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Cloning, heterologous expression and characterization of three thioredoxin h isoforms (OsTrx1, OsTrx20 and OsTrx23) from rice

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Abstract

Thioredoxin h (Trx h) is a major cytoplamic and mitochondrial disulfide reductase. In plants, Trx h isoforms are encoded by a multigenic family of genes. The multiplicity of these isoforms raises the question of their functional specificity. In this study, we describe isolation and cloning of three cDNAs encoding different Trx h isoforms, namely OsTrx1, OsTrx20 and OsTrx23. Three Trx h were heterologously expressed in *Escherichia coli* and their activities were compared using DTT-dependent insulin assay. OsTrx23 and OsTrx1 demonstrated highest (0.05 Δ650/min) and lowest (0.016 Δ650/min) activity, respectively. In contrast to OsTrx1 and OsTrx20 isoforms, OsTrx23 was efficiently reduced by NADPH-dependent thioredoxin reductase from barley (HvNTR2). The gene expression of three Trx h was analyzed in one, two and three-week old rice seedlings. The transcripts of OsTrx23 with high intensity and OsTrx20 with low intensity were expressed in both root and shoot, whereas OsTrx1 was only expressed in root. All of three isoforms were appeared partially dimerized under non-reducing conditions suggesting that disulfide bridges were responsible for dimerization.

Keywords: rice; thioredoxin h; cloning; heterologous expression; insulin assay; Nadph-dependent thioredoxin reductase.
Abbreviations: BSA- Bovin Serum Albumin; DTNB- Dithio-bis (2-nitrobenzoic acid); DTT- Dithiothreitol ; FTR-Ferredoxin Thioredoxin Reductase; IPTG-Isopropyl-b-D-thiogalactopyranoside; MPK-Mitogen-activated Protein Kinase; NCBI-National Centre for Biotechnology Information; NTR-NADPH Thioredoxin Reductase; TNB: 2-nitro-5-thiobenzoic acid; Trx-Thioredoxin.

Introduction

Thioredoxins (Trxs) are ubiquitous, low-molecular-mass proteins that are characterized by the presence of an exposed active site with the amino acid sequence of WC(G/P)PC (Schürmann and Jacquot, 2000). Trxs participate in numerous redox processes via the reversible disulfide/dithiol reduction reaction involving active site cysteines. Plants have multiple isoforms of Trx as opposed to animals, bacteria and fungi. The multiple Trx isoforms in plants are classified on the basis of both their primary structures and subcellular localization. Trxs f, m, x, and y are found in the chloroplasts, Trx o is localized in mitochondria and Trx h isoforms are distributed in multiple cell compartments: cytosol, nucleus, endoplasmic reticulum, and mitochondria (Gelhaye et al., 2004; Meyer et al., 2002, 2005). The oxidized form of each Trx contains a disulfide (S-S) bridge that is reduced to the sulfhydryl (SH) group. In cytoplasms and mitochondria, the reduction of disulfide bond of Trxs is accomplished by NADPH via flavin-containing NADPH-dependent Trx reductase (NTR) (Bréhélin et al., 2004). By contrast, the chloroplastic type of Trxs is reduced by ferredoxin via ferredoxin thioredoxin reductase (FTR). Ferredoxin itself is reduced by photosynthetic electron transport (Hirasawa et al., 1999). The reduced Trxs then act as electron donor for the reduction of disulfide bonds of many proteins involved in different cellular pathways (Montrichard et al., 2009). Multiple h-type Trxs are present in higher plants. For instance, in the genome of Arabidopsis (*Arabidopsis thaliana*) eight genes encoding Trx h have been identified (Meyer et al., 2002, 2005). Many proteomic-based studies in the cereal seeds have identified several putative Trx h-target proteins indicating the critical role of this protein in seed development and germination (Buchanan and Balmer, 2005). Upon germination, Trx h plays a key role to reduce disulfide bonds in seed storage proteins, making them soluble and more accessible for degradation by proteases (Kobrehel et al., 1991). The inactivation of inhibitors such as α-amylase and trypsin inhibitor (Kobrehel et al., 1992), and activation of a calcium-dependent substrate specific protease thiocalsin are other suggested roles for this system leading to promotion of seed germination (Besse et al., 1996). The presence of several isoforms of Trx h in plants has raised the question of whether different isoforms have different physiological roles. However to date there has been less progress in assigning specific functions to individual Trx h isoforms in plants. Functional complementation studies of six Trx h isoforms from Arabidopsis using Trx-deficient yeast mutant have shown that different Trx h isoforms may involve in different cellular processes (Mouaheb et al., 1998). In addition, two isoforms of barley (*Hordeum vulgare*) and three isoforms of wheat (*Triticum aestivum*) Trx h are differentially expressed in different seed tissues suggesting that different Trx h isoforms may have specific roles in cereal seeds (Maeda et al., 2003; Cazalis et al., 2006). In the genome of rice (*Oryza sativa*), 30 loci with 54 gene models encoding putative Trx

