

# Sequence and expression analysis of the thioredoxin protein gene family in rice

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**Abstract** Thioredoxin (Trx) proteins play important biological functions in cells by changing redox via thioldisulfide exchange. This system is especially widespread in plants. Through database search, we identified 30 potential Trx protein-encoding genes (*OsTrx*) in rice (*Oryza sativa* L.). An analysis of the complete set of *OsTrx* proteins is presented here, including chromosomal location, conserved motifs, domain duplication, and phylogenetic relationships. Our findings suggest that the expansion of the Trx gene family in rice, in large part, occurred due to gene duplication. A comprehensive expression profile of Trx genes family was investigated by analyzing the signal data of this family extracted from the whole genome microarray analysis of Minghui 63 and Zhenshan 97, two *indica* parents, and their hybrid Shanyou 63, using 27 different tissues representing the entire life cycle of rice. Results revealed specific expression of some members at germination transition as well as the 3-leaf stage during the vegetative growth phase of rice. *OsTrx* genes were also found to be differentially up- or down-regulated in rice seedlings subjected to treatments of phytohormones and light/dark conditions. The expression levels of the *OsTrx* genes in the different tissues and under different treatments were also checked by RT-PCR

analysis. The identification of *OsTrx* genes showing differential expression in specific tissues among different genotypes or in response to different environmental cues could provide a new avenue for functional analyses in rice.

**Keywords** *Oryza sativa* · Phylogenetic analysis · Expression profile · Phytohormone

## Abbreviations

Trx	Thioredoxin
SH	Sulfhydryl
ATP	Adenosine triphosphate
GA <sub>3</sub>	Gibberellic acid
NAA	Naphthalene acetic acid
KT	Kinetin
RT-PCR	Reverse transcription polymerase chain reaction
Cys	Cysteine
FBPase	Fructose 1, 6-bis phosphatase
NADPH	Nicotinamide adenine dinucleotide phosphate
TIGR	The Institute for Genomic Research

## Introduction

Thioredoxins (Trxs) are small proteins (around 12 kDa) that are presented in virtually all organisms from Archea to humans, and function in a spectrum of biochemical pathways. Trx protein was first identified as the source of reducing equivalents to ribonucleotide reductase in *Escherichia coli* (Laurent et al. 1964; Holmgren 1981, 1985). Trxs are ubiquitous proteins that perform an important role during various aspects of plant life, including enzymatic activation, photosynthesis, photorespiration, reactions associated with the citric acid cycle, lipid metabolism, electron transport,

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ATP synthesis/transformation, membrane transport, translation, protein assembly/folding, nitrogen metabolism, sulfur metabolism, hormone synthesis and stress responses (Gelhay et al. 2004; Raines 2005; Balmer et al. 2006a, b).

Trx proteins in the extracellular environment or on the cell surface are rich in stabilizing disulfides (reflecting oxidizing conditions), kept in reduced forms with many free sulfhydryl (SH) groups and rare disulfides inside of cells (Gilbert 1990). According to Holmgren (1985), Trxs are the major ubiquitous disulfide reductase responsible for maintaining proteins in their reduced state with electrons from NADPH.

Because Trx, with a dithiol/disulfide active site (CGPC), acts as a cellular protein disulfide reductase, it exchanges reactions via redox active disulfides and plays important roles in electron transport for essential redox enzymes such as ribonucleotide reductase, which is required to provide deoxyribonucleotides for DNA synthesis. Thiol-disulfide exchange reactions are rapid, readily reversible and also ideally suitable to control protein function via the change of redox state of structural or catalytic SH groups. Oxidation of a critical SH group generally leads to a change in biological function. The basic processes of thiol modulation have been established based on extensive research on both the Calvin cycle and other redox-sensitive enzymes such as glyceraldehyde 3-phosphate dehydrogenase, FBPase, sedoheptulose 1, 7-bis phosphatase, and phosphoribulokinase (Buchanan 1991). This mechanism of thiol redox control (Holmgren 1985) is emerging as a major regulatory mechanism in signal transduction. Increased production of reactive oxygen species oxidizes protein thiols and does balance by Trx- and glutathione glutaredoxin-dependent reactions have a wide range of functions in cellular physiology (Arner and Holmgren 2000). Although numerous variants of Trx proteins exist, especially in plants, they have a common function in the formation of disulfide bond between the two active-site Cys residues of the disulfide-dithiol exchange reaction, allowing Trx to reduce disulfide bonds on a wide range of target proteins (Hisabori et al. 2007). Because the regulatory mechanism commonly consists of oxidation and reduction of dithiols in the protein molecule, Trx-target enzymes are called “thiol enzymes” and the regulatory system is referred to as “thiol modulation.”

Trx proteins are located in the chloroplast, mitochondria and cytosol of higher plants. In *Arabidopsis*, members of the Trx family are divided into six major groups (*f*, *m*, *h*, *x*, *y* and *o* type) based on their primary structure. The *m*, *x* and *y* types are related to prokaryotic Trx, whereas *f*, *h* and *o* types are specified to eukaryotic organisms (Gelhay et al. 2004). This classification has been widely applied in gene expression profiling (Stoughton 2005). Trx *f*, *m*, *x* and *y* are chloroplastic proteins. Trx *f* is able to reduce FBPase,

activate MDH, and supply reducing power to 2-Cysteine (Cys) peroxiredoxins (Prx). Trx *m* can activate MDH and FBPase and reduce 2-Cys Prx, whereas Trx *x* and *y* are only able to reduce 2-Cys-Prx (Collin et al. 2003; Jaramillo et al. 1997). Location and function of Trx *h* are complicated. The specificity of Trx *h* is linked to tissue and sub-cellular localization and specificity of reduction pathway (Gelhay et al. 2004). In plants, Trx *h* plays a significant role in early seedling growth of germinating cereals seeds. It serves as an electron donor for several enzymes to protect oxidative stress, and involved in biological processes such as self-incompatibility, carbon and nitrogen metabolism (Wong et al. 2003). Trx *o* is a mitochondrial protein and probably plays an important role in plant redox homeostasis and principally in stress resistance induction (Dutilleul et al. 2003). Nearly 200 potential Trx-linked proteins have been identified in different plant metabolisms (Gelhay et al. 2004; Balmer et al. 2006a, b).

In the presence of light, the photosynthetic electron transfer chain of thylakoid membranes allows reduction of ferredoxin (Fd) which can subsequently transfer electrons to Trx through a specific Iron-Sulfur containing enzyme called ferredoxin thioredoxin reductase (FTR). Trx is then able to reduce key disulfide bridges on their target enzymes. The bridge formed between 2-Cys of the Trx protein is reduced by ferredoxin via ferredoxin-Trx reductase (FTR) in the chloroplast, whereas cytosolic Trx is reduced by NADPH via NADPH-Trx reductase in other organelles and different parts of the plant. These enzymes are mainly inactivated in the dark but activated on illumination by the Fd/Trx system. Since the discovery of Trx *f* and *m* in chloroplasts, many studies have been carried out to investigate the molecular mechanisms underlying the Trx-dependent regulation of several target enzymes, such as NADP malate dehydrogenase (NADPMDH) (Miginiac-Maslow and Lancelin 2002; Dai et al. 2004). Within this photosynthetic organelle, both Trx *f* and *m* are known to regulate the enzymes of the Calvin cycle as well as key metabolism namely malate dehydrogenase, glucose 6-phosphate dehydrogenase (Scheibe and Anderson 1981), and ATP synthase (Mills et al. 1980). The NADP/Trx system is widely distributed among organisms and thought to be ubiquitous in aerobes. The biological role of NADP-linked Trx protein is currently an interesting area of extensive research in plants and animals.

