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Molecular cloning and characterization of two isoforms of cytoplasmic/mitochondrial type NADPH-dependent thioredoxin reductase from rice (*Oryza sativa* L. ssp. *indica*)

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Abstract

An NADP/thioredoxin system, consisting of NADPH, NADPH-dependent thioredoxin reductase (NTR) and thioredoxin (Trx) plays a post-translational regulatory role by reducing disulfide bonds in target proteins involved in various cellular processes. Plants have a complex NTR/Trx system comprising several Trxs and NTR isoforms. Three genes encoding NTR were found in the genome of rice. OsNTRC is chloroplastic type NTR whereas OsNTRA and OsNTRB are cytoplasmic/mitochondrial type NTR. The presence of two isoforms of cytoplasmic/mitochondrial type NTR with high amino acid identity raises the question of their functional specificity. In the present study, we describe isolation and cloning of two cDNAs encoding OsNTRA and OsNTRB. Both isoforms were heterologously expressed in *Escherichia coli* and purified using affinity chromatography, enabling comparison of their NADPH-dependent reduction activities. OsNTRB and OsNTRA exhibited almost similar *in vitro* activities. OsNTRB was highly expressed in roots and shoots of one, two and three week-old rice seedlings, whereas the expression of OsNTRA in shoots was much higher than roots. Differential expression of OsNTRA in different tissues suggests that this isoform may have different functions in different tissues.

Keywords: Cloning; Heterologous expression; NADPH-dependent thioredoxin reductase; *Oryza sativa*; Purification; RT-PCR.; Thioredoxin.
Abbreviations: BSA- bovine serum albumin; DTNB- 5,5´- dithio-bis (2-nitrobenzoic acid); IPTG- isopropyl-b-D-thiogalactopyranoside; NCBI- National Center for Biotechnology Information; NTR**-** NADPH-dependent thioredoxin reductase; TNB- 2-nitro-5-thiobenzoic acid; Trx- thioredoxin.

Introduction

Thioredoxins (Trxs) are small and powerful disulfide reductases with two close and reactive Cys residues in the conserved motif WCG/PPC (Jacquot et al., 1997). In their reduced state they act as electron donor to a large number of cellular proteins involved in various cellular processes including carbon and sulfur assimilation (Buchanan, 1991), protection from oxidative stresses (Balmer et al., 2004), self incompatibility (Cabrillac et al., 2001), seed germination and development (Alkhalfioui et al., 2008; Marx et al., 2003; Shahpiri et al., 2008). In contrast to other organisms, plants contain a wide range of Trx isoforms which are classified in six subgroups based on their primary structure and their cell localization (Gelhaye et al., 2005). Chloroplastic Trxs (f, m, x and y) are members of the ferredoxin/ Trx system whereby light-driven photosynthetic electrons reduce ferredoxin, which then serves as an electron donor for Trxs via the ferredoxin thioredoxin reductase (Dai et al., 2004; Balmer et al, 2006). In contrast, the plant cytosolic/mitochondrial type Trxs (Trx h / Trx o) in common with bacterial and mammalian Trxs, are part of an NADPH/Trx system, where the reducing power of NADPH is transferred to Trx via the NADPH-dependent Trx reductase (NTR, Laloi et al., 2001; Gelhaye et al., 2004). In plants, NTRs are homodimeric flavoenzymes consisting of 310-330 amino acids in each monomer and with a monomeric molecular weight of about 35 kDa (Dai et al., 1996). Each subunit has two domains namely FAD- and NADP binding domain. A non-covalently bound FAD-molecule is formed in the FAD-binding domain and the conserved active site sequence CAT(V)CD is located in the NADP-binding domain (Dai et al., 1996). The cysteins in the active site form a disulfide bond in the oxidized NTR and receive electrons from NADPH via the coenzyme FAD (Wang et al., 1996). For reduction of Trxs, two cystein thiols in the reduced NTR transfer electron to cysteins in active site of Trxs (Wang et al., 1996). The presence of multiple Trx h and NTR isoforms in plants makes the NADPH/Trx system particularly complex compared with other organisms. For instance, in the genome of Arabidopsis (*Arabidopsis thaliana*) eight genes encoding Trx h (Meyer et al., 2002; Meyer et al., 2005) and three genes encoding NTR (AtNTRA, AtNTRB and AtNTRC) have been identified (Laloi et al., 2001; Reichheld et al., 2005). AtNTRA and AtNTRB are the major cytoplasmic and mitochondrial isoforms, respectively (Reichheld et al., 2005). However, AtNTRC with a C-terminal thioredoxin domain is a chloroplastic type NTR. Similar to Arabidopsis, multiple genes encoding putative h and o-type Trx are present in rice (*Oryza sativa*) genome database (Nuruzzamn et al., 2008). Also in this database three genes encoding putative NTR are available which were previously assigned as OsNTRA,

