



The thioredoxin gene family in rice: Genome-wide identification and expression profiling under different biotic and abiotic treatments

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ABSTRACT

Thioredoxin (TRX) is a multi-functional redox protein. Genome-wide survey and expression profiles of different stresses were observed. Conserved amino acid residues and phylogeny construction using the OsTRX conserved domain sequence suggest that the TRX gene family can be classified broadly into six subfamilies in rice. We compared potential gene birth-and-death events in the OsTRX genes. The Ka/Ks ratio is a measure to explore the mechanism and 3 evolutionary stages of the OsTRX genes divergence after duplication. We used 270 TRX genes from monocots and eudicots for synteny analysis. Furthermore, we investigated expression profiles of this gene family under 5 biotic and 3 abiotic stresses. Several genes were differentially expressed with high levels of expression and exhibited subfunctionalization and neofunctionalization after the duplication event response to different stresses, which provides novel reference for the cloning of the most promising candidate genes from OsTRX gene family for further functional analysis.

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1. Introduction

Thioredoxin (TRX) is a general designation for small proteins and conserved WC(G/P)PC motif to catalyze thioldisulfide interchanges [1]. According to their intrinsic biochemical activity, TRX proteins can regulate various redox biochemical pathways and play a significant role in the maintenance of cellular redox homeostasis [2]. Although numerous variants of TRX proteins exist, in plants, two cysteine (Cys-X-X-Cys) residues in the active site provide sulfhydryl (SH) groups that are involved in a reducing activity. The reduced form of thioredoxin, free TRX-(SH)₂, reduces disulfide bonds of target proteins, whereas the oxidized form, TRX-S₂, contains a disulfide bond within the active site, which is reduced to TRX-(SH)₂ dithiol by NADPH and TRX reductases [3].

Six different types (*m*, *f*, *x*, *y*, *o*, and *h*) of TRXs have been reported in plants according to their function and localization within the cell. The four types of TRX (*f*, *m*, *x*, and *y*) are chloroplastic proteins, while the type *h* has a wide distribution in cytoplasm and is reduced by mitochondrial or cytoplasmic NADPH-dependent TRX reductases [4]. Thioredoxins play a vital role during diverse functions of plant life, including translation, protein assembly/folding, protein repair after damage by oxidation, hormone synthesis, metabolisms, enzymatic activations, pathogen, plant development and seed germination, mitogen-activated protein (MAP) kinase pathway,

self-incompatibility reactions, biotic, and abiotic stress responses [3,5–13]. The aim of this study was to determine the TRX genes classification based on domain and protein sequences, gene evolution, synteny, and gene expression profiles (GEPs) under different kinds of biotic and abiotic treatments. Expression of selected differentially expressed genes (DEGs) was validated using semi-quantitative and quantitative PCR. These results provide a solid basis for future functional genomic research of the TRX genes.

2. Materials and methods

2.1. Database searches and phylogenetic analysis

HomoloGene is a system for automated detection of homologs among the genes of completely sequenced eukaryotic genomes. In this study we conducted extensive searches for TRX homologs in public databases (see in details in the [Supplementary materials](#)).

Multiple alignment analyses were performed using CLUSTAL_X version 1.83 [[Supplementary reference, SR1](#)]. The unrooted phylogenetic tree was constructed with MEGA3.1 [[SR2](#)] by the neighbor-joining method and bootstrap analysis (1000 replicates).

2.2. Gene locations on chromosomes and duplications

OsTRX was located on rice chromosomes according to the positions specified in the <http://rice.plantbiology.msu.edu> [[SR3](#)]. We

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identified genome duplications of rice in the <http://rice.plantbiology.msu.edu> with a maximum permitted distance between collinear gene pairs of 500-kb (http://rice.plantbiology.msu.edu/segmental_dup/index.shtml). We considered genes to be tandemly duplicated if two *OsTRX* genes were separated by three or fewer gene loci according to the <http://rice.plantbiology.msu.edu>. These genes were not included previously [SR4].

2.3. Synteny analysis and Ka/Ks computing

Syntenic information from all examined genes was collected from the Plant Genome Duplication Database (PGDD, <http://chibba.agtec.uga.edu/duplication>) and using the Codeml procedure of the PAML program [SR5] the rate of non-synonymous substitution (K_a), the rate of synonymous substitutions (K_s) and K_a/K_s were determined. The dates of the duplication events were calculated by the equation $T = K_s/2\lambda$, for rice, the $\lambda = 6.5 \times 10^{-9}$ [SR6].

