Target Proteins of the Cytosolic Thioredoxins in \textit{Arabidopsis thaliana}

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Possible target proteins of cytosolic thioredoxin in higher plants have been investigated in the cell lysate of dark-grown \textit{Arabidopsis thaliana} whole tissues. We immobilized a mutant of cytosolic thioredoxin, in which an internal cysteine at the active site was substituted with serine, on CNBr activated resin, and used the resin for the thioredoxin-affinity chromatography. By using this resin, the target proteins for thioredoxin in the higher plant cytosol were efficiently acquired. The obtained proteins were separated by two-dimensional gel electrophoresis and analyzed by matrix-assisted laser desorption ionization time-of-flight mass spectrometry. Thus we have identified proteins of the anti-oxidative stress system proteins (ascorbate peroxidase, germin-like protein, and monomeric type II peroxiredoxin), proteins involved in protein biosynthesis (elongation factor-2 and eukaryotic translation initiation factor 4A), proteins involved in protein degradation (the regulatory subunit of 26S proteasome), and several metabolic enzymes (alcohol dehydrogenase, fructose 1,6-bis phosphate aldolase-like protein, cytosolic glyceraldehyde 3-phosphate dehydrogenase, cytosolic malate dehydrogenase, and vitamin B$_6$-independent methionine synthase) together with some chloroplast proteins (chaperonin 60-$\alpha$ and 60-$\beta$, heat shock protein 70, and glutamine synthase). The results in this study and recent proteomics studies on the target proteins of chloroplast thioredoxin indicate the versatility and the physiological significance of thioredoxin as reductant in plant cell.

Keywords: \textit{Arabidopsis thaliana} — Cytosolic thioredoxin — Redox regulation — Thioredoxin.

Abbreviations: APX, ascorbate peroxidase; CDSP32, chloroplastic drought-induced stress protein of 32 kDa; Cpn60-$\alpha$ and Cpn60-$\beta$, rubisco-binding protein $\alpha$ subunit and $\beta$ subunit; DTT, dithiothreitol; EF-2, elongation factor 2; eIF4A, eukaryotic translation initiation factor 4A; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; Hsp70, heat shock protein 70; MDH, malate dehydrogenase; MALDI, matrix-assisted laser desorption ionization; PMF, peptide mass fingerprint; Prx, peroxiredoxin; RT-PCR, reverse transcriptase PCR; Trx, thioredoxin.

This paper is dedicated to Professor Heinrich Strotmann on his sixty-fifth birthday.

Introduction

Thioredoxin (Trx) is a small, ubiquitous, disulfide oxidoreductase with two redox active cysteines (WCGPC) in a conserved domain (Buchanan 1991, Schürmann and Jacquot 2000, Buchanan et al. 2002). In higher plants, three different types of Trxs are known based on their localization in the cell; Trx-$f$ and Trx-$m$ in chloroplasts (Jacquot et al. 1997), and Trx-$h$ in the cytosol (Rivera-Madrid et al. 1995). In addition, several additional Trx groups are reported based on the whole genome data of \textit{Arabidopsis thaliana} (Laloï et al. 2001, Meyer et al. 2002). Trx can modulate the enzyme activity of target proteins by reducing disulfide bonds. In some cases, Trx also acts as hydrogen donor, for example, to reduce peroxiredoxin (Prx). The reduced form Prx reduces hydrogen peroxide, lipid peroxides, or peroxynitrite and functions as anti-oxidative stress system in the cell (Rouhier and Jacquot 2002). Thus Trx acts as electron donor to various enzymes. The specificity of Trxs for their targets in chloroplasts are reported to be determined mainly by the surface charges around the active site (Morga-Garcia et al. 1998, Capitanì et al. 2000, Collin et al. 2003).

In the cytosol of plants, five isoforms of Trx-$h$ have been identified (Rivera-Madrid et al. 1995). They were further confirmed by the whole genome analysis of \textit{A. thaliana} (Arabidopsis Genome Initiative 2000), and in total eight isoforms of Trx-$h$ are reported (Meyer et al. 2002). Interestingly, three isoforms, Trx-$h3$, $h4$ and $h5$, have a slightly different active site sequence, WCPC, instead of the common Trx active site sequence WCGPC. Although the relevance between the active site variety and the target specificity of Trx-$h$ isoforms were
studied by Bréhélin et al. (2000) using complementation test of Trx-deficient yeast, it is not clear whether the differences may reflect a different target specificity due to the lack of sufficient information on Trx target proteins.

