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OsTZF1 regulates senescence and stress tolerance

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**ABSTRACT**

OsTZF1 is a member of the CCCH-type zinc finger gene family in rice (*Oryza sativa*). Expression of OsTZF1 was induced by drought, high-salt stress and H$_2$O$_2$. OsTZF1 gene expression was also induced by abscisic acid (ABA), methyl jasmonate (MeJA) and salicylic acid (SA). Histochemical activity of β-glucuronidase (GUS), in transgenic rice plants containing the promoter of OsTZF1 fused with GUS, was observed in callus, coleoptile, young leaf and panicle tissues. Upon stress, OsTZF1-GFP localization was observed in the cytoplasm and cytoplasmic foci. Transgenic rice plants over-expressing OsTZF1 driven by a maize *ubiquitin* promoter (*Ubi*:OsTZF1-OX) exhibited delayed seed germination, growth retardation at the seedling stage and delayed leaf senescence. RNAi knocked-down plants (OsTZF1-RNAi) showed early seed germination, enhanced seedling growth and early leaf senescence compared to controls. *Ubi*:OsTZF1-OX plants showed improved tolerance to high-salt and drought stresses and vice versa for OsTZF1-RNAi plants. Microarray analysis revealed that genes related to stress, reactive oxygen species (ROS) homeostasis and metal homeostasis were regulated in the *Ubi*:OsTZF1-OX plants. RNA binding assays indicated that OsTZF1 binds to U-rich regions in the 3'-UTR of mRNAs, suggesting that OsTZF1 might be associated with RNA metabolism of stress-responsive genes. OsTZF1 may serve as a useful biotechnological tool for the improvement of stress tolerance in various plants through the control of RNA metabolism of stress-responsive genes.
INTRODUCTION

Plants encounter various abiotic stresses during their life cycle, such as cold, drought and high salinity. Many genes induced by various abiotic stresses have been identified using genome-wide analysis techniques, including microarray analysis (Bartels and Sunkar 2005; Fowler and Thomashow, 2002; Seki et al., 2002; Rabbani et al., 2003; Yamaguchi-Shinozaki and Shinozaki, 2006) and proteomics (Pechanova et al., 2010; Kosová et al., 2011). These induced genes either directly protect plants against stresses by the production of important metabolic proteins (functional proteins) or regulate the genes for signal transduction in the stress response (regulatory proteins). It is of immense importance to elucidate the exact function of genes involved in signal transduction and their regulation to understand their responses to different environmental stresses in plants (Cui et al., 2002; Ronald and Leung, 2002).

Zinc finger protein genes comprise a large and diverse gene family. They are involved in many aspects of plant growth and development and play important roles in many cellular functions, including transcriptional regulation, RNA binding and protein–protein interactions (Ciftci-Yilmaz and Mittler, 2008). Zinc finger proteins have been classified into several different types, including C2H2, C2C2, C2HC, C2C2C2C2, C2HCC2C2 and CCCH, according to the number and order of the cysteine (C) and histidine (H) residues binding the zinc ion in the secondary structure of the finger (Berg and Shi, 1996; Mackay and Crossley, 1998; Moore and Ullman, 2003; Schumann et al., 2007). A number of zinc finger proteins have been found to be involved in abiotic and biotic stress responses (Sakamoto et al., 2004; Mukhopadhyay et al., 2004; Ciftci-Yilmaz et al., 2002). Zinc finger proteins containing a tandem zinc finger (TZF) domain, characterized by two CCCH zinc fingers separated by 18 amino acids, are well documented in humans and a number of them have been associated with RNA metabolism (Varnum et al., 1991; Shimada et al., 2002; Carballo et al., 1998, 2000; Al-Souhibani et al., 2010; Jeong et al., 2010).

A genome-wide annotation analysis of CCCH zinc finger proteins found 67
genes in rice (*Oryza sativa*) and 68 genes in Arabidopsis (Wang et al., 2008). Analysis of zinc finger proteins in Arabidopsis and rice identified a plant unique tandem CCCH zinc finger containing Cx_{7.8}Cx_{5}Cx_{3}Hx_{16}Cx_{5}Cx_{4}Cx_{3}H and an uncharacterized N-terminal side C-x_{5}-H-x_{4}-C-x_{3}-H motif (Wang et al., 2008; Pomeranz et al., 2010). The functions of most of the genes in this unique subfamily are unknown, but recently a few genes of Arabidopsis, *PEII*, *AtSZF1/AtSZF2*, and *SOMNUS*, were characterized to function in embryogenesis (Li and Thomas, 1998), salt stress response (Sun et al., 2007), and light-dependent seed germination (Kim et al., 2008), respectively. *AtTZF1* was reported to shuttle between the nucleus and cytoplasmic foci and be involved in ABA/GA-mediated growth and abiotic stress responses (Pomeranz et al., 2010; Lin et al., 2011). Rice OsDOS (*Oryza sativa* Delay of the Onset of Senescence) was reported to be nucleus localized and involved in delaying leaf senescence (Kong et al., 2006). Here, we report the isolation and functional characterization of *OsTZF1* (*Os05g10670*), which is induced under abiotic stress conditions and a homolog of *AtTZF1* (Pomeranz et al., 2010) in rice. We show that *OsTZF1* acts as a negative regulator of leaf senescence in rice under stress conditions and confers abiotic stress tolerance by delaying stress response phenotypes, possibly through the control of RNA metabolism of stress responsive genes.

**RESULTS**

**Stress-induced and Spatial Expression Profiles of OsTZF1 in Rice**

To identify important genes under abiotic stress conditions we monitored the expression profiles of rice (cv. Nipponbare) genes under drought conditions using a 44K rice microarray (Maruyama et al., unpublished). The expression of *OsTZF1* was induced by drought, high-salt stress and ABA, and was selected for further characterization. Expression of *OsTZF1* in response to dehydration, ABA, NaCl and H$_2$O$_2$ was analyzed by RNA gel-blot analysis to investigate time-dependent induction patterns. As shown in
Fig. 1, the OsTZF1 transcript accumulated in seedlings within 2 h following ABA, NaCl and H₂O₂ treatments. In contrast, expression of the OsTZF1 gene induction peaked after 5 h and then decreased slightly over 24 h of dehydration stress treatment (Fig. 1A). There was no significant accumulation of OsTZF1 mRNA in seedlings treated with H₂O only (Fig. 1A). The expression of OsTZF1 in rice seedlings was also up regulated following treatment with exogenous SA or JA (Fig. 1B).

To assess the effect of the promoter region on the expression of OsTZF1 in rice seedlings under abiotic stresses, we generated transgenic rice plants containing a 1,417 bp OsTZF1 promoter fragment fused to the β-glucuronidase (GUS) reporter gene (P_{OsTZF1}:GUS). Quantitative analysis of GUS activity in the P_{OsTZF1}:GUS plants confirmed that the promoter region of OsTZF1 regulates the induction of this gene in response to ABA and NaCl (Fig. 1C). Histochemical GUS activity was mainly detected in the aerial parts of P_{OsTZF1}:GUS plants and its intensity increased in response to ABA and NaCl (Fig. 1D).