2.4. Biotic and abiotic treatments

Rice seedlings were grown on absorbent tissue paper at 28 °C under a 16-h light:8-h dark photoperiod for 10 days and were exposed to the various stress treatments: cold (24-h, 48-h, and 72-h, incubation at 10 °C); drought (1-h, 09-h, and 24-h, with the addition of 25% polyethylene glycol 6000 to the planter box), and laid-down submergence (6-h, 24-h, and 48-h). Control and treatment seedlings were harvested and stored at –80 °C until RNA extraction. Methods used to infect rice plants with biotic stresses were published by Satoh et al. [SR7].

2.5. Microarray experiment

Cyanine-3 (Cy3) and cyanine-5 (Cy5)-labeled target complementary RNA (cRNA) samples were prepared from 850 ng total mRNA using a fluorescent linear amplification kit (Agilent Technologies) in accordance with the manufacturer's instructions. Transcriptome profiles specific to stressed plants were examined by the direct comparison of transcription activities between stressed and non-stressed plants on the same oligoarray. We hybridized 1-μg of fluorescent linear-amplified, Cy3- and Cy5-labeled cRNA (825 ng) to a customized rice oligo microarray. The fragmented cRNAs were added to the hybridization buffer, applied to the microarray, and hybridized for 17-h at 60 °C. The scanned microarray images were analyzed using Feature Extraction 6.1.1 software (Agilent Technologies), and the dye-normalized, background-subtracted intensity and ratio data were exported to a text file. This software flags corrupted spots and detects a lack of differences between sample spots and the background.

2.6. Data analysis

For five biotic [*Rice grassy stunt virus* (RGSV; GSE25217), and *Rice dwarf virus* (RDV; 3-strain RDV-D84, -O, and -S; GSE24937), *Rice black-streaked dwarf virus* (RBSDV), *Rice ragged stunt virus* (RRSV), and *Rice transitory yellowing virus* (RTYV)] and three abiotic stress treatments (cold and drought, GSE2415; submergence, GSE7532), we used 44 K and 22 K-microarray data available at NCBI-GEO except RBSDV, RRSV, and RTYV. The Cy3 and Cy5 signal intensities were normalized using rank-consistency filtering and the LOWESS method, processed by Feature Extraction version 9.5 (Agilent Technologies). Expression patterns of all biotic (3-repeat) and abiotic samples (2-repeat) were transformed into \log_2 -based numbers and normalized according to the quantile method for standardization of among array slides by EXPANDER version 5.0 [SR8]. Expression of a gene (up-or down-regulated) was defined as a gene with a \log_2 -based ratio (stressed samples/control) > 0.585 or < –0.585;

and a significant difference in GEPs between the treated plants and the control indicated by $P \leq 0.05$ by paired t -test (permutations, all possible combinations; FDR collection, adjusted Bonferroni method). Data processing was done with MeV version 4.3.

2.7. RT-PCR and qRT-PCR

DEGs were analyzed using RT-PCR and qRT-PCR and methods of these PCR were described previously [SR7,SR9]. The gene primers are listed in Supplementary Table 1.

3. Results

3.1. Classification of *OsTRX* proteins

Based on the detailed results of Pfam search, the sixty-one *OsTRX* proteins were classified into 6 subfamilies based on their domain compositions (Fig. 1). Thirty-nine members merely with one TRX domain (e.g., domain 1) belonged to subfamily TRX-A, while seven members contained two TRX domains (e.g., domain 1 and domain 2) and assigned to TRX-B. Besides TRX domain, *OsTRX* proteins containing several other known functional domains were classified into the following subfamilies. Three members containing the tetratricopeptide repeat domain were identified as TRX-TPR subfamily; 2 members containing the endoplasmic reticulum protein domain were identified as subfamily TRX-ERp29; 3 members with the ferredoxin thioredoxin reductase variable alpha chain domain were identified as TRX-FeThRedA subfamily; TRX-O subfamily (7 members) contained other domains including Evr1_Alr, PAPS_reduct, COPII coated_ERV, Pyr_redox_2, tify_CCT_2, Glutaredoxin, and NB-ARC (Supplementary Fig. 1). The differences and comparison of classification of *OsTRX* and *TRX* gene family among 6 species were investigated (Table 1).

3.2. Phylogenetic analysis

To evaluate the phylogenetic relationship among the *OsTRX* genes and infer the evolutionary history of this gene family, a combined phylogenetic tree was constructed with the aligned *OsTRX* protein sequences and classified into five groups according their domain organization (Supplementary Fig. 2). In addition, we observed that most members in the same groups shared one or more domains outside the TRX domain, further supporting the subfamily definition above. For example, all members of the TRX-ERp29, TRX-FeThRedA and, TRX-TPR subfamilies were assigned to groups A and C, respectively. However, the members of TRX-A and TRX-O were distributed in almost all the groups speculating that the functions of those members were diversified. In general, most of the closely related members in the phylogenetic tree had very similar domain composition.