were identified. On the basis phylogenetic relationship among the amino acid sequences of putative rice Trxs, nine isoforms are grouped as h-type Trx which were stated as OsTrx1, OsTrx18, OsTrx20, OsTrx15, OsTrx30, OsTrx26, OsTrx10 and OsTrx24 (Nuruzzaman et al., 2008). To gain more insight into the functional specificity of different Trx h isoforms in rice, we cloned three h-type isoforms OsTrx1, OsTrx20 and OsTrx23. The production of recombinant proteins and their subsequent extraction and purification enabled us to make a comparison between biochemical properties of different isoforms. In addition, the gene expression profiling of genes encoding these isoforms were analyzed in root and shoot during development of rice seedlings.

Results

Isolation, cloning and sequence analysis of three genes encoding three Trx h isoforms from rice

The cDNA sequences obtained here correspond to LOC_Os01g07376.1, LOC_Os05g40190.1 and LOC_Os07g08840.1 which were stated before as OsTrx1, OsTrx20 and OsTrx23, respectively (Nuruzzaman et al., 2008). OsTrx23 corresponds to previously reported rice Trx h which is abundant in rice phloem sap (Ishiwatari et al., 1995). A BlastP search against the National Centre for Biotechnology Information database (NCBI) using the deduced amino acid sequences of OsTrx1, OsTrx20 and OsTrx23 as queries was accomplished separately. In deed the amino acid sequences of these three isoforms were more similar to Trx h sequences from other plants than to each other. The amino acid sequence of OsTrx1 was 82% identical with Trx h from *Lymus chinensis* (AAO16555). OsTrx23 was 37% identical with OsTrx1, but 76% identical with wheat Trx h (AAL67139) and 74% identical with barley Trx h (AAP72290). OsTrx20 shared only 42% and 37% identity with OsTrx1 and OsTrx23, respectively. A multiple alignment between these three isoforms demonstrated some remarkable differences between OsTrx20 with two other isoforms. The active site sequences of OsTrx1 and OsTrx23 correspond to classical Trx-active site motif (WCG/PPC), whereas Gly/Pro was replaced with Thr in the active site of OsTrx20 (Fig.1, gray box). OsTrx1 and OsTrx23 contain a third Cys residue besides the two Cys residues at active site, whereas there are two additional Cys residues in the sequence of OsTrx20 (Fig.1. asterisk). The presence of one additional Cys has been reported previously for Trx h from poplar (Gelhaye et al., 2003), barley (Maeda et al., 2003) and wheat (Cazalis et al., 2006). The replacement of this Cys with Ser in wheat Trx h showed that this residue has no effect on the activity but is essential for dimerization (Cazalis et al., 2006).

