Analysis of Arabidopsis thioredoxin-h isotypes identifies discrete domains that confer specific structural and functional properties

Young Jun JUNG*1, Yong Hun CHI*1, Ho Byoung CHAE*, Mi Rim SHIN*, Eun Seon LEE*, Joon-Yung CHA*, Seol Ki PAENG*, Yunoo LEE*, Jin Ho PARK*, Woe Yoon KIM*, Chang Ho KANG*, Kyun Oh LEE*, Keun Woo LEE*, Dae-Jin YUN* and Sang Yeol LEE*2

*Division of Applied Life Science, PMBBRC, Gyeongsang National University, Jinju 660-701, Republic of Korea

INTRODUCTION

Thioredoxins (Trxs) are small heat-stable proteins that are present in all organisms and participate in a wide range of biological functions [1]. The active site of all Trxs contains two cysteine residues that are involved in a disulfide/dithiol exchange between the Trx and its target proteins. The reversible formation or breakage of the disulfide bridge involves an electron transport reaction, which can in turn activate multiple redox-dependent signalling pathways, including the modulation of transcription factors, activation of ribonucleotide reductase and enhancement of photosynthetic activity in plant cells [2–5].

Trxs also control several redox-independent cellular reactions and biological processes by regulating protein conformation and stability, such as the assembly of T7 DNA polymerase complex, formation of filamentous phage and protection of proteins from denaturation against external stresses [6–8]. The mechanisms of these reactions remain to be elucidated.

In comparison with non-photosynthetic organisms, there are relatively large numbers of Trx isotypes in plants. In the genome of Arabidopsis thaliana, Trxs-m, -f, -x, -y, -z, -o and -h subgroups have been identified [9]. Whereas most of these plant Trxs are located in chloroplasts and mitochondria [10,11], the h-type Trxs typically localize to the cytoplasm as well as the endoplasmic reticulum, mitochondria, plasma membrane and nucleus [8,12,13]. Although Trx-hs comprise the largest group of Trxs in plants, they demonstrate distinct and overlapping expression patterns and only a few physiological functions have been identified [14].

Multiple isoforms of Arabidopsis thaliana h-type thioredoxins (AtTrx-hs) have distinct structural and functional specificities. AtTrx-h3 acts as both a disulfide reductase and as a molecular chaperone. We prepared five representative AtTrx-hs and compared their protein structures and disulfide reductase and molecular chaperone activities. AtTrx-h2 with an N-terminal extension exhibited distinct functional properties with respect to other AtTrx-hs. AtTrx-h2 formed low-molecular-mass structures and exhibited only disulfide reductase activity, whereas the other AtTrx-h isoforms formed high-molecular-mass complexes and displayed both disulfide reductase and molecular chaperone activities. The domains that determine the unique structural and functional properties of each AtTrx-hs protein were determined by constructing a domain-swap between the N- and C-terminal regions of AtTrx-h2 and AtTrx-h3 (designated AtTrx-h-2N3C and AtTrx-h-3N2C respectively), an N-terminal deletion mutant of AtTrx-h2 [AtTrx-h2-N(Δ19)] and site-directed mutagenesis of AtTrx-h3. AtTrx-h2-N(Δ19) and AtTrx-h-3N2C exhibited similar properties to those of AtTrx-h2, but AtTrx-h-2N3C behaved more like AtTrx-h3, suggesting that the structural and functional specificities of AtTrx-hs are determined by their C-terminal regions. Hydrophobicity profiling and molecular modelling revealed that Ala100 and Ala106 in AtTrx-h3 play critical roles in its structural and functional regulation. When these two residues in AtTrx-h3 were replaced with lysine, AtTrx-h3 functioned like AtTrx-h2. The chaperone function of AtTrx-hs conferred enhanced heat-shock-resistance on a thermosensitive trx1/2-null yeast mutant.

Key words: Arabidopsis thaliana, disulfide reductase, heat-shock-resistance, molecular chaperone, thioredoxin h.
In the present study, we prepared five AtTrx-h recombinant proteins, AtTrx-h1–AtTrx-h5, and compared their protein structures and dual functions of disulfide reductase and molecular chaperone. To probe further the structure–function relationship, domain-swapped proteins switching the N- and C-terminal regions of AtTrx-h2 and AtTrx-h3 (designated AtTrx-h-2N3C and AtTrx-h-3N2C) were constructed and their properties were compared. In addition, to investigate the specific domain and amino acid residues responsible for the structural and functional properties of AtTrx-h2 and AtTrx-h3, we performed site-directed mutagenesis on the C-terminal residues of AtTrx-h3 on the basis of the amino acid sequence, hydrophobic property and molecular modelling data. The results showed that Ala100 and Ala106 play critical roles in the regulation of the AtTrx-h3 protein structure and function. Furthermore, the physiological role of the WT (wild-type) and mutant forms of AtTrx-hs were also investigated by overexpressing them in a thermosensitive mutant yeast line lacking endogenous Trx1 and Trx2, designated yΔtrxl/2.

MATERIALS AND METHODS

Materials

A. thaliana (Columbia ecotype, Col-0) was grown under conditions of 100–120 μmol m−2·s−1 photosynthetic flux at 22 °C, 70% humidity and a 16 h light/8 h dark cycle. Molecular markers used for PAGE were purchased from iNtRON. Saccharomyces cerevisiae, strains of EMY60 (WT) and EMY63 (yΔtrxl/2), were used for thermostability experiments [21]. The anti-His-tag monoclonal antibody was purchased from Abcam and DTNB was purchased from Sigma-Aldrich. The Superdex 200 HR 10/30 column for FPLC was obtained from GE Healthcare.