3.3. Evolutionary expansion of the *TRX* genes

Gene duplication has greatly impacted the amplification of this gene family in the genome. Gene duplication is the primary driving force in the development of new gene functions in the evolution of genetic systems and genomes [14]. Sixty-one *OsTRX* genes were localized on the 12 chromosomes with an obviously uneven distribution. *Os06g22140* gene was positioned around the centromere on chromosome 6, whereas *Os02g01010* was located near a telomeric region on chromosome 2 (Supplementary Fig. 3). To elucidate the potential mechanism of evolution of the *OsTRX* gene family, we analyzed segmental and tandem duplication events, identifying 7 segmental duplication events in rice (Supplementary Table 2). Approximately 21% of the *OsTRX* family might have evolved from

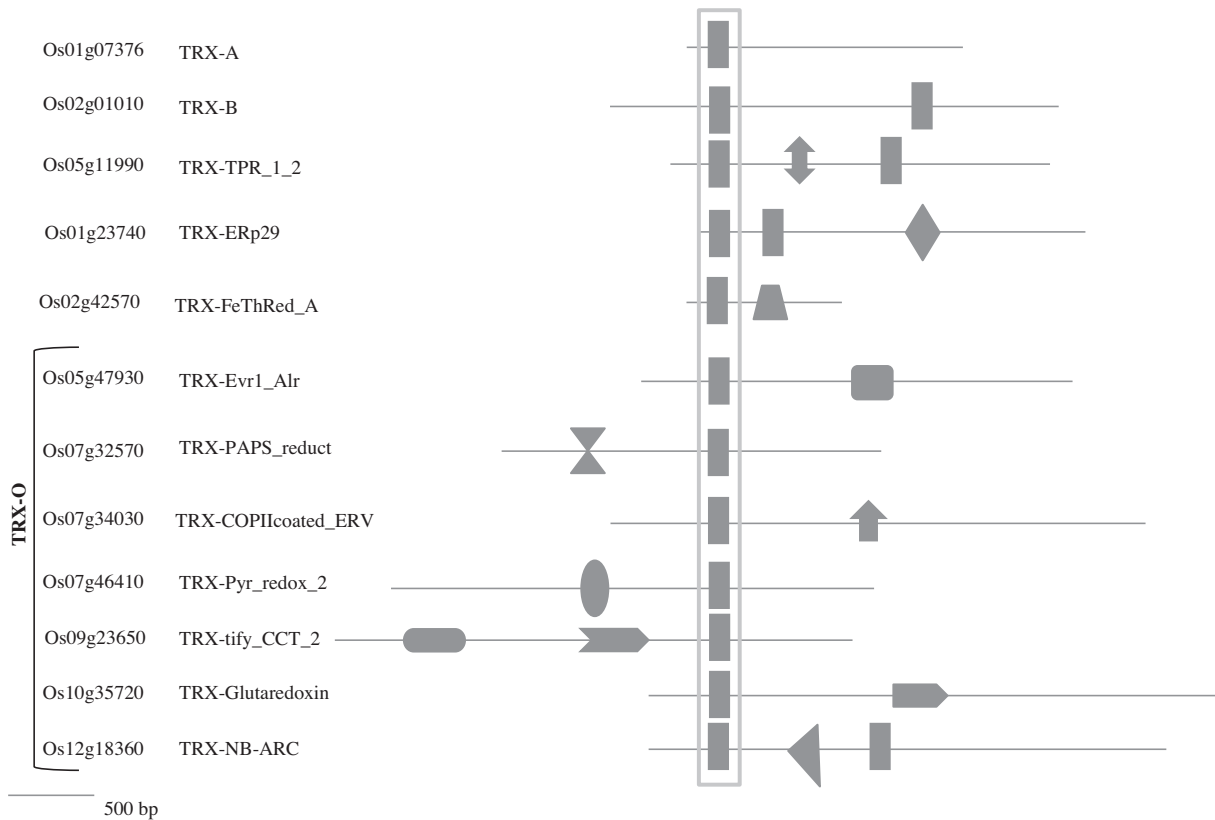


Fig. 1. Structure of representative OsTRX protein from each subfamily. Subfamily and gene name are given on the left side and in Table 1.

Table 1

Number of each subfamily of TRX proteins in different species.