Historically, enzymes interacting with Trx were identified by biochemical studies on target enzymes. Hence four Calvin cycle enzymes in chloroplasts [glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (Baalmann et al. 1995), fructose 1,6-bis-phosphatase (Clancey and Gilbert 1987), sedoheptulose 1,7-bis phosphatase (Cadet et al. 1987), and phosphoribulokinase (Wolosiuk and Buchanan 1978)], chloroplast ATP synthase (Mills et al. 1980, Schwarz et al. 1997, Stumpp et al. 1999), and the stromal enzymes, NADP-dependent malate dehydrogenase (MDH) (Scheibe and Anderson 1981), glucose 6-phosphate dehydrogenase (Scheibe and Anderson 1981), Rubisco activase (Zhang and Portis 1999), and acetyl CoA carboxylase (Sasaki et al. 1997) were reported to be regulated by reduction or reoxidation of the internal disulfides, and thus to be Trx target proteins. However, recent progress of the genome sequencing projects for various organisms made it possible to survey the candidate proteins, which can interact with Trx, by comprehensive and systematic efforts. In 2001, we reported a new method to capture the target protein candidates of Trx comprehensively using immobilized mutated Trx, which is able to form stable mixed-disulfide intermediates with target proteins but not to facilitate the reduction of the disulfides of the target proteins (Motohashi et al. 2001). Proteins eluted from the resin with dithiothreitol (DTT) were identified by N-terminal Edman sequencing. This way we identified three potential target proteins (Prx-Q, cyclophilin, and Rubisco small subunit) together with five known proteins (Rubisco activase, sedoheptulose-1,7 bis-phosphatase, GAPDH, glutamine synthase, and 2-Cys type Prx) for chloroplast Trx-m. Within the newly identified proteins in that study, reduction of Prx-Q by Trx-m was biochemically confirmed (Motohashi et al. 2001). In addition, redox-regulation of the peptidyl-prolyl cis-trans-isomerase activity of cyclophilin and the relevant disulfide bonds in the molecule was also clarified (Motohashi et al. 2003). Similar immobilized Trx resin was used to purify 2-Cys Prx from cell lysate of green algae Chlamydomonas reinhardtii (Goyer et al. 2002). In addition, Balmer et al. recently analyzed target proteins in spinach chloroplasts using the immobilized mutated Trx-f and Trx-m (Balmer et al. 2003). By combining two-dimensional gel electrophoresis and LC-mass spectrometry, they reported 26 proteins in spinach chloroplasts as potential targets for chloroplast Trx.

In the case of cytosolic Trx-h from plants, Yano et al. (2001) reported a method to identify targets of Trx-h using subtraction display of the proteins in which cysteines were labeled with fluorescent-dye before or after the incubation with Trx and DTT. They detected more than 20 targets in peanut seeds on two-dimensional gel electrophoresis and five of them were partially sequenced. Finally they identified three allergens (Ara h2, Ara h3, and Ara h6) as novel targets of Trx-h. Following this work, they reported several Trx-h targets in barley embryos, which are related to germination and seedling development: globulin 1, 1-Cys type Prx, and the acidic ribosomal protein P3 (Marx et al. 2003). In contrast, Verdoucq et al. expressed a mutant Trx-h3(C42S) mutant of A. thaliana in yeast to find the counter part proteins of this mutant Trx-h, and successfully captured the Trx-dependent peroxidase (YLR109) (Verdoucq et al. 1999). In addition, they reported the existence of the homolog of YLR109 in A. thaliana genome based on sequence homology analysis.

In the present study, we intended to enumerate the target proteins of the cytoplasmic Trx, Trx-h, in the plant cell for the understanding of their physiological significance. For this purpose, we adopted the method using the immobilized Trx mutant resin again, and tried to capture the target proteins of Trx-h directly. The genes for Trx-h isoforms were cloned from total RNA of A. thaliana by RT-PCR and the active site cysteine mutants of each isoforms were prepared. Finally we successfully identified nine novel Trx target candidate proteins in the cytosol together with several target proteins for chloroplast Trx. Accumulation of the information on the target proteins for Trx-h certainly may assist the elucidation of the significance of Trx-h and the whole redox cascade in the cell.

Results

Cytosolic Trx-h isoforms in A. thaliana

Five cDNAs for Trx-h isoforms (Trx-h1 to Trx-h5) in A. thaliana have been cloned and their cellular expression was
confirmed on the transcriptional level by Rivera-Madrid et al. (1995). In the present study, we obtained these genes by reverse transcriptase PCR (RT-PCR) method from the total RNA prepared from whole tissues of A. thaliana. In addition, RT-PCR analysis showed that there are no remarkable differences of the amounts of mRNA for these five Trx-h isoforms as compared from dark-grown cultures (data not shown).

We constructed Escherichia coli expression vectors for the five Trx-h isoforms, expressed the recombinant Trx-h proteins, and purified them (Fig. 1, inset). To study the ability of the recombinant Trx-h as reductant, we utilized the insulin reduction assay. All of the recombinant Trx-h isoforms were able to reduce insulin, indicating the correct folding of the expressed Trxs in E. coli (Fig. 1).