Rice has become a model plant for genomic research of monocot species because of its small genome size and economic importance, but our knowledge of the Trx gene family in rice is rather limited. The findings presented here are the first step toward understanding the function of the Trx family in rice. We presented the results of bioinformatics analysis and expression profiling at several stages of development in rice, including the vegetative growth and seed

development stages. In addition, expression analysis was performed for a number of rice Trx genes under hormone stress and light/dark treatments.

## Materials and methods

### Database searches

To obtain sequences of putative Trx proteins of rice genome, the number PF00085 was used to search the TIGR (version 5) database (<http://www.tigr.org>). In cases where there were several gene models for one locus, only one complete gene model was selected for further sequence analysis. According to one gene model of Trx, we found 30 *OsTrx* genes in rice. PF00085 was also used to search against The *Arabidopsis* Information Resource (<http://www.arabidopsis.org/index.jsp>) database (Huala et al. 2001) to retrieve sequences of putative Trx proteins in *Arabidopsis*. Sequences of Trx proteins from other plants were identified with a BLAST search against the National Centre for Biotechnology Information database (<http://www.ncbi.nlm.nih.gov/BLAST>) using conserved Trx sequences of *Arabidopsis* and rice as queries. A similar approach was used for algae (<http://genome.jgi-psf.org/cgi-bin/blastOutput?db=Chlre3&jobId=852284&jobToken> and <http://bioinformatics.psb.ugent.be/blast/public/index.php?project=ostreococcus&dbLIB=Ostreo>). The PFAM database (<http://www.sanger.ac.uk/Software/Pfam>) was used to confirm the predicted domain of Trx protein sequences.

### Sequence analysis

Putative domains of Trx proteins were identified by SMART and PFAM analysis with an *E* value smaller than  $1E-5$ . The unknown conserved motifs were predicted by MEME version 3.5.2 (<http://meme.sdsc.edu>). MEME is a tool for discovering motifs in a group of related DNA or protein sequences (Bailey and Elkan 1995). Multiple alignment analyses were performed using CLUSTAL\_X version 1.83 (Thompson et al. 1997). The unrooted phylogenetic trees were constructed with MEGA3.1 (Kumar et al. 2004). Exon-intron organization was determined by comparing the genomic cDNA sequences of the Trx genes.

### Plant materials and growth conditions

Two parental lines Minghui 63 (MH63) and Zhenshan 97 (ZS97), and their elite hybrid Shanyou 63 (SY63), were used to study the Trx genes expression level at different developmental stages under different stress conditions using Affymetrix rice microarray. Seeds were soaked in water and germinated at room temperature (28°C). For the

light/dark treatments, seedlings at the plumule and radicle stages were placed under 48-h continuous light or darkness. For the hormone treatments, seedlings at the 3-leaf stage were treated with 0.1 mM of the phytohormones gibberellic acid (GA<sub>3</sub>), naphthalene acetic acid (NAA), or kinetin (KT) and the treated leaves were collected after 5, 15, 30 and 60 min, respectively.

### Expression profile analysis

Expression profiles data were produced within a rice transcriptome project (Wang et al., unpublished) using Affymetrix Rice GeneChip microarray. These data can be retrieved from CREP (<http://crep.ncpgr.cn>), a database of the rice transcriptome project. In this study we extracted the expression signal values of the rice Trx genes from the database for 34 tissues of the three rice genotypes. All tissues of different developmental stages of the plant life cycle used for microarray are presented in Supplementary Table S1. A tissue-specific expression profile tree was constructed by CLUSTER version 3.0 (De Hoon et al. 2004). Mean signal values of two biological replicates for each tissue were used for analysis, except for five tissues (embryo and radicle after germination; root and leaf at the trefoil stages; young panicle at stages 3, 4, and 5) which had six biological replicates each. To identify the differentially expressed genes, a Student's *t*-test was performed and only those genes that exhibited *P* value less than 0.05 were considered to be significant in specific tissues or under hormone and light/dark treatment conditions. For tissue specific expression analysis and different treatments conditions (hormone and light/dark), we studied 29 *OsTrx* genes because one gene (*OsTrx4*) had no probe set in the Affymetrix rice chip. For this gene, we performed the RT-PCR to examine its expression level in nearly all of the tissues studied.

RT-PCR was performed to confirm the differential expression of representative Trx genes identified by microarray data analysis using gene-specific primers. Total RNA was extracted from various tissues or organs from throughout the life cycle of the three rice genotypes using an RNA extracting kit (TRIzol reagent, Invitrogen) according to the manufacturer's instructions. Before the RT reaction, total RNA was treated with amplification grade DNase I (Invitrogen) for 15 min to degrade possibly contaminated residual genomic DNA. RT was performed using SuperScriptII reverse transcriptase (Invitrogen) according to the manufacturer's instructions to synthesize first-strand cDNA from the DNase I-treated total RNA. About 1/20 of the first-strand cDNA generated from 1 µg total RNA was used as template for PCR in a reaction volume of 50 µl with rTaq DNA polymerase (Takara). PCR was performed in an ABI 9700 Thermocycler (Applied Biosystems) with the following cycling profile: 94°C for 3 min; 25–35 cycles (depending on

the expression levels of different genes) at 94°C for 50 s, 55°C for 50 s, and 72°C for 1 min. Fifteen microliters of the PCR product was separated in a 1.2% agarose gel and stained with ethidium bromide for visualization. We used a pair of primers specific to the rice *Actin* gene (accession number AK100267) for RT-PCR as an internal control. We checked the transcript level of the *Actin* gene and there was no significant variation in different RNA samples in this study. This gene was also used as the internal control in other RT-PCR experiments (Huang et al. 2007). A pair of primers for each Trx gene (Supplementary Table S2) with 500–600 bp amplicon was used for RT-PCR. All RT-PCRs were repeated three times with independently reverse-transcribed templates.

#### Vector construction and transformation

The native promoter (an upstream fragment (1,500 bp) starting from the base next to the start codon of *OsTrx3* and *OsTrx14*) was amplified from genomic DNA and placed into pCAMBIA1381xb-GFP to control GFP expression. The following PCR primers were designed: *OsTrx3F* and *OsTrx14F* (5'-CGCTGAATTCGATCGATTCGATCACA CGGA-3'; 5'-GCTAGAATTCGCATAGTTGGGACTCT GT-3') *OsTrx3R* and *OsTrx14R* (5'-TAGCGGATCCGG CTGTTAGCATCAGGAGCA-3'; 5'-ACTGAAGCTTCA TTGCTGACGTTGCCGAAGCT-3'). The constructs were transformed into the *japonica* rice Nipponbare by the *Agrobacterium*-mediated transformation method (Hiei et al. 1997). Leaves, roots and endosperm of transgenic rice were observed by confocal microscopy (TCS SP2, Lecia).