Subfamily	Description	Rice	Arabidopsis	Sorghum	Maize	Soybean	Poplar
TRX-A	Thioredoxin domain 1	39	32	2	15	25	16
TRX-B	Thioredoxin domain 1 and domain 2	7	10	5	8	12	9
TRX-TPR_1_2	Tetratricopeptide repeat	3	6	–	2	5	3
TRX-ERp29	Endoplasmic reticulum protein Erp29 (C-terminal domain)	2	1	2	3	4	3
TRX-FeThRed_A	Ferredoxin thioredoxin reductase variable alpha chain	3	3	–	3	3	3
TRX-O	TRX protein with other domain	7	22	2	3	2	2
Total		61	74	11	36	51	36

putative rice genome duplication events. In rice, six *OsTRX* genes were involved in tandem duplications, consisting of 3 clusters in rice (Supplementary Table 3). Therefore approximately 9% of *OsTRX* are organized in clusters and likely have evolved via tandem duplications.

3.4. Duplication and evolution analysis of the *OsTRX* genes

The K_a/K_s ratio is a measure to explore the mechanism of gene divergence after duplication. $K_a/K_s = 1$ means neutral selection, $K_a/K_s < 1$ means purifying selection, and $K_a/K_s > 1$ means accelerated evolution with positive selection. We calculated 10 duplicated pairs in the *OsTRX* gene family (Supplementary Table 4). The K_a/K_s ratio of 9 pairs (e.g., *Os01g07376/Os05g07690*) were less than 1, suggesting purifying selection on these 9 duplicated pairs; however, the K_a/K_s ratio of *Os04g35150/Os04g35290* was more than 1, suggesting positive selection on the one duplicated pair.

Duplication events for 3 pairs (*Os01g07376/Os05g07690*, *Os02g42700/Os04g44830*, and *Os03g21000/Os07g48510*) occurred within last 70 to 50 million years, after origin of grasses and before divergence of rice and maize, according to the first whole-genome duplication events of grass genomes [15]. Duplication events for

4 pairs (*Os02g42570/Os04g44650*, *Os03g58630/Os07g09310*, *Os02g42570/Os02g42700*, and *Os04g44650/Os04g44830*) occurred within last 50 to 20 million years, after divergence of rice and maize, but before Zizaniinae and Oryzinae were separated from each other [16]. Duplication events for the other three pairs (*Os02g34940/Os04g35600*, *Os01g23740/Os05g06430*, and *Os04g35150/Os04g35290*) occurred within last 20 to 9 million years, after Zizaniinae and Oryzinae were separated, and before *Oryza* genus was branched off from the remaining genera of Oryzeae [16]. Therefore, evolutionary origin of the 61 TRX genes might undertake 3 evolutionary stages (Supplementary Table 4).

Genes in different species and related by a speciation event are defined as orthologs. PGDD is a public database that identifies and catalogs plant genes in terms of cross-genome syntenic relationships [17]. As data only of syntenic relationships within the angiosperm are available, 270 TRX genes from monocots and eudicots were used in this analysis. We found that redundant 38 *OsTRX* genes containing a TRX domain can be detected in syntenic blocks in selected species. The number of duplicated genes detected by synteny analysis is 15 of TRX-A, 9 of TRX-B, 5 of TRX-ERp29 and of TRX-O, and 4 of TRX-FeThRed_A (Supplementary Table 5). It is suggested that many plant genomes underwent one to several

large scale duplication events in their long evolutionary history, in which duplicated functional genes were preferentially retained. This view provides an explanation for the expansion of many families in the plant kingdom. Whole genome duplication events within or between species can account for most of the expansion of the TRX family.

3.5. GEPs under different treatments

To establish infection in plants, viruses require host factors for their replication, cell-to-cell and long-distance movement. To counterattack virus infection, the plant has evolved different defense mechanisms including up-or down-regulating specific genes with different functions [18]. The TRX protein localized exclusively to chloroplasts in coordination with the maintenance of cellular reducing conditions, which accompanied an elevation in the

glutathione disulfide couple ratio. Viral infection might trigger a rapid induction of reactive oxygen species (ROS) [19]. During defense responses, ROS are produced by plant cells because of the enhanced enzymatic activities of plasma-membrane-bound NADPH oxidases, cell-wall bound peroxidases [20]. To gain insight into the comprehensive roles of the OsTRX family members in response to various stresses, their expression patterns were investigated in rice seedlings subjected to biotic (RBSDV, RGSV, RRSV, RTYV, and RDV) and abiotic (cold, drought, and laid-down submergence) by microarray analysis. Only the genes, whose expression change was at least 1.5-fold increase or decrease, were considered response to above stresses. Among the OsTRX 61, twenty-four genes showed differential expression in at least one stress or at least one time point of treatment compared to the control. The number of genes up-regulated was highest at 28 DAI under RRSV infection, followed by 28, 24, and 21 DAI (listed in decreasing order) under

