Isolation and characterization of thioredoxin and NADPH-dependent thioredoxin reductase from tomato (Solanum lycopersicum)

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To investigate the pathways of oxidoreductases in plants, 2 key components in thioredoxin systems i.e. thioredoxin h (Trx h) and NADPH-dependent thioredoxin reductase (NTR) genes were first isolated from tomatoes (Solanum lycopersicum). Subsequently, the coding sequences of Trx h and NTR were inserted into pET expression vectors, and overexpressed in Escherichia coli. In the UV-Visible spectra of the purified proteins, tomato Trx h was shown to have a characteristic ‘shoulder’ at ∼290 nm, while the NTR protein had the 3 typical peaks unique to flavoenzymes. The activities of both proteins were demonstrated by following insulin reduction, as well as DTNB reduction. Moreover, both NADPH and NADH could serve as substrates in the NTR reduction system, but the catalytic efficiency of NTR with NADPH was 2500-fold higher than with NADH. Additionally, our results reveal that the tomato Trx system might be involved in oxidative stress, but not in cold damage. [BMB reports 2011; 44(10): 692-697]

INTRODUCTION

The thioredoxin system, including thioredoxins (Trxs), thioredoxin reductases (TrxRs) and NADPH, is present in both prokaryotes and eukaryotes, and is involved in several processes including defense against oxidative damage, signaling, and regulation of apoptosis (1). The Trx proteins are low molecular mass proteins (12-13 kDa) which contain 2 conserved cysteines in their ‘CGPC’ redox sites, and act as electron donors for numerous enzymes (1, 2). The TrxR proteins are pyridine nucleotide-disulfide oxidoreductases that contain a FAD-binding site, a NADPH-binding site, and a central catalytic domain (3).

In plants, 3 types of Trxs, i.e. Trx m, f, and h, have been well characterized. Trx m and f are catalyzed by a ferredoxin-dependent TrxR with power derived from photosynthetic carbon metabolism, while the h classes of Trxs are reduced by NADPH-thioredoxin reductase (NTR) using NADPH as an electron donor (2). The NADPH-dependent thioredoxin system (NTS) and NADPH-dependent glutathione/glutaredoxin system are reported to play vital roles in maintaining cellular redox equilibrium and antioxidant defense by serving as electron donors and regenerating numerous enzymes (4). Recently, numerous NTSs have been identified from a variety of organisms such as Deinococcus radiodurans, Arabidopsis thaliana, and Triticum aestivum (2, 3, 5). Moreover, the activities of MSRs from A. thaliana and poplar (Populus trichocarpa) were demonstrated in thioredoxin systems (6, 7), suggesting that the NTS is an important oxidation-reduction system. However, there have only been a few studies on redox systems from tomato (Solanum lycopersicum).

To characterize targeted proteins related to tomato thioredoxin systems and to simulate the internal environment in S. lycopersicum, we isolated, expressed, and purified the critical components of the NTS, including tomato Trx h and NTR. In addition, the kinetic parameters of these recombinant proteins and their function in response to oxidative stress were also investigated.

RESULTS AND DISCUSSION

Identification of Trx h and NTR in tomato

The h types of Trx, which are encoded by a multiple gene family in plants, have been shown to be involved in the NADPH-dependent thioredoxin system, and have many functions including being involved in seed germination, and as electron donors for MSRs (1, 2). Based on the sequences of Trx h and NTR from Arabidopsis, we searched and identified these 2 genes in tomato Trx h and NTR. In addition, the kinetic parameters of these recombinant proteins and their function in response to oxidative stress were also investigated.
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gene (3,759 bp) encodes a predicted polypeptide of 342 amino acids with a calculated MW of 36.5 kDa and pI of 5.28 (Supplementary Table 1). The Trx h gene is located on tomato chromosome 4 and possesses 3 exons and 2 introns, while the NTR gene composed of 2 exons and 1 intron is found to exist on chromosome 3 (Fig. 1A and Supplementary Table 1). The amino acid sequences of the Trx h polypeptide aligned from Trx proteins isolated from other species revealed that it contains the typical dithiol/disulfide active site ‘WCPC’ at the N-terminus (Fig. 1B). In wheat (T. aestivum), the thioredoxin h reductase as well as Arabidopsis NTRs were reported to contain 3 characteristic domains; including a FAD-binding, a site NADPH-binding, and a catalytic site (2, 5). A comparative alignment of the tomato NTR sequence with thioredoxin reductase from other organisms showed that the tomato peptide also possesses these key domains (Fig. 1C). Phylogenetic analysis indicated that tomato Trx and NTR were similar in their predicted amino acid sequences with the Trx and NTR of other selected organisms, especially plant species (Fig. 1D and E).