OsNTRB and OsNTRC (Serrato et al., 2004). On the basis of sequence identity, OsNTRA and OsNTRB are cytoplasmic and mitochondrial type NTR, whereas OsNTRC is chloroplastic type NTR. OsNTRC has been previously characterized and its expression was compared with OsNTRB (Serrato et al., 2004). However, little is known about the individual roles of two cytoplasmic/mitochondrial NTR isoforms. Therefore, in this work we cloned and heterologously expressed both isoforms, OsNTRA and OsNTRB, in *E. coli*. The purification of both isoforms enabled us to compare their *in-vitro* activities. Their gene expression patterns were also analyzed in roots and shoots of one-, two- and three- week rice seedlings.

Results

Cloning and sequence analysis of three genes encoding two NTR isoforms from rice

The cDNA sequences obtained here were corresponding to open reading frame of genes OsNTRB (Q6ZFU6) with 996 bp and OsNTRA (Q69PS6) with 1107 bp which are located on chromosomes 2 and 6, respectively. The deduced amino acid sequences from OsNTRB and OsNTRA have theoretical molecular mass (kDa)/pI of 34.676/6.18 and 38.218/7.05, respectively. A multiple alignment between amino acid sequences of OsNTRB and OsNTRA with the amino acid sequences available in NCBI from other plants was used for generation of phylogenetic tree (Fig. 1A). Both OsNTRA and OsNTRB were classified with mitochondrial/cytoplasmic type of monocotyledon plants. The amino acid sequences of OsNTRA and OsNTRB had 88% identity. Nevertheless, OsNTRA and OsNTRB had more identity with the amino acid sequence of *Hordeum vulgare* NTR1 (HvNTR1, 94% identity) and NTR2 (HvNTR2, 91%), respectively. OsNTRA and OsNTRB contain the NADP-binding motif GXGXXA (Figure 1B, Box 3), FAD-binding motifs GXGXXA (Figure 1B, Box1) and TXXXXVFAAGD (Figure 1B, Box 4) and two active-site Cys residues in the motif CAVC (Figure1B, Box 2).

Heterologous expression and purification of OsNTRA and OsNTRB

The coding sequences of two NTR genes were subcloned into pET28a vector to produce recombinant proteins in *E. coli* with a His-tag at the N-terminus. Recombinant His-OsNTRA and His-OsNTRB were found in both soluble and insoluble fractions of the *E. coli* transformant culture after induction with isopropyl-b-D-thiogalactopyranoside (IPTG). The theoretical molecular mass kDa/pI of His-OsNTRB and His-OsNTRA were 38.49/6.9 and 42.03/8.2, respectively. SDS-PAGE of cell extracts showed a prominent polypeptide band of the expected molecular mass (Fig.2A, lane 2 and 5), and the recombinant OsNTRB and OsNTRA were purified from the crude extracts by nickel affinity chromatography (Fig. 2A, lanes 3 and 6) in yields of 10 and 5 mg/L culture, respectively. On the basis of SDS-PAGE, the intensity of band corresponding to OsNTRB in soluble fraction was almost similar to the intensity of band in insoluble fraction when the bacteria were grown in 37 ^{0}C (Figure 2B, lane 1). However, the soluble fraction of OsNTRB was increased when temperature was decreased to 25 ^{0}C (Figure 2B, lane 3). At this temperature the amount of total protein was almost similar with the addition of either 50 or 100 µM IPTG (Fig. 2B, lane 4). The effect of temperature on the solubility was similarly observed on the solubility of OsNTRA (data not shown). The purified proteins were yellow with maxima absorption pattern at 270, 360, and 440 nm for OsNTRB and 270, 370 and 450 nm for OsNTRA (Fig. 2C) which are typical for flavoproteins (Jacquot et al., 1994).