Cloning of AtTrx-h isoforms and expression in Escherichia coli

AtTrx-h isoforms were cloned from an Arabidopsis cDNA library and their deletion or point-mutated constructs were generated by PCR, as described in [8,15]. After confirming the entire sequences, the amplified products were ligated into the BamHI/HindIII sites of the pGEX expression vector (NEB), and the resulting DNA constructs were transformed into E. coli BL21(DE3) cells. The transformants were grown in LB broth containing 50 g/ml ampicillin and 12.5 g/ml chloramphenicol at 37 °C. The culture was diluted 1:50 in LB medium containing 50 g/ml ampicillin and was grown at 30 °C until a D600 of 0.5 was reached. Expression of recombinant proteins was induced by the addition of 0.5 mM IPTG and was grown further for 4 h. The cells were harvested by centrifugation at 5000 g for 5 min, and the pellet was resuspended in PBS (140 mM NaCl, 2.7 mM KCl, 10 mM Na2HPO4 and 1.8 mM KH2PO4, pH 7.6) containing 1 mM PMSF. The GST–AtTrx-h fusion proteins were applied to GSH–agarose columns and then the mature proteins were cleaved from the column with thrombin, and purified as described in [8]. DnaK, a possible contaminant from the GSH column chromatography, was removed by ATP–agarose affinity chromatography. The recombinant AtTrx-hs (AtTrx-h1–AtTrx-h5) and chimaeric proteins were purified further by FPLC using a Superdex 200 HR 10/30 column. The purity of AtTrx-hs and their mutant proteins were examined by SDS/PAGE. Trx in E. coli (EcTrx) used as a control was obtained by the same procedures as AtTrx-hs.

Disulfide reductase activity assay

Disulfide reductase activity was measured using DTNB as a substrate, as described in [8,22] with a slight modification. Briefly, 100 mM DTNB dissolved in 500 mM potassium phosphate buffer (pH 7.0) was added to the reaction mixture containing AtTrx-hs, 50 mM NADPH, 0.3 μM thioredoxin reductase and 500 mM EDTA in a final volume of 0.3 ml. After thorough mixing of the reaction, changes in A340 were measured using a DU800 spectrophotometer (Beckman).

Molecular chaperone activity

Chaperone activity of AtTrx-hs was measured by heat-induced MDH denaturation. Aggregation of MDH was initiated by the addition of recombinant AtTrx-hs into 50 mM Hepes/KOH (pH 8.0) buffer at 45 °C. Turbidity due to substrate aggregation was monitored with a DU800 spectrophotometer equipped with a thermostatic cell holder, as described in [8,19,22].

SEC (size-exclusion chromatography)

FPLC was carried out at 25 °C on a Superdex 200 HR 10/30 column equilibrated with 50 mM Hepes/KOH (pH 8.0). Proteins were eluted with the same buffer at a flow rate of 0.5 ml/min. Fractions corresponding to protein peaks (A280) were collected and concentrated at 4 °C using a Centricon YM-10 filter.

Measurement of bis-ANS fluorescence

Reaction mixtures consisting of 10 μM AtTrx-hs in 50 mM Hepes (pH 7.0) added to 10 μM bis-ANS were incubated for 30 min at 25 °C. The fluorescence of bis-ANS was analysed using a SFM25 spectrofluorimeter (Kontron), as described in [8,22]. The excitation wavelength was set at 380 nm and the emission was scanned between 400 and 600 nm.

Aliphatic and instability indexes

Physicochemical properties of AtTrx-hs were computed by the ProtParam program using their protein sequences. Relative volumes of alanine (1.0), valine (2.9), isoleucine (3.9) and leucine (3.9) were used to calculate the aliphatic index, which provided an estimate of the thermostability of globular protein [23]. The instability index was calculated using the DIWW (dipeptide instability weight value) assigned to each of 400 different dipeptides [24].

Computational details of MD and protein–protein docking simulations

The protein structures of AtTrx-h2 and AtTrx-h3 were built using the Homology Modeling protocols in Discovery studio (DS) 2.5 (Discovery Studio 2.5, Accelrys). The NMR structure of AtTrx-h1 (PDB code 1XFL) was used as a template. The MD simulation of the modelled structures were carried out using the GROMACS program (version 4.5.1) [25] and the AMBER-03 force field [26]. The structures were immersed in an orthorhombic water box (1.2 nm thickness) and the net charge of the system was neutralized by the addition of Na+ counterrions. The two production runs at 500 K and a normal pressure constant (100 kPa) for 5 ns were performed under periodic boundary conditions with NPT ensemble and V-rescale thermostat [27]. The initial decameric structure of the AtTrx-h3 was constructed.
Structural and functional specificity of AtTrx-hs

by superimposition of bacterial Prx AhpC (PDB code 1YF0) using DS 2.5 software. To refine the two different types of interfaces, a 10 ns MD simulation of the initial structure was performed with the same parameters described above without high temperature. The simulated structure of the B-type interface can only be used for interaction analysis. To investigate the proper binding conformation of the A-type interface, protein–protein docking simulation of two B-type dimers was performed using the ZDOCK program in DS 2.5 software [28]. Almost 54,000 different translated orientations of one B-type dimer were obtained from the stationary molecule. Additional electrostatic and desolvation energy terms were used to obtain a more accurate ranking. The top 100 poses were categorized into ten clusters, and the top pose in the highest populated cluster 1 was selected for further analysis.

Thermotolerance assay of yΔtrx1/2 mutant yeast overexpressing various AtTrx-hs

Thermotolerance of WT (EMY60) and yΔtrx1/2 mutant yeast was measured as described in [21] with a slight modification. WT and yΔtrx1/2 yeasts were transformed with various DNA constructs encoding His6-tagged recombinant proteins. Transformants were incubated for 1 day in SD (synthetic defined) liquid minimal medium (Clontech) to induce plasmid gene expression from the GAL1 promoter. To examine thermotolerance of the transformed yeasts, 7 × 10^5 cells/ml were serially diluted and then heat-treated at 30°C or 48°C for 1 h. After immediate cooling on ice, 100 μl aliquots of the cells were plated on SD minimal medium and incubated at 30°C for 2 days, and the surviving colonies were counted. The survival rate (%) was measured by calculating the cells that survived at 48°C compared with cells grown at 30°C. Cell density was determined using a spectrophotometer to measure the D_600.

Statistical analysis

Statistics of the data for cell survival and growth were analysed by one-way ANOVA (SPSS 12.0.1 for Windows) and presented as the means ± S.D. Tukey’s test (P < 0.05) was used for mean separation.