Fig. 1. Characterization of tomato Trx h and NTR genes. (A) Exons/introns analysis of Trx h and NTR genes from tomato. Black boxes indicating the exons and introns are shown with thin lines. The scale-bar represents 400-bp length nucleotide sequences; (B) Amino acid sequence alignments of Trx h with other Trx proteins. SbTrx (Solanum berthaultii, ABD65296), NtTrx h (Nicotiana tabacum, P29449), AtTrx1 (A. thaliana, NP 190672), E.coli (E.coli thioredoxin, M54881). Conserved catalytic sequences are marked with box; (C) Multiple alignment of SINTR from NTR protein in A. thaliana (AtNTRA, NP 179334), Medicago truncatula (MtNTRA, ABH10138), Oryza sativa (OsNTR, BAD33510), and E.coli (E.coli thioredoxin reductase, P09625). Active site, NADPH-binding domain, FAD-binding domain, and cysteines in active site are indicated by box, ↓, *, and ▲, respectively. (D) Phylogenetic tree of Trx homologs in S. berthaultii, N. tabacum, Ipomoea batatas (IbTrx, AAQ23135), Plantago major (PmTrx, CAH94430), A. thaliana, E.coli, and S. cerevisiae (ScTrx, CAA42258). (E) A phylogenetic tree showing the relationship between SINTR and other NTRs from O. sativa, A. thaliana, M. truncatula S. cerevisiae (ScNTR, AAA64747), and E.coli.
Expression and purification of recombinant Trx h and NTR proteins

To further investigate these 2 components, Trx h and NTR were subcloned into pET 22b and 28 a(+) expression vectors, respectively. After introduction into Escherichia coli BL21 (DE3) cells, recombinant protein expression was induced using 0.1 mM of isopropyl β-D-1-thiogalactopyranoside (IPTG). In the denaturing SDS-PAGE assay, high expression levels of Trx h and NTR were observed after the addition of IPTG when compared with the groups in the absence of IPTG (Fig. 2A). The recombinant Trx h protein with an apparent MW of 14 kDa was the major band in SDS-PAGE analysis and the purity of the protein was >95% after Ni²⁺-affinity column purification using the Gel-pro Analyzer 4.0 analysis program (Lane 3, Fig. 2A). For the tomato NTR recombinant protein, a single polypeptide corresponding to ~36.5 kDa was observed and its purity was calculated to be 98% of the total purified protein (Lane 7, Fig. 2A). Finally, the yields of the recombinant Trx h and NTR were 12.1 mg and 8.5 mg of pure protein per liter, respectively.

UV-scanning of purified Trx h and NTR from tomato

Due to the existence of 2 tryptophan (Trp) residues, the absorption spectrum of Trx in Chlamydomonas reinhardtii contained a typical ‘shoulder’ peak at ~290 nm (8). In this study, a sharp ‘shoulder,’ which resulted from Trp20, Trp42, and Trp80 in tomato Trx h, was also observed at 290 nm, while the absorption spectrum of BSA exhibited a unique peak at 280 nm.
(Fig. 2B). Sequence alignments suggested that tomato NTR was a flavoprotein (Fig. 1C). To verify this initial finding, the UV-Vis spectrum of the fusion NTR protein was determined in the range of 250 nm to 600 nm (Fig. 2C). Typical absorbance peaks at 270, 378, and 454 nm were detected, further demonstrating that SINTR was a flavoprotein. This result was consistent with scanning spectra of recombinant proteins from Arabidopsis and T. aestivum, respectively (2, 5).

Catalytic properties of tomato Trx h and NTR

Trx h was shown to be a disulfide reductase that catalyzed the reduction of insulin in the presence of DTT. An increase of absorbance at 650 nm, which was derived from precipitation of the insulin β-chain, was observed, indicating that the recombinant Trx was active (9). The insulin reduction assay revealed a negative correlation between the amount of Trx h and the initial time of increase in turbidity at 650 nm (Fig. 2D). The $V_{\text{max}}$ and $K_m$ values for Trx h were determined to be 0.094 (μM/650/ min) and 6.7 μM, respectively (Fig. 2E). The $V_{\text{max}}$ value for tomato Trx h was higher than the $V_{\text{max}}$ value reported in Lolium perenne (10).

Because recombinant Trx h could serve as a disulfide reductase in the insulin reducing system, we further examined whether tomato Trx h played a role in the NADPH-dependent thioredoxin system. In these experiments, the absorbance at 340 nm, which was due to NADPH, significantly decreased with the addition of 50 nM or 100 nM of purified NTR, when compared with the reaction in the absence of NTR (Fig. 2F). The $V_{\text{max}}$ and $K_m$ values were determined to be 1.13 μmol/min and 0.31 μM, respectively; based on a Lineweaver-Burk plot generated from the Trx h data (Fig. 2G). We found that the $K_m$ value for tomato NTR was lower than those reported for Arabidopsis (1.1 μM) and T. aestivum (7.6 μM) (2, 5).

