Site-directed mutagenesis substituting cysteine for serine in 2-Cys peroxiredoxin (2-Cys Prx A) of Arabidopsis thaliana effectively improves its peroxidase and chaperone functions

Eun Mi Lee¹, Seung Sik Lee¹,¹, Bhumi Nath Tripathi¹, Hyun Suk Jung², Guang Ping Cao³, Yuno Lee³, Sudhir Singh³, Sung Hyun Hong¹, Keun Woo Lee³, Sang Yeol Lee³, Jae-Young Cho⁴ and Byung Yeoup Chung¹*¹

¹Advanced Radiation Technology Institute, Korea Atomic Energy Research Institute, 29 Geumgu-gil, Jeongeup 580-185, Republic of Korea, ²Department of Biochemistry, College of Natural Sciences, Kangwon National University, Chuncheon 200-701, Republic of Korea, ³Division of Applied Life Science (BK21 Program), Systems and Synthetic Agrobiotech Center (SSAC), Plant Molecular Biology and Biotechnology Research Center (PMBBRC), Research Institute of Natural Science (RINS), Gyeongsang National University, 501 Jinju-daero, Jinju 660-701, Republic of Korea and ⁴Department of Bioenvironmental Chemistry, Chonbuk National University, Jeonju 561-756, Republic of Korea

* For correspondence. E-mail bychung@kaeri.re.kr
† These authors contributed equally to this work.

INTRODUCTION

Peroxiredoxins (Prxs) are ubiquitous thiol-based peroxidases capable of reducing a broad range of toxic peroxides and peroxinitrites in almost all living organisms (Tripathi et al., 2009; Dietz, 2011). Based on their subunit composition and position and the number of conserved cysteine (Cys) residues, Prx can be divided into the four sub-classes 1-Cys Prx, 2-Cys Prx, Prx II and Prx Q (Bhatt and Tripathi, 2011). Distinct locations, transcriptional regulation and structural and functional variations of Prx suggest specific biological roles of these sub-classes of Prx in the plant cell (Park et al., 2000; Dietz, 2003). Among these sub-classes, 2-Cys Prx has received greater attention due to its capability to act as chaperone, protein-binding partner, enzyme activator and redox sensor, along with its peroxide detoxification property (Muthuramalingam et al., 2009).

2 Plant 2-Cys Prx is nuclear-encoded but exclusively located in chloroplasts, the most active site of the redox metabolism in plant cells (Baier and Dietz, 1997). Two isoforms of 2-Cys Prx, viz., 2-Cys Prx A and 2-Cys Prx B, have been identified in Arabidopsis thaliana (Kirchsteiger et al., 2009) and account for ~0.6% of total chloroplast protein (Baier et al., 1996; Baier and Dietz, 1996). Although 2-Cys Prx is an obligate...
homodimer, it frequently changes its conformation (reduced dimer, reduced oligomer, oxidized dimer and hyperoxidized dimer) depending on the redox status (Muthuramalingam et al., 2009). 2-Cys Prx is considered a functional hub in redox-dependent regulation and signalling in the chloroplast (Bhatt and Tripathi, 2011). Earlier studies suggested a protective role of 2-Cys Prx in chloroplasts against oxidative damage by inactivation and regulation of H₂O₂ at an optimal level. Suppression of 2-Cys Prx A has been shown to impair photosynthetic capacity in plants and increase the susceptibility of chloroplast proteins to oxidative damage (Baier and Dietz, 1999; Baier et al., 2000). Pulido et al. (2010) have demonstrated altered redox homeostasis and a marked increase in H₂O₂ content in chloroplasts of Arabidopsis thaliana 2-Cys Prx A-2-Cys Prx B (Δ2ep) double mutants. Furthermore, over-expression of 2-Cys Prx increased tolerance of heat and oxidative stresses in tall fescue and potato plants (Kim et al., 2010, 2011) and increased the tolerance of reactive nitrogen species in yeast cells (Sakamoto et al., 2003).