NTR assay

NADPH-dependent reduction of DTNB for both OsNTRA and OsNTRB was followed by measuring the increase of absorbance at 412 nm (Fig. 3A). Both OsNTRA and OsNTRB were able to reduce DTNB in the presence of NADPH compared to a control reaction containing NADPH and DTNB with no addition of NTR. The velocity of DTNB reduction for OsNTRB (0.019 Δ412 /min) was almost similar to OsNTRA (0.015 Δ412/min). Since the natural electron acceptor for NTR is Trx, the activity of NTR isoforms were also compared in the presence of *E. coli* Trx. It should be mentioned that in this assay less amounts of NTR and DTNB (20 nM and 200 µM) were used as compared to the above assay. Hence in the control reaction containing NADPH, NTR and DTNB without Trx the reduction of DTNB was not absolutely detected. However with the addition of *E. coli* Trx both OsNTRA and OsNTRB were able to reduce *E. coli* Trx as determined with the reduction of DTNB. The velocity of DTNB reduction for OsNTRB (0.037 Δ412/min) was slightly higher than OsNTRA (0.028 Δ412/min) in the presence of *E. coli* Trx (Fig. 3B).

Expression analysis of OsNTR genes in root and shoot of seedlings

Analysis of expression of genes encoding OsNTRA and OsNTRB was accomplished by semi-quantitative RT-PCR using total RNA from root and shoot of one-week, two-week and three-week old seedlings (Fig. 4). Transcripts of OsNTRB were accumulated at similar amounts in shoot and root of one-week, two-week and three-week old seedlings. The transcripts of OsNTRA were accumulated in shoot at similar amount during the growth of seedlings but they were hardly detected in root tissues.

Discussion

The presence of disulfide bonds provides an increase in stability on the one hand and decrease in solubility on the other hand in seed storage proteins. Both features provide protection of proteins against proteolysis. There are several reports showing that disulfide bonds in storage proteins are converted to reduced state to facilitate mobilization during germination. The cytoplasmic/mitochondrial type NTR/Trx system appears to play a critical role in reduction of disulfide bonds in storage proteins in both cereal (Kobrehel et al., 1992) and legume seeds (Alkhalfioui et al., 2007) during germination. Transcripts encoding both HvNTR1 and HvNTR2 which are homologs of OsNTRA and OsNTRB, respectively were weakly detected in mature barley seed embryo. These isoforms were accumulated up to 48 h after imbibition and then remained at a constant level, indicating the importance of the NTR/Trx system during germination (Shahpiri et al., 2008). Accordingly, the level of HvTrxh protein increased slightly in embryos up to 24 h after imbibition (Shahpiri et al., 2008). In the present study, we demonstrated that the NTR isoforms are expressed in root and shoot of rice seedlings even in three-week rice seedlings, suggesting that this system actively functions in seedling tissues. In addition, Trx h has been identified as the major protein in the phloem sap of rice seedlings (Ishiwatari et al.,