Heterologous expression and purification of OsTrx1, OsTrx20 and OsTrx23

The coding sequences of three Trx h genes were subcloned in pET15b vector to produce recombinant proteins in *Escherichia coli* with a His-tag at the N-terminus. Recombinant His-OsTrx1, His-OsTrx20 and His-OsTrx23 were found in the soluble fraction of the *E. coli* transformant culture after induction with isopropyl-b-D-thiogalactopyranoside (IPTG). The theoretical molecular mass (KDa)/pI of His-OsTrx1, His-OsTrx20 and His-OsTrx23 were 16.86/5.9, 17.54/5.01 and 15.31/6.29, respectively. SDS-PAGE of cell extracts showed a prominent polypeptide band of the expected molecular mass (Fig.2A, lanes 4,5 and 6), and the recombinant proteins were purified from the crude extracts by nickel affinity chromatography (Fig. 2A, lanes 7, 8 and 9) in yields of 23, 13 and 25 mg/L culture, respectively.SDS-PAGE analysis of three purified recombinant OsTrxh under non-reducing conditions (Fig. 2B) showed that all three isoforms are partially dimer. The intensity of bands corresponding to monomeric form of OsTrx23 is much higher than dimer band, whereas the intensity of monomer and dimer bands is almost similar in OsTrxh20 and OsTrxh1. Under reducing conditions, all three isoforms were detected as monomers, suggesting that dimerization was produced by disulfide formation. Further analysis using site-directed mutagenesis is required to understand the function of one additional Cys residue in OsTrx1 and OsTrx23 as well as two additional Cys residues in OsTrx20.

Insulin assay

The recombinant barley Trx h isoforms were analyzed for their ability to reduce insulin (Fig. 3). In reaction mixtures containing 5 µM OsTrx1, OsTrx20 or OsTrx23, insulin was aggregated compared to control reaction containing all reagents except Trx, demonstrating that all three recombinant proteins could catalyze insulin reduction. However, the rate of reaction was not similar when different isoforms were added. The precipitation of insulin was started 15 min after addition of OsTrx23, whereas insulin in the presence of OsTrx20 and OsTrx1 was precipitated after 27 and 37 min, respectively. The insulin reduction activity of OsTrx23 (0.05 Δ650/min) was higher than activity of OsTrx20 (0.03 Δ650/min) and OsTrx1 (0.016 Δ650/min).

NTR assay

In NADPH/Trx system, the electrons flow from NADPH to NTR that in turn reduce Trx. The reduced Trx then reduce its target proteins. In the present study, we examined the ability of reduction of OsTrx isoforms by NTR from barley (HvNTR2, Shahpiri et al., 2008) in the presence of NADPH using DTNB [5,5´- dithio-bis (2-nitrobenzoic acid)] as the final electron acceptor. HvNTR2 was able to reduce OsTrx23 efficiently as it was monitored by increasing the absorbance at 412 nm with the rate of 0.03 Δ412/min in compared to control reaction containing all reagents except OsTrxs (Fig. 4). In contrast, OsTrx1 and OsTrx23 were not reduced by HvNTR2 even when higher amounts of HvNTR2 (up to 80nM) were added to the reaction mixture.

Gene expression analysis of OsTrx genes in root and shoot of seedlings

The different biochemical properties of the three rice Trx h isoforms indicated that each of these Trx h isoforms might have different functions, which might be reflected in various expression patterns. This possibility was addressed by analysis of expression of genes encoding OsTrx1, OsTrx20 and OsTrx23 by semi-quantitative RT-PCR using total RNA from root and shoot of one-week, two-week and three-week old seedlings (Fig. 5). Transcripts of OsTrx23 were highly accumulated in both roots and shoots of seedling at various developmental stages whereas the transcripts of OsTrxh1 were poorly appeared in roots and were not detected in shoots. Transcripts of OsTrxh20 were poorly detected in both roots and shoots at different stages of seedling development.