## Results

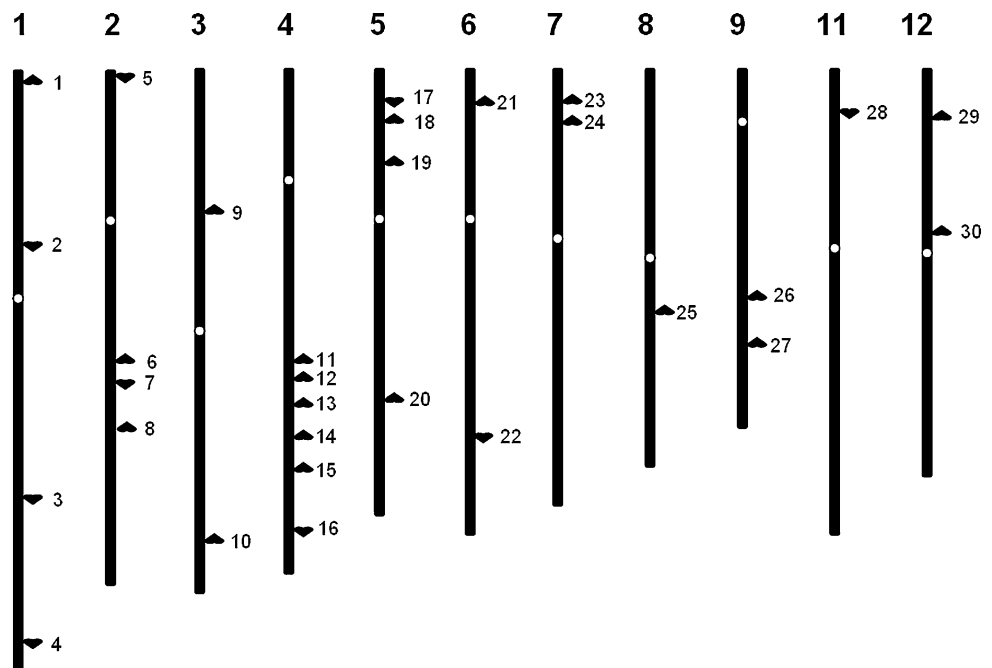
### Identification of Trx genes in the rice genome

A total of 30 loci (*OsTrx1–30*) with 54 gene models encoding putative Trx proteins were identified in the rice genome. The BAC or PAC clones carrying the Trx protein-encoding genes were identified based on the information of rice chromosomal pseudomolecules available at TIGR. The approximate chromosome map positions of BACs/PACs (in cm) from the top of the chromosome and their nearest markers obtained from the rice physical map are listed in Supplementary Table S3. The chromosomal locations and directions of transcription of Trx protein-encoding genes are given in Fig. 1. The 30 *OsTrx* genes are distributed on all of the rice chromosomes except chromosome 10: six genes on chromosome 4, four genes each on chromosomes 1, 2, and 5, two genes each on chromosomes 3, 6, 7, 9 and 12, and one gene each on chromosomes 8 and 11 (Fig. 1).

### Protein sequence and phylogenetic analysis of the Trx family

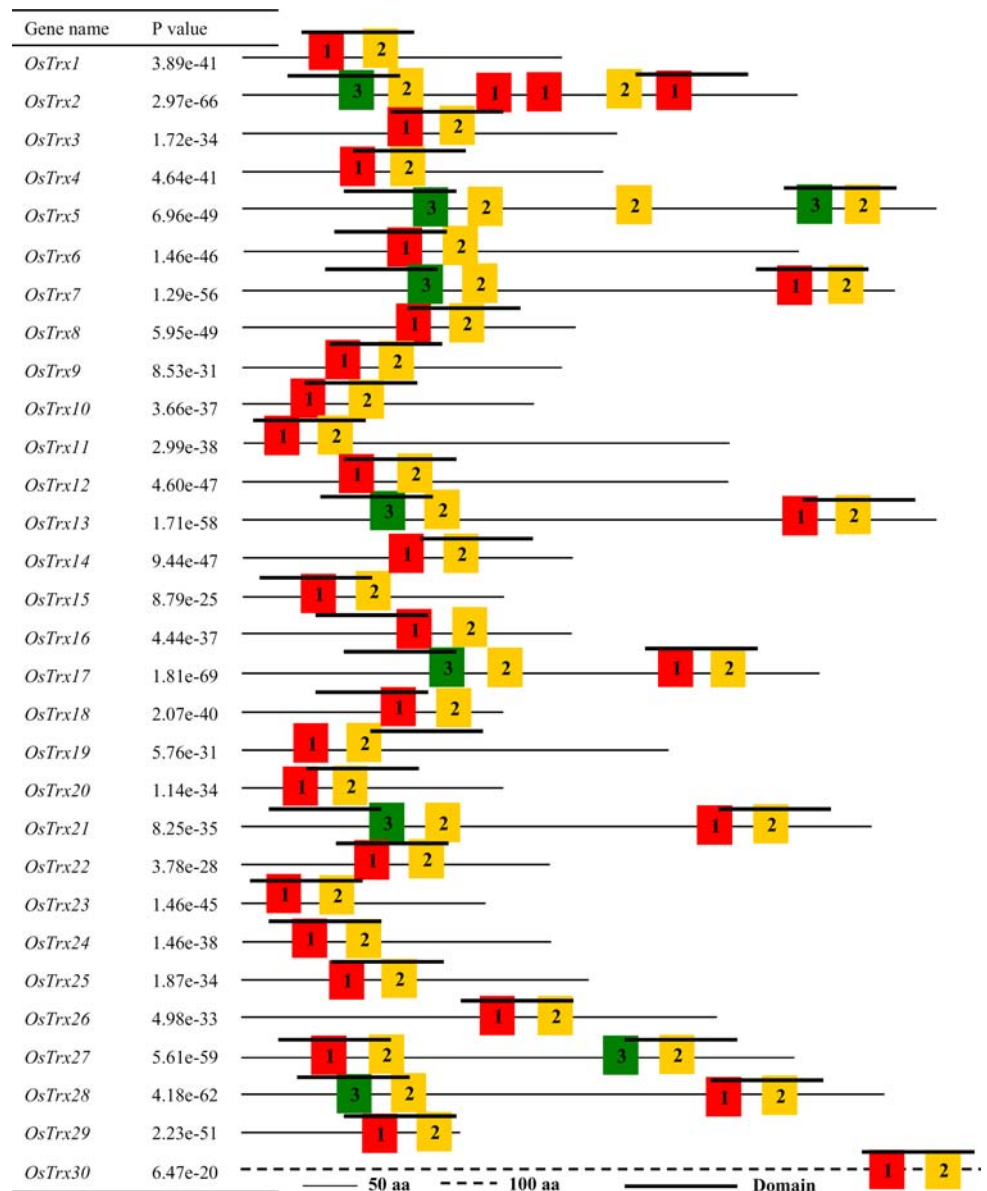
By using MEME program, three motifs (1, 2 and 3) of Trx proteins with E values less than  $1.00E-30$  were identified (Table 1, Fig. 2). Motif 1 was presented in all the members except *OsTrx5*. Duplication of motif 1 or 2 was found for 22 Trx sequences. *OsTrx2* showed inverted repeat of a motif 1–2 block. Motif 3 and 2 were present in 8 genes, and *OsTrx5* had an inverted repeat of motif 3–2 block interrupted by an additional motif 2 (Fig. 2). All three motifs