The activity of tomato NTR was further examined by measuring its ability to reduce DTNB. Kinetic parameters corresponding to NTR with various substrates are shown in Table 1. It has been reported that both NADPH and NADH could serve as substrates in the wheat NADPH-dependent thioredoxin system, and the $K_m$ value for NADPH was much higher than the value for NADPH (2). Analogous results were observed in this study for the tomato thioredoxin system when NADPH or NADH was used as the reductant (Table 1). However, the enzyme efficiency ($K_m$/$K_m$) of NTR with NADPH as a substrate was ~2500-fold greater than when NADH was used as the substrate. The $K_m$ value for DTNB was 18.60 μM at a saturating concentration of other substrates, indicating a relatively higher affinity than that shown by thioredoxin reductase (660 μM) from rat liver (11).

Table 1. Kinetics parameters of tomato NTR. The kinetics parameters were determined under standard reactions consist of 500 μl of 50 mM potassium phosphate, pH 7.0, 10 mM EDTA, 6 μM recombinant Trx h, 100 nM NTR. $K_m$ values for various substrates were measured at saturating concentrations of the other substrates.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>$K_m$ (μM)</th>
<th>$V_{\text{max}}$ (μmol/min)</th>
<th>$K_m$/$K_m$ (μS⁻¹)</th>
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<td>NADPH</td>
<td>2.35</td>
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<td>0.06</td>
<td>6.0 × 10⁻⁵</td>
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<tr>
<td>$SITrx h$</td>
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<td>0.38</td>
<td>1.23 × 10⁻⁵</td>
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*The $K_m$ for DTNB was calculated in the presence of 100 μM NADPH indicating by the increase of absorbance at 412 nm. $K_m$ values for Trx h was measured by following insulin reducing procedure for instance, a high transcript level of Trx h was detected in rice seedlings after methyl viologen (MV) treatment (12). Laloi and co-authors reported that Arabidopsis Trx h5 mRNA had accumulated in response to treatment with oxidative elicitors and incompatible pathogens (13). To test whether the NTS system functions to protect the organism against oxidative damage, Northern blotting assays were performed on tomato seedlings exposed to various types of stress (Fig. 3A). In these experiments, the $SITrx h$ transcript levels were significantly increased after 3 h of treatment with hydrogen Peroxide (H₂O₂) and salinity, but subsequently declined to the original level after 12 h of treatment. Expression of $SITrx h$ was increased after 3 h exposure to MV stress and was maintained at a high level after 12 h. In cold-treated tomatoes, the expression of $SITrx h$ was not induced and it remained at a similar level after 3 and 12 h of treatment. These results indicate that tomato Trx h was involved in defense against oxidative stress, but its regulated patterns were distinct.

Thioredoxin reductase deficient strains of E.coli and Saccharomyces cerevisiae were highly sensitive to oxidative stress. Subsequent studies claimed that the transcription of yeast thioredoxin reductase increased after exposure to H₂O₂ (14). NTS has also been shown to regulate numerous oxidative-related pathways (1). In this report, we found that the expression of SINTR was significantly increased by H₂O₂ and NaCl treatment when compared with the untreated tomato seedlings. For the MV-treated seedlings, accumulation of SINTR transcription could also be detected, but its level was relatively lower than that observed in H₂O₂ and NaCl treated plants. Similar to the expression pattern of $SITrx h$, SINTR transcription did not change relative to the control (Fig. 3B). These results indicated that the NTS system responded to some environmental damages, such as H₂O₂, MV, and salinity, but not to chilling-induced stress.

In conclusion, we developed a NADPH-dependant thioredoxin system using an E.coli expression system. The purified Trx h and NTR proteins were shown to be capable of catalyzing corresponding substrates. Using the tomato thioredoxin system, they could further function as electron donors in vitro
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A

B

Fig. 3. Expressions of SlTrx h and SlNTR in response to abiotic stress. Tomato seedlings were exposed to H2O2, MV, salinity (NaCl), and cold for various time periods. Total RNAs (20 μg) were isolated and loaded on each lane. The cDNAs of the genes were synthesized from 2 μg of total RNA by M-MLV Reverse Transcriptase (Invitrogen, CA, USA). The primers used for amplification were purchased from Sigma (St.Louis, MO, USA).

MATERIALS AND METHODS

Materials
A His-Bind purification kit was purchased from EMD Biosciences Company (San Diego, CA, USA). DTNB [(5,5'-dithiobis (2-nitrobenzoic acid)], DTT, NADH, NADPH, and bovine insulin were purchased from Sigma (St.Louis, MO, USA).

Gene search and sequence analysis
The tomato Trx h and NTR genes were identified using the BLAST tool to search for homologs of these genes from Arabidopsis in a tomato genomics database. Amino acid sequence alignments were determined using the DNASAN program. Phylogenetic trees were constructed using TreeTop software (http://www.genebee.msu.su/services/phtree_full.html).