To determine the organ-specific expression of the OsTZF1 gene, we isolated total RNA from callus, roots, shoot bases, seedling leaves, four expanded leaves at bolting stage, nodes, internodes and two different stages of the rice panicle. RNA gel-blot analysis showed that the expression of OsTZF1 was high in callus, moderate in shoot bases, seedling leaves, internodes and panicles after dehiscence, but no or low expression was observed in roots, expanded leaves, nodes and panicles before dehiscence (Fig. 2A). Consistent with the results of RNA gel-blot analysis, histochemical GUS activity in the P_{OsTZF1}:GUS plants was observed in rice callus, germinating seedlings, seedling leaves, and panicles after dehiscence, while no or low histochemical GUS activity was observed in roots (Fig. 2, B–F).

Putative sequences of various cis-acting elements involved in the response to abiotic stresses were identified in the 1,417 bp promoter region of OsTZF1 (Supplemental Fig. 1) using the PLACE signal scan program (Higo et al., 1999). We found five ABA-responsive elements (ABRE, ACGTG; Simpson et al., 2003), three
MYB core sequences (MYBCOR, CNGTTR; Urao et al., 1993) and four recognition sites for MYC (MYCRSs, CANNTG; Abe et al., 2003). The OsTZF1 promoter also contained some putative cis-acting elements involved in the response to biotic stresses, including three WRKY71OS sequences (TGAC-containing W-box; Eulgem et al., 2000) and seven W-boxes of different types (data not shown), which are known as recognition sites for WRKY transcription factors.

Sub-cellular localization of OsTZF1

To determine the sub-cellular localization of OsTZF1, transgenic rice plants expressing an OsTZF1-sGFP chimeric gene driven by either the native OsTZF1 promoter or by the maize ubiquitin promoter were generated. Under normal growth conditions, OsTZF1 was predominantly localized in the cytoplasm of root meristem cells, and was occasionally observed in cytoplasmic foci (Fig. 3A). OsTZF1-GFP was rarely observed in the nucleus of root cells at young seedling stage (Fig. 3A). To examine whether these OsTZF1-associated cytoplasmic foci are stress-related, seedlings were treated with 10 µM ABA or 100 mM NaCl for 12 h. As shown in Fig. 3A, ABA and NaCl enhanced the formation of cytoplasmic foci in root cells. The size, shape, and number of these cytoplasmic foci varied depending on the condition of plants and the type of roots. These cytoplasmic foci resembled p-bodies (PBs) and stress granules (SGs) where mRNA turnover and translational repression take place (Parker and Sheth, 2007; Balagopal and Parker, 2009).

To confirm whether OsTZF1 is associated with PBs and SGs, co-localization analysis using Arabidopsis marker genes for PBs and SGs was performed. OsTZF1 was fused in frame with YFP and the marker genes were fused with CFP. DCP2 is an mRNA-decapping enzyme and localizes to p-bodies (Iwasaki et al., 2007; Weber et al., 2008). Both constructs were introduced into protoplasts prepared from rice mesophyll cells. OsTZF1-YFP co-localized with DCP2 in cytoplasmic foci resembling PBs (Fig. 3B). Co-localization of OsTZF1 was also tested in SGs using the SG marker PABP8.
(Newbury et al., 2006; Anderson and Kedersha, 2008). OsTZF1 and PABP8 co-localized in comparatively large cytoplasmic foci, considered to be SGs (Fig. 3B).

In onion (*Allium cepa*) epidermal cells, OsTZF1 was localized in the cytoplasm and cell membrane (Supplemental Fig. 2A). Furthermore, OsTZF1 localization could be observed in the nucleus in comparatively old OsTZF1-sGFP plants (Supplemental Fig. 2B). These results indicate that OsTZF1 has dynamic sub-cellular localization patterns in the cytoplasm and probably the nucleus.

**In Vivo Functional Analysis of OsTZF1 Using OsTZF1-OX and OsTZF1-RNAi Plants**

To analyze the function of OsTZF1 in rice, transgenic rice plants over-expressing OsTZF1 driven by the maize *ubiquitin* promoter (OsTZF1-OX) were generated. Seven OsTZF1-OX transgenic rice plants (T2 generation) were analyzed by RNA gel-blot analysis and two lines (OsTZF1-OX#6 and OsTZF1-OX#9) exhibiting moderate levels of transgene expression (Supplemental Fig. 3A) were selected for phenotypic and functional characterization. OsTZF1-RNAi transgenic rice plants were also produced to further evaluate the function of OsTZF1. Seven OsTZF1-RNAi plants were analyzed by RNA gel-blot analysis and two lines (OsTZF1-RNAi#7 and OsTZF1-RNAi#9) were selected for further functional analysis (Supplemental Fig. 3B).

The OsTZF1-OX plants showed a phenotype of repressed seed germination. The OsTZF1-OX seeds required more than two days after imbibition (DAI) for coleoptile plumule emergence compared to 24 to 30 h in control seeds, whereas OsTZF1-RNAi seeds showed an enhanced seed germination phenotype, with an elongated coleoptile plumule at 2 DAI (Fig. 4A). The difference in seed germination and growth became more obvious at 4 to 5 DAI (Fig. 4A). Differences in seed germination time affect subsequent growth; the growth of OsTZF1-OX seedlings was slow while OsTZF1-RNAi seedlings showed enhanced growth compared to controls at
10 days after seed germination (Fig. 4B). These results indicated that OsTZF1 affects germination and subsequent rice seedling growth. The OsTZF1-OX seedlings planted in soil grew normally and there was no difference in the growth of OsTZF1-OX, OsTZF1-RNAi and control plants. At the bolting stage, the OsTZF1-OX and control plants were indistinguishable (Supplemental Fig. 3). The OsTZF1-OX plants exhibited delayed senescence of leaves and retained more photosynthetic activity (Fv/Fm) compared to controls after the start of seed setting (Supplemental Fig. 4). Interestingly, during the heading and seed setting stages, OsTZF1-OX leaves were greener but developed a number of brown lesions that increased in frequency over time (Fig. 4C). The seeds of OsTZF1-OX were also brownish in appearance compared to controls at harvest (Fig. 4D). The above results indicated that constitutive over-expression of OsTZF1 caused pleiotropic effects on the phenotype of rice plants.

**OsTZF1-OX Plants Exhibit High-salt and Drought Stress Tolerance**

The expression of OsTZF1 was induced by NaCl and dehydration stress (Fig. 1A); therefore, OsTZF1-OX and OsTZF1-RNAi plants were evaluated for high-salt stress and drought tolerance. Two-week-old rice seedlings grown in soil were treated with 250 mM NaCl for three days. After high-salt stress treatment, plants were allowed to recover under normal growth conditions (Fig. 5). OsTZF1-RNAi seedlings showed severe damage to leaves and ultimately died, whereas the OsTZF1-OX transgenic seedlings showed good tolerance to high-salt stress and continued to grow. More than 65% of the OsTZF1-OX plants survived, whereas only 33% of the control plants survived (Fig. 5, left panels). Conversely, the survival rate of the OsTZF1-RNAi plants was around 19% to 21% compared to 50% in the controls (Fig. 5, right panels). The OsTZF1-OX plants had higher photosynthetic activities than the control or OsTZF1-RNAi plants according to Fv/Fm measurements (Supplemental Fig. 5). Similarly, two-week-old rice seedlings grown in soil were subjected to drought stress. Plants were allowed to recover under normal growth conditions after stress treatment (Supplemental Fig. 6). More than 60%
of the OsTZF1-OX rice plants survived compared to only 38% in the control. The survival rate of the OsTZF1-RNAi plants was around 21% to 23% compared to 46% in the controls (Supplemental Fig. 6). These results demonstrated that OsTZF1 positively regulates high-salt stress tolerance and drought stress tolerance in rice plants.