RESULTS

Structural and functional comparison of the five AtTrx-hs

Previously, using SEC MALDI–TOF screening, we identified AtTrx-h3 from heat-treated suspension cells that exhibited structural shifts from a low-molecular-mass protein species to high-molecular-mass complexes accompanying its functional switch from a disulfide reductase to a molecular chaperone [8]. As there are numerous Trx-hs in Arabidopsis, we tried to analyse the specific properties of the five representative AtTrx-hs, AtTrx-h1–AtTrx-h5, all of which have high amino acid sequence similarities (Figure 1A). The AtTrx-hs contained 109–133 amino acids and two conserved cysteine residues at their active site, except for AtTrx-h2, which uniquely has a 19-amino-acid extension at its N-terminus.

To investigate whether the five AtTrx-hs, similarly to the previous results of AtTrx-h3 [8], exhibited dual disulfide reductase and molecular chaperone activities with high-molecular-mass polymeric structures, we expressed AtTrx-h1–AtTrx-h5 in E. coli and purified the proteins to homogeneity by affinity chromatography (Figure 1B). Disulfide reductase activity of the recombinant AtTrx-hs was examined using DTNB as a substrate in the presence of NADPH and thioredoxin reductase as reductants and found that all of the AtTrx-hs exhibited DTNB-reduction activity (Figure 1C). The AtTrx-h2 had the highest, but AtTrx-h3 had the lowest, specific activity of DTNB reduction. Since AtTrx-h3 was shown previously to have strong chaperone activity [8], we also examined the chaperone activity of the five AtTrx-hs using MDH as a substrate. Incubation of MDH alone or with ovalbumin at 45°C resulted in a rapid increase in the A_600 due to heat-induced denaturation and aggregation of MDH. This aggregation of MDH was significantly inhibited by all AtTrx-hs, with the exception of AtTrx-h2, which suggested that the four AtTrx-hs had dual functions as both disulfide reductase and molecular chaperone (Figure 1D). The chaperone activity of the four AtTrx-hs was inversely related to disulfide reductase activity, in the order of AtTrx-h3 > AtTrx-h5 > AtTrx-h4 > AtTrx-h1. In contrast, despite its strong disulfide reductase activity, AtTrx-h2 was not able to prevent denaturation of MDH from heat shock, indicating that only AtTrx-h2 did not exhibit chaperone function.

Since the formation of homo-oligomeric protein complexes is a well-conserved feature of molecular chaperones [29], we analysed protein structures of AtTrx-hs by SEC as a possible underlying mechanism for the different functional properties of AtTrx-h2 compared with other AtTrx-hs (Figure 1E). On the basis of SEC and SDS/PAGE data, we found that AtTrx-h2 was present as a low-molecular-mass protein species that was eluted as a single protein peak in SEC, and the other AtTrx-hs formed multiple homo-oligomeric structures. The monomeric size of AtTrx-hs analysed by SDS/PAGE was shown to be approximately 12–13 kDa. Representative results of SDS/PAGE for AtTrx-h2 and AtTrx-h3 are shown in Figure 1E (inset). These results indicated that the AtTrx-hs examined, with the exception of AtTrx-h2, formed discretely sized homopolymeric structures through self-association, and this correlated well with their activity as molecular chaperones [19,30].

Construction of deletion and domain-swapping variants of AtTrx-h2/AtTrx-h3 and their structural and functional characterization

Compared with the other isotypes of AtTrx-hs, AtTrx-h2 specifically exhibited different structural and functional properties (Figure 1). Comparison of amino acid sequences of the five AtTrx-hs demonstrated that AtTrx-h2 contained 19 additional amino acids at its N-terminus (Figure 1A). To investigate whether this N-terminal extension was responsible for the differences between AtTrx-h2 and the other AtTrx-hs, we constructed a variant of AtTrx-h2 deleting the N-terminal 19 amino acids (Figure 2A) designated AtTrx-h2-N(Δ19), and analysed its protein structure by SEC (Figure 2B). We compared protein structures of AtTrx-h2-N(Δ19) with those of AtTrx-h2 as well as AtTrx-h3, as the latter exhibited the strongest chaperone activity and adopted highly oligomerized structures. However, there was no structural difference between AtTrx-h2 and AtTrx-h2-N(Δ19) in SEC (Figure 2B). Their structural similarity was confirmed again by bis-ANS fluorescence assay, which measures the binding of a fluorescent bis-ANS probe to hydrophobic regions of proteins. In contrast with the marked difference in bis-ANS fluorescence between AtTrx-h2 and AtTrx-h3, no difference was detected in fluorescence between AtTrx-h2 and AtTrx-h2-N(Δ19) (Figure 2C). Furthermore, AtTrx-h2 and AtTrx-h2-N(Δ19) showed similar activities of disulfide reductase and chaperone activities (Figure 2D). The results strongly suggested that the N-terminal extension of 19 amino acids in AtTrx-h2 is not responsible for the structural and functional differences between AtTrx-h2 and AtTrx-h3.
To investigate further the differences between AtTrx-h2 and AtTrx-h3, we engineered two chimaeric proteins of AtTrx-h-2N3C and AtTrx-h-3N2C by exchanging the N- and C-terminal motifs of AtTrx-h2 with AtTrx-h3 (Figure 3A). The N-terminal motifs of AtTrx-h2 and AtTrx-h3 were switched at their active sites without disturbing the proper reading frame of the proteins, which differed in amino acid sequence (WCGPC and WCPPC respectively). When we analysed molecular structures of the
Figure 2 Effect of the N-terminal 19-amino-acid extension of AtTrx-h2 on its protein structure and functions

(A) Schematic representation of the structure of AtTrx-h2, AtTrx-h2-N(Δ19) and AtTrx-h3. a-a, amino acids. (B) Structural comparison of AtTrx-h2, AtTrx-h2-N(Δ19) and AtTrx-h3 analysed by SEC on a Superdex 200 column. (C) Hydrophobicity of the proteins. After incubating bis-ANS (10 μM) with AtTrx-h2 (●), AtTrx-h2-N(Δ19) (○) and AtTrx-h3 (●), or without the protein (■), the fluorescence of bis-ANS bound to the proteins was measured at an excitation wavelength of 390 nm and emission wavelength of 400–600 nm. (D) Comparison of the disulfide reductase and chaperone functions of AtTrx-h2, AtTrx-h2-N(Δ19) and AtTrx-h3. The activities were measured using the same procedures as described in the legend to Figure 1, and were compared with those of AtTrx-h3, which was set as 100%. Results are means ± S.D. for at least three independent experiments.

chimaeric proteins by SEC, the AtTrx-h-3N2C eluted in a low-molecular-mass fraction like the AtTrx-h2, but the AtTrx-h-2N3C polymerized into higher-molecular-mass complexes than those of WT AtTrx-h3 and eluted in void volume of the column (Figure 3B). The results indicated that the C-terminal region of At-Trx-hs is crucial for the formation of polymeric structures.