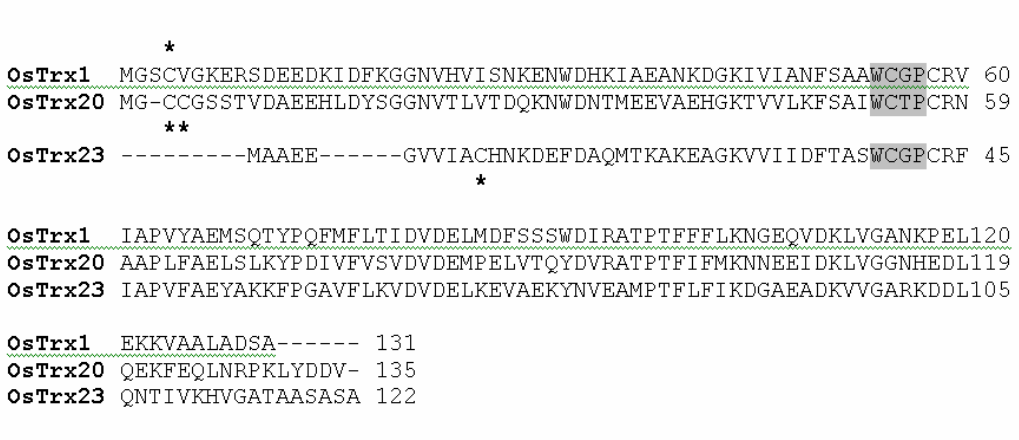

Fig 1. Multiple alignment between OsTrx1, OsTrx20 and OsTrx23 using the ClustalW software. (http://www.ebi.ac.uk/Tools/msa/clustalw2/). The active site is shown with gray box and N-terminal Cysteins are marked with asterisk.

Differential expression of these three genes in different seedling tissues may reflect the various functions of isoforms. OsTrx1 might have a specific role in root as its expression was only detected in this tissue.

Discussions

An NADPH/Trx system, consisting of NADPH, NTR and Trx (Trx h or Trx o) plays a post-translational regulatory role by reducing disulfide bonds in target proteins involved in different cellular process (Shahpiri et al., 2009; Buchanan and Balmer., 2005; Jamal et al., 2010). Plants have a complex NADPH/Trx system comprising several Trx h isoforms. Therefore, one important question to be addressed is whether different isoforms of Trx h have individual roles in plants. Here using the sequence data available for rice as a model plant for monocot species, we isolated and cloned three genes encoding three different rice Trx h isoforms (OsTrx1, OsTrx20 and OsTrx23) with less than 50 % amino acid sequence identity to each other. The recombinant proteins were then produced in *E. coli* as fusion proteins with His-tag at their N-terminal. This allowed comparing their biochemical properties using DTT-dependent insulin assay. The rate of reduction of insulin which was used as Trx h target varied in the presence of the same amount of different isoforms (OsTrx23>OsTrx20>OsTrx1). This suggests that the rate of electron flux from each Trx h isoform to Trx h-target proteins may also vary *in vivo* reflecting individual roles for each isoform in rice. An important aspect for comparing the biochemical properties of different Trx h isoforms is their interaction with NTR, which catalyzes the electron transfer from NADPH to Trx h. Therefore, in the present study the question was addressed with using a cytoplasmic/mitochondrial type NTR from barley, HvNTR2, which is almost 90% identical with putative amino acid sequence of OsNTRA (Q69PS6) and OsNTRB (Q6ZFU6). HvNTR2 was efficiently able to reduce OsTrx23, whereas the reduction of OsTrx1 and OsTrx20 by this enzyme was indistinguishable. These differences in interaction with HvNTR2 may indicate that each isoform of Trx h is differentially reduced in plant cells. However, for better understanding the source of reduction for each OsTrxh isoform, future studies are necessary by analysis of interaction between different cytoplasmic/mitochondrial type NTR isoforms from rice with these OsTrxh isoforms.

On the basis of our enzyme assay, OsTrx23 seems to be the most active and efficient isoform in rice. This isoform has long been known as the major protein in rice phloem sap and recently was found as a negative regulator of two stress-related MAPKs of rice, OsMPK3 and OsMPK6 (Xie et al., 2009). The role of NTR/Trx system in seed development and germination has been very well documented in both cereals (Lozano et al., 1996; Kobrehel et al., 1991) and legumes (Alkhalfioui et al., 2007; Renard et al., 2011). However, the knowledge lags behind of the function of this system in seedling tissues. In the present study, we demonstrated that three Trx h isoforms are expressed in rice seedling tissues even after three weeks, suggesting Trx h isoforms may have function in seedling tissues. The gene encoding OsTrx23 and OsTrx20 were expressed in both root and shoot tissues whereas the transcripts of OsTrx1 were not detected in shoots. Therefore, OsTrx1 may have specific role in root. According to a theoretical analysis by PSORT program (Nuruzzaman et al., 2008), all these three genes are predicted to express in cell cytoplasm and chloroplast. However, the predicted expression of gene encoding OsTrx1 in nucleous indicates a new role for this isoform. Therefore, future studies for determination of intracellular localization of individual Trx h isoforms in conjugation with analysis of protein appearance pattern of isoforms using specific antibodies in different rice tissues may clarify the specific role of different isoforms.