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OsTZF1 Confers Increased Tolerance to Oxidative Stress

In plants, the primary symptoms of senescence are largely mediated by dramatic increases in reactive oxygen species (ROS; Thompson and Lake, 1987; Leshem, 1988). Using rice leaf fragments (4–5 cm in length), we tested the response of OsTZF1-OX, control and OsTZF1-RNAi plants to dark-induced leaf senescence, UV-C exposure, and 20 mM H₂O₂. After six days dark treatment, leaf yellowing was observed in the control, whereas the leaves of the OsTZF1-OXs remained green (Fig. 6A). In contrast, the OsTZF1-RNAi leaf fragments exhibited more yellowing than controls (Fig. 6A). Under normal growth conditions, no diaminobenzidine (DAB) staining was observed in either controls or OsTZF1-OXs, but enhanced DAB staining was observed in the OsTZF1-RNAi leaf fragments (Fig. 6A). Exposure to UV-C for three days caused significant yellowing and accumulation of DAB in the OsTZF1-RNAi leaves compared to controls whereas less yellowing or DAB staining was observed in the OsTZF1-OX plants (Fig. 6B). The role of ROS in rice leaf senescence was confirmed directly by treating rice leaf fragments with 20 mM H₂O₂ solution for three days. There was less or no effect of H₂O₂ treatment on the OsTZF1-OX leaf fragments, while enhanced leaf yellowing and DAB staining was observed in OsTZF1-RNAi plants (Fig. 6C). These results suggested that the delayed leaf senescence phenotype of OsTZF1-OX plants was because of enhanced tolerance to oxidative stress.

We evaluated the effect of darkness on Fv/Fm in detached leaf fragments. When leaf fragments were kept in the dark for six days, the photochemical efficiency of the OsTZF1-RNAi, control, and OsTZF1-OX dropped to 18 ± 1.6%, 38.0 ± 4.5%, and
74 ± 2.7%, respectively (Fig. 6D). These data were in agreement with the phenotypes of leaves (Fig. 6A–C).

The oxidation of lipids and proteins during membrane injury by ROS was evaluated by measuring plasma membrane electrolyte leakage (Fig. 6E). Senescing leaf fragments of OsTZF1-RNAi showed a marked increase in ion leakage, from an average of 11% to 40% and 42%, indicating membrane damage, whereas the increase in the ion leakage of controls was 12% to 36%. In contrast, the OsTZF1-OX leaf fragments maintained low electrolyte leakage (18% and 20%) even after six days of dark treatment. These results provided strong evidence of an enhanced tolerance of OsTZF1-OX plants to membrane damage during leaf senescence.

The effect of darkness alone or with JA, ABA and NaCl on leaf senescence was also evaluated using leaf fragments from Ubi:OsTZF1-OX and P_{OsTZF1}:OsTZF1-OX plants (Supplemental Fig. 7). In each case, OsTZF1-OX leaves showed delayed senescence compared to controls. Surprisingly, the delayed senescence in \( P_{OsTZF1}:OsTZF1 \)-OXs under these conditions was more pronounced than in \( Ubi:OsTZF1 \)-OXs (Supplemental Fig. 7).

**Global Gene Expression Changes Regulated by OsTZF1**

To elucidate the molecular mechanism of stress tolerance conferred by OsTZF1 in rice, microarray analyses were carried out using the 44K rice oligo microarray (Agilent Technologies, Inc.). Two independent OsTZF1-OX lines (OsTZF1-OX#6 and OsTZF1-OX#9) were used for the microarray analysis. A total of 198 genes showed > 2.0-fold changes in the OsTZF1-OXs compared to the control. Among them, 119 genes were up regulated and 79 genes were down regulated in the OsTZF1-OXs (Supplemental Table S1). Based on microarray analysis of stress-inducible genes (Maruyama et al., unpublished), of the 119 up regulated genes in OsTZF1-OX rice plants, 27 genes were induced by high-salt stress (Supplemental Table S1) and 55 genes were induced by dehydration stress (Supplemental Fig. 8; Supplemental Table S1). Of
the 79 down regulated genes in the OsTZF1-OXs, 28 and 32 genes were down regulated by high-salt and dehydration stress, respectively (Supplemental Fig. 8; Supplemental Table S1). Among the up regulated genes in the OsTZF1-OXs were genes encoding proteins with predicted functions in stress tolerance, such as dehydrins, detoxification (GSTs), redox homeostasis (Ferritin and Metallothionin), NAD(P)H-dependent oxidoreductases and transcription factors (Supplemental Table S2). We also identified genes encoding proteins with predicted functions in biotic stress responses, such as pathogenesis-related proteins, chitinases and peroxidases (Supplemental Table S2). Among the 79 down regulated genes in OsTZF1-OXs were genes involved in heavy metal and oligopeptide transport (OPT), polyamine and secondary metabolism (Supplemental Table S3). Quantitative RT-PCR (qRT-PCR) analysis confirmed the regulation of some of these genes in OsTZF1-OXs (Fig. 7).

The OsTZF1-OX plants showed improved high-salt stress tolerance (Fig. 5). To examine the role of OsTZF1 in the transcriptional network in response to high-salt stress, we compared the expression profiles of OsTZF1-OX and control plants grown in soil for two weeks and then treated with 250 mM NaCl solution for two days using the 44K rice oligo microarray. After two days of high-salt stress treatment, 2,051 genes were up regulated and 2,141 genes were down regulated in the OsTZF1-OXs (Fig. 8A; Supplemental Table S4). Surprisingly, out of 2,051 up regulated genes in OsTZF1-OXs, 885 and 1,459 genes were normally down regulated by high-salt and dehydration stresses, respectively (Fig. 8A; Supplemental Fig. 8; Supplemental Table S3). Conversely, out of 2,141 down regulated genes in the OsTZF1-OX, 1,048 and 1,561 genes were normally up regulated by high-salt and dehydration stresses, respectively (Fig. 8A; Supplemental Fig. 8; Supplemental Table S3). According to gene expression atlas data sources (http://signal.salk.edu/RiceGE/RiceGE_gAtlas_Source.html) and our unpublished microarray data (Maruyama et al., unpublished), a significant number of genes showing decreased expression levels in the OsTZF1-OX plants are normally responsive to salt stress and dehydration, indicating that either OsTZF1-OXs are
impaired in stress responsive gene expression or that they exhibit attenuated stress response and in turn attenuated stress responsive gene expression.