Because the length of C-terminal regions in the chimaeric proteins was quite similar, the results suggested that the differential ability of AtTrx-h oligomerization was most likely to be due to variations of the amino acid sequences in their C-terminal domains. This was also supported by comparison of the hydrophobicity between AtTrx-h2 and AtTrx-h3 (Supplementary Figure S1 at http://www.biochemj.org/bj/456/bj4560013add.htm) analysed with the ProtScale database (http://www.expasy.org/tools/protscale.html). In contrast with the N-terminal region, the hydrophobic score of the C-terminal domain in AtTrx-h3 was higher than that of AtTrx-h2. This suggested that AtTrx-h3 can polymerize into higher oligomeric complexes through its hydrophobic C-terminal region. This type of oligomerization mechanism was reported previously for α/β-crystallin [31] and HSP33 (heat-shock protein 33) chaperones [30]. In the case of HSP33, a single mutation of the protein resulted in a complete loss of post-translational regulation of its chaperone function.

In addition to structural changes, the WT and chimaeric proteins exhibited significant differences in disulfide reductase and chaperone activities. The reductase activity of AtTrx-h-3N2C was much higher than that of AtTrx-h-2N3C (Figure 3C), whereas AtTrx-h-2N3C displayed stronger chaperone activity than AtTrx-h-3N2C (Figure 3D). This also agreed well with the previous observation that higher reductase activity was associated with lower chaperone function (Figure 1). Thus the chaperone activity of the AtTrx-h chimaeras correlated inversely with disulfide reductase activity. The structural and functional differences between WT and chimaeric proteins were also confirmed by bis-ANS fluorescence assay (Figure 3E). As expected, chimaeric AtTrx-h-2N3C exhibited the highest fluorescence. To eliminate the possible effects of the N-terminal 19 amino acids in AtTrx-h2, we constructed a chimaeric protein of AtTrx-h2-N(Δ19)3C by swapping the AtTrx-h2-N(Δ19) with AtTrx-h3 (Supplementary Figure S2A at http://www.biochemj.org/bj/456/bj4560013add.htm). However, using the chimaeric protein, we found no structural and functional differences between AtTrx-h2-N(Δ19)3C and AtTrx-h-2N3C (Supplementary Figures S2B, S2C and S2D), indicating that the 19 additional amino acids of AtTrx-h2 did not confer any effect on its physicochemical properties.

Prediction of AtTrx-h’s thermostability by sequence-based calculation and molecular modelling

Protein thermostability can be predicted by using the ProtParam program (http://web.expasy.org/protparam) which calculates
Figure 3  Effect of domain swapping between AtTrx-h2 and AtTrx-h3 on protein structure and function

(A) Schematic representation of the domain swapping of AtTrx-h2 with AtTrx-h3. a-a, amino acids. (B) Structural analysis of WT and chimaeric proteins of AtTrx-h2 and AtTrx-h3 by SEC. (C) DTNB reduction activity of the proteins. Reduction of DTNB (5 mM) was measured in the presence of NADPH, thioredoxin reductase and 10 μM AtTrx-hs. In (C)–(E), AtTrx-h2 (▲), AtTrx-h3 (●), AtTrx-h-2N3C (△) and AtTrx-h-3N2C (○) were used. The disulfide reduction of DTNB was followed by measuring the A412 (Abs412). (D) Chaperone activity of the proteins. The activity was measured at 45 °C for 20 min in the presence of AtTrx-hs. Thermal aggregation of MDH (20 μg) was followed by measuring the A650 (Abs650). The molar ratio of AtTrx-h to MDH was set at 5:1. In (C) and (D), substrate alone (○) or 30 μM ovalbumin (□) was used instead of AtTrx-h as a control. (E) Comparison of hydrophobicity. Bis-ANS (10 μM) was incubated with AtTrx-hs or without the protein (▲). Results are means for at least three independent experiments.

The aliphatic index is defined as the relative volume occupied by four aliphatic amino acids (alanine, valine, isoleucine and leucine) in the polypeptides [23]. In the prediction of AtTrx-h’s thermostability, AtTrx-h2 showed the lowest aliphatic index, 82.86, and AtTrx-h3 exhibited the highest value (95.08), indicating that AtTrx-h3 is more stable than AtTrx-h2 (Table 1). Protein stability was also assessed by calculating the DIWW for each of 400 different dipeptides [24]. When the instability index was estimated using this protocol, AtTrx-h2 had the highest instability index (38.88), suggesting that the AtTrx-h2 was more unstable than the other AtTrx-hs. Also, the relative composition of aliphatic residues in AtTrx-h2 (30.8) was significantly lower than the other AtTrx-hs (Table 2). In addition, although all the N-terminal domains of the AtTrx-hs had similar aliphatic values within the range 10–12, the aliphatic composition of the C-terminal domain of AtTrx-h2 was the lowest (18.8) among the proteins. These results suggest that the AtTrx-h2 has the lowest thermostability owing to its C-terminal domain.

Table 1  Physicochemical characteristics of AtTrx-hs

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Table 2  Relative number and composition of aliphatic side chains (alanine, valine, isoleucine and leucine) in various forms of AtTrx-hs

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The degree of thermostability of AtTrx-h2 and AtTrx-h3 was also compared by MD simulation using the GROMACS program [25] with adjusting for high temperature (500 K). For this simulation, the initial structures of AtTrx-h2 and AtTrx-h3 were constructed by a homology modelling approach using the NMR structure of AtTrx-h1 (PDB code 1XFL) as a template (Figures 4A and 4C). When the proteins were subjected to heat shock, most of the C-terminal structure of AtTrx-h2 was completely denatured, but only a small part of the corresponding region of AtTrx-h3 was unfolded (Figures 4B and 4D). The results indicated that the C-terminal region of AtTrx-hs might play an important role in determining their functional and physicochemical properties. They agree well with the data of Brehelin et al. [15], who found that the highly conserved C-terminal region of AtTrx-hs, but not the divergent N-terminal region, is a critical determinant for their functional specificity.