Materials and methods

Plant materials

Rice (*oryza sativa*) seeds from line 2 selected from local variety of Lenjani were provided by Isfahan Center for Research of Agriculture Science and Natural Resources. The seeds were washed twice with water and soaked for 24 hours. The seeds were germinated at room temperature in hydroponic culture for three weeks. The roots and shoots were harvested from one-week, two- week and three-week old seedlings, frozen and stored in –80 ^{0}C until use.

Isolation and cloning of three OsTrxh encoding genes

Total RNA was extracted from shoots and roots of one-week old seedlings using the High Pure RNA isolation Kit (Roche)

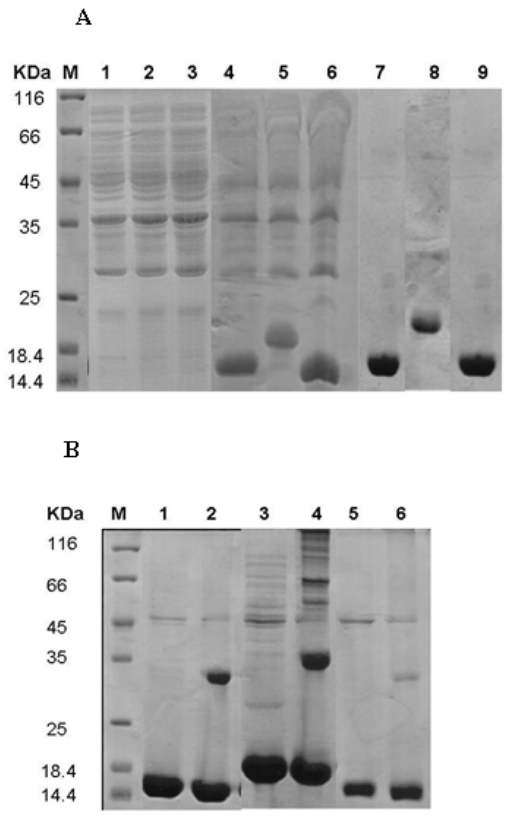

Fig 2. Overexpression of rice Trx h isoforms in *E. coli.* (A) SDS-PAGR analysis for verification of expression and purification. Total soluble protein extracted from *E. coli* harboring pET15b-OsTrx1 (lanes 1,4), pET15b-OsTrx20 (lanes 2,6) and pET15b-OsTrx23 (lanes 3,6). Before addition IPTG (lanes 1,2 and 3) and 4 hours after addition of IPTG (lanes 4,5 and 6). Purified His-OsTrx1, His-OsTrx20 and His-OsTrx23, respectively (7, 8 and 9). (B) Effesct of reducing (lanes 1, 3 and 5) and non-reducing conditions (lanes 2, 4 and 6) on dimerization of OsTrx1 (lanes 1 and 2), OsTrx20 (lanes 3 and 4) and OsTrx23 (lanes 5 and 6).

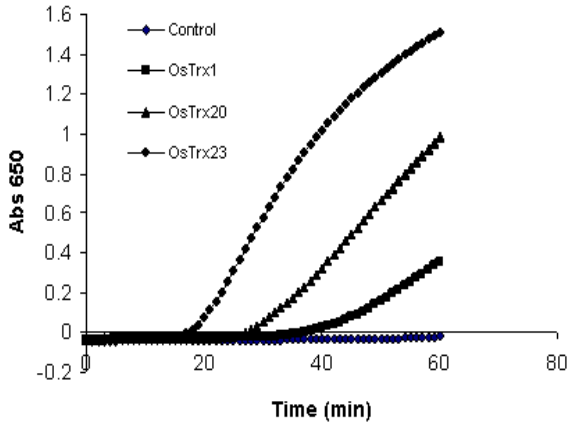

Fig 3. Time course of insulin reduction by purified recombinant His-OsTrx1, OsTrx20 and OsTrx23. Control shows the reaction containing insulin and DTT without addition of Trx h.