Cloning, expression, and purification of tomato Trx h and NTR
Total RNA was isolated from the 5-week old tomato leaves using the Trizol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer’s instructions. First, strand cDNA was synthesized from 2 μg of total RNA by M-MLV Reverse Transcriptase (Invitrogen, CA, USA). The primers used for amplification of Trx h cloning were 5′-GACAATGTATGGCCTCATCTGAGGA-3′ (Forward primer, NdI enzyme site is underlined) and 5′-CTCGAGAGCAGTCACAACACGAGGAG-3′ (Reverse primer, Xho I site is underlined). For the NTR fragment, the sequences of the forward and reverse primers with Nco I and Xho I restriction enzyme sites (underlined), were 5′-CCATGGCGGACATAAGACCAAAG-3′ and 5′-CTCGAGGATCCTCTTCTCTCTTGTGCC-3′, respectively.

The Trx h and NTR fragments were digested with the corresponding restriction enzymes and ligated to pET 22b and pET 28a (+) expression vector, respectively. The recombinant plasmids were introduced into the E. coli BL21 (DE3) strain and successful transfection was determined by colony PCR using the corresponding primers. The transformants were initially grown at 37°C in 1 L of Luria-Bertani medium supplemented with 50 mg/L kanamycin. When the optical density (OD600) reached 0.6, IPTG was added to a final concentration of 0.1 mM and the cells were further cultured at 20°C for 20 h. The cells were harvested and resuspended in 1× binding buffer (500 mM NaCl, 20 mM Tris-HCl, 5 mM imidazole, pH 7.9). The cells were then passed twice through a French press at 12,000 psi, and centrifuged at 14,000 rpm for 30 min. The filtered supernatant was loaded on to a Ni2+ affinity column. The eluted fraction was dialyzed twice against 20 mM Tris-HCl and 1 mM EDTA. The purified proteins were pooled, concentrated, and stored at -80°C.

The recombinant proteins were visualized using 15% SDS-PAGE gels. Protein concentrations were determined by absorbance at 595 nm using a protein assay regent (Bio-Rad, California, CA, USA) (16). The UV-Visible spectrum was recorded over a range of 250 to 600 nm using a HP 8453 DAD Spectrophotometer.

Assay of Trx h and NTR activities
Trx h activity was measured using an insulin reduction assay reported by Juttner et al. (10). The reaction mixture (600 μl) containing 100 mM potassium phosphate, pH 7.0, 2 mM EDTA, 0.13 mM insulin, 0.33 mM DTT, and different concentrations of recombinant Trx h, was monitored at 340 nm in a standard mixture (500 μl) containing 50 mM potassium phosphate, pH 7.0, 1 mM EDTA, 0.1 mM NADPH, 80 μM insulin, 50 mM NTR, and various amounts of recombinant Trx h. The Km and velocities (V) were calculated as previously described (3). In addition, NTR activity was also tested using the DTNB reduction assay following a previously described procedure (3) with minor modification. In brief, the activity was tested in a final volume of 500 μl containing 50 mM potassium phosphate, pH 7.0, 10 mM EDTA, 6 μM Trx h, and 100 mM NTR. Varying concentrations of NADH, NADPH, and DTNB were added to initiate the reactions.

RNA isolation and Northern blot analysis
Tomato plants (5-weeks old) were grown at a stable temperature of 25°C. The seedlings were then exposed to 10 mM...
H₂O₂, 50 μM MV, and 200 mM NaCl for 3 and 12 h, respectively. For low temperature treatment, the seedlings were kept in a cold chamber at 4°C under dim light. Various stress-treated plants were harvested, frozen, and stored at -80°C.

RNA isolation and Northern blot analysis were carried out as described by Dai et al. (17). SlTrx h and SlNTR cDNA labeled with [α-32P]-dCTP were used as probes during hybridization.

Acknowledgements
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REFERENCES

**Supplementary Table 1.** Summary of *Trx h* and *NTR* gene from tomato

<table>
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<tr>
<th>Name</th>
<th>Exons (Introns)</th>
<th>AA&lt;sup&gt;a&lt;/sup&gt;</th>
<th>MW (KDa)&lt;sup&gt;b&lt;/sup&gt;</th>
<th>pI&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Chromosome</th>
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<td>123</td>
<td>13.6</td>
<td>5.92</td>
<td>4</td>
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<tr>
<td><em>SINTR</em></td>
<td>2 (1)</td>
<td>342</td>
<td>36.5</td>
<td>5.28</td>
<td>3</td>
</tr>
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</table>

<sup>a</sup> Predicted number of amino acids

<sup>b</sup> Protein molecular weight and pI was predicted with ProtParam program