Identification of specific residues in the C-terminal region of AtTrx-h3 that are responsible for its oligomerization and chaperone function

Because the C-terminal part of AtTrx-h3 was shown to play crucial roles in determining its oligomerization and chaperone function, we tried to identify the specific region or residues by comparing its amino acid sequences, hydrophobicity plots and 3D structure with those of AtTrx-h2. On the basis of the results of sequence alignment and hydrophobicity difference between AtTrx-h2 and AtTrx-h3 (Figure S1), residues Ala100–Ala106 (AKEEIIA) in AtTrx-h3 involved in its thermostability were selected as the most important region for elucidating their structural difference. As shown in Table 2, the C-terminal region of AtTrx-h3 had the highest numbers of aliphatic residues among the AtTrx-hs, with a 26.3 % composition (31 residues). In particular, the number of alanine residues in the C-terminal region of AtTrx-h3 was higher than that of AtTrx-h2, whereas the numbers of isoleucine residues in the C-terminal region of AtTrx-h3 was the same as that of the AtTrx-h2. The results suggest that alanine residues in AtTrx-h3 should be the most important for the regulation of its oligomerization and chaperone function.

In addition, to investigate the interface regions of oligomeric structures of AtTrx-hs at the molecular level, molecular modelling analyses were carried out with protein–protein docking and MD simulations. The 3D structure of AtTrx-h3 was constructed on the basis of the NMR structure of AtTrx-h1 (PDB code 1XFL) as a template. Subsequently, the AtTrx-h3 oligomeric structure was built by superimposition of the ten AtTrx-h3 monomers with the Trx-scaffold in the subunit of bacterial AhpC (PDB code 1YF0), because AhpC is a typically well-known molecular chaperone containing Trx-fold with high-molecular-mass structures (Supplementary Figure S3 at http://www.biochemj.org/bj/456/bj4560013add.htm) [33] as well as sharing a number of similar biochemical properties [34–36]. It was reported that the AhpC, including members of the AhpC/Prx1 subfamily, formed a dimeric structure through the two different, A and B, types of interfaces [37]. Because of the common structural fold between β-sheets in the B-type interface of Prx and Trx, the B-type interface is relatively more reasonable than the A-type interface. We aligned the B-type interface structure of AtTrx-h3 and analysed its oligomeric structural properties at the molecular level. To improve co-ordinates of the aligned structure of AtTrx-h3, 10 ns MD simulation was implemented and a refined structure
was obtained, especially at the interface region. Analysis of interface interaction in B-type dimer structure showed that the aliphatic residues Ala\textsuperscript{100} and Ala\textsuperscript{106} tightly interacted with each other by hydrophobic forces (Figure 5B). Thus, when we mutated the two alanine residues of AtTrx-h3 to its counterpart residue, lysine, in AtTrx-h2 (Figure 5A), we observed that the stable interactions in the B-type interface were collapsed by electrostatic repulsions because of the many lysine residues in the C-terminal region (Figure 5C).

Considering the results of sequence alignment, hydrophobicity plot and molecular modelling study, we constructed various AtTrx-h3 mutant proteins, such as AtTrx-h3-A100K, AtTrx-h3-A106K, AtTrx-h3-A100K/A106K in which one or both of the alanine residues were replaced by lysine, as well as its active-site cysteine mutants, AtTrx-h3-C39S/C42S and AtTrx-h3-C39S/C42S/A100K/A106K, in which both of the cysteine residues were replaced by serine. Using the purified recombinant proteins, we investigated the effect of alanine and/or cysteine residues on their protein structures and disulfide reductase/chaperone functions (Figure 6A, and Supplementary Figure S4A at http://www.biochemj.org/bj/456/bj4560013add.htm).

From the experiment, single- or double-mutant proteins of AtTrx-h3, such as AtTrx-h3-A100K, AtTrx-h3-A106K and AtTrx-h3-A100K/A106K showed no effect on their disulfide reductase function, whereas the active site cysteine-free mutant proteins, such as AtTrx-h3-C39S/C42S and AtTrx-h3-C39S/C42S/A100K/A106K, showed no reductase function (Supplementary Figures S4B and S4C). In contrast, only a small difference was detected between AtTrx-h3-C39S/C42S, AtTrx-h3-A100K and AtTrx-h3-A106K proteins in terms of chaperone activity and protein structure (Figures 6B and 6C, and Supplementary Figure S5A and S5B at http://www.biochemj.org/bj/456/bj4560013add.htm). However, the double-mutant protein AtTrx-h3-A100K/A106K showed a significant decrease in chaperone activity with low-molecular-mass protein structures (Figures 6B and 6C). This result suggested that the single mutation of each amino acid, Ala\textsuperscript{100} or Ala\textsuperscript{106} residue in C-terminal region of AtTrx-h3, did not confer any structural changes, but the double-mutant protein of AtTrx-h3-A100K/A106K produced a significant change in oligomerization and in chaperone function. Furthermore, the mutation of C39S/C42S in AtTrx-h3-A100K/A106K (producing the quadruple mutant AtTrx-h3-C39S/C42S/A100K/A106K) resulted in a complete loss of chaperone function and oligomerization activity, suggesting that
Cys39/Cys42, the active-site residues of AtTrx-h3, also participate in its oligomerization and chaperone function, together with Ala100/Ala106 residues.

To resolve the structural information for supporting the above results, proper binding conformation of A-type interface region for AtTrx-h3 was predicted by conducting protein–protein docking simulation with two B-type dimers (Supplementary Figure S6 at http://www.biochemj.org/bj/456/bj4560013add.htm). In the docking result of A-type interface, Arg43 located nearby to the active-site cysteine residues was shown to interact with Glu14 by hydrogen bonding (Supplementary Figure S6A). Hence we expected that if we only mutate the cysteine residues to serine, no significant changes would be observed in the interface. For predicting the effect of mutation C39S/C42S, the cysteine residues were mutated to serine and the structure was subjected to energy minimization. As we expected, this result shows the subtle difference including loss of hydrogen-bond interaction between Arg43 and Glu14 (Supplementary Figure S6B). Although the subtle difference does not induce significant functional changes, adding this effect along with A100K/A106K can lead to the interruption of oligomer formation in a synergistic manner. Our observation was matched by the results of oligomeric structure and chaperone activity of AtTrx-h3-C39S/C42S/A100K/A106K protein (Figure 6). Additionally, we generated the oligomeric structure of AtTrx-h3 using the A- and B-type dimers to predict the dimer conformations have been predicted successfully. From this result, we can conclude that the protein can have strong chaperone activity. On the basis of our modelling work, it was possible to confirm the structural and functional changes of AtTrx-h3 and its mutant proteins.