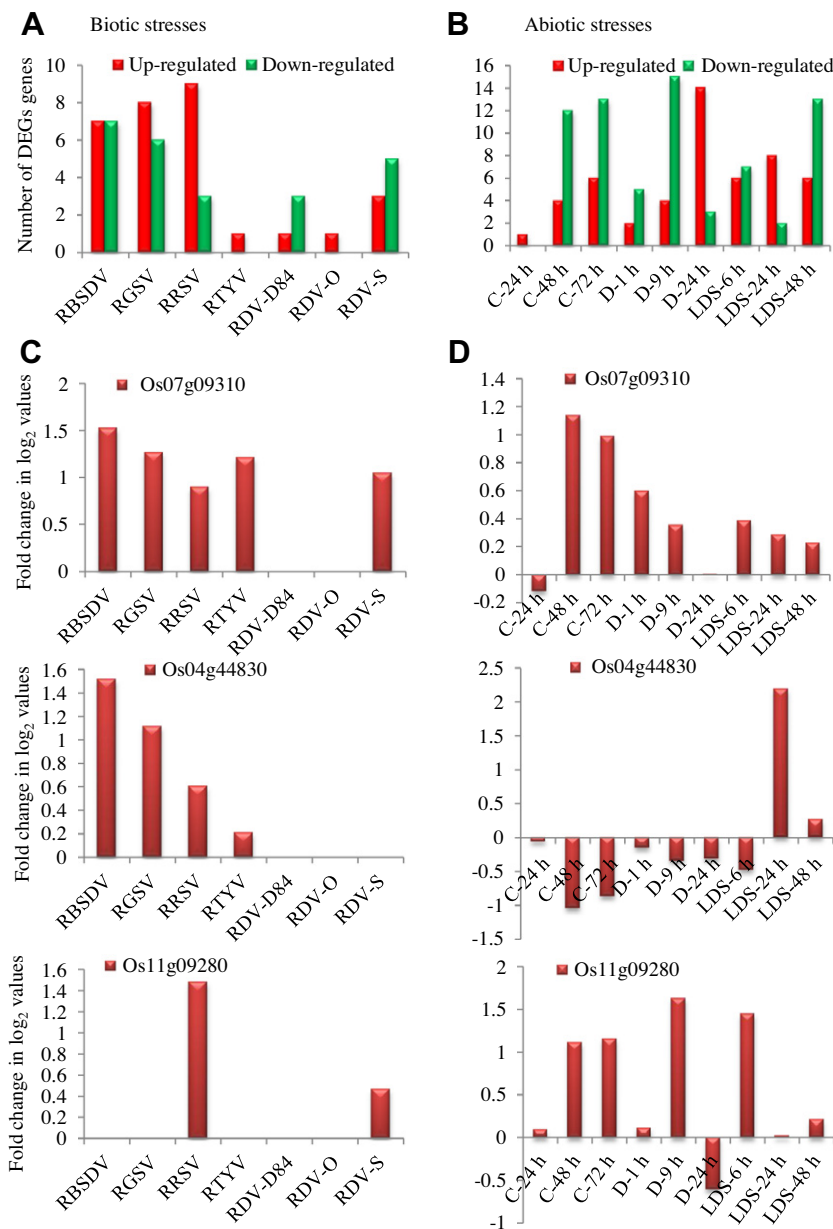


Fig. 2. Number of DEGs is under (A) Biotic and (B) Abiotic stresses. Identification of the reference genes exhibited highest expression under (C) Biotic and (D) Abiotic stresses. Y-axis represents the number of DEGs and fold changes in log₂ values and treatments are indicated on the X-axis. Elaborations of treatments are in the Supplementary Tables 6 and 7.