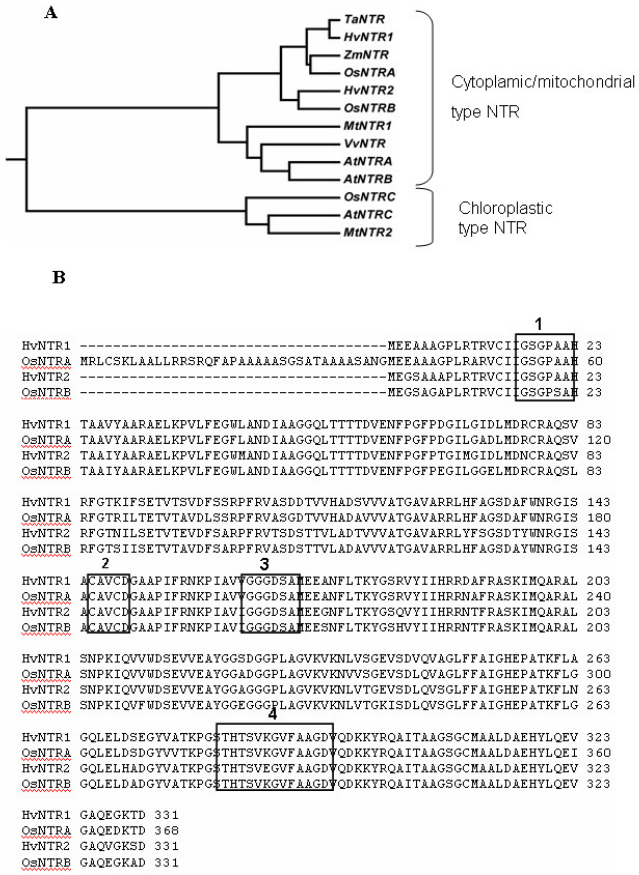

Fig 1. Multiple alignment and phylogenetic tree. (A) phylogenetic tree of NTR sequences. (B) Multiple alignment between cytoplasmic/mitochondrion type NTRs from barley and rice. FAD-binding motifs (Box1 and Box4), NADP-binding motif (Box 3), and two Cys residues in the active site motif (Box 2). For accession numbers and organisms, see "Materials and Methods".

1995) supporting the importance of this system in the rice seedlings. Whereas both OsNTRA and OsNTRB were expressed in shoot, OsNTRA was hardly detected in root and therefore OsNTRB was the predominant isoform in this tissue. This distinct transcript accumulation pattern observed for these isoforms indicates that they are differentially expressed in different tissues and therefore they may have different physiological roles in different rice seedling tissues. However, further analysis using protein appearance pattern with specific antibodies in different rice tissues in conjugation with their intracellular localization are required for better understanding the individual role of these two cytoplasmic/mitochondrial isoforms. The recombinant forms of OsNTRA and OSNTRB were produced in *E. coli* as fusion proteins with His-tag at their N-terminal. The two isoforms exhibited almost similar DTNB reduction activity both in the absence and presence of *E. coli*Trx. These results are in accordance with the high sequence identity of these two isoforms and the conservation of residues surrounding their active sites. The similarity in activity of cytoplasmic NTR isoforms was also previously observed for HvNTR1 and HvNTR2 toward two barley Trx h isoforms (HvTrxh1 and HvTrxh2). However it should be noted that *E. coli* Trx can not be a good substrate for plant NTR (Shahpiri et al., 2008). Therefore the low velocity of reduction of DTNB in the presence of *E. coli* Trx may not reflect the differences between these two NTR isoforms. To understand the specific role of each NTR isoform further study is necessary by analyzing the interaction of each NTR isoform toward different recombinant rice Trx h.

Materials and methods

Plant Materials

Rice (*Oryza sativa* L. ssp. *indica*) seeds from line 2 selected from local variety of Lenjani were provided by Isfahan Center for Research of Agriculture Science and Natural Resources. Germination of seeds was performed as previously described (Pourhadian et al., 2010) with minor modifications. The seeds were washed twice with distilled water and soaked for 24 hours. Then seeds were placed in 0.1×0.08×0.06 m plastic containers on a stainless steel wire mesh lined with bandage and 0.01 m above distilled water. The containers were kept at room temperature for one, two and 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.