Chaperone function of AtTrx-h complements the thermosensitive phenotype of mutant yeasts lacking Trx1 and Trx2, yΔtrx1/2

It is a well-known property of molecular chaperones that they confer enhanced resistance to external stresses including heat shock, oxidative stress and heavy metal toxicity [38,39]. To distinguish the effect of chaperone function from the reductase function of AtTrx-hs, we prepared AtTrx-h3-C39S/C42S variants and used it for the complementation of a thermosensitive mutant yeast, yΔtrx1/2. AtTrx-h2, AtTrx-h3 and various chimaeric proteins (AtTrx-h-2N3C and AtTrx-h-3N2C in Figure 3) were overexpressed in yΔtrx1/2 yeast and the protein expression was confirmed by immunoblotting with the use of an anti-His-tag antibody (Figure 7A, inset). Under optimal growth conditions, the survival rate of yΔtrx1/2 was similar to WT yeasts, but the yΔtrx1/2 mutant exhibited a high sensitivity to heat shock (Figure 7A). However, the heat-sensitive phenotype of yΔtrx1/2 mutant was significantly recovered by the expression of AtTrx-h3 or AtTrx-h-2N3C, which contained strong chaperone function (Figure 7A). However, the overexpression of AtTrx-h2 and AtTrx-h-3N2C exhibiting no chaperone activity (Figure 3) failed to complement the thermosensitive phenotype of yΔtrx1/2. The results were confirmed by comparing the growth rate of the yΔtrx1/2 yeast cells overexpressing the various chimaeric AtTrx-hs with WT yeasts under heat-shock conditions (Figure 7B). Whereas the growth rate of yΔtrx1/2 overexpressing AtTrx-h2 and AtTrx-h-3N2C was arrested, the yΔtrx1/2 overexpressing AtTrx-h3 or AtTrx-h-2N3C was able to grow further at 48°C.
Figure 7 Chaperone function of AtTrx-hs confers enhanced heat-shock-tolerance of \( y_{\Delta \text{trx}1/2} \) mutant yeasts

(A) AtTrx-h2, AtTrx-h3, AtTrx-h3-C/S and chimaerie proteins AtTrx-h-2N3C and AtTrx-h-3N2C were overexpressed in \( y_{\Delta \text{trx}1/2} \) mutant yeast and the cells were grown under optimal growth conditions to a \( D_{600} \) of 0.8. The yeasts were exposed to heat shock (48°C) for 30 min, serially diluted, plated on to agar and then incubated at 30°C for 2 days. Cell survival was estimated by counting the colony numbers produced. The results were compared with that of WT cells grown at 30°C and set as 100%. Immunoblot analysis of the His6-tagged proteins expressed in \( y_{\Delta \text{trx}1/2} \) yeast was shown in the inset. (B) Growth curve of \( y_{\Delta \text{trx}1/2} \) yeast overexpressing the various forms of AtTrx-hs, as described in (A). Each strain was cultured at 30°C with shaking in SD minimal medium supplemented with the appropriate amino acids until a \( D_{600} \) of 0.8 was reached. WT (\( \Delta H17040 \)) and \( y_{\Delta \text{trx}1/2} \) mutant yeast overexpressing vector (\( \Delta H17003 \)), AtTrx-h3 (\( \Delta H17033 \)), AtTrx-h3-C39/42S (\( \Delta H17039 \)), AtTrx-h3-A100K (\( \Delta H17009 \)) and AtTrx-h3-C39S/C42S/A100K/A106K (\( \Delta H17003 \)) were subjected to heat shock (48°C), and cell growth was monitored periodically by measuring the \( D_{600} \). (C) Various forms of AtTrx-hs were overexpressed in \( y_{\Delta \text{trx}1/2} \) mutant yeasts. As described in (A), cell survival was estimated by counting the number of colonies formed. Immunoblot analysis of the His6-tagged proteins expressed in \( y_{\Delta \text{trx}1/2} \) mutant yeast was shown in the inset. (D) Growth curve of \( y_{\Delta \text{trx}1/2} \) yeast overexpressing the various forms of AtTrx-hs, as described for (C). WT (\( \Delta H17033 \)) and \( y_{\Delta \text{trx}1/2} \) yeasts overexpressing vector (\( \Delta H17003 \)), AtTrx-h3 (\( \Delta H17033 \)), AtTrx-h3-C39/42S (\( \Delta H17039 \)), AtTrx-h3-A100/106K (\( \Delta H17009 \)) and AtTrx-h3-C39S/C42S/A100K/A106K (\( \Delta H17003 \)) were subjected to heat shock (48°C) and then cell growth was monitored by measuring the \( D_{600} \). Results are means ± S.D. for four independent experiments. Results in (A) and (C) for cell survival rates were analysed using one-way ANOVA method, and the differences for cell survival were assessed using Tukey’s test. Ab, antibody; OD600 = \( D_{600} \); Vec, vector.