RBSDV, RGSV, RTYV, and RDV infections (Fig. 2A), although multiplication of viruses may be inhibited by plant defense system under RRSV infection. It may suggest that the defense system in the host was not activated in timely manner to suppress virus replication. Furthermore, to elucidate the basis of differences in symptom severity caused by three RDV strains at the GEPs, the number of DEGs was different among the plants infected with three RDV strains (Fig. 2A). Of these DEGs in seedlings under RDV infections, none of the genes were shared in common in strains (Supplementary Table 6). Generally, the degree of gene response to RDV-O infection was lowest and the response to RDV-S infection was highest (Fig. 2A). The degree of gene activation by RDV-D84 infection was closer to that by RDV-S infection than to that by RDV-O infection, whereas the degree of gene suppression by RDV-84 was closer to that by RDV-O infection (Fig. 2A). The number of activated genes was highest in RDV S-strain and then decreased in the order of strain -D84 and strain -O (Fig. 2A). In case of RBSDV, RGSV, RRSV, and RTYV up \geq down, whereas in the case of RDV, up $<$ down. Defense systems are activated in RBSDV, RGSV, and RRSV, but in RTYV and RDV (except RDV-S) host was nearly death. One of the host defense systems against virus infection is the gene silencing system. The expression of the genes involved in the gene silencing system is often activated by virus infection [21]. Comparatively speaking, the result of GEPs analysis for plants infected with five viruses suggested inter-correlations among the numbers of DEGs, the degrees of gene responses.

In response to abiotic treatments, the greatest number of genes was up-regulated under drought stress (D-24-h), and the lowest number was up-regulated under cold stress (C-24-h) (Fig. 2B, Supplementary). Out of 24 DEGs, 9 (e.g., *Os04g44830*) were up-regulated at least one virus infections or one time point of abiotic stress conditions, whereas *Os02g01010*, *Os03g58130*, and *Os02g53400* were down-regulated or not differentially expressed under abiotic treatments. The specific TRX genes that were activated during the different biotic and abiotic stresses response are of particular interest. This study focused on DEGs with high levels of expression in response to different stresses. We identified *Os07g09310*, *Os04g44830*, and *Os11g09280* the most promising candidate genes for novel reference in the specific stress conditions (Fig. 2C,D), which might play functional roles in the specific stress conditions. This may be because proteolytic processing is a major scheme regulating the OSTRX activities. Further work is required to distinguish these possibilities.

3.6. GEPs of duplicated *OsTRX* genes

Divergence of GEPs plays a very vital role in the preservation of duplicated genes and demonstrated three novel functions: non-functionalization, neofunctionalization, and subfunctionalization [22]. We performed the expression patterns of segmentally and tandemly duplicated genes under different abiotic stress conditions. Probes were matched to 4 of the 7 genes located in segmentally duplicated regions. One pair (*Os03g58630* and *Os07g09310*) of genes exhibited highly similar expression patterns under all the stresses, indicating subfunctionalization after the duplication events (Fig. 3A). Similarly, we found three clusters of tandemly duplicated *OsTRX* genes. Two clusters of the genes probe set were found in our 22 K-microarray data and their (e.g., *Os02g42570* and *Os02g42700*) expression profiles were dissimilar; indicate neofunctionalization (Fig. 3B). Based on the diverse roles, these duplicated genes have dissimilar functions under stress conditions.

3.7. RT-PCR/qRT-PCR

We selected 11 DEGs from various stresses, and examined the similarity between gene responses observed by microarray and

those by RT-PCR/qRT-PCR. Most cases of activation or suppression of GEPs detected by microarray were also observed by RT-PCR/qRT-PCR, although the degree of the response was different for some genes (Fig. 4).

4. Discussion

The objectives of this study were to determine the expression patterns of members of this gene family under different stresses and select the most appropriate candidate genes for further func-