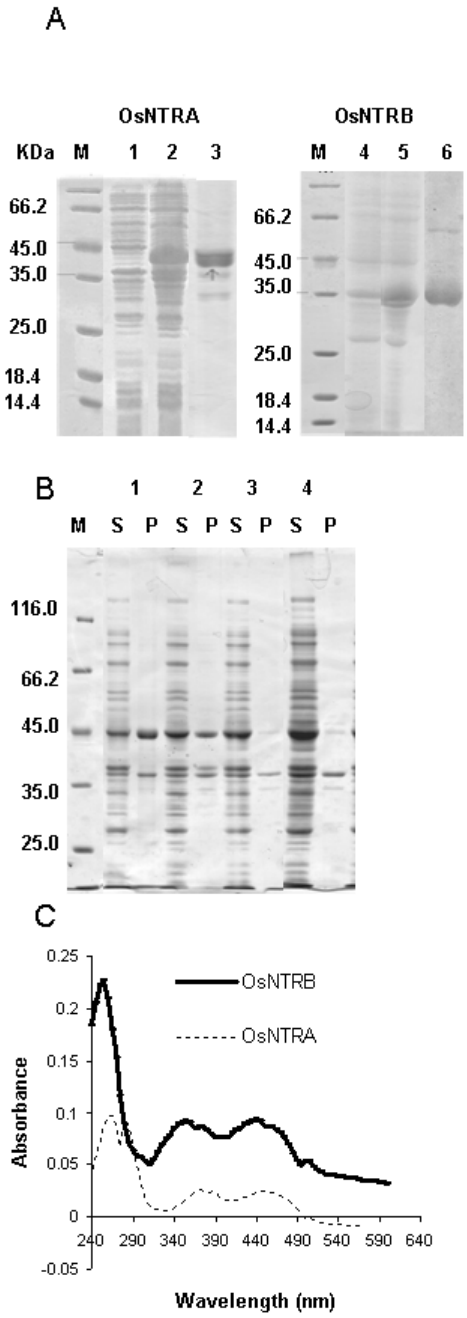

Fig 2. Overexpression of rice NTR isoforms in *E. coli.* (A) SDS-PAGE analysis for verification of expression and purification. Total soluble protein extracted from *E. coli* harboring pET28a-OsNTRA (lanes 1,2) and pET28a-OsNTRB (lanes 4,5). Before addition of IPTG (lanes 1, 4) and 4 hours after addition of IPTG (lanes 2,5). Purified His-OsNTRA (lane 3) and purified His-OsNTRB (lane 6). (B) Effects of temperature and IPTG on the solubility of OsNTRA and OsNTRB. The insoluble fractions (P) and soluble fraction (S) of OsNTRA after addition of 100 µM IPTG at 37 ^{0} C (lane 1), 30 ^{0} C (lane 2) and 25 ^{0} C (lane 3) and after addition of 50 µM IPTG at 25 ^{0} C (lane 4). (C) Absorption spectrum of recombinant OsNTRA and OsNTRB.

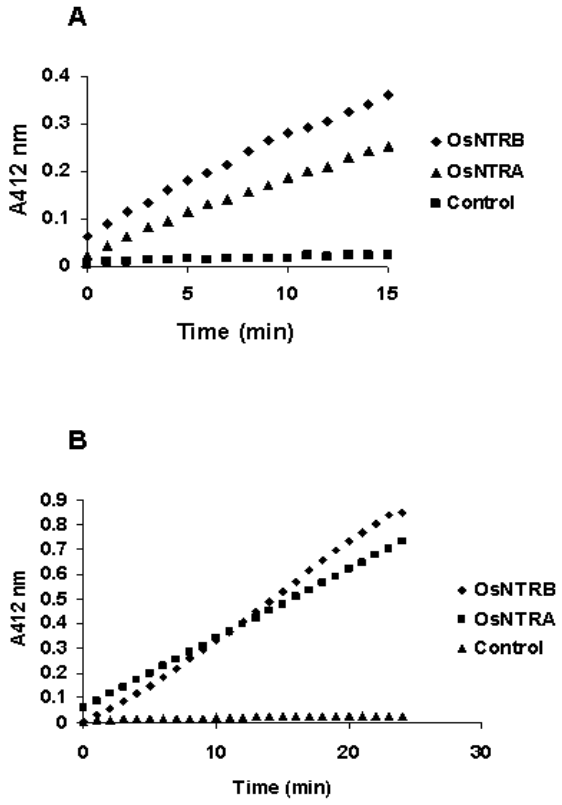

Fig 3. Enzyme assay of OsNTRA and OsNTRB. (A) NADPH-dependent reduction of DTNB for OsNTRA and OsNTRB. Control shows the reaction containing NADPH and DTNB with out addition of NTRs. (B) Reduction of recombinant *E. coli* Trx by OsNTRA and OsNTRB, monitored via the reduction of DTNB. Control shows the reaction containing NADPH, NTR and DTNB without addition of *E. coli* Trx.