and treated with RNase-Free DNase (Fermentas) to remove the genomic DNA contamination. Total RNA (0.1 µg) was reverse transcribed (RT) to synthesize first strand cDNA using AMV reverse transcriptase (Fermentas) and oligo dT primer (Fermentas) according to the manufacturer's recommendations. cDNA samples were subsequently amplified with proof reading *pfu* polymerase (Fermentas) and specific primers for coding sequences OsTrx1(5'CATATGATGGGGTCCTGTGTGGGA3';5'GGATCCTCAGGCGCTGTCAGCAAG3'),OsTrx20(5'CATATGATGGGTTGCTGTGGCAGCAG3';**5'**GGATCCTTATACATCATATAAC3') and OsTrxh23(5´-CATATGGCCGCCGAGGAGG-3´and5´-GGATCCTTAGGCAGAAGCAGATGCAGC -3´). The specific primers were designed using software invitrogen Oligo Perfect™ Designer (http://tools.invitrogen.com/content.cfm). A *Nde*I restriction site (underlined in forward primers) at the start codon and a *Bam*HI restriction site after stop codon (underlined in reverse primers) were introduced in the primers. The RT-PCR product of OsTrxh23 coding sequence was cloned in pJET cloning vector (Fermentas) to give pJET-OsTrxh23. The RT-PCR products of OsTrx20 and OsTrx1 were cloned in pUC57 (Genscript) to give pUC57-OsTrx20 and pUC57-OsTrx1, respectively. The constructs were introduced to *E. coli* DH5α. The sequences of the inserts were determined on both strands.

Protein expression and purification of recombinant rice Trx h isoforms

For construction of expression vectors inserts were isolated from pJET-OsTrx23, pUC57-OsTrx20 and pUC57-OsTrx1 by digestion with *Nde*I and *Bam*HI and ligated into the pET15b expression vector linearized with *Nde*I and *Bam*HI, to give pET15b-OsTrxh23, pET15b-OsTrxh20 and pET15b-OsTrx1, respectively. Sequences were verified and constructs were used to transform *E. coli* strain Rosetta (DE3). Cells were grown at 37^0C in Luria-Bertani medium supplemented with 100 µg/ml ampicillin and 5 µg/mL chloramphenicol to an OD_{600} of 0.6. Cultures were induced by 100 µM Isopropyl-β-D-thiogalactopyranoside (IPTG, Fermentase) for 4 h. Cells were harvested by centrifugation and frozen at -80^0C until use. Frozen pellets were resuspended in Bugbuster protein extraction reagent including Benzonase (Novagen) and shaken for 30 min at room temperature. After centrifugation (14,000 g for 20 min, 4^0C), recombinant proteins in the supernatants were applied onto His-Trap HP columns (Amersham Biosciences) preequilibrated with loading buffer (10 mM imidazole, 500 mM NaCl, 30 mM Tris-HCl, pH 8.0) and eluted in a gradient of 10–200 mM imidazole. The concentration of His_6-OsTrxh1, His_6-OsTrxh20 and His_6-OsTrx23 were determined by A_{280} and Beer-lambert law with molar extinction coefficient 19542.5, 17085 and 8542.5, respectively.

Activity of OsTrxh isoforms using insulin assay

Thioredoxin activity was determined using insulin assay as described (Holmgren, 1979) with slightly changes. A 1-mL reaction mixture contained 100 mM potassium phosphate, pH 6.5, 0.2 mM EDTA, 1 mg insulin, 5µM His-OsTrxh1, His-OsTrxh20, or His-OsTrxh23, and 1 mM dithiothreitol. The reactions were initiated by addition of dithiothreitol. Reactions proceeded at room temperature and were followed