**Fig. 1** Distribution of Trx protein-encoding genes on rice chromosomes. White ovals on the chromosomes (vertical bar) indicate the position of centromeres. The arrows next to the gene names show the direction of transcription. Chromosomes numbers are indicated at the top of each bar



**Table 1** Annotation of putative motifs of Trx protein-encoding genes identified by MEME

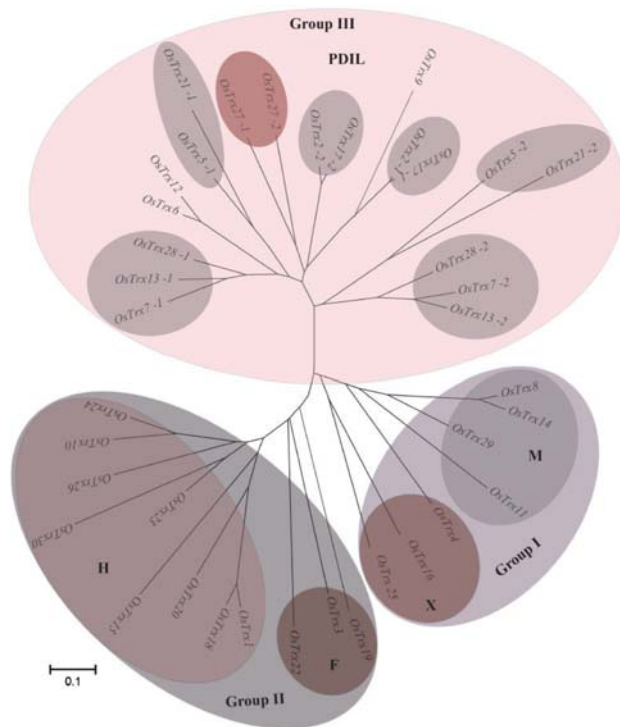
Motif	<i>E</i> -value	Width	Site	Log likelihood ratio	Best possible match for the motif
1	3.2E-331	29	30	1,448	VLVEFYAPWCGHCRMMAPIYEEMAQYEYPG
2	9.8E-173	24	30	1,019	DENKSLASQYGIRGMPFTMFFKNG
3	3.9E-079	29	8	531	LVEFYAPWCGHCKQLAPEYEKAAAHLRKH

**Fig. 2** Motif comparison of all the Trx protein-encoding genes by MEME. *Thick black line* indicates the position of conserved Trx domain of the each gene

also existed in the Trx proteins in other studied plant species (Supplementary Fig. 1).

In PFAM, Trx proteins have a marked domain “PF00085”. Multi sequence alignment indicated that the PF00085 domain is divergent in Trx family. Eight genes showed two Trx domains 1 and 2 in PFAM database search. Thirty-eight *OsTrx* domains were used for phylogenetic analysis. The unrooted phylogenetic tree showed that

*OsTrx* family can be classified broadly into three major groups (I, II and III) (Fig. 3). Group I and II each can be further divided into two subgroups based on putative functions (TIGR data base). For subcellular localization the PSORT program (Horton et al. 2007; <http://psort.nibb.ac.jp>) was used (Supplementary Table S4). Group III is also named as PDIL (protein disulfide isomerase like PDI) and 8 PDIL proteins in this group constitute a well supported



**Fig. 3** Phylogenetic relationship among the rice Trx protein-encoding genes. The unrooted tree was generated using MEGA 3.1 version. Domain 1 and 2 showed in group III, for example *OsTrx7-1*, and *OsTrx7-2*

active site PWC (data not shown). PDIL is a family of oxidoreductase typically containing two Trx domains involved in the formation of S–S bonds (Frandsen et al. 2000). Domains 1 and 2 in the same Trx genes are divided into different branches. The phylogenetic relationships of these genes are the same except for *OsTrx6*, *OsTrx9* and *OsTrx12* (Fig. 3). For example, *OsTrx28*, *OsTrx7* and *OsTrx13* showed the same phylogenetic relationship in domain 1 and domain 2-specific phyta. It is indicated that domains of these 3 proteins duplicated before the *OsTrx* genes duplication. *OsTrx27* showed domain duplication but no gene duplication. This is not surprising because Trx is a very old and important functional family.

A comparison of the full-length cDNA sequences with the corresponding genomic DNA sequences revealed diverse exon-intron structures of the *OsTrx* gene family in terms of both the location and the number of introns for each gene. The coding sequences of all the *OsTrx* genes are disrupted by introns, and the number of introns varies from one to 11 (Supplementary Fig. 2). Based on phylogenetic tree, we analyzed the intron/exon structure of *OsTrx* genes belonging to the groups I, II and III. There was no obvious difference among different classes, although the genes in the group I has fewer introns than the other two groups. In each group, at least one *OsTrx* gene has more introns than others (Supplementary Fig. 2). Meyer et al. (2002) classified plant

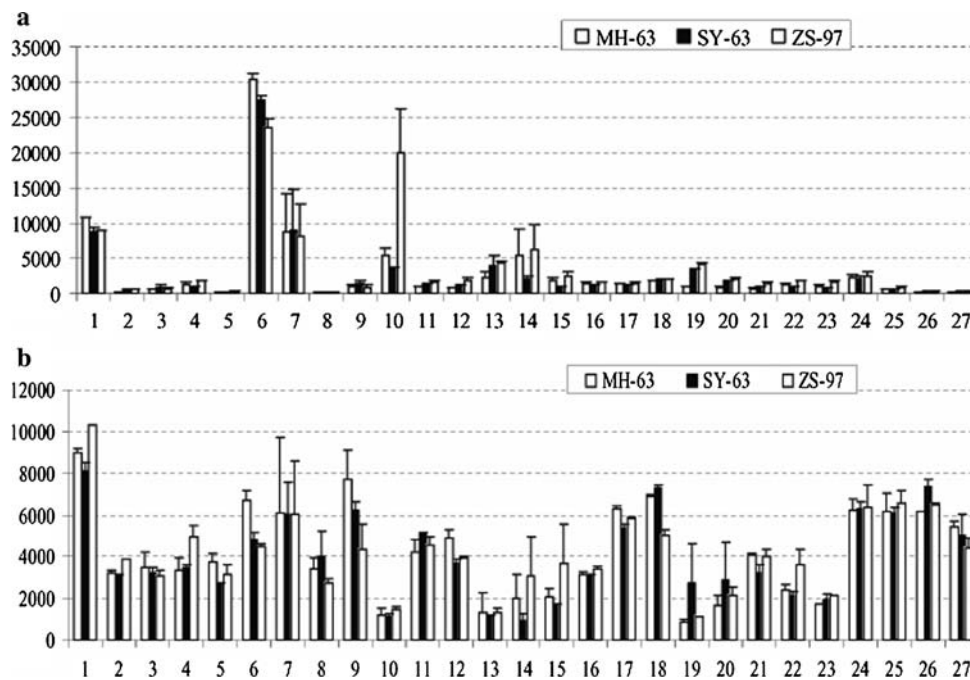
thioredoxins by sequence similarity and intron position and related them with common origins. Recent researches in rice have shown that the rate of intron loss is bigger than intron gain after segmental duplication (Roy and Penny 2006; Lin et al. 2006). Therefore, it seems that the genes in the group I may represent young members in this gene family and in each group the genes with more introns may be the original gene of that group.

