by salinity (Table 1). In addition to salt stress, other stimuli, such as mannitol, H_2O_2, cold, ABA, and $CaCl_2$, elicited a response, which indicates a significant role for these genes in abiotic stress-triggered signaling and stress tolerance in rice plants (Fig. 1). Moreover, the stress-induced expression of rice *LtpII.3* and *LtpII.6* was also tissue-specifically regulated in rice seedlings. RNA transcripts of *LtpII.3* and *LtpII.6* were not detected in root tissues before and after stress treatments. However, the expression of *LtpII.3* in shoots was induced by NaCl, mannitol, and H_2O_2, and that of *LtpII.6* was induced by NaCl and mannitol. In contrast, the stress response of *LtpV.2* and *LtpL2* was restricted to shoot tissues, and constitutive in root tissues. *LtpV.2* and *LtpL2* expressions were not detected in root tissues before and after stress treatments (Fig. 1). In shoot tissues, the salt-inducible genes showed distinct responses to ABA: *LtpII.5, LtpV.1* and *LtpV.2* were upregulated by ABA, but *LtpII.3, LtpII.6*, *LtpL1*, and *LtpL2* were not affected by ABA. Therefore, the expression of *Ltp* genes was mediated by an ABA-dependent or ABA-independent pathways.

Gene expression of *Ltps* regulated by external stimuli was not parallel between roots and shoots. For example, the expression of *LtpV.1* was induced by salt, cold, ABA, and $CaCl_2$ in shoots, but its expression was up-regulated by NaCl and cold but not ABA and $CaCl_2$ in roots. The expression of *LtpV.2* was induced by ABA, whereas that of *LtpL2* did not respond to ABA. Interestingly, both genes showed constitutive expression in root tissues (Fig. 1). These data indicate that ABA-dependent or independent gene expression was also tissue-specific. The expression patterns of *LtpII.3* (formerly named *LTP2*) and *LtpV.2* (formerly named Os04g33920) were previously reported by Garcia-Garrido *et al.* (1998) and Tapia *et al.* (2013). The tissue-specific and stress-induced expression patterns of *LtpII.3* were mostly consistent with previous study except the response to ABA. In this study, *LtpII.3* was not induced by ABA, but the previous study showed that *LTP2* was induced by ABA (Garcia-Garrido *et al.* 1998). The inconsistency may result from the use of different rice cultivars or plant materials at different stages for gene expression analysis. Rice seeds imbibed for 3 d were used for ABA treatment in the Garcia-Garrido *et al.* (1998) study, whereas 2-week-old rice seedlings were used for stress treatment in this study. In agreement with earlier study (Wang *et al.* 2012), *LtpV.2* transcripts were increased by dehydration in shoots. Conversely, the response to salinity showed distinct expression patterns. Possible reasons for inconsistent result can be attributed to the different cultivars used or treatments with different NaCl concentrations.

Apart from understanding the effect of different stresses on the transcription of the five *Ltp* and two *LtpL* genes, we checked the expression of these genes in different tissues at seedlings and reproductive stages. All seven genes showed distinct expression patterns with significant induction or repression or no change in expression at various stages of development, specifically or in common. Spatial and temporal fluctuations in the expression of these genes suggest that these genes might affect specific developmental stages positively or negatively and ultimately could affect overall rice growth and development. Distinct patterns of expression also indicate non-redundancy and possible function in a coordinated manner to regulate rice development. Most interestingly, we observed that three *LtpII* group genes (*LtpII.3, LtpII.5, LtpII.6*) showed specific expression in flowers and seeds, and very strong expression of *LtpV.1* in stem tissue (Fig. 3).

From the *in-silico* analysis of promoters for the five *Ltp* and two *LtpL* genes, we found five important *cis*-regulatory elements (ABRE, MYB, MYC, DRE/CRT, and ROSE motifs) known to regulate abiotic stress mediated responses of various genes (Fig. 4). Analysis of the promoter elements supports the expression patterns of these genes under diverse stress conditions. Gene expression patterns of the salt-inducible *Ltp* and *LtpL* genes revealed that they contained elements associated