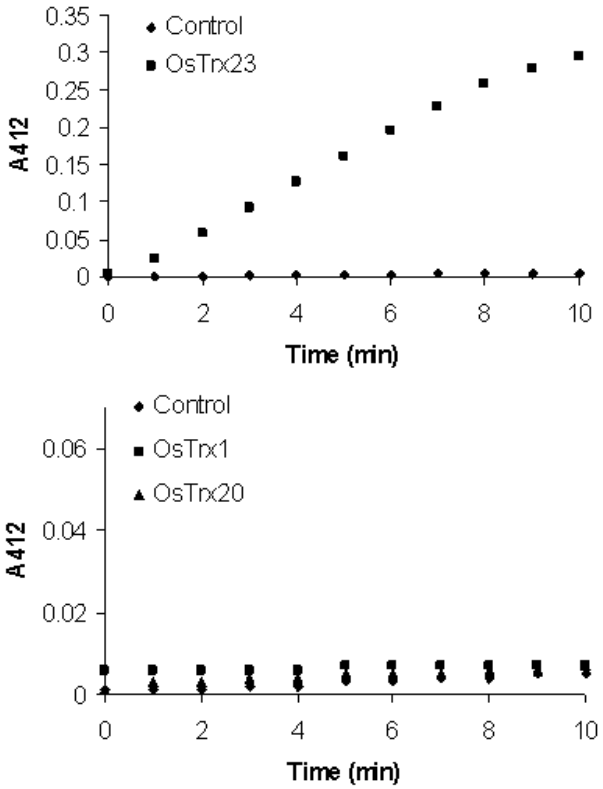

Fig 4. Time course of reduction of OsTrx1, OsTrx20 and OsTrx23 by HvNTR2 as monitored by reduction of DTNB. Control shows the time course of DTNB reduction by HvNTR2 without addition of OsTrx.

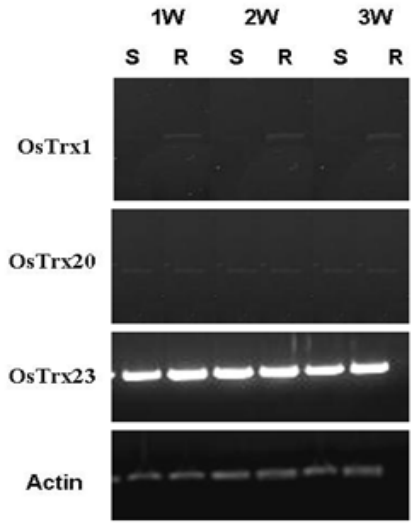

Fig 5. Gene expression analysis of genes encoding OsTrx1, OsTrx20 and OsTrx23 in Shoot (S), Root (R) in one, two and three-week (W)-old rice seedlings. One representative gel is shown from three independent replicates. Rice actin showing invariant expression across the samples was used as internal standard.

by measuring A_{650} on a spectrophotometer (Beckman DU 530).

NTR assay

Recombinant OsTrx1, OsTrx20 and OsTrx23 were tested and compared as substrate for HvNTR2. The assay mixture contained 100 mM potassium phosphate (pH 7.5), 10 mM EDTA, 0.1 mg/ml bovine serum albumin (BSA), 200 µM DTNB (Sigma-Aldrich], 200 µM NADPH (Sigma-Aldrich). The reactions containing of 5 µM OsTrx1, OsTrxh20 or OsTrx23 were started with the addition of 40 nM HvNTR2. Reactions proceeded at room temperature and were followed by measuring the rate of increase of absorbance at 412 nm reflecting the formation of TNB (2-nitro-5-thiobenzoic acid).

RT-PCR analysis

The extraction of total RNA from different tissues and synthesis of cDNA were performed as described above. The cDNA samples were reverse transcribed by Taq DNA polymerase (SinaClon) using specific primers for sequences OsTrx1(5'TGGGAAAGGAACGCA GTG3'; 5'TGCTGGC-AACGTCTGAAC3'), OsTrx20 (5'CTCTTGCAGAGTGGA-GCAGA3';5'TCAGTAGAATGAGCTCTC3') and OsTrx23 (5'ATCGCCTGCCACAACAA G3'; 5'ACACCCAAGCCA-AACTGA3'). The primers were designed by software Invitrogen Oligo Perfect™ Designer (http://tools.invitrogen.com/content.cfm). Rice actin showing invariant expression across the samples was amplified in parallel. The optimal number of amplification cycles (between 15 and 45) for each set of primers was determined at the exponential phase range of amplification. To control for possible genomic DNA contamination, parallel PCR reactions were carried out using RNA samples instead of cDNA samples. A negative control lacking template RNA was included for each set of RT-PCR reactions. Reactions were performed in triplicate and amplification products were separated by agarose gel electrophoresis.

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