To study the evolutionary relationships of Trx protein-encoding genes by phylogenetic analysis, we collected a data set of 129 putative Trx protein sequences, including 30, 67, 20 and 12 sequences from rice, *Arabidopsis*, unicellular algae (*Chlamydomonas* and *Ostreococcus*) and other plant species (*Zea mays*, *Hordeum vulgare*, *Triticum aestivum*, *Medicago truncatula*, *Pisum sativum*, *Brassica napus* and *B. oleracea*), respectively. The numbers of Trx genes are highly different between rice, *Arabidopsis* and algae. We constructed the phylogenetic tree based on the protein sequences of Trx domain. The phylogenetic tree suggests that the plant Trx family was diversified during evolution (Supplementary Fig. 3). The 129 plant Trx were classified into three groups (I, II and III) similar to that for *OsTrx* family above. *Arabidopsis* and rice Trx members are almost evenly distributed in the three groups. Half of the sequences containing two domains (1 and 2) belong to group III (Supplementary Fig. 3).

#### Expression profiles of Trx protein-encoding genes in different tissues of rice

To preview the expression profiles of *OsTrx* genes, we searched the CREP database (<http://crep.ncpgr.cn/>), which was based on Affymetrix DNA chip expression files for 39 tissues for the three genotypes, for the expression levels of the *OsTrx* genes in major rice tissues/organs (callus, plumule, radicle, shoot, root, seedling, leaf, stem, panicle, endosperm, sheath, hull, spikelet, and stamen). The signal values for all the 29 *OsTrx* genes are given in Supplementary Table S5.

Hierarchical cluster analysis based on the signal values showed that the *OsTrx* genes had very diverse expression patterns (Supplementary Fig. 4). Most of the *OsTrx* genes are expressed in at least one of the 27 rice tissues investigated. *OsTrx3* had a significantly higher expression in leaves and roots at the trefoil stages in MH63 than in other tissues (Fig. 4a), whereas *OsTrx14* had high expression in the embryo and radicle after germination in ZS97 (Fig. 4b). However, none of the genes showed tissue-specific differential expression in the hybrid rice SY63. *OsTrx3* and *OsTrx14* have one Trx domain each (PFAM data search Supplementary Table S3), and belong to *f* and *m* type, respectively, according to TIGR data base search. Trx *f* and *m* are mainly located in chloroplast. Recently, Barajas-Lopez



**Fig. 4** Trx protein-encoding genes **a** *OsTrx3* and **b** *OsTrx14* showed significantly high expression in the root and leaf at trefoil stages, and after germination of embryo and radicle stage under MH63 and ZS97 among three materials (MH63, SY63, and ZS97) in the following 27 tissues: (1) embryo and radicle after germination; (2) calli, 15 days after subculture; (3) calli, screening stage; (4) calli, 5 days after regeneration; (5) seed, 72 h after imbibition; (6) root and leaf at trefoil stages; (7) shoot, seedling with two tillers; (8) root, seedling with two tillers; (9) stem, 5 days before heading; (10) flag leaf, 5 days before

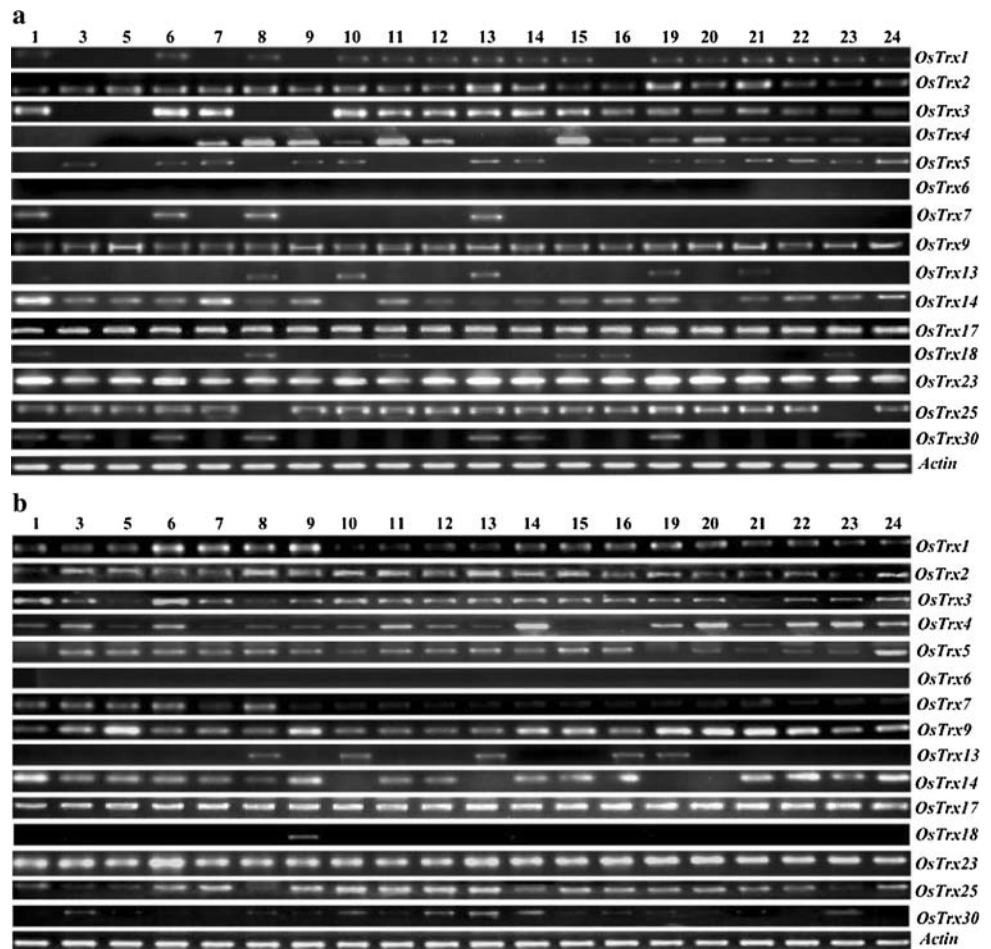
heading; (11) stem, heading stage; (12) panicle, heading stage; (13) flag leaf, 14 days after heading; (14) leaf, young panicle at stage 3; (15) sheath, young panicle at stage 3; (16) young panicle at stage 3; (17) young panicle at stage 4; (18) young panicle at stage 5; (19) leaf, 4–5 cm young panicle; (20) sheath, 4–5 cm young panicle; (21) panicle, 4–5 cm young panicle; (22) hull, 1 day before flowering; (23) stamen, 1 day before flowering; (24) spikelet, 3 days after pollination; (25) endosperm, 7 days after pollination; (26) endosperm, 14 days after pollination; (27) endosperm, 21 days after pollination