**Acquisition of the potential target proteins of Trx-h by the mutant Trx immobilized resin**

Based on the reduction pathway of the proteins by Trx, we generated the cysteine mutant Trx isoforms, which can capture the target protein as a mixed disulfide intermediate (Verdoucq et al. 1999), as shown in Table 1. By using the immobilized mutant proteins of these Trx isoforms, we successfully captured the potential target proteins in the cell lysate of dark-grown culture of A. thaliana. Fig. 2A shows the representative result using the mutant Trx-h3 immobilized resin. A number of proteins were eluted from the Trx mutant immobilized resin after the resin was incubated with DTT following the repeated NaCl washings. Similar SDS-PAGE profiles were observed for other Trx-h isoforms (data not shown) in spite of the differences of the active site sequences as shown in Table 1. The variety of the obtained proteins on the gel were apparently much higher than those from chloroplast Trx immobilized resin (see Fig. 2 of Motohashi et al. 2001 for reference). One may consider that the acquisition of such a variety of proteins are the results of non-specific binding of the proteins to the physiologically inactive Trx-h mutants on the resin, which were defected by immobilization. To rule out this possibility, we confirmed the activity of the wild-type Trx-h isoforms, which was immobilized on the same resin, by the insulin reduction assay. The immobilized Trx-h could show certain level of reduction activity (Fig. 2B). These activities were not much different from that of chloroplast Trx-m immobilized resin used for the former study (Motohashi et al. 2001).

By N-terminal Edman sequencing, we could identify three proteins, ascorbate peroxidase (APX), 2-Cys Prx, and germin-like protein in the captured proteins (Fig. 2A, Table 2). In a former study (Motohashi et al. 2001), we could identify most of the target protein candidates by this way. However, this method was not effective this time because the N-terminus of many proteins was blocked and could not be analyzed by Edman sequencing.

**Specificity of the immobilized mutant Trx-h isoforms**

To analyze the composition of the captured proteins thoroughly, the proteins acquired by the immobilized mutant Trx-h isoforms were separated by two-dimensional gel electrophoresis. Although slight differences of the protein patterns were observed on the two-dimensional gels when the different Trx-h

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**Table 1** Active site sequence of Trx-h isoforms and their mutants

<table>
<thead>
<tr>
<th>Isoforms</th>
<th>Active site</th>
<th>Mutants</th>
<th>Primers for mutation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Trx-h1</td>
<td>39WCGPC</td>
<td>C43S (WCGPS)</td>
<td>5’-TTACCGGCTTCTTGGTGGAGCATGCTTTTCTTCATGCTTTTGCACATGTT3’</td>
</tr>
<tr>
<td>Trx-h2</td>
<td>58WCGPC</td>
<td>C62S (WCGPS)</td>
<td>5’-TTCTCGGCTACATTGGTCGGAGACATGCTTTTCTTCATGCTTTTGCACATGTT3’</td>
</tr>
<tr>
<td>Trx-h3</td>
<td>38WCPC</td>
<td>C42S (WCPPS)</td>
<td>5’-TTACGCTCTGTTCTTCATGCTTTTCTTCATGCTTTTGCACATGTT3’</td>
</tr>
<tr>
<td>Trx-h4</td>
<td>39WCPC</td>
<td>C43S (WCPPS)</td>
<td>5’-TTACGCTCTGTTCTTCATGCTTTTCTTCATGCTTTTGCACATGTT3’</td>
</tr>
<tr>
<td>Trx-h5</td>
<td>38WCPC</td>
<td>C42S (WCPPS)</td>
<td>5’-TTACGCTCTGTTCTTCATGCTTTTCTTCATGCTTTTGCACATGTT3’</td>
</tr>
</tbody>
</table>

*Residue numbers are indicated based on Meyer et al. 2002.*

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**Fig. 2** Availability of Trx-immobilized resin. (A) 1 mg of Trx-h3(C42S) was immobilized on 0.5 ml of the CNBr-activated resin and the target protein candidates were captured. The obtained protein samples were separated by 15% (w/v) SDS-PAGE. Three proteins identified by N-terminal Edman sequencing were indicated with arrows; 2-Cys Prx, APX, and germin-like protein from top to bottom. (B) The oxidoreductase activity of the immobilized Trx-h3 (wild type) was confirmed by insulin reduction assay as in Fig. 1. Trx-immobilized resin was prepared as for Fig. 2A and 75 µl of the resin was used for the assay. DTT (330 µM) was added to initiate the reaction. Spinach wild-type Trx-m immobilized on the resin was used as control.
Target proteins of cytosolic thioredoxin-h

isoform mutants were used (data not shown), it was difficult to assign the characteristics of the captured proteins for each Trx-h isoforms. Here, we showed the results from Trx-h1(C43S) isoform as a representative of the series of our experiments (Fig. 3A). From the comparison of the protein profiles of the captured proteins by the Trx mutant immobilized resin (Fig. 3A, B) with those of whole cell lysate proteins (Fig. 3C), it is obvious that the target protein candidates were concentrated by this procedure. The quantity and the variety of the proteins captured by the different cysteine mutant Trx-h1(C40S), in which the first reactive cysteine was substituted with serine, was remarkably less than those by Trx-h1(C43S) mutant (Fig. 3A, B). However, several proteins within the captured proteins by Trx-h1(C43S) were also captured by this C40S mutant, although their amounts were much less than those by C43S mutant. This result suggests that the major determinant of the interaction with the target proteins were basically similar in both Trx mutants but the efficiency to form the stable mixed disulfide intermediate via the remaining cysteine residue was different. As another control experiment, activated thiol Sepharose 4B resin was treated with DTT in advance and the theoretically equal amounts of exposed SH group as those of the Trx mutant immobilized resin used for the experiments shown in Fig. 3A and 3B, was incubated with cell lysate proteins. Then the captured proteins by this thiol Sepharose 4B resin were eluted with DTT. In this case, only the faint protein bands were observed (data not shown).