To confirm these results, the expression of genes encoding representative stress-related proteins identified by the microarray analysis, including isocitrate lyase, β-glucanase, RD22, YSL6, an N-amino transporter, the beta-subunit of SnRK1 (akin-β) and allene oxide synthase (AOS), were analyzed during high-salt stress in OsTZF1-OX and control plants (Fig. 8B). Soil-grown two-weeks-old plants were subjected to 250 mM NaCl stress for three days. The stress-responsive expression of the above genes was delayed or reduced in OsTZF1-OX compared to control plants (Fig. 8B, left panel).

The OsTZF1-OX plants exhibited a delayed senescence phenotype; therefore, the expression of above genes was also examined in relation to dark-induced senescence. Two-week-old OsTZF1-OX and control plants grown in soil were subjected to dark-induced senescence and expression of the above genes was examined using RNA gel-blot analysis. Interestingly, reduced senescence-induced expression of the above genes was observed in OsTZF1-OXs compared to controls (Fig. 8B, right panel). Similar results were obtained when the expression of these genes was analyzed in UV-C exposed OsTZF1-OX and control plants (data not shown).

Consistent with the above results, the induction of native OsTZF1 expression in response to high-salt stress was also lower in OsTZF1-OXs than control plants treated with 250 mM NaCl stress for three days; however positive upregulation of some stress-related genes was also observed (Supplemental Fig. 9). Of the 2,051 up regulated genes in OsTZF1-OX treated with NaCl for two days, only 51 and 86 genes are normally up regulated by high-salt and dehydration stresses, respectively (Fig. 8A; Supplemental Fig. 8; Supplemental Table S3). Expression analysis of the representative up regulated stress responsive genes, including Tic32, RNS4 and WRKY45, showed positive regulation by high-salt stress in OsTZF1-OX plants (Supplemental Fig. 9). These results reveal that the majority of stress-related genes show an inverse relationship in the microarray analysis of OsTZF1-OX and control plants treated with
250 mM NaCl.

**OsTZF1 Regulates Metal and Oligopeptide Transport Related Genes**

The microarray analysis revealed that many genes related to biotic stress response were up regulated by OsTZF1 (Supplemental Table S1). Furthermore, genes like Ferritin, MT-type1 and ChaC-like were up regulated and OsNRAMP1, Feroportin1 and oligopeptide transporters (OPT) were down regulated (Fig. 7; Supplemental Table S2, S3). The OsTZF1-OX plants consistently showed a lesion-mimicking phenotype after heading and during seed setting. To test if the lesion-mimicking phenotype of the OsTZF1-OXs was from nutrient deficiency or toxicity, we grew OsTZF1-OX and control plants in two sets of soil. One set was watered with tap water and the other set was maintained with water that had been pH adjusted to 9.5, as the availability of many nutrients including P, N, Mn, Fe, Zn and Cu depends on optimal soil pH (Islam et al., 1980). The OsTZF1-OXs watered with tap water developed lesions upon entering the heading and seed setting stages. The other set of OsTZF1-OXs, maintained with water at pH 9.5, developed no or few lesions (Supplemental Fig. 10A). We determined the contents of Fe, Mn and Zn and metalloid B, in the leaves of controls and OsTZF1-OXs at the heading stage. We found that OsTZF1-OX had slightly reduced Fe accumulation and greater accumulation of Mn, however, these differences were not significant between the OsTZF1-OX and control plants (Supplemental Fig. 10B).

**OsTZF1 Binds RNA In Vitro**

Previously, it was shown that plant TZFs bind DNA or RNA through *in vitro* bead binding assays (Pomeraz et al., 2010). We performed *in vitro* binding assays on recombinant OsTZF1-GST protein and homopolymers of A, U, C, and G using the RNA gel electrophoresis mobility shift assay (REMSA). We observed that OsTZF1 could only bind to U homopolymers, whereas GST alone could not bind to any of the homopolymers (Fig. 9A).
Down regulated genes with U-rich sequences in their 3’-UTR were selected from the microarray analysis of OsTZF1-OX. Two such genes were the beta-subunit of SnRK1 (akin-β) and allene oxide synthase (AOS). The expression of these genes was increased under high-salt stress in control plants; however, their expression remained relatively low in OsTZF1-OXs compared to controls (Fig. 8B). Because akin-β and AOS were down regulated in OsTZF1-OX plants and contain U-rich and AU rich elements (ARE-like motifs; akin-β 5’-UUUUUUUUUAAUUCUCUCUUGUUUCUU-3’, AOS-I 5’-GUUCGAUUCUAAUUGUAAUUUGUUGUAAU-3’ and AOS-II 5’-UUGUUGCUUGAAUACGAUGUGUAUUUA-3’) in their 3’-UTRs, we performed REMSA to determine whether OsTZF1 binds ARE and ARE-like motifs. As shown in Fig. 9B, OsTZF1 formed a complex with an ARE sequence used as a positive control, which caused a band shift. A band shift was also observed with the ARE-like motifs of akin-β and AOS (Fig. 9B). No band shift was observed when GST protein was used (Fig. 9B). Using a protein gradient of OsTZF1, an increase in the band shift was observed for the ARE-like motifs of akin-β and AOS (Fig. 9C, left panel). Furthermore, this shift was abolished when the respective competitors of akin-β and AOS were included in the experiment (Fig. 9C, right panel). Together, these results demonstrated that OsTZF1 binds to U-rich regions, AREs and ARE-like motifs.

DISCUSSION

In humans, TZFs control the expression of cytokines whereas in yeast TZF homologs (Cth1 and Cth2) promote reprogramming of Fe-dependent metabolism and Fe-storage through control of target mRNA half-lives (Blackshear et al., 2005; Puig et al., 2008). Characterization of plant-unique TZF genes has revealed their importance in development and different environmental responses (Li and Thomas, 1998; Kim et al., 2008; Grabowska et al., 2009; Guo et al., 2009; Lin et al., 2011; Lee et al., 2012), but
their exact molecular mechanisms are still unknown. To determine the function of OsTZF1, we examined its expression profiles and investigated the gain- and loss-of-function phenotypes. Our results indicated that OsTZF1 is involved in seed germination, seedling growth, leaf senescence and oxidative stress tolerance. The OsTZF1-OXs exhibited phenotypes of reduced and delayed seed germination (Fig. 4A). Young rice seedlings were smaller, but grew normally once transplanted into soil (Fig. 4B; Supplemental Fig. 3). In contrast, OsTZF1-RNAi plants showed early and enhanced seed germination and grew faster than controls (Fig. 4A, B), suggesting that OsTZF1 plays a negative regulatory role during seed germination and seedling growth.

Arabidopsis SOMNUS (AtC3H2), a homolog of OsTZF1, was reported to be a negative regulator of phytochrome-mediated seed germination and a positive regulator of ABA accumulation/negative regulator of GA accumulation through its effects on ABA/GA metabolic genes (Kim et al., 2008). AtTZF1 was also considered to affect ABA/GA mediated growth and stress responses (Lin et al., 2011). Since ABA/GA metabolic or signaling related genes were not detected in our microarray analysis, it is not clear at this stage whether OsTZF1 is involved in ABA/GA mediated growth responses.