Cloning of genes encoding OsNTRA and OsNTRB

Total RNA was extracted from shoots of one-week old seedlings using the High Pure RNA isolation Kit (Roche). Quality of total RNA was checked by ratios of A_{260}/A_{280} and A_{260}/A_{230}. To remove the genomic DNA contamination, extracted RNA was treated with RNase-Free DNase (Fermentas). Total RNA (0.1 µg) was reverse transcribed 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* DNA polymerase (Fermentas) and specific primers for coding sequences OsNTRB (5'GAATTCATGGAGGGATC-CGCCG 3´ and 5'AAGCTTTCAATCGGCCTTGCCCT 3´). The specific primers were designed using Oligo Perfect^{TM} Designer (http://tools.invitrogen.com/content.cfm). An *Eco*RI restriction site (underlined in forward primers) at the start codon and a *Hind*III restriction site after stop codon

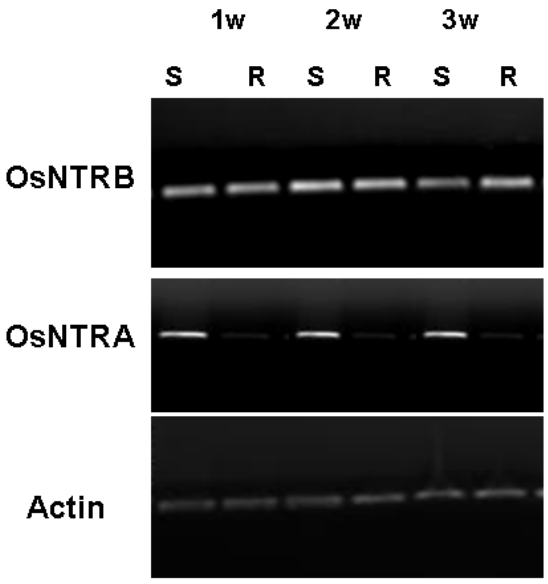

Fig 4. Gene expression analysis of genes encoding OsNTRA and OsNTRB in Shoot (S), Root (R) in rice one, two and three week (W)-old seedlings.

(underlined in reverse primers) were introduced in the primers. The RT-PCR product of OsNTRB coding sequence was cloned in pJET cloning vector (Fermentas) to give pJET-OsNTRB. The construct was introduced into *E. coli* DH5α. The sequence of the insert was determined on both strands.
The coding sequence of OsNTRA was synthesized (Genscript) and cloned in PUC 57 (Genscript) between two restriction sites *Eco*RI and *Hind*III to give PUC57-OsNTRA.

Sequence analysis

Multiple sequence alignment was performed using ClustalW (http://www.ebi.ac.uk Tools/msa/ clustalw2). A phylogenetic tree was constructed using software MEGA 4. National Center for Biotechnology Information (NCBI) accession numbers used for the analysis were: At, Arabidopsis (*Arabidopsis thaliana*), AtNTRA (Q39242), AtNTRB (NP_195271.2), AtNTRC (NP_565954); Hv, *Hordeum vulgare*, HvNTR1(EU314717), HvNTR2 (ABX09990); Zm, *Zea mays,* Zm NTR (NP_001150415), Ta, *Triticum aestivum,* TaNTR (CAD19162); Mt, *Medicago truncatula,* MtNTR1 (ABH10138), MtNTR2 (ABH10139); Os, *Oryza sativa,* OsNTRA (Q69PS6), OsNTRB (Q6ZFU6), OsNTRC (Q70G58.2), Vv, *Vitis vinifera,* VvNTR (XP_002263864.1).