et al. (2007) found that these types of genes are highly expressed in various parts of non photosynthetic organs. The promoters for these genes (*f* and *m*) were found to contain *cis*-elements that are required for light-dependent expression and negative regulation of tissue-specific expression of Trx *m* in the hypocotyls of *Arabidopsis* plants (Kim and Mayfield 2002; Barajas-Lopez et al. 2007). RT-PCR was performed to confirm the expression level of 15 genes, in 20 tissues of the MH63 and ZS97 parental lines as listed in Fig. 5. A relatively high expression was found in roots and leaves at the trefoil stages (*OsTrx3*) and in the post-germination embryo and radicle (*OsTrx14*) compared with other tissues in MH63 and ZS97, respectively. Two genes (*OsTrx17* and *OsTrx23*) showed high expression in all the tissues of MH63 and ZS97. *OsTrx6* and *OsTrx18* had no or very low expression, respectively (Fig. 5). Generally, the RT-PCR results matched very well with the DNA microarray data. The green fluorescence protein (GFP) reporter gene under the control of the native promoter of *OsTrx3* and *OsTrx14* genes was transformed into rice. The expression pattern of GFP in rice (Supplementary Fig. 5) also supported the expression features of the genes revealed by DNA chip and RT-PCR analysis.

#### Expression of Trx protein-encoding genes under hormone stress conditions

The CREP database also contains genomic expression files of rice (seedling at trefoil stages for the three genotypes) subjected to different hormone treatments (GA<sub>3</sub>, NAA, and KT). Therefore, we also checked the expression of *OsTrx* genes under these treatments. The average signal values for 29 *OsTrx* genes are given in Supplementary Table S6. Hierarchical cluster analysis based on the signal values showed very diverse expression patterns of these genes (Supplementary Fig. 6). Several *OsTrx* genes showed high or low expression in at least one hormone treatment compared with the control. Some genes changed expression level significantly in response to hormone treatments (Fig. 6a–f). Six genes (*OsTrx2*, *OsTrx5*, *OsTrx8*, *OsTrx19*, *OsTrx22* and *OsTrx25*) showed a higher expression in different hormone treatments compared with the control in the three genotypes, while seven genes (*OsTrx1*, *OsTrx3*, *OsTrx10*, *OsTrx17*, *OsTrx21*, *OsTrx23* and *OsTrx29*) showed a lower expression in different hormone treatments compared with the control. The RT-PCR results of differentially expressed genes under hormone treatment conditions are consistent

**Fig. 5** RT-PCR analysis of 15 genes for differential expression in 20 studied tissues in **a** MH63 and **b** ZS97. See Fig. 4 for explanation of different tissues



with the DNA chip data (Fig. 7). For example, *OsTrx19*, *OsTrx22* and *OsTrx25* showed a significantly higher expression in MH63 and SY63, while *OsTrx1*, *OsTrx3* and *OsTrx23* showed a significantly lower expression compared with the control in different hormone treatments in all the materials. Some genes showed low expression which was very similar to the results of chip data analysis (Fig. 7).

#### Expression of *OsTrx* genes in the light and dark treatments at the plumule and radicle stages

Expression profiles of *OsTrx* genes in seedlings (plumule and radicle stages) in the light and dark treatments were also investigated (the average signal values of all the *OsTrx* genes are given in Supplementary Table S7). We identified 5 genes (*OsTrx3*, *OsTrx19*, *OsTrx20*, *OsTrx25* and *OsTrx29*) which showed a significantly higher expression under the light treatment in MH63 and ZS97. In contrast, these five genes were not significantly up-regulated by light in the hybrid SY63 (Fig. 8a). All these five genes are located in chloroplast according to PSORT analysis (Supplementary Table S4). None of the *OsTrx* genes showed any significant expression at the radicle stage under the

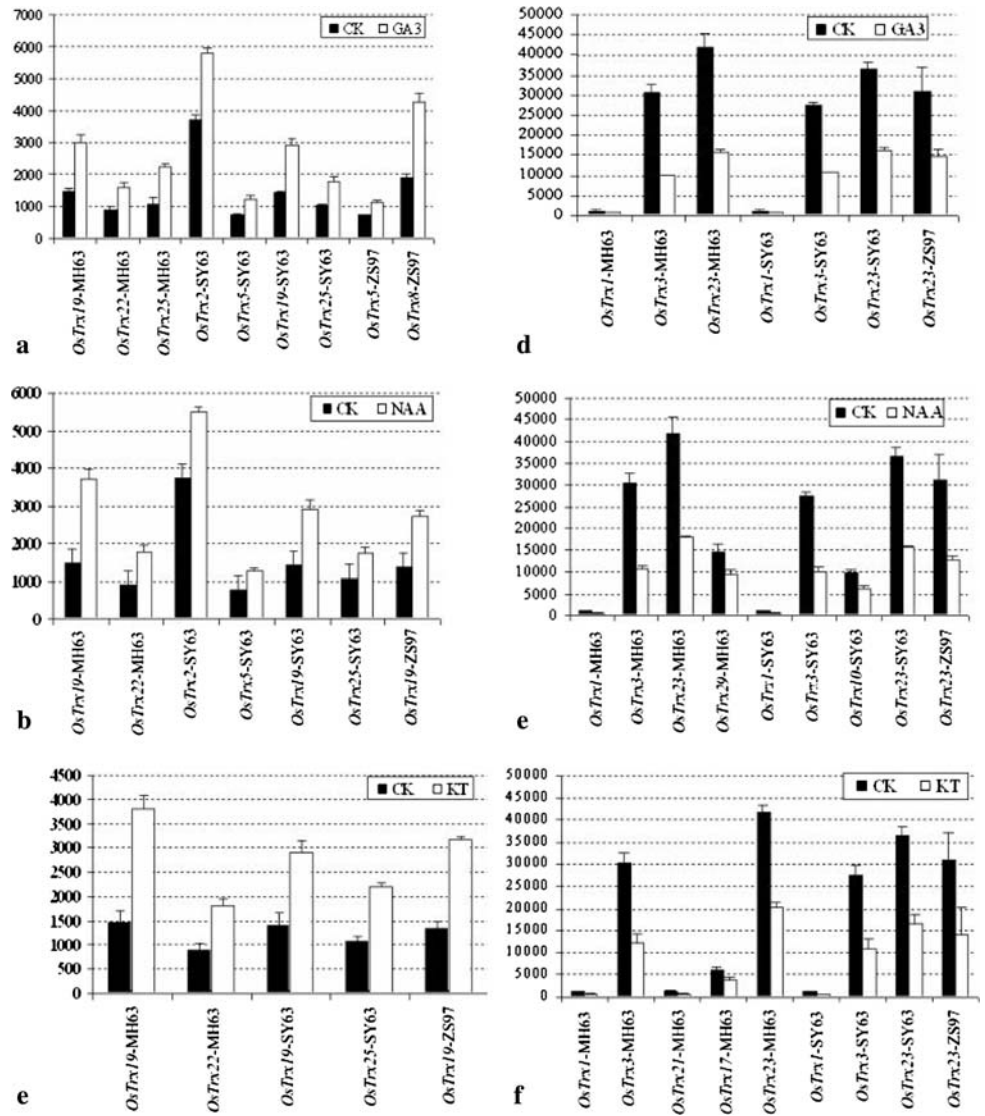
dark treatment. The high expression of *OsTrx* genes (*OsTrx3* and *OsTrx25*) at the plumule stage revealed by microarray data analysis was confirmed by RT-PCR. The RT-PCR results matched very well with the DNA chip data. For example, *OsTrx1* and *OsTrx6* genes showed very little or no expression by RT-PCR analysis and these genes had very small signal values in the chip data (Fig. 8b).