Delayed senescence of the OsTZF1-OX rice was supported by the testing of different senescence-inducing factors (Fig. 6; Supplemental Fig. 7). The degrees of leaf yellowing and the rates of chlorophyll loss induced by darkness, UV-C and H2O2 in detached OsTZF1-OX leaves were significantly lower than in the controls (Fig. 6). Senescence is affected not only by age but also by multiple other factors (Lim et al., 2007). JA-, ABA- and stress (high-salt)-induced senescence was also delayed in OsTZF1-OX plants (Supplemental Fig. 7), indicating that the delayed senescence might be due to a common factor like tolerance to oxidative stress.

The expression of OsTZF1 was induced by ABA, JA, SA, H2O2 and several abiotic stresses (Fig. 1A, B), suggesting that it is involved in multiple stress responses. High-salt stress at the seedling stage caused OsTZF1-RNAi plants to exhibit chlorosis and damage to leaves whereas OsTZF1-OXs showed enhanced tolerance (Fig. 5,
Supplemental Fig. 7). DAB staining revealed higher H$_2$O$_2$ accumulation in control and OsTZF1-RNAi plants compared to OsTZF1-OXs (Fig. 6C). Furthermore, OsTZF1-OX seedlings showed enhanced tolerance to drought stress compared to control or OsTZF1-RNAi plants (Supplemental Fig. 6). Analysis of Arabidopsis mutants with delayed senescence phenotypes has revealed that extended longevity results in increased stress tolerance, for example, *gigantea*, *ore1*, *ore3*, *ore9* and *jub1* mutants showed increased stress tolerance (Kurepa et al., 1998; Woo et al., 2004; Wu et al., 2012). Due to an imbalance between production and scavenging of ROS, senescence is triggered by oxidative stress and delayed senescence in plants is correlated with increased tolerance to oxidative stress (Muller et al., 2007; Wu et al., 2012). Our results suggest that OsTZF1 confers tolerance to abiotic stresses in rice by enhancing tolerance to oxidative stress.

Transcriptome analysis of OsTZF1-OX plants revealed the upregulation of many biotic and abiotic-stress-related genes, including pathogenesis-related proteins and transcription factors, peroxidases, dehydrins, metal detoxifying proteins and ROS homeostasis genes (Supplemental Table S2). On the other hand, genes involved in metal and oligopeptide transport, and polyamine and secondary metabolism were down regulated in the OsTZF1-OXs (Supplemental Table S3). Our results indicate that OsTZF1 functions in conferring tolerance to high-salt, drought and oxidative stress in addition to delaying leaf senescence in rice. At the moment it is not known precisely how TZFs regulate gene expression changes in plants. We showed that OsTZF1 binds to the RNA of some down regulated genes containing U-rich and ARE-like motifs in their 3’-UTR (Fig. 9). It is possible that OsTZF1 is involved in RNA turnover processes, leading to changes in gene expression.

The analysis of transcriptome expression profiles of OsTZF1-OX and control plants treated with 250 mM NaCl for two days revealed that out of the 2,051 up regulated genes in OsTZF1-OX, 885 and 1,459 genes are normally down regulated by high-salt stress and dehydration stress, respectively (Fig. 8A; Supplemental Fig. 8B).
Interestingly, among the 2,141 down regulated genes in the OsTZF1-OX rice plants, 1,048 and 1,561 genes are normally up regulated by high-salt stress and dehydration stress, respectively. The inverse relationships of these stress inducible genes upon high-salt stress emphasize that OsTZF1-OX exhibits attenuated stress response and in turn attenuated stress response gene expression (Fig. 8A, B; Supplemental Fig. 8B). In other words, OsTZF1-OX and control plants exhibited different induction ratios of stress-inducible genes under two-day salt stress (Fig. 8B). The atszf1-1 atszf2-1 double mutant is sensitive to high-salt, but exhibits higher induction of stress-inducible genes than controls (Sun et al., 2007). In Arabidopsis, a series of hos (high expression of osmotic responsive genes) mutants, including hos1 (Lee et al., 2001), hos5 (Xiong et al., 1999), hos9 (Zhu et al., 2004) and hos10 (Zhu et al., 2005), have shown higher expression of stress-responsive genes than controls in response to abiotic stresses. The Arabidopsis mutants fiery1 (fry1) and fiery2 (fry2) displayed induction of ABA- and stress-responsive genes (Xiong et al., 2001; Xiong et al., 2002). Surprisingly, all of these mutants were compromised in their tolerance to the tested abiotic stress conditions. These mutants are reminiscent of our microarray data showing an inverse relationship between the induction of stress-responsive genes and stress tolerance.

Leaves at different stages of development respond differently to identical treatments, for example older leaves respond to ethylene but younger leaves do not (Grbic and Bleecker, 1995; Louis et al., 1998). The chloroplast biogenesis protein Tic32 was up regulated in OsTZF1-OX plants under both normal and stress conditions (Supplemental Fig. 9). This might lead to differential chloroplast development in OsTZF1-OX and control plants and subsequently differential retrograde responses. Taking into consideration the delayed senescence phenotype and tolerance to ROS of the OsTZF1-OXs, their stress-hyposensitive response is understandable.

The OsTZF1-OX plants displayed a lesion-mimicking phenotype during heading and seed setting (Fig. 4C). Microarray analysis revealed many genes related to biotic stress response (Supplemental Tables S2, S3). The products of these genes might
be responsible for the lesion-mimicking phenotype. Furthermore, the up regulation of Ferritin, and MT-type1 and ChaC-like proteins and the down regulation of OsNRAMP1, Feroportin1 and oligopeptide transporters (OPT) points towards a role for metal ions in the lesion-mimicking phenotype of OsTZF1-OX plants (Fig. 7; Supplemental Tables S2, S3). High pH (9.5) water rescued the lesion-mimicking phenotype in OsTZF1-OX plants (Supplemental Fig. 10A). The toxicities of Fe and Mn produce necrotic phenotypes and the absorption of Fe and Mn are particularly perturbed by high pH. To address the question directly, we determined the metal contents of Fe, Mn, Zn and metalloid B in the leaves of control and OsTZF1-OX plants at the heading stage. The results showed that OsTZF1-OXs had slightly reduced Fe accumulation and higher accumulation of Mn, however, these differences were not significant between OsTZF1-OXs and controls (Supplemental Fig. 10B). Furthermore, high pH treatment lowered the overall content of the above metals, but there was little difference between OsTZF1-OXs and controls (data not shown). This inconsistency might be due to the differential accumulation of metal ions in different tissues, organs and cells of rice plants. It is also possible that other undetermined metal ions, unknown xenobiotics or oligopeptides are responsible for the lesion-mimicking phenotype of OsTZF1-OXs. For example, rice Sekiguchi lesion (sl) mutants accumulate tryptamine, a candidate substrate for serotonin biosynthesis (Fujiwara et al., 2010). Expression of the SL gene (Os12g0268000) was down regulated in OsTZF1-OX plants treated with NaCl for two days (Supplemental Table S4). Further work is required to determine the precise function of OsTZF1 in relation to this lesion-mimicking phenotype in rice.