These results strongly suggest that the protein profiles obtained from our capture experiments should represent the overall profiles of the target protein candidates of Trx-h.

Identification of the captured proteins by mass spectrometry

We used a proteomics approach to identify the separated proteins on a two-dimensional gel using peptide mass fingerprint (PMF) analysis by Matrix-assisted laser desorption ionization (MALDI) time-of-flight mass spectrometry. Again, the two-dimensional gel profile of Trx-h1 is shown as a reference in Fig. 4. This way fourteen proteins were successfully identified in the examined spots and are listed in Table 2. Some protein spots obviously contained several proteins and identification only by PMF analysis was impossible. For these proteins (Spot Nos. 11 and 15), a measurement by tandem MS/MS spectrometry was applied and they were directly identified from their internal amino acid sequences together with the PMF analysis (see Table 2). Although two protein spots gave several candidates from the PMF data analyses (Spot Nos. 18 and 19 in Fig. 4), the protein at the Spot No. 18 is seemed to be the same one as fructose 1,6-bisphosphate aldolase-like protein (Spot No. 13) and the other one (Spot No. 19) will be APX1 (Spot No. 1), respectively, because these enzymes were cited as expected candidates from PMF data analysis and the theoretical molecular weights of them almost coincided with those calculated from SDS-PAGE mobility.

Identification of the proteins on a two-dimensional gel. The proteins were captured by Trx-h1(C43S) immobilized resin and separated by two-dimensional gel electrophoresis. The protein spots, which were used for PMF analysis and MS/MS analysis, were given the identification numbers. The identified proteins were listed in Table 2.
**Table 2** List of the target protein candidates of Trx-h captured in the present study

<table>
<thead>
<tr>
<th>Spot no.</th>
<th>Name (conserved cys/whole cys)</th>
<th>Accession</th>
<th>Interaction with Trx</th>
<th>Method</th>
<th>Determinants</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Anti-oxidative stress system</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>APX1 (3/5)</td>
<td>AT1G07890</td>
<td>This study</td>
<td>ES</td>
<td>TKNYPTVSEDYXAV (TKNYPTVSEDYKKAV)</td>
</tr>
<tr>
<td>2</td>
<td>Germin like protein (2/3)</td>
<td>AT5G20630</td>
<td>This study</td>
<td>ES</td>
<td>SVQDFXLADPX5(SVQDFPCADPKG)</td>
</tr>
<tr>
<td>3</td>
<td>Type II Prx (2/2)</td>
<td>AT1G60740</td>
<td>Suggested (Choi et al. 1999)</td>
<td>ES</td>
<td>APIAVGDXVPGTITQQFXDN (APITVGDVVPDGITISSFFDEN)</td>
</tr>
<tr>
<td><strong>Protein biosynthesis</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>4</td>
<td>Elongation factor, EF-2 (15/16)</td>
<td>AT1G56070</td>
<td>This study</td>
<td>PMF</td>
<td>926.604, 1203.710, 1496.807, 1969.936, 1985.929, 2177.111, 2420.253</td>
</tr>
<tr>
<td>5</td>
<td>Elongation factor, EF-2 (15/16)</td>
<td>AT1G56070</td>
<td>This study</td>
<td>PMF</td>
<td>890.468, 1007.501, 1039.634, 1324.630, 1430.671, 1634.806, 1762.903, 1807.887, 2079.970, 2132.013, 2298.003, 2399.110</td>
</tr>
<tr>
<td>6</td>
<td>Elongation factor, EF-2 (15/16)</td>
<td>AT1G56070</td>
<td>This study</td>
<td>PMF</td>
<td>955.515, 1111.660, 1145.518, 1634.911, 1799.937, 2116.143, 2132.116</td>
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<td>7</td>
<td>Eukaryotic translation initiation factor, eIF4A (5/6)</td>
<td>AT1G54270</td>
<td>This study</td>
<td>PMF</td>
<td>1113.680, 1446.756, 1574.846, 1583.766, 1783.757, 1783.766, 1783.757, 2076.888</td>
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<tr>
<td><strong>Protein folding and degradation process</strong></td>
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<td>10</td>
<td>HSP70 (chloroplast) (2/2)</td>
<td>AT4G24280</td>
<td>Suggested (Balmer et al. 2003)</td>
<td>PMF</td>
<td>870.533, 915.498, 970.531, 1024.624, 1373.742, 1444.750, 1461.782, 1580.824, 1589.882, 1735.943, 1737.896, 1865.958</td>
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<tr>
<td>11</td>
<td>26S Proteasome regulatory subunit, RPN12 (4/4)</td>
<td>AT1G64520</td>
<td>This study</td>
<td>PMF</td>
<td>1110.53, 1259.68, 1732.92, 1872.186, 2188.22</td>
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<td><strong>Metabolic enzymes</strong></td>
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<td>12</td>
<td>Alcohol dehydrogenase (11/12)</td>
<td>AT1G77120</td>
<td>This study</td>
<td>PMF</td>
<td>915.492, 1148.641, 1274.592, 1901.063, 2240.123</td>
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<td>13</td>
<td>Fructose 1,6-bis phosphate aldolase-like protein (4/6)</td>
<td>AT3G52930</td>
<td>This study</td>
<td>PMF</td>
<td>1020.544, 1061.541, 1311.681, 1336.725, 1458.755, 1488.800, 1614.866, 1750.901, 2223.049, 2263.088, 2392.210, 2416.265</td>
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<tr>
<td>15</td>
<td>Glyceraldehyde 3-phosphate dehydrogenase (2/2)</td>
<td>AT3G04120</td>
<td>Suggested (Anderson et al. 1995)</td>
<td>PMF</td>
<td>834.45, 1038.57, 1102.65, 1199.56, 1148.68, 1498.90, 1677.04, 1705.00, 1734.82, 1869.84, 1869.10, 2035.14, 2171.99</td>
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<tr>
<td>16</td>
<td>Malate dehydrogenase (cytosolic) (6/6)</td>
<td>AT1G04410</td>
<td>This study</td>
<td>PMF</td>
<td>873.474, 1187.643, 1376.733, 1649.988, 2015.041, 2266.093, 2349.158</td>
</tr>
<tr>
<td>17</td>
<td>Vitamin B&lt;sub&gt;12&lt;/sub&gt;-independent methionine synthase (5/6)</td>
<td>AT5G17920</td>
<td>This study</td>
<td>PMF</td>
<td>1095.569, 1180.624, 1198.562, 1505.753, 1997.925, 2074.08, 2383.208, 2860.42, 3012.407</td>
</tr>
</tbody>
</table>