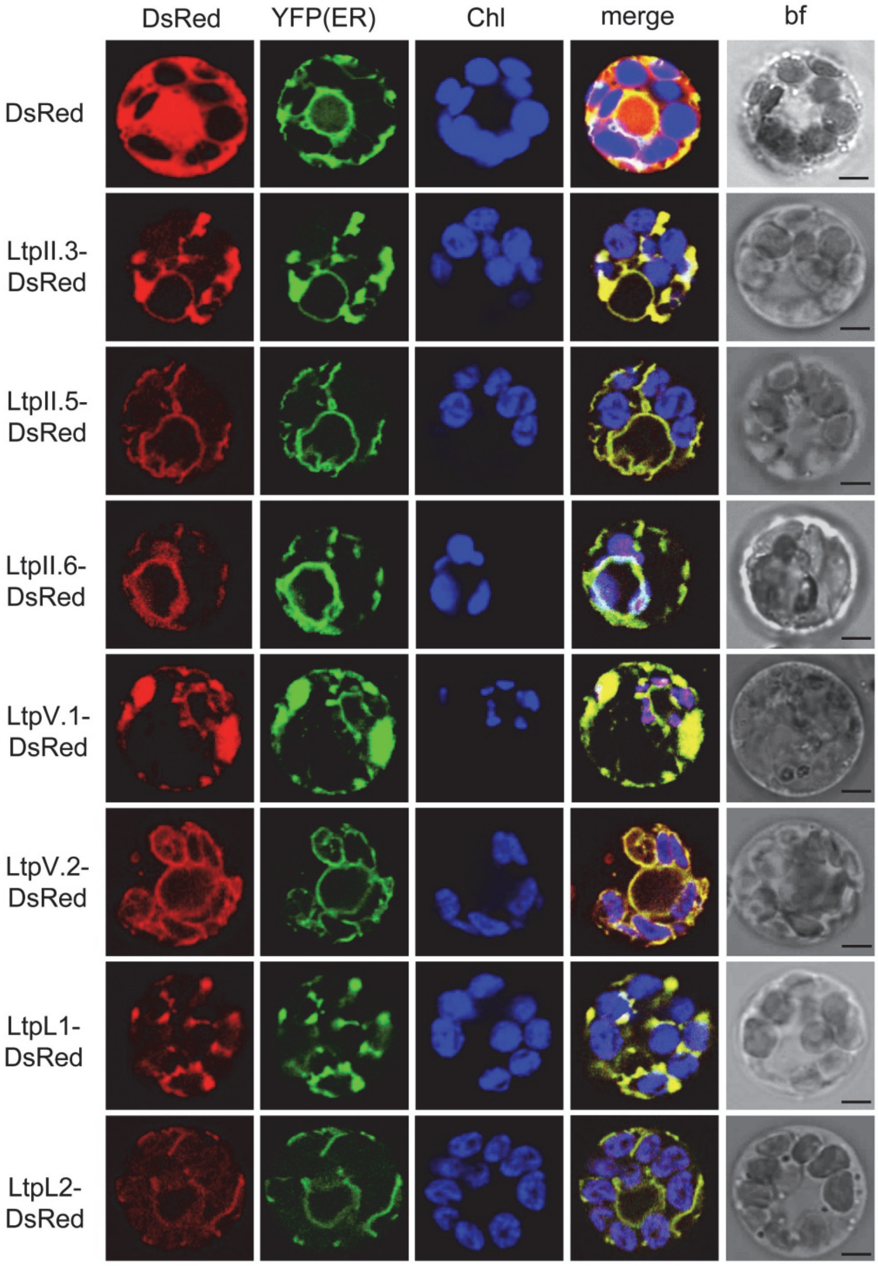

Fig. 4. Subcellular localization of LTPs in rice protoplasts. Rice leaf sheath protoplasts were transiently transformed with C-terminal DsRed fusions to full length *Ltps* or *LtpLs*. LTP-DsRed fusion proteins were co-expressed with an endoplasmic reticulum (ER) marker. Maximum intensity signals from confocal images are shown for DsRed fluorescence (DsRed), YFP fluorescent ER marker (YFP(ER)**,** GFP fluorescence (GFP), chlorophyll autofluorescence (Chl), an overlay of all fluorescence (merge), and bright-field image of the selected protoplast (bf). The constructs are depicted on the left. *Bars* = 2.5 μm.

with ABA-dependent and -independent stress responses. Previous report indicated that gene response to ABA and calcium shares the same element (ABRE) in the promoter region (Kaplan *et al*. 2006). *LtpII.5* possesses six ABREs on the 1.5-kb promoter region. Surprisingly, *LtpII.5* showed a distinct response to ABA and calcium in roots and shoots: *LtpII.5* was induced by both ABA and calcium in roots but only by ABA and not by calcium in shoots (Fig. 1), so gene expression mediated by ABA and calcium may also be tissue-specific.

The localization of LTP has been reported in different organelles or tissues. Wheat LTPs are localized in membrane and cytoplasm (Wang *et al*. 2014) or cell walls (Safi *et al*. 2015). By immunocytochemical methods, *Arabidopsis* LTP1 was found to be localized at cell walls and the cytoplasm (Potocka *et al*. 2012). *Arabidopsis* LTP3 protein was localized to the cytoplasm (Guo *et al*. 2013), with another AZI1 protein expressed in endoplasmic reticulum (ER) and plasmodesmata (Yu *et al*. 2013). A GPI-anchored LTP in *Physcomitrella patens* was localized at the plasma membrane (Edstam *et al*. 2014). Several reports revealed LTPs localized in secretory vesicles of *Brassica chinensis* (Wang *et al*. 2008) and *Capsicum annuum* (Diz *et al*. 2011), and glyoxysomes of *Ricinus communis* (Tsuboi *et al*. 1992) and *Helianthus annuus* (Pagnussat *et al*. 2012). We found the five LTP and two LTPL are localized at the ER (Fig. 4). These results are consistent with the existence of N-terminal signal peptide sequences in LTPs identified in this study (Fig. 2 Suppl).

In conclusion, we identified and validated five *Ltp* and two *Ltp*-like genes that were highly up-regulated under salt stress by microarray analysis. Differential gene expression patterns under various abiotic stress treatments and different development stages in both shoots and roots highlights the important biological role of these seven genes in overall rice growth and development. Subcellular localization studies showed that LTP and LTPL were all targeted to the endoplasmic reticulum. Future studies involving GUS histochemical analysis to check the tissue-specific expression and knock-out/knock-down studies would provide a clear idea about the specific site of action and the functional role of these genes in rice growth and development.

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