Most of the CCCH finger proteins reported to date (e.g. OsDOS, HUA1, PEI1, AtSZF1 and SOMNUS) are localized in the nucleus and function as transcriptional regulators, except HUA1, which is an RNA-binding protein (Li and Thomas 1998, Li et al., 2001; Kong et al., 2006; Sun et al., 2007; Kim et al., 2008). We showed that OsTZF1 is predominantly localized in the cytoplasm and cytoplasmic foci under stress conditions (Fig. 3). Although OsTZF1 was occasionally observed in the nucleus
(Supplemental Fig. 2), no transcriptional activation was observed in relation to OsTZF1 (data not shown), and it probably does not function as a transcriptional regulator. Some studies have revealed that CCCH zinc finger proteins affect mRNA stability, as mentioned earlier (Addepalli and Hunt, 2008; Pomeranz et al., 2010); thus, it can be speculated that OsTZF1 participates in RNA metabolism. To address whether OsTZF1 has RNA binding activity, we carried out REMSA. The results showed that OsTZF1 binds U-rich, ARE and ARE-like motifs (Fig. 9). This observation suggests that the OsTZF1 protein is likely to be involved in RNA metabolism. During the preparation of this manuscript, Lee et al. (2012) reported that the Arabidopsis CCCH-type zinc finger proteins AtC3H49/AtTZF3 and AtC3H20/AtTZF2 are involved in ABA and JA responses and that their recombinant proteins displayed RNase activity in vitro, suggesting that they might be involved in the mRNA turnover process. Although OsTZF1 could bind to U-rich and ARE-like motifs in the 3’-UTR regions of akin-β and AOS, it remains to be established whether they are OsTZF1’s real target mRNAs and how it affects their metabolism.

Based on the available experimental data showing that OsTZF1 transcription is activated by ABA, JA, SA, H₂O₂ and abiotic stresses such as high-salt or drought stress and it confers stress tolerance; we suggest that OsTZF1 assists in regulating cellular ROS homeostasis. OsTZF1 expression lowers ROS concentration in plant tissues, through redox homeostasis genes like Ferritins/MTs, and ROS scavenging enzymes such as peroxidases and GSTs (Supplemental Table S1), while the opposite effect may be speculated in the OsTZF1-RNAi plants. Concomitant with enhanced OsTZF1 expression and reduced ROS accumulation, the induction ratio of significant numbers of stress-related genes was compromised in two-day-NaCl treated OsTZF1-OX plants. We propose that OsTZF1 lowers the cellular ROS level, thereby minimizing the stimulatory effect on stress-related genes. In addition, there is the possibility that OsTZF1 regulates stress tolerance and senescence through other target genes of currently unknown molecular function. Future work will be required to address the intricacies of the
underlying regulatory network in greater detail.

METHODS

Plant Materials and Growth Conditions
Rice (Oryza sativa ssp. japonica var. Nipponbare) was used for various experiments in this study, including expression analysis, transformation and hormone treatments as described previously (Ito et al., 2006; Nakashima et al., 2007). Seedlings of rice were grown in basal nutrient solution (Makino et al., 1988). Two-week-old rice plants were dehydrated in a plastic box for dehydration treatment. Two-week-old rice plants were transferred from the basal nutrient solution to nutrient solutions containing 250 mM NaCl, 10 mM H$_2$O$_2$, 10 µM ABA, 10 µM methyl jasmonate (MeJA), or 100 µM salicylic acid (SA) for stress treatments.

RNA Gel-blot Analysis and Quantitative PCR analysis
Isolation of total RNA, RNA gel-blot hybridization and qRT-PCR analysis were performed as described previously (Nakashima et al., 2007). The 5' end-specific DNA fragment (ca. 300 bp) of the OsTZF1 full-length-cDNA was used as probe for hybridization. Gene-specific probes were used for the other genes in the RNA gel-blot analysis (see supplemental Table-S5). The primers used in qRT-PCR are listed in Supplemental Table S5.

Analysis of Transgenic Rice Plants Containing OsTZF1 promoter:GUS fusions
The promoter region of OsTZF1, 1,417 bp upstream of ATG (P$_{OsTZF1}$), was amplified from the rice genome by PCR using KOD DNA polymerase (Toyobo, http://www.toyobo.co.jp/bio). The primers used are listed in Supplemental Table S5. The OsTZF1 promoter fragment was inserted into the Smal site of the GUS expression vector pGreenII 0129 (Hellens et al., 2000), generating P$_{OsTZF1}$:GUS. Confirmation of
the fusion constructs, transformation of rice and growth of the transgenic plants were performed as described previously (Ito et al., 2006; Nakashima et al., 2007). Histochemical activity of GUS in \( P_{OsTZF1} \)-GUS transgenic plant materials was detected according to Jefferson et al. (1987). Quantitative analysis of GUS activity was performed as described previously (Nakashima and Yamaguchi-Shinozaki, 2002).

**Transient and Stable Expression of the sGFP Proteins**

The OsTZF1 coding region was fused to sGFP (Chiu et al., 1996; Nakashima et al., 1997) using the pGreenII 0129 vector (Hellens et al., 2000) either with an ubiquitin or OsTZF1 native promoter, as indicated in the text. These constructs were used for transient and stable expression of sGFP proteins. For transient expression, the DNA constructs were introduced into onion (Allium cepa) epidermal cells as described previously (Fujita et al., 2004). After incubation at 22°C for 12 h, GFP fluorescence was observed using a confocal laser scanning microscope LSM5 PASAL (Carl Zeiss; http://www.zeiss.com/). Stable transgenic rice plants expressing OsTZF1-sGFP under the native OsTZF1 promoter were generated as described above. Cellular localization of the OsTZF1-sGFP proteins in the lateral root cells of the transgenic rice plants was observed as described above. For co-localization, the OsTZF1 coding region was fused with YFP, and the DCP2 and PABP8 coding regions were fused with CFP in-frame in the pGreenII vector. These constructs were introduced into rice mesophyll protoplasts and observed as described above.

**Analysis of OsTZF1-OX and OsTZF1-RNAi Plants**

To generate transgenic rice plants over-expressing OsTZF1 (OsTZF1-OX), we used the pBIG-ubi vector (Becker, 1990; Ito et al., 2006). For the generation of RNA knock-down plants (OsTZF1-RNAi), the pANDA vector was used (Miki et al., 2005). The primers used are listed in Supplemental Table S5. We introduced the constructs into wild-type rice cv. Nipponbare by Agrobacterium-mediated transformation (Goto et
al., 1999), and T2 and T3 seeds were used in further experiments. Transgenic plants (OsTZF1-OX and OsTZF1-RNAi) and vector controls were grown in the isolation greenhouse under normal growth conditions (14 h light at 28°C/ 10 h dark at 25°C).