* Locus nomenclatures named by AGI (Arabidopsis Genome Initiative) for each of the obtained proteins were shown.
* Determinants for each of the obtained proteins were as follows; for Edman sequencing (ES), N-terminal sequences together with the corresponding sequences of identified proteins in parenthesis was shown; for PMF, the queried mass peak data, which coincided with the value of the database, were shown; for tandem mass spectrometry (MS/MS), the determined sequence, which was completely matched with the desired sequence together with the obtained mass and the calculated mass in parenthesis, was shown.
* The number of the conserved cysteines per the total number of the cysteines in the sequence of each protein was shown. The results of the multiple alignment with the sequences from the various organisms were shown in the supplemental figures (Fig. 5A–M).
* Only from the PMF data, we could not specify the protein within the isoforms.
* The PMF data for these proteins were obtained by AXIMA-CFR, which was also used for tandem mass spectrometry.
Target proteins of multiple cytosolic Trx isoforms

In plants, not very much is known about the role of the cytosolic Trx-\(h\). In addition, the existence of the multiple Trx-\(h\) isoforms is very enigmatic because all of them are closely related although three in five Trx-\(h\) isoforms have a different active site sequence, WCPCP, instead of the common Trx active site sequence WCGPC as listed in Table 1 (Brehélin et al. 2000, Meyer et al. 2002). To understand the physiological significance of these multiple Trx-\(h\) isoforms in the cytosol, we intended to identify their target proteins comprehensively. The usefulness of the active site cysteine mutant Trx, which was immobilized on the resin, to capture the target proteins has already been examined by us (Motohashi et al. 2001) and by others (Goyer et al. 2002, Balmer et al. 2003, Broin et al. 2002).

Consequently we have successfully identified at least fifteen proteins including four chloroplast proteins, heat shock protein 70 (Hsp70), Rubisco binding protein \(\alpha\) subunit (Cpn60-\(\alpha\)) and \(\beta\) subunit (Cpn60-\(\beta\)), and glutamine synthase, which were already reported as Trx-target proteins in chloroplasts (Motohashi et al. 2001, Balmer et al. 2003). These chloroplast proteins must be contaminated from the plastids in the dark-grown tissue. Unfortunately, we could not obtain the assertive results to give a certain conclusion on the difference of the specificity of these Trx-\(h\) isoforms. In addition, the existence of the multiple Trx-\(h\) isoforms in the cytosol should be confirmed.

An isoform of APX was also captured as a potential Trx-targeted protein. APX is thought to be a key enzyme in the hydrogen peroxide-detoxifying system in the cytosol, and uses ascorbate for reduction (Shigeoka et al. 2002). In A. thaliana five APX isoforms have been already identified in the whole genome; two cytoplasmic isoforms, APX1 and APX2, the microsomal enzyme APX3, the chloroplast stroma enzyme sAPX, and the thylakoid membrane-bound tAPX (Arabidopsis Genome Initiative 2000). Here we identified APX1 as a possible Trx-target protein, suggesting the possibility that a disulfide bond in APX1 can be reduced by the NADPH-Trx system in the cytosol though APXs normally need ascorbate as a reductant to reduce hydrogen peroxide. Whether the suggested interaction is necessary for the activity of APX or for the regulation should be confirmed.

It is reported that an extracellular germin-like protein has superoxide dismutase activity in bryophyte Barbula unguiculata (Yamahara et al. 1999). Although the superoxide dismutase activity depends on divalent metal-ions and there is no information about the relevance of cysteines in this reaction, germin-like proteins may be reduced by Trx and act as a part of the anti-oxidative stress system in the cytosol.