DISCUSSION

The functional multiformity of plant Trx-hs can also be regulated by their structural shift, which changes their target substrates [15]. The structural changes of AtTrx-hs from a low-molecular-mass species to high-molecular-mass complexes are mediated by the association of individual subunits into polymeric structures. Interaction of hydrophobic patches of the proteins that are exposed by external stress plays a critical role in their functional diversities [8,22]. The structural changes allow AtTrx-hs to respond to different stresses through induced-functional switching [40]. This for 2 days. Likewise, important results were obtained from the experiments analysed by point-mutated AtTrx-hs. Expression of the mutant proteins in yeasts was confirmed by immunoblotting with the use of an anti-His-tag antibody (Figure 7C, and the inset of Supplementary Figure S7A at http://www.biochemj.org/bj/456/bj4560013add.htm). When we expressed the mutant proteins of AtTrx-h3 harbouring chaperone function, such as AtTrx-h3-C39/42S and the single-mutant proteins of AtTrx-h3-A100K and Trx-h3-A106K (Figure 6B and Supplementary Figure S5A) in the \( y_{\Delta \text{trx}1/2} \) yeast line, the cells showed heat-tolerant phenotypes measured by cell survival and cell growth (Figures 7C and 7D, and Supplementary Figure S7). However, the \( y_{\Delta \text{trx}1/2} \) yeast cells expressing the double-mutant proteins displayed no chaperone function, such as AtTrx-h3-A100K/A106K and AtTrx-h3-C39S/C42S/A100K/A106K proteins (Figure 6B) displayed heat-sensitive phenotypes (Figures 7C and 7D). From the results, it can be concluded that the chaperone function of AtTrx-hs was critically responsible for enhancing heat-shock-tolerance of yeasts.
phenomenon was initially described for AtTrx-h3, which was shown to form high-molecular-mass complexes against heat shock, accompanied with a reversible functional switching from a disulfide reductase to a molecular chaperone. The structural and functional switch is highly important to protect their substrate aggregation from heat shock, thereby endowing eukaryotic cells with heat-shock-tolerance. This type of activity regulation has also been demonstrated for PDI, which exhibits concentration-dependent shifts in chaperone and anti-chaperone functions [41]. On the basis of these precedents in other proteins, we investigated the structural and functional properties of five typical AtTrx-h proteins. Four of the AtTrx-hs exhibited the dual functions of disulfide reductase and molecular chaperone in response to heat shock, concomitant with structural changes. The exception was AtTrx-h2 which displayed only the disulfide reductase activity. Because AtTrx-h2 exhibited quite different structural and chemical properties compared with the other AtTrx-hs, we tried to investigate its protein thermostability through domain swapping and molecular modelling analyses. Then, we found that the C-terminal domain of AtTrx-h2 had lower aliphatic value than the other AtTrx-hs, whereas the N-terminal domains of the AtTrx-hs had similar values, suggesting that the C-terminal domain of AtTrx-h2 is more unstable than the others. Domain swapping of the C-terminal motif of AtTrx-h2 with AtTrx-h3 resulted in discernible changes in the hydrophobic surfaces of the proteins and the ability to form higher-ordered structures. In particular, probably due to structural changes and the exposure of hydrophobic surfaces by domain swapping, there was a functional switching between disulfide reductase and molecular chaperone of the chimaeras. Thus the enhanced chaperone activity of AtTrx-h2N3C should be attributed to a greater availability of hydrophobic surfaces compared with the parent proteins, AtTrx-h2 and AtTrx-h3. In addition, from the molecular modelling studies and specific site-directed mutagenesis, we identified two aliphatic residues in AtTrx-h3, Ala100 and Ala106, playing key roles in governing its oligomer formation and chaperone function.

To analyse the role of functional switching in AtTrx-hs from disulfide reductase to molecular chaperone in vivo, we examined the ability of the five AtTrx-hs and chimaeras to complement the thermosensitive mutant yeast, yATRX1/2, under heat-shock conditions. The mutant has been used to search the functional specificity, determinants of specificity and interacting partner from plant Trxs [15,42–46]. From the experiment, we found that, in contrast with AtTrx-h2, AtTrx-h3N2C and AtTrx-h-A100K/A106K containing reductase with no chaperone function, AtTrx-h3 and AtTrx-h2N3C were able to enhance heat-shock-resistance of yATRX1/2. In addition, mutant yeast overexpressing AtTrx-h3-C39S/C42S which displayed only chaperone with no reductase function showed a similar level of heat-shock-tolerance as the cells overexpressing the WT form of AtTrx-h3. These results clearly demonstrated that the recovery of heat-shock-tolerance of mutant yeast was due to the chaperone function of AtTrx-hs. The results were similar to what has been reported for PDI [47], namely that a cysteine-deficient variant of PDI exhibiting chaperone function, but no disulfide isomerase activity improved the viability of PDI-null yeast.

Through a sophisticated mechanism of regulation involving both structural and functional switches, AtTrx-hs are able to functionally substitute for AtTrx-h5 [16]. Also, the two Trx-hs from pea (Pisum sativum), designated PsTRX-h1 and PsTRX-h2, exhibited different binding properties to their substrates [49]. In conclusion, we demonstrate in the present study that, although the C-terminal region of the AtTrx-hs shares relatively higher amino acid sequence similarities than their N-terminal domain [50,51], the structural and functional specificity of the AtTrx-hs predominantly resides in the C-terminus (Figure 3). In addition, the active-site cysteine residues of AtTrx-h3 play a critical role in disulfide reductase function, but the specific amino acids of AtTrx-h3, Ala100 and Ala106, were shown to control its chaperone function, which resulted in a significant influence on stress resistance of living organisms. These data provide important insights into the as-yet-unidentified specific regulatory mode for functional specificity of individual AtTrx-hs in plants. In particular, the identification of a role in vivo for the N-terminal extension of AtTrx-h2 in plant cells should be highly valuable to study. The physiological significance of this stress-dependent structural and functional switching of AtTrx-h warrants further investigation.

**AUTHOR CONTRIBUTION**

Young Jun Jung, Yong Hun Chi and Sang Yeol Lee designed the experiments and wrote the paper. Young Jun Jung, Yong Hun Chi, Ho Byoung Chae, Mi Rim Shin, Eun Seon Lee, Seol Ki Paeng, Jin Ho Park, Woe Yeon Kim, Chang Ho Kang, and Kyun Oh Lee performed the experiments. Yun Lee and Keun Woo Lee performed molecular modelling. Joon-Yung Cha and Dae-Jin Yun provided critical comments for data analysis.