5 Stress Tolerance of the Transgenic Rice Plants
For high-salt stress treatment, two-week-old rice seedlings grown in soil were transferred to 250 mM NaCl solution for three days. After the stress treatment, plants were allowed to recover under normal growth conditions for two weeks. The number of plants that survived and continued to grow were counted. The statistical significance of the values was determined using the chi-squared test. Photosynthetic activity (Fv/Fm) was measured according to the method described by Kasuga et al. (2004). For drought stress treatment, two-week-old rice seedlings grown in soil were dehydrated. After the stress treatment, plants were allowed to recover under normal growth conditions for two weeks. The numbers of plants that survived and continued to grow were counted. The statistical significance of the values was determined using the chi-squared test.

Senescence Testing Using Leaf Fragments
Leaf fragments from six-week-old OsTZF1-OX and OsTZF1-RNAi rice plants were used in experiments with dark, UV-C and H2O2 treatments. For UV-C treatment, leaf fragments in petri plates covered with lids were incubated under 15 W UV-C tube light (254 nm) for three days. For H2O2 treatment, detached leaf fragments were incubated in water (mock) and solution containing 10 mM H2O2 for the time periods indicated. The respective leaf fragments were used for detection of ROS by 3,3’-diaminobenzidine (DAB) staining (Thordal-Christensen et al., 1997). For dark treatment, leaf fragments from six-week-old OsTZF1-OXs were incubated in water (mock) or solutions containing 10 µM ABA, 10 µM MeJA or 250 mM NaCl in the dark for five days.

Microarray Analysis
Two-week-old seedlings of OsTZF1-OX and control rice grown in soil, untreated or treated with 250 mM NaCl for two days, were harvested, and total RNA was isolated by the TRIzol method (Invitrogen; http://www.invitrogen.com/). Total RNA was used for the preparation of Cy5- and Cy3-labeled cRNA, and these were subjected to microarray experiments using the 44K rice oligo microarray (Agilent Technologies Inc., http://www.home.agilent.com). All microarray experiments, including data analysis, were carried out as described previously (Nakashima et al., 2007). The microarray data for "OsTZF1_untreated" were assigned accession number E-MEXP-3700 and the microarray data for "OsTZF1_NaCl 2days" were assigned accession number E-MEXP-3701 in MIAMExpress (www.ebi.ac.uk/miamexpress/).

Measurement of Fe, Mn, Zn and B in OsTZF1-OX and control plants.
Metal contents in OsTZF1-OX and control plants were measured by ICP-OES (SPS-3500, SII, Chiba, Japan). Shoots were dried for 24 h and dissected into pieces by a pair of ceramic scissors. The dried samples (ca. 100 mg) were carefully weighed in Teflon cups and decomposed by nitric acid in Teflon bombs heated at 150 °C for 10 h. The sample solutions were made to 10 mL with 0.1 M nitric acid and filtrated by PTFE-membrane (0.45 mm) before analysis.

RNA Binding Assay
REMSAs were conducted as described by Brewer et al. (2004). RNA probes were synthesized by cloning probe sequences into the pBluescript KS+ containing an upstream T7 promoter. The primers used are listed in Supplemental Table S5. Plasmid vectors were linearized using the KpnI restriction enzyme and then used for RNA synthesis labeled with [32P]α-UTP using the Promega T7 Riboprobe Kit. Binding reactions consisted of each protein being incubated with $1 \times 10^6$ cpm of various radiolabeled RNA probes. Each reaction was assembled in a final volume of 20 µL containing 10 mM Tris-HCl (pH 8.0), 40 mM KCl, 2 mM dithiothreitol, 3 mM MgCl$_2$,
50 µM ZnCl₂, 10 mM HEPES, 10% glycerol, and heparin (20 µg µL⁻¹). Binding reactions were incubated at room temperature for 10 min and then separated using electrophoresis through a 6% (40:1 acrylamide:bis-acrylamide) native gel at 4°C. After electrophoresis, gels were exposed using phosphorescence imaging (Fuji Film, Japan).

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**Figure legends**

**Figure 1.** Expression profiles of OsTZF1 under different stress and hormone treatments. (A) RNA gel-blot analysis of OsTZF1 expression under different stress conditions. Hydroponically grown two-week-old rice plants were dehydrated or transferred to solutions containing 10 μM ABA, 250 mM NaCl, 10mM H2O2 or water (H2O) for the time periods indicated. (B) Hormone-dependent expression analysis of OsTZF1. Two-week-old rice plants grown hydroponically were transferred to nutrient solution containing 10 μM ABA, 10 μM methyl jasmonate (MeJA) or 100 μM salicylic acid (SA) for the time periods indicated. (C) Quantitative GUS analysis of control and \( P_{OsTZF1} \)-GUS transgenic rice plants under ABA and NaCl treatments. Plants were treated...
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Figure 2. Expression profiles of OsTZF1 in different rice organs. (A) RNA gel-blot analysis of OsTZF1 expression in different organs. For organ-specific expression, total RNA was extracted from callus, roots, shoot bases, seedling leaves, four leaves at bolting stage, nodes, internodes, panicles before dehiscence (PBD) and panicles after dehiscence (PAD). Histochemical analysis of $P_{\text{OsTZF1}}:\text{GUS}$ gene activities in different tissues and organs of rice (B–F). Callus (B), three-days old seedlings (C), 10-day old seedlings (D), panicles before dehiscence (E) and panicles after dehiscence (F) derived from the $P_{\text{OsTZF1}}:\text{GUS}$ transgenic plants were incubated in GUS staining solution for 12 h. Bars = 2 mm.

Figure 3. Sub-cellular localization of OsTZF1 and co-localization with cytoplasmic foci marker genes. (A) Sub-cellular localization of OsTZF1. Stably transformed transgenic rice plants expressing the OsTZF1-sGFP chimeric gene, driven by the native promoter, were analyzed by fluorescence microscopy. OsTZF1-sGFP transgenic rice plants were either untreated or treated with 10 µM ABA or 50 mM NaCl for 12 h. (B) Co-localization of OsTZF1 with cytoplasmic foci marker genes. OsTZF1 is localized in cytoplasmic foci that resemble p-bodies (PBs) and stress granules (SGs). OsTZF1 co-localizes with PB marker DCP2 (upper panel). OsTZF1 and SG marker PABP8 co-localize in SGs (lower panel).

Figure 4. Phenotypic analysis of OsTZF1-OX and OsTZF1-RNAi rice plants. (A) Seed germination phenotypes of control, OsTZF1-OX and OsTZF1-RNAi plants during the five days after imbibition (DAI). Graph curves indicate the growth of the control,
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**Figure 5.** Analysis of high-salt stress tolerance in control, OsTZF1-OX and OsTZF1-RNAi plants. Seeds in equivalent germination states were sown in soil and subjected to 250 mM NaCl salt stress as described in the experimental procedures. The results are the average of four independent experiments with 12 plants per experiment. Plants marked with asterisks (*) had significantly higher survival rates than the controls as determined using the chi-squared test ($P < 0.01$).