Anti-oxidative stress system

Prx was found in bacteria, yeast, animals and higher plants as a common anti-oxidative stress protein (Rouhier and Jacquot 2002, Dietz et al. 2002), which requires reducing equivalents for the hydroperoxide reducing activity via cysteine residues, and Trx was identified as a natural reductant (Cheong et al. 1999). Plant Prx is classified according to the number of conserved cysteine residues. In general they are called 1-Cys Prx and 2-Cys Prx. 2-Cys Prxs are further classified into three types, dimeric 2-Cys Prx, monomeric type I Prx, which is called Prx-Q, and monomeric type II Prx (Baier and Dietz 1999, Kong et al. 2000, Motohashi et al. 2001, Rouhier and Jacquot 2002). We identified monomeric type II Prx as a target protein of Trx-\(h\). This Prx was first identified in the plant cytosol by Choi et al. (1999). However, our finding is the first report that indicated the direct interaction between the type II Prx in higher plants and the cytosolic Trx-\(h\). We have already obtained the homologous protein from the survey of the target proteins of Trx in Synechocystis sp. PCC6803 (Matsuda and Hisabori, unpublished results).

An isofrom of APX was also captured as a potential Trx-targeted protein. APX is thought to be a key enzyme in the hydrogen peroxide-detoxifying system in the cytosol, and uses ascorbate for reduction (Shigeoka et al. 2002). In A. thaliana five APX isoforms have been already identified in the whole genome; two cytoplasmic isoforms, APX1 and APX2, the microsomal enzyme APX3, the chloroplast stroma enzyme sAPX, and the thylakoid membrane-bound tAPX (Arabidopsis Genome Initiative 2000). Here we identified APX1 as a possible Trx-target protein, suggesting the possibility that a disulfide bond in APX1 can be reduced by the NADPH-Trx system in the cytosol though APXs normally need ascorbate as a reductant to reduce hydrogen peroxide. Whether the suggested interaction is necessary for the activity of APX or for the regulation should be confirmed.

Is Trx involved in the protein biosynthesis process?

In the captured proteins, we identified the elongation factor (EF-2) and the eukaryotic translation initiation factor (eIF4A). In the case of EF-2, partially degraded fragments were also captured (Spot Nos. 5 and 6 in Fig. 4). The elongation factor plays a central role during the translocation step in the peptide chain elongation. Recent studies revealed that oxidative stress inhibits protein synthesis at the elongation step in mammals (Ayala et al. 1996). Nishiyama et al. (2001) also suggested that the elongation step on the process of translation of psbA gene in Synechocystis is inhibited by reactive oxygen species. Furthermore the elongation factor in chloroplasts has been captured by the chloroplast Trx (Balmer et al. 2003). Taken together, the redox state of this protein must strongly relate to the elongation of peptides both in chloroplasts and in cytosol.

In contrast, there is no additional evidence for the redox sensitivity of eIF4A so far, although eIF4A has five conserved cysteines (Table 2, supplemental Fig. 5E). Further investigation is necessary to determine whether the observed interaction between Trx and the initiation factor has a physiological significance.

Possible role of Trx on the protein folding and degradation process

The proteins important for protein folding, chloroplast chaperonin 60-\(\alpha\) and 60-\(\beta\) subunits and Hsp 70 were also captured (Table 2, Fig. 5F–H in the supplement) as in a former study using chloroplast Trx-\(f\) and Trx-\(m\) (Balmer et al. 2003), although they must be the contaminated plastid proteins in our case. Physiological significance of the redox change of these proteins and the relevance to their function should be further confirmed by biochemical study.

In the present stage, there is no additional information on the relevance between the redox conditions in the cytosol and the regulation of 26S proteasome activity. RPN12 certainly has
Several metabolic enzymes were captured in this study. Based on the homology analyses of the amino acid sequences, higher plant alcohol dehydrogenase has eleven conserved cysteines (Fig. 5j in the supplement). Inactivation of plant alcohol dehydrogenase due to the oxidation of two cysteines had been investigated by Kang et al. (1986). However, the molecular details of this inactivation remain unknown. In the case of fructose 1,6-bis-phosphate aldolase, four cysteines are conserved in the enzyme from higher plants. Within them, Cys130 and Cys173 of fructose 1,6-bis-phosphate aldolase from Arabidopsis thaliana are conserved in the mammalian enzyme, too. In addition, the distance between the corresponding cysteines of them [Cys134 and Cys177 of human aldolase (Dalby et al. 1999), PDB code: 4ALD] in the three-dimensional structure is 4.6 Å, which is the closest distance within the all cysteines in the molecule (see Fig. 5k in the supplement). This possible disulfide bond might be the reason why this enzyme was captured by mutant Trx immobilized resin.