#### Discussion

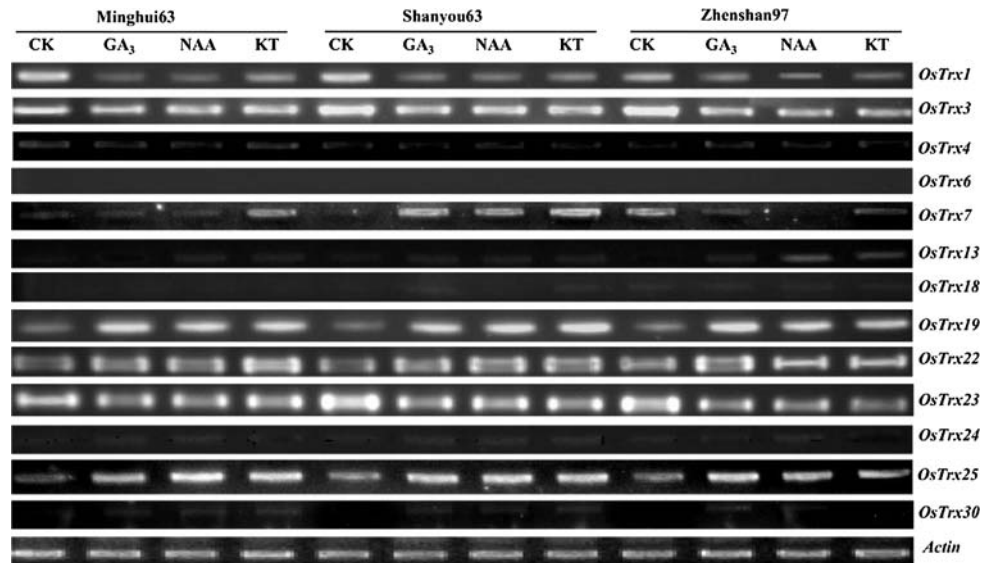
The rice genome size and gene number are about 3.7 times (450 Mb vs. 130 Mb) and 1.5 times (37,000 vs. 25,000) larger than those of *Arabidopsis*, respectively. However, we identified fewer Trx genes in rice than in *Arabidopsis* (Supplementary Fig. 3). Multiple members of a specific gene family in a particular organism appear to be the natural outcome of a long evolutionary history of that organism. The number of members of a gene family reflects a succession of genomic rearrangements and expansions due to extensive duplication and diversification that frequently occurs in the course of evolution (Wang et al. 2007). Research suggests that gene duplication plays a major role in evolution (Taylor and Raes 2004).



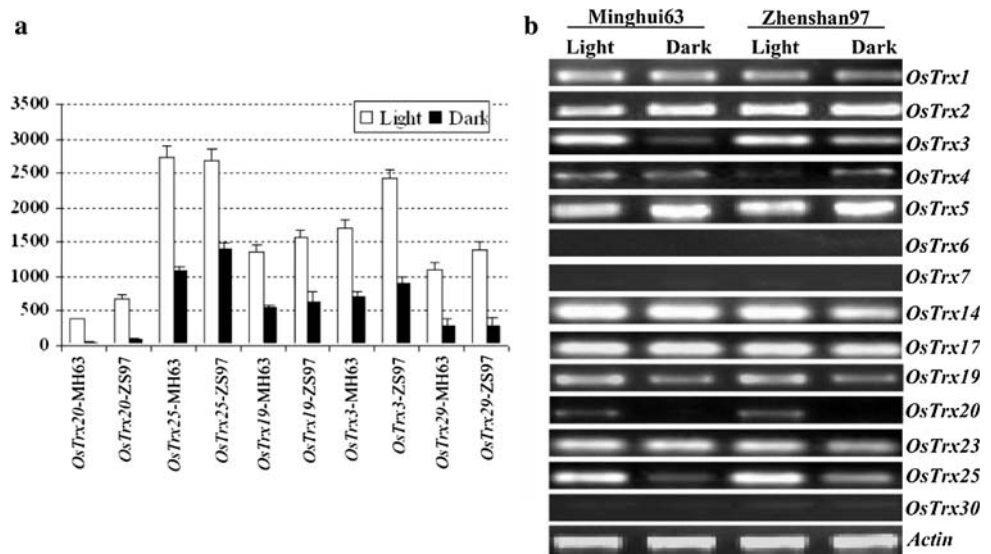
**Fig. 6 a–f** Expression of *OsTrx* genes under  $GA_3$ , NAA, and KT treatments based on microarray analysis. Number of genes expressed in the three rice (named below each bar) is given under different hormone treatment at the trefoil stages. CK control;  $GA_3$  gibberellic acid; NAA naphthalene acetic acid; KT kinetin



**Fig. 7** RT-PCR analysis of differential gene expression under hormone treatments. CK control;  $GA_3$  gibberellic acid; NAA naphthalene acetic acid; KT kinetin



**Fig. 8** **a** Expression of *OsTrx* genes at the plumule stage (48 h after emergence) under light and dark treatments. **b** RT-PCR analysis of high-expression genes based on chip data in the two rice parental lines MH63 and ZS97



Several rounds of whole-genome duplication have been reported in both the *Arabidopsis* and rice genomes (Lynch and Conery 2000; Simillion et al. 2002; Raes et al. 2003; Wang et al. 2005). Two rounds of large-scale genome duplications have taken place in most of the rice chromosomes; one was predicted to have occurred around 40–50 million years ago (MYA) and another before the divergence of monocots and dicots at about 120–150 MYA (Goff et al. 2002; Yu et al. 2005). The relationship of domain duplication and genes duplication of Trx genes indicated that Trx genes may be derived from common ancestors, and the domains are duplicated in these ancestors, but some of them may have been differentiated separately in monocot and dicot species. This pattern has been observed for other large gene families as well (Bai et al. 2002; Zhang et al. 2005; Jain et al. 2006a, b). Our results indicate that Trx proteins harboring similar domains expanded in a species-specific manner; probably only a few members originated from the common ancestral genes that existed before the divergence of monocots and dicots (Supplementary Fig. 3). A high level of variation exists in the intron sequences among the 3 groups (Supplementary Fig. 2), indicating that some of these introns may have been involved in the evolution and diversification of Trx proteins. Phylogenetic analysis based on the protein sequences of Trx domains suggests that the Trx domain may also contribute to the diversification of plant Trx genes. Two putative Trx domains of group III (Fig. 3) may be important for interaction of Trx proteins with others proteins, which remains to be elucidated in further studies.