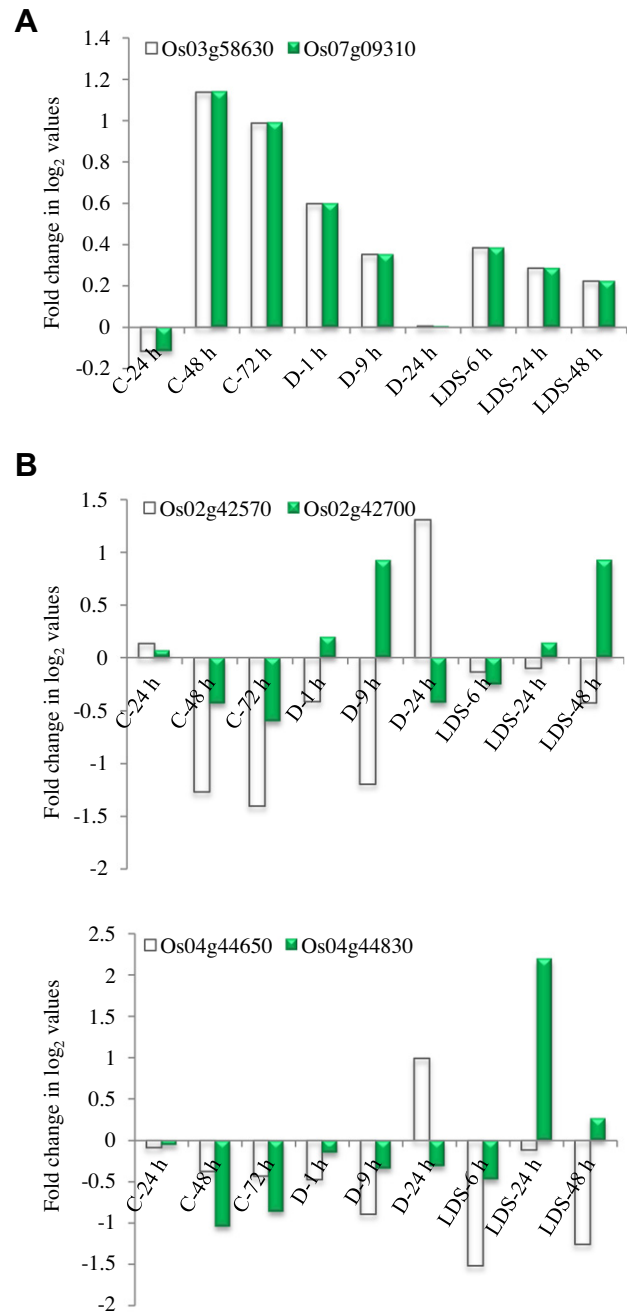


Fig. 3. Examples of expression patterns of *OsTRX* genes found in duplicated regions of the rice genome. Expression patterns of two *OsTRX* genes found in (A) Segmentally and (B) Tandemly duplicated *OsTRX* genes. The different stresses are shown on the X-axis and the fold change in \log_2 values on the Y-axis. Elaboration of abiotic stresses is in the Supplementary Table 7.

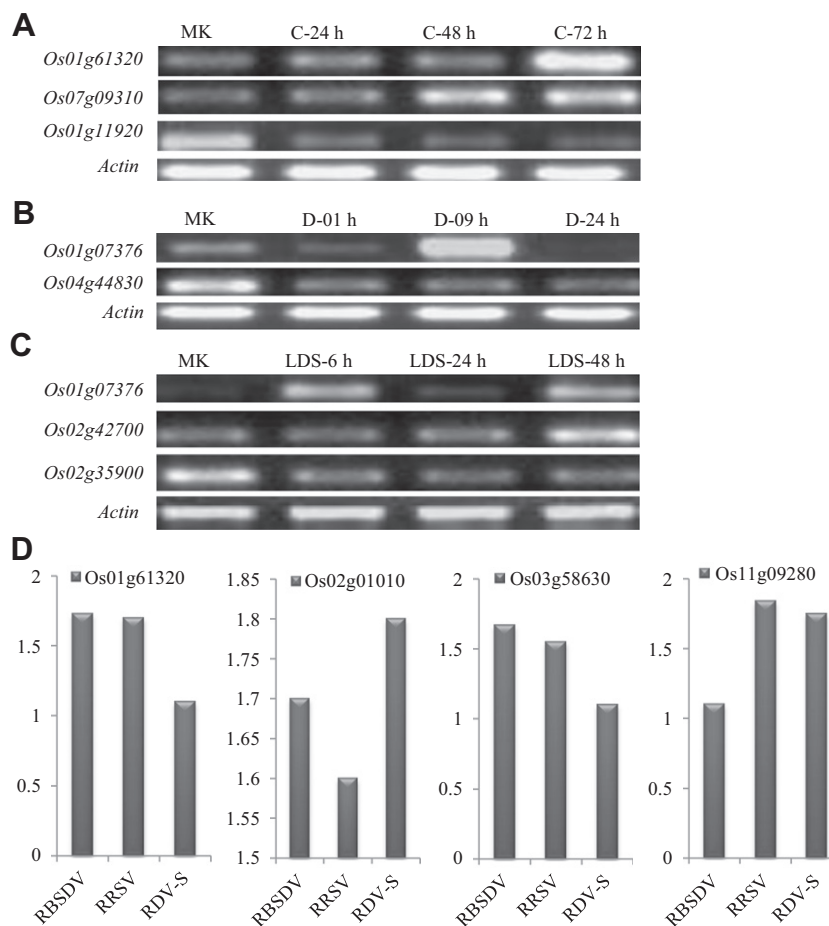


Fig. 4. (A–C) RT-PCR and (D) qRT-PCR analysis of DEGs is under abiotic and biotic stresses. MK = control; Elaborations of treatments are in the [Supplementary Tables 6 and 7](#).

tional analysis. Our results show that the TRX domain is such kind of common and conserved domain and is widely present in all land plants and other vascular plants.