Protein expression and purification of recombinant rice NTR isoforms

For construction of expression vectors, inserts were isolated from pJET-OsNTRB and pUC57-OsNTRA by digestion with *Eco*RI and *Hind*III and ligated into the pET28a expression vector linearized with *Eco*RI and *Hind*III to give pET-OsNTRB and pET-OsNTRA, respectively. Sequences were verified and constructs were used to transform *E. coli* strain Rosetta (DE3). Cells were grown in 500 ml Luria-Bertani medium supplemented with 50 µg/ml kanamycin and 5 µg/ml chloramphenicol to an OD_{600} of 0.6. For optimization of expression, cultures were grown at different temperatures (37 ^{0}C, 30 ^{0}C, and 25^{0}C) and induced with different concentration of IPTG 50 and 100 µM) for 4 h. Cells were harvested by centrifugation and frozen at -80 ^{0}C until use. For extraction of cytoplasmic soluble proteins, the frozen pellets were resuspended in Bugbuster protein extraction reagent (Novagen) and shaken for 30 min at room temperature. After centrifugation (14000g for 20 min, 4^{0}C), recombinant proteins in the supernatants were harvested. The extracted soluble proteins were then purified using 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 with a gradient of 10–200 mM imidazole. For extraction of insoluble fraction the remaining pellet was resuspended in 1× protein loading buffer (60 mM Tris-HCl, 2% SDS, Glycerol 10 %, 0.01 % bromophenol blue). The analysis of protein fractions was performed on SDS-PAGE after addition of DTT and gels were stained with coomassie Brilliant Blue R-250. The concentration of His_6-OsNTRA and His_6-OsNTRB were determined by A_{280} and Beer-lambert law with molar extinction coefficients of 21617.5 M^{-1} cm^{-1} and 28545 M^{-1} cm^{-1}, respectively.
The absorption spectrum was recorded for 7.8 µM OsNTRB and 3.5 µM OsNTRA in 30 mM Tris-HCl (pH 8.0) at room temperature.

NTR activity with DTNB in the absence of Trx

Activities of OsNTRA and OsNTRB were determined using DTNB (Holmgren, 1977). The reaction containing 100 mM potassium phosphate (pH 7.5), 10 mM EDTA, 0.1 mg/ml bovine serum albumin (BSA), 2 mM DTNB (Sigma-Aldrich), and 200 µM NADPH (Sigma-Aldrich) was started with the addition of 240 nM NTR. 100 mM DTNB was prepared in 96% ethanol as stock solution. The reaction was monitored by measuring the absorbance at 412 nm reflecting the formation of 2-nitro-5-thiobenzoic acid (TNB).

NTR assay with Trx

The ability of NTR to catalyze the reduction of Trx by NADPH was examined in a NTR assay (Krause and Holmgren, 1991) with slight modifications. The reaction mixture was prepared as described above but with much lower concentrations of DTNB (200 µM) and NTR (40 nM). The reaction was started with the addition of 24 µM *E. coli* Trx (Sigma-Aldrich). The reaction was monitored by measuring the rate of increase of absorbance at 412 nm reflecting the formation of TNB.

RT-PCR analysis

The extraction of total RNA from shoots and roots of one-week, two-week and three-week old seedlings and synthesis of cDNA were performed as described above. The cDNA samples were amplified by Taq DNA polymerase (Cinaclon) using specific primers for sequences OsNTRB (5'-GACTCCACCACCGTGCTC-3', 5'-ATCTTCCCAGTAAC-CAAGTTCTTC-3') and OsNTRA (5'-TGGT GAGCGGC-GAGGTCT-3', 5'-CGGAGTCCAGCTCGAGCT-3'). The specific primers were designed based on the coding sequence of OsNTRB and OsNTRA by Oligo Perfect^{TM} Designer (http://tools.invitrogen.com/content.cfm). Specificity of the primers was checked by BLAST search and confirmed by sequencing of amplified PCR products. Rice actin was used as standard control and amplified by primers 5´-

GAAGATCACTGCCTTGCTCC-3´ and 5´-CGATAACA-GCTCCTCTTG GC-3´. 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. Amplification products were separated by agarose gel electrophoresis. RT-PCR products were purified by Silica Bead DNA Extraction Kit (Fermentas) and cloned in pJET cloning vector (Fermentas).

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