**Figure 6.** Analysis of delayed leaf senescence and ROS tolerance in control, OsTZF1-OX and OsTZF1-RNAi plants. (A) OsTZF1-OXs showed a delay in dark-induced leaf senescence. Detached leaf fragments from control, OsTZF1-OX, and OsTZF1-RNAi plants were incubated in water for six days in the dark. The treated leaf fragments were stained with 3,3’-diaminobenzidine (DAB, 1 mg/ml) solution to visually detect H$_2$O$_2$. (B) The OsTZF1-OXs showed delayed UV-C induced leaf senescence. Detached leaf fragments from control, OsTZF1-OX, and OsTZF1-RNAi plants were incubated in water for three days under UV-C. The treated leaf fragments were stained with DAB solution to visually detect H$_2$O$_2$. (C) OsTZF1-OXs showed delayed H$_2$O$_2$-induced leaf senescence. Detached leaf fragments from control, OsTZF1-OX, and OsTZF1-RNAi plants were incubated in 10 mM H$_2$O$_2$ solution for three days under dark conditions. The treated leaves were stained with DAB solution to visually detect H$_2$O$_2$. (D) OsTZF1-OXs showed high photosynthetic activities during dark-induced leaf senescence. The photosynthetic activities (Fv/Fm) of detached leaf fragments from control, OsTZF1-OX, and OsTZF1-RNAi plants were measured at 0, 2,
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**Figure 7.** qRT-PCR analysis of metal homeostasis-related genes identified in OsTZF1-OX plants by microarray analysis. Two-week-old, soil grown OsTZF1-OX and control plants were used to isolate RNA. The analyzed genes, whose expression was up regulated in OsTZF1-OX, were Ferritin (Os11g0106700), MT-type1 (Os12g0568500), ChaC-like (Os02g0465900). The down regulated genes analyzed were OsNRAMP1 (Os07g0258400), Ferroportin1 (Os06g0560000) and OPT (Os03g0751100).

**Figure 8.** Transcriptome analysis of genes regulated in OsTZF1-OX under high-salt stress. (A) Venn diagram showing the relationship between genes regulated in OsTZF1-OX treated with 250 mM NaCl for two days and NaCl stress inducible genes. In total, 2,051 and 2,141 genes were up and down regulated, respectively, in OsTZF1-OXs treated with 250 mM NaCl for two days in relation to similarly treated controls. (B) RNA gel-blot analysis of genes regulated in OsTZF1-OX identified by microarray analysis. Two-week-old rice plants treated with 250 mM NaCl for 0, 1, 2 and 3 days or subjected to dark-induced senescence for 0, 3, 6 and 9 days were used to isolate RNA. Probes were prepared from the 3’-UTRs of Isocitrate lyase (Os07g0529000), 1,3;1,4 β-glucanase (Os05g0375400), RD22 (Os10g0409400), YSL6 (Os08g0508000), akin-β (Os05g0491200), N-amino transporter (Os02g0191300) and AOS (Os03g0225900) as described in the experimental procedures.

**Figure 9.** RNA binding studies of OsTZF1. (A) OsTZF1 binds U homopolymers. REMSA showed that OsTZF1 forms a complex with U-homopolymers. GST alone did not bind any homopolymers. (B) OsTZF1 binds U-rich ARE-like sequences in the
3’-UTR of akin-β and AOS. An ARE sequence was used as a positive control (left). (C) OsTZF1 protein gradient and competition assays. For the protein gradient, the OsTZF1 protein was increased to 4-fold. For competition assays, corresponding cold probes were used. OsTZF1-RNA complexes are marked with arrowheads.
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(A) OsTZF1-OXs showed a delay in dark-induced leaf senescence. Detached leaf fragments from control, OsTZF1-OX, and OsTZF1-RNAi plants were incubated in water for six days in the dark. The treated leaf fragments were stained with 3,3'-diaminobenzidine (DAB, 1 mg/ml) solution to visually detect H₂O₂. (B) The OsTZF1-OXs showed delayed UV-C induced leaf senescence. Detached leaf fragments from control, OsTZF1-OX, and OsTZF1-RNAi plants were incubated in water for three days under UV-C. The treated leaf fragments were stained with DAB solution to visually detect H₂O₂. (C) OsTZF1-OXs showed delayed H₂O₂-induced leaf senescence. Detached leaf fragments from control, OsTZF1-OX, and OsTZF1-RNAi plants were incubated in 10 mM H₂O₂ solution for three days under dark conditions. The treated leaves were stained with DAB solution to visually detect H₂O₂. (D) OsTZF1-OXs showed high photosynthetic activities during dark-induced leaf senescence. The photosynthetic activities (Fv/Fm) of detached leaf fragments from control, OsTZF1-OX, and OsTZF1-RNAi plants were measured at 0, 2, 4 and 6 days during dark-induced leaf senescence. (E) OsTZF1-OXs showed reduced ion leakage during dark-induced leaf senescence. The ion leakage of detached leaf fragments from control, OsTZF1-OX, and OsTZF1-RNAi plants were measured at 0, 2, 4 and 6 days during dark-induced leaf senescence.
Figure 7. qRT-PCR analysis of metal homeostasis-related genes identified in OsTZF1-OX plants by microarray analysis.

Two-week-old, soil grown OsTZF1-OX and control plants were used to isolate RNA. The analyzed genes, whose expression was up regulated in OsTZF1-OX, were Ferritin (Os11g0106700), MT-type1 (Os12g0568500), ChaC-like (Os02g0465900). The down regulated genes analyzed were OsNRAMP1 (Os07g0258400), Ferroportin1 (Os06g0560000) and OPT (Os03g0751100).
Figure 8. Transcriptome analysis of genes regulated in OsTZF1-OX under high-salt stress.

(A) Venn diagram showing the relationship between genes regulated in OsTZF1-OX treated with 250 mM NaCl for two days and NaCl stress inducible genes. In total, 2,051 and 2,141 genes were up and down regulated, respectively, in OsTZF1-OXs treated with 250 mM NaCl for two days in relation to similarly treated controls. (B) RNA gel-blot analysis of genes regulated in OsTZF1-OX identified by microarray analysis. Two-week-old rice plants treated with 250 mM NaCl for 0, 1, 2 and 3 days or subjected to dark-induced senescence for 0, 3, 6 and 9 days were used to isolate RNA. Probes were prepared from the 3'-UTRs of isocitrate lyase (Os07g0529000), (1,3;1,4) β-glucanase (Os05g0375400), RD22 (Os10g0409400), YSL6 (Os08g0508000), akin-β (Os05g0491200), N-amino transporter (Os02g0191300) and AOS (Os03g0225900) as described in the experimental procedures.
Figure 9. RNA binding studies of OsTZF1. (A) OsTZF1 binds U homopolymers. REMSA showed that OsTZF1 forms a complex with U-homopolymers. GST alone did not bind any homopolymers. (B) OsTZF1 binds U-rich ARE-like sequences in the 3’-UTR of akin-β and AOS. An ARE sequence was used as a positive control (left). (C) OsTZF1 protein gradient and competition assays. For the protein gradient, the OsTZF1 protein was increased to 4-fold. For competition assays, corresponding cold probes were used. OsTZF1-RNA complexes are marked with arrowheads.