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SUPPLEMENTARY ONLINE DATA

Analysis of *Arabidopsis* thioredoxin-h isotypes identifies discrete domains that confer specific structural and functional properties

Young Jun JUNG*1, Yong Hun CHI*1, Ho Byoung CHAE*, Mi Rim SHIN*, Eun Seon LEE*, Joon-Yung CHA*, Seol Ki PAENG*, Yuno LEE*, Jin Ho PARK*, Woe Yeon KIM*, Chang Ho KANG*, Kyun Oh LEE*, Keun Woo LEE*, Dae-Jin YUN* and Sang Yeol LEE*2

*Division of Applied Life Science, PMBBRC, Gyeongsang National University, Jinju, 660-701, Republic of Korea

Figure S1 Comparison of hydrophobicity between AtTrx-h2 and AtTrx-h3

Hydrophobicity plots of AtTrx-h2 (red line) and AtTrx-h3 (blue line) were generated by the Kyte–Doolittle analysis. Broken boxes indicate the hydrophobicity domains that exhibited critical differences between the two proteins. The y-axis indicates the hydrophobicity scores; positives score on the y-axis indicate hydrophobic regions. In bold letters, WCGPC (red) and WCPCC (blue) represent the active sites of the two proteins. a-a, amino acids.

1 These authors contributed equally to this work.
2 To whom correspondence should be addressed (email sylee@gnu.ac.kr).
Figure S2  Effect of the N-terminal 19-amino-acid extension of AtTrx-h2 on its biochemical properties analysed by domain swapping with AtTrx-h3

(A) Schematic representation of the chimaeras of AtTrx-h2, AtTrx-h2-N(Δ19) and AtTrx-h3. a·a, amino acids. (B) Structures of AtTrx-h-2N3C and AtTrx-h2-N(Δ19)3C analysed by SEC on a Superdex 200 column. (C) Hydrophobicity of AtTrx-h-2N3C and AtTrx-h2-N(Δ19)3C was compared by using bis-ANS (10 μM). Fluorescence of the protein-bound bis-ANS was measured at an excitation wavelength of 390 nm and emission wavelength of 400–600 nm. (D) Comparison of the disulfide reductase and chaperone functions of AtTrx-h3, AtTrx-h2-N(Δ19)3C and AtTrx-h-2N3C. DTNB reduction activity was followed by measuring the A412 and chaperone activity was measured at A650, using the same procedures as described in the legend to Figure 1 of the main text. Activities are expressed relative to AtTrx-h3, which was set at 100 %. Results are means for at least three independent experiments.

Figure S3  Aligned structure of AtTrx-h3 on the decameric structure of E. coli Prx, AhpC

Each subunit of AtTrx-h3 (yellow) is superimposed with the respective Trx scaffold in a subunit of decameric E. coli Prx, AhpC (grey). The B-type dimer structure is highlighted by a box. The rotated dimer structures of AtTrx-h3 and E. coli Prx, AhpC, proteins are shown enlarged in the right-hand panel.
Figure S4  Comparison of disulfide reductase activity of AtTrx-h3 with the point-mutated AtTrx-h3s using DTNB as a substrate

(A) Schematic representations of the mutant AtTrx-h3s. Left: single-mutant proteins. Right: double-mutant proteins. Mutated residues are indicated with asterisks (*). (B) Disulfide reductase activity of the single-mutant proteins of AtTrx-h3 including AtTrx-h3 (∗), AtTrx-h3-A100K (∗) and AtTrx-h3-A106K (∗). (C) Disulfide reductase activity of the double-mutant proteins of AtTrx-h3, such as AtTrx-h3 (∗), AtTrx-h3-C39S/C42S (∗), AtTrx-h3-A100K/A106K (∗) and AtTrx-h3-C39S/C42S/A100K/A106K (∗). (B-C) Reduction of DTNB (5 mM) was measured by light scattering at 412 nm (Abs412) in the presence of NADPH, TR and AtTrx-h3 proteins. Substrate alone (∗) or 30 μM ovalbumin (∗) was used instead of AtTrx-h3 as a control.

Figure S5  Comparison of protein structure and chaperone functions of AtTrx-h3 with the point-mutated AtTrx-h3s

(A) Chaperone activity of the proteins. Chaperone activity was examined at 45°C for 20 min in the presence of AtTrx-h3 (∗), AtTrx-h3-A100K (∗) and AtTrx-h3-A106K (∗), using the same procedures as described in the legend to Figure 1 of the main text. In the reaction, substrate alone (∗) or 30 μM ovalbumin (∗) was used instead of AtTrx-h3 as a control. (B) Structural analysis of the proteins by SEC. Molecular masses of standards are indicated in kDa.
Figure S6  Comparison of the A-type interface interaction between AtTrx-h3 and AtTrx-h3-C39S/C42S

A-type dimer structures of WT AtTrx-h3 (A) and AtTrx-h3-C39S/C42S (‘C39/42S’) (B) showing A-type interface (also known as the ‘alternative’ or ‘A’ interface) interactions. To clarify the subunits, each subunit is shown in green and yellow respectively. Detailed view of each interface is shown on the right-hand side with the key electrostatic (purple box) interacting residues indicated. (C) Quaternary structures of AtTrx-h3 constructed by the two different types of interfaces: the A-type interface and B-type interface.
Figure S7  Effect of Ala<sup>100</sup> or Ala<sup>106</sup> in AtTrx-h3 on complementing the thermosensitivity of yΔtrx1/2 mutant yeasts

(A) AtTrx-h3, AtTrx-h3-A100K and AtTrx-h3-A106K were overexpressed in yΔtrx1/2 yeasts and then the cells were grown under optimal growth conditions to a D<sub>600</sub> of 0.8. Yeasts were exposed to heat shock (48°C) for 30 min, serially diluted, plated on to agar and then incubated at 30°C for 2 days. Cell survival was estimated by counting the number of colonies formed. The results are expressed as a percentage relative to WT cells. Immunoblot analysis of the His<sub>6</sub>-tagged proteins expressed in yΔtrx1/2 mutant yeast is shown in the inset. (B) Growth curve of yΔtrx1/2 yeast overexpressing the various forms of AtTrx-hs, as described in (A). Each strain was cultured at 30°C with shaking in SD minimal medium supplemented with the appropriate amino acids until a D<sub>600</sub> (OD<sub>600</sub>) of 0.8 was reached. WT (○), yΔtrx1/2 mutant yeast overexpressing vector (■), AtTrx-h3 (●), AtTrx-h3-A100K (■) and AtTrx-h3-A106K (▲) were subjected to heat shock (48°C), and cell growth was monitored periodically by measuring the D<sub>600</sub>. Results are means±S.D. for four independent experiments. Results in (A) for cell survival rates were analysed using one-way ANOVA and differences for cell survival were assessed using Tukey’s test. Means with different letters are significantly different from each other at P < 0.05.