4.1. TRX gene multiplication and purifying selection

Most gene families have multiple members and the reason for this could be the variable status of whole genome duplications in plants [23]. Therefore, we consider the number of TRX genes to have increased rapidly during the course of evolution that whole genome duplication and tandem/segmental duplication played a key role in the expansion of TRX genes in rice ([Supplementary Fig. 3](#)) and that have been shown in the F-box family [24]. In particular, the number of genes of subfamily TRX-A in rice is higher among 6 species, conversely the genes belonging to TRX-O subfamily are three times in Arabidopsis than rice, suggesting that genes of those subfamilies gone multiple duplications to gain more members ([Table 1](#)). We suggested the evolutionary expansion of the *OsTRX* gene family in rice genome underwent three stages based on the dates of the duplication events ([Supplementary Table 4](#)). After the duplication events of these genes, gene retention and loss always occur in the long evolutionary history. The retention and loss of TRX genes varied in each subfamily. We believe that the genes in the TRX family were under purifying selection for their functional importance ([Fig. 3](#)). Increased genes in these subfamilies might be the evolutionary consequences of adjusting to environmental changes and the gene duplication is species-specific.

4.2. *OsTRX* genes roles under different stresses

Regulation of protein activity by changes in the redox status thiol groups (reduced by NADPH and ferredoxin) is now known to occur in all organisms. Virus genomes often encode a protein to inhibit the gene-silencing process in host cells (silencing suppressor) in order for viruses to propagate in host cells [22]. Virus infection activated many genes likely related to the RNAi process. For example, *SHOOTLESS4 (SHL4)* in rice is the gene encoding a component of the trans-acting siRNA process for endogenous genes, which is one of the post-transcriptional gene silencing processes [6]. The greatest number of genes of TRX family was up-regulated by RRSV infection and lowest number gene was activated by RTYV. Jasmonic acid and hormones are signal molecule for the regulation of a defense system against biotic stress. Gibberellic acid induced *GAST*-like genes in petunia encode proteins containing putative catalytic disulfide bonds (redox-active cysteines) and involved in redox regulation [7]. Several TRX genes that confer enhanced disease resistance to *Tobacco/Cucumber mosaic virus* when overexpressed, e.g., *NtTRXh3* [8]. Gene responses by three RDV strains can be largely categorized into two types; i) responses that are similar in plants infected with RDV-D84 and RDV-S, and ii) responses that are similar in plants infected with RDV-O and RDV-D84 ([Fig. 2A](#)). The first response is mainly found in genes inactivated by RDV infection, such as the degree of suppression in plants infected with RDV-D84 is lower than that with RDV-S. The suppression of host gene expression compared in *Nicotiana* plants infected with RNA virus *Cymbidium ringspot virus* showed that the severe suppression of host genes was associated with

the development of severe symptoms [9]. The amino acid changes in virus proteins are also associated with the disease symptoms. Second, the gene is activated by RDV infection and such genes involved in stress and defense processes and correlated with RDV-D84 and -O strains. Therefore, the activation of genes for defense processes may be related to symptom development. The functions of *TRX* genes are dependent on the types of domain encoded in the genes. The responses of *TRX* genes seem to be dependent on the encoded domain types, which may be related to distinctive gene functions.

Transcriptome analyses have generated considerable data, which show extensive overlapping on GEPs between biotic and abiotic stresses. Recently, thioredoxin-regulated *BAM1* activates a starch degradation pathway in illuminated mesophyll cells upon osmotic stress in *Arabidopsis* [10]. MAP kinases are key signal-transducing enzymes that are activated by a wide range of extracellular stimuli. *AtMPK3* and *AtMPK6* have been implicated in multiple abiotic stresses tolerance [11]. Rice orthologs of *AtMPK3* and *AtMPK6*, *OsMPK3/OsMAP1/OsWIPK* and *OsMPK6/OsSIPK*, respectively, are also involved in stress responses [12]. A cold-induced *TRX-h* of rice, *OsTrx23*, has an inhibitory activity on stress-activated MAP kinases of *OsMPK3* and *OsMPK6* in vitro [13]. We found that certain members of *TRX* gene family are involved in response to stresses (Fig. 4), which are bioinformatically predicted. Overexpression and RNAi analyses of those genes are underway in our laboratory and these experiments will help to the deeper understanding of gene regulatory network pathways.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.bbrc.2012.05.142>.

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