#### Expression profiles of Trx protein-encoding genes in different tissues

In *Arabidopsis*, at least four Trx isoforms (*m*, *f*, *x*, and *y*) have been found in chloroplast (Collin et al. 2003; Gelhaye

et al. 2005). Chloroplastic Trx is associated with light regulation of carbon metabolism through regulation of the reducing pentose phosphate as well as the C<sub>4</sub> pathway (Gelhaye et al. 2005). Based on the results from the TIGR data base search, *OsTrx3* gene is considered to be the *f* type form of Trx. Consistent with earlier reports regarding chloroplastic Trx, we also observed that *OsTrx3* was highly expressed in roots and leaves at the 3-leaf stage (Figs. 4a, 5), suggesting that *OsTrx3* may participate in regulating the enzyme activities in photosynthesis (Calvin cycle) and metabolism (FBPase, MDH, and FTR). Similar results have also been found in pea chloroplast and roots (Barajas-Lopez et al. 2007; Jacquot et al. 1997; Meyer et al. 2005; Schurmann 2003).

Germination of cereal grain is accompanied by comprehensive changes in the redox state of seed proteins. Proteins present in the oxidized form in dry seeds are converted to the reduced state following imbibition. Trx appears to play a key role in this transition in cereals (Alkhalfioui et al. 2007). Trx (*h* type) occurring in plant cell cytoplasm has been found to function in several capacities during the seed germination stage (Schurmann and Jacquot 2000). The NADP/Trx system, consisting of NADPH, Trx *h*, and NADP-Trx reductase NTR (Johnson et al. 1987; Florencio et al. 1988), functions in the reduction of the major storage proteins of the grain endosperm, gliadins and glutenins in rice and wheat, converting disulfide (S–S) bonds to the reduced (SH) state during the germination process (Kobrehel et al. 1992; Lozano et al. 1996; Yano et al. 2001; Yano and Kuroda 2005). Trx proteins are electron donors for several enzymes involved in the protection against oxidative stress, such as peroxiredoxin and methionine sulfoxide reductase (Jung et al. 2002; Gelhaye et al. 2003). Trx accelerated seed germination in wheat, barley, and pea (Serrato et al. 2001; Wong et al. 2002; Montrichard et al. 2003).

According to TIGR database search, *OsTrx14* belongs to the *m* type form of Trx. This gene is highly expressed in heterotrophic tissues such as leaves, seeds and flowers in pea (Barajas-Lopez et al. 2007). Similarly, we also found *OsTrx14* promoter driven GFP expression in those tissues (Supplementary Fig. 5). The role of Trx *m* might be directly related to the redox regulation of processes in the tissues with high proportions of cells undergoing division. In non photosynthetic organs, Balmer et al. (2006a) reported a complete ferredoxin-Trx system in amyloplasts from wheat starchy endosperm that was involved in metabolism (Fd, and FTR; Barajas-Lopez et al. 2007). A search of Genevestigator microarray gene expression databases revealed similar patterns of expression of *Arabidopsis* chloroplastic Trx in the tissues (Zimmermann et al. 2004). Our analysis revealed that *OsTrx14* had a strikingly high expression in embryo and radicle after germination (Figs. 4b, 5).

#### Expression of Trx protein-encoding genes under various treatment conditions

Plants frequently encounter external stresses conditions that adversely affect growth and development. Abiotic stresses trigger a wide range of plant responses, from alteration of gene expression and cellular metabolism to changes in plant growth rates and crop yields. Trx gene expression under hormone and light/dark treatments requires extensive cross-talk between the response pathways, and there are likely substantial physiological connections between Trx protein production and hormone/abiotic stresses. A high expression suggests that the genes may play an important protective role against stressful hormone and light/dark conditions. In contrast, a low expression of the important genes in response to abiotic/hormone treatments, although possibly functioning as a survival strategy, may make the plants more vulnerable to those treatment conditions. Different kinds of proteins were increased or decreased by the GA<sub>3</sub> treatment in rice leaf sheath (Shen et al. 2002). Previously identified GA<sub>3</sub>-induced *GAST*-like genes in petunia encode proteins containing putative catalytic disulfide bonds (putative redox-active cysteines) and might be involved in redox regulation (Wigoda et al. 2006). These could derive from the cell activation of reducing systems in response to oxidative stress induced by ROS (reactive oxygen species). Alternatively, Crawford et al (1989) showed that GA<sub>3</sub> could activate protein disulfide reductases such as the ferredoxin-thioredoxin reductase. The rapid alterations of the expression levels of the regulatory elements, such as genes involved in signal transduction and transcription regulation, represent the primary response of the regulatory machinery to different hormone treatments. The patterns of both induced and repressed expression exhibited by the regulatory elements are consistent with the up- or down-

regulation of functional genes of various classes identified, suggesting a cohesive nature of the expression network. Although it is not possible to configure such a complex regulatory network based only on the expression profiles obtained in this study, understanding the changes in the regulatory elements may be a key point for understanding the responses to different hormones and light/dark treatments. Global comparison of expression profiles between rice and *Arabidopsis* reciprocal best-matched gene pairs revealed a higher correlation of genome expression patterns in constant light than in darkness (Jiao et al. 2005). Two chloroplastic Trx *f* and *m*, which were originally identified as light-dependent regulators of several carbon metabolism enzymes, including Calvin cycle enzymes, have a function in oxidative stress responses (Lemaire et al. 2007). In our present study, some Trx genes showed a high or low expression under different hormones and light/dark treatments at the 3-leaf and plumule stages, respectively (Figs. 7, 8b).

In conclusion, our data suggest that the Trx gene family has been greatly expanded and diversified in monocot and dicot species. Phylogenetic analysis revealed duplication of Trx genes in both rice and *Arabidopsis*. *OsTrx* genes showed temporal and spatial patterns of expression, and responded differentially to hormone treatments. In addition, Trx proteins appear to serve as the key components of the machinery involved in regulating plant growth and development throughout the life cycle, and their expression was influenced by light and hormone treatments. *OsTrx3* showed a significantly higher expression in particular tissues and various treatments (hormone and light). However, further research using, for example, RNAi strategy or insertion mutagenesis is required to understand the exact biochemical roles and molecular mechanisms of Trx proteins.

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