Potential disulfide bonds and their relevance for the regulation of cytosolic GAPDH have already been discussed by Anderson et al. based on the analysis of position of the conserved cysteines in the molecule (Anderson et al. 1995). The chloroplast GAPDH has four conserved cysteines, and N-terminal and C-terminal cysteines are suggested to be involved in the redox regulation of this enzyme from the molecular modeling (Li et al. 1994). Additional two cysteines at the middle region, Cys156 and Cys160 of the cytosolic GAPDH of A. thaliana, are conserved completely in the prokaryotic and eukaryotic GAPDH (Fig. 5m in the supplement) and are involved in catalysis. Based on the crystal structure of the homologous molecule (Yun et al. 2000, PDB code: 1DC4), the distance between them, 8.8 Å, is not far to form the disulfide bond. Indeed, the partial disulfide formation under the oxidizing conditions are also suggested (Brodie and Reed 1987, Brodie and Reed 1990, Souza and Radi 1998) though the enzyme inactivation by the oxidation of catalytic cysteine to sulfenic or sulfonic acid in the presence of peroxynitrite (Souza and Radi 1998) and by S-glutathionylation of the cysteine in the presence of glutathion disulfide (Lind et al. 1998) have been reported. The suggested partial disulfide formation might be a possible reason why the cytosolic GAPDH has been captured by the immobilized Trx mutant in the present study.

Interestingly, the six cysteines conserved in the cytosolic MDH at 79, 125, 155, 252, 292, and 331 (these residue numbers are of the cytosolic MDH of A. thaliana; AT1G04410) is not observed in the chloroplast MDH (for details, see Fig. 5n in the supplement). Within these six cysteines, Cys125 and Cys292 seem to be close enough (3.8 Å) to form the disulfide bond based on the three-dimensional structure of MDH from E. coli as reference (Hall et al. 1992, PDB code: 1IB6).

Vitamin B12-independent methionine synthase, catalyzes the transfer of a methyl group from methyltetrahydrofolate to homocysteine and produces tetrahydrofolate and methionine. The gene for this enzyme was cloned from E. coli, expressed and purified (Gonzalez et al. 1992). The methionine synthase has two cysteines and one histidine for zinc binding. Inhibition of zinc binding to the enzyme impaired the activity (Gonzalez et al. 1996). Therefore, it is reasonable to assume that Trx controls the redox state of these cysteines and acts as a regulator.

**Conclusions and future perspectives**

Identification of target proteins of Trx is important both for understanding the role of each Trx isoforms in the various cell compartments and for understanding the whole redox network in the cell as a regulation system. In higher plant chloroplasts, information on the thiol enzymes has recently been accumulated by the comprehensive survey of the target proteins (Motohashi et al. 2001, Balmer et al. 2003). In contrast, the role of Trx-h in the cytosol is not very well known. In addition, there are several reports that Trx-h and its counterpart in vertebrates can act as an intercellular messenger protein (Ishiwatari et al. 1995, Ishiwatari et al. 1998, Pekkari et al. 2001).

When we were preparing this manuscript, Wong et al. reported the variety of proteins which are Trx targets in the starchy endosperm of mature wheat seeds (Wong et al. 2003). By using the fluorescent-dye labeling method, they suggested that 93 proteins including cytosolic GAPDH, aldolase, cytosolic MDH and 26S proteasome regulating subunit S6 are potential target proteins of Trx. In addition, they confirmed the Trx dependent regulation of the enzyme activities of several proteins in their list using the protein aliquots prepared from the wheat endosperm. These data are a good confirmation that the proteins listed in the present study must be the certain candidate proteins as Trx targets, although we ourselves do not examine the redox regulation of the captured enzymes.

In this study, we suggested a relation between the redox state in the cytoplasm and the regulation of protein biogenesis and folding. The anti-oxidative stress system in cytosol must be promoted by Trx-h and several metabolic enzymes must be reduced by Trx-h. Thus the relevance of cytosolic Trx on various phenomena in the plant cell was suggested. Although we do not know how the entire redox state of the plant cytosol controls the redox state of the captured proteins, the combination of the proteomics approach and biochemical research of the identified proteins provides important knowledge for the understanding of the whole redox network system in cytoplasm.

**Materials and Methods**

**Materials**

CNBr-activated Sepharose 4B, activated thiol Sepaharose 4B and immobilized pH gradient gel (IPG) strips were purchased from Amer-
sham Bioscience (Piscataway, NJ, U.S.A.). The Bradford protein assay system was from Bio-Rad Inc. (Hercules, CA, U.S.A.). 3-[3-cholamidopropyl] dimethylammoniomio)-l-propane-sulfonate (CHAPS), DTT, protease inhibitor cocktail for plants (catalog code, P9599), and bovine insulin (catalog code, 15500) were from Sigma (St. Louis, MO, U.S.A.). Trypsin (catalog code, V5111) was from Promega (Madison, WI, U.S.A.). Reverse transcriptase and LA-Taq polymerase were from Takara (Kyoto, Japan). Other chemicals were of the highest grade commercially available.

**Preparation of the recombinant Trx-h isoforms and their mutants**

From the whole genome sequence data of *A. thaliana*, eight genes for cytosolic Trx-h isoforms were identified (Meyer et al. 2002). Within them, we cloned the genes for Trx-h1, Trx-h2, Trx-h3, Trx-h4, and Trx-h5 by RT-PCR method using reverse transcriptase, LA-Taq polymerase, and the oligonucleotides, which were constructed based on the registered DNA sequences for Trx-h1 (Z14084), Trx-h2 (Z35475), Trx-h3 (Z35474), Trx-h4 (Z35473), and Trx-h5 (Z35476). The amplified DNA fragments were cloned into the NdeI and Xhol sites (for Trx-h1, h2, and h5), the NdeI and EcoRI sites (for Trx-h3 and h4) of pET21c (for Trx-h1, h2 and h5, Novagen) or pET23c (for Trx-h3 and h4, Novagen), and the DNA sequences were confirmed by DNA sequencing (PRISM 310, Applied Biosystems). The recombinant Trxs were then overexpressed in E. coli strain BL21-Gold (DE3). The expressed Trx was purified using the same method as described (Motohoshi et al. 2003).

Cysteine mutants of the Trx-h isoforms were prepared by the megaprimer method (Sarkar and Sommer 1990) using the oligonucleotides listed in Table 1. The expressed mutant proteins were purified by the method used for the wild-type Trx (Motohoshi et al. 2003).

**Cell lysate**

To minimize the contamination of the chloroplasts into the cell lysate preparation, 80 mg of the seeds of *A. thaliana* ecotype Columbia were surface sterilized with 5% (w/v) sodium hypochlorite, rinsed, sown in 2 liters of Murashige and Skoog medium (Murashige and Skoog 1962) containing 2% (w/v) sucrose, and cultivated at 23°C in the complete dark. The obtained plant whole tissue was homogenized in 50 mM Tricine-KOH, pH 8.0 containing 400 mM sucrose, 50 mM NaCl and 2% (v/v) protease inhibitor cocktail for plants. The homogenate was passed through four layers of gauze and centrifuged at 7,000 g for 30 min at 4°C and supernatant was further centrifuged at 100,000 g for 50 min at 4°C. The obtained supernatant (300 mg proteins) was used as cell lysate.

**Trx activity assay**

To confirm the oxidoreductase activity of the recombinant Trx, we measured change of the turbidity of the insulin solution due to the precipitation of the free insulin B chain by reduction by the reduced Trx activity assay initiated by adding 330 μM DTT. Isoelectric focusing was performed at 0–300 V linear gradient for 10 min, 300–3,500 V linear gradient for 3.5 h and 3,500 V for 3 h. The strips were then equilibrated with 50 mM Tris-HCl, pH 6.8, 6 M urea, 30% (v/v) glycerol, 16 mM DTT and 1% (w/v) SDS, and then incubated with 50 mM Tris-HCl, pH 6.8, 6 M urea, 30% (v/v) glycerol, 240 mM iodoacetamide and 1% (w/v) SDS. For the two-dimensional separation, vertical gradient slab SDS-PAGE (7.5–15% (w/v) gradient gel, BioCraft, Tokyo, Japan) was used. The separated proteins were visualized by Coomassie Brilliant Blue R-250 or silver staining.

**Two-dimensional electrophoresis**

Eluted proteins were precipitated with trichloroacetic acid [final concentration, 10% (w/v)], washed with acetone and suspended with sample buffer containing 8 M urea, 2% (w/v) CHAPS, 40 mM DTT, 0.5% (w/v) IPG buffer (Amersham Bioscience) and 0.001% (w/v) bromophenol blue. Isoelectric focusing was performed by using the Multiphor II system (Amersham Bioscience) according to the manufacturer’s instructions. IPG strips (pH 4–7, 7 cm) were rehydrated with 8 M urea, 2% CHAPS, 10 mM DTT, 2% IPG buffer and 0.001% bromophenol blue. Isoelectric focusing was performed at 0–300 V linear gradient for 10 min, 300–3,500 V linear gradient for 3.5 h and 3,500 V for 3 h. The strips were then equilibrated with 50 mM Tris-HCl, pH 6.8, 6 M urea, 30% (v/v) glycerol, 16 mM DTT and 1% (w/v) SDS, and then incubated with 50 mM Tris-HCl, pH 6.8, 6 M urea, 30% (v/v) glycerol, 240 mM iodoacetamide and 1% (w/v) SDS. For the two-dimensional separation, vertical gradient slab SDS-PAGE (7.5–15% (w/v) gradient gel, BioCraft, Tokyo, Japan) was used. The separated proteins were visualized by Coomassie Brilliant Blue R-250 or silver staining.

**Preparation of the Trx immobilized resin**

Trx-h mutants in 100 mM sodium carbonate buffer, pH 8.3, containing 0.5 M NaCl were incubated with CNBr-activated Sepharose 4B resin, which had been swelled in the same solution for 2 h at room temperature according to the manufacturer’s instruction. After termination of the coupling reaction by centrifugation, the unreacted side chains on the resin were blocked by incubation with 50 mM Tris-HCl, pH 8.0 for 12 h at 4°C.
try was performed with a MALDI-time-of-flight mass spectrometer, AXIMA-CFR (Shimadzu, Kyoto, Japan), and the internal sequences of the peptides were directly determined.

**Supplementary Data**

Supplementary data (Figs 5A-M) is available to members with the online version of the journal at: http://pcp.oupjournals.org.

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**References**


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