3 Peroxide released during oxidative stress causes hyperoxidation of 2-Cys Prx, leading to a reversible conformational change from a low molecular weight (LMW) form to a high molecular weight (HMW) form (Muthuramalingam et al., 2009). However, this conformational change impairs the peroxidase activity of 2-Cys Prx (predominantly in the LMW form), but enables 2-Cys Prx to act as a molecular chaperone to prevent misfolding or aggregation of intracellular macromolecules caused by stress (Chuang et al., 2006). The chaperone function of 2-Cys Prx has already been established in bacteria (Chuang et al., 2006), yeast (Jang et al., 2004) and mammalian cells (Yang et al., 2002) and has subsequently been reported from the plant kingdom (Tripathi et al., 2009). It has been reported that 2-Cys Prx efficiently prevents the heat-dependent aggregation of citrate synthase and reduction-dependent aggregation of insulin (Jang et al., 2004; Kim et al., 2009; Muthuramalingam et al., 2009; An et al., 2010, 2011a). Thus, depending on its conformation, 2-Cys Prx may function as a peroxidase or molecular chaperone to protect the plant cell from either oxidative damage or denaturation or aggregation of intracellular proteins during stress conditions (König et al., 2013). The dual functions of these proteins are associated with dynamic reversible changes in their quaternary structure and also depend on the oxidation of peroxidatic Cys (Cₚ), phosphorylation, oxidative and/or heat stress, irradiation, etc., which induce a structural change in 2-Cys Prx from the LMW form to the HMW form (König et al., 2013). Based on this description, it is assumed that 2-Cys Prx evolved as a multi-functional protein that switches between a high-affinity but low-turnover peroxidase function and a low-affinity but high-turnover chaperone function (König et al., 2013).

4 The enhancement of one of these activities (peroxidase and chaperone) of 2-Cys Prx may be an effective strategy to harness the potential of these molecules to the greatest advantage. However, earlier attempts to manipulate 2-Cys Prx to enhance one of these activities have compromised the efficiency of the other activity (An et al., 2011a; Hong et al., 2012). Recently, however, Park et al. (2014) have reported that proton irradiation can enhance both the peroxidase and the chaperone activity of PaPrx (2-Cys Prx). It has also been observed that the dual function of 2-Cys Prx can be regulated by an additional Cys on the α-helix region between two active Cys residues (An et al., 2011b). The presence of additional Cys residues promotes the oligomerization of 2-Cys Prx from the LMW form to the HMW form and thereby affects the switching of this protein between the two physiological functions. Therefore, further research is warranted to get better insight into the regulation and improvement of the dual function of 2-Cys Prx.

5 Based on the above, the present study investigated: (1) the production of various Ser → Cys substituted mutants of 2-Cys Prx A; (2) the regulatory mechanism of 2-Cys Prx A and in turn ways of enhancing its dual functions; and (3) the role of helix formation in Bas1 using molecular dynamics (MD) simulation studies for improvement of the two activities.

In the molecular modelling part of this study, we focused on observing the structural differences between wild-type (WT) 2-Cys Prx A and six mutants developed by point mutation (substitution of Cys for Ser) at residues 127, 131, 150, 180, 182 and 219. Wild-type 2-Cys Prx A had low chaperone and peroxidase activities, and in this it differed from six of the 2-Cys Prx A mutants, which demonstrated better chaperone or peroxidase activity. Mutants S127C, S131C and S150C showed high chaperone activity and S150C, S180C and S182C showed high peroxidase activity. However, S219C showed low chaperone and peroxidase activity. Hence, an investigation to identify the protein structural changes responsible for improvement in these activities was performed, employing the molecular modelling approaches of homology modelling and MD simulation.

MATERIALS AND METHODS
Site-directed mutagenesis of 2-Cys Prx A and protein expression
A cDNA of Arabidopsis thaliana encoding 2-Cys Prx A was cloned following standard procedures and was used to generate the mutants S127C, S131C, S150C, S180C, S182C and S219C by substituting Cys for Ser¹²⁷, Ser¹³¹, Ser¹⁵⁰, Ser¹⁸⁰, Ser¹⁸² and Ser²¹⁹, respectively, by PCR-mediated site-directed mutagenesis. 2-Cys Prx A and all mutants were subsequently cloned in pGEM-T Easy vector (Promega, Madison, USA) and the point mutations were verified by automated DNA sequencing.

We cloned 2-Cys Prx A and its mutants in pET28a(+) expression vector (Novagen, Madison, USA), subsequently transformed and over-expressed in Escherichia coli. Then, His-tagged protein was purified from E. coli KRX (Promega) as described by An et al. (2010). In brief, 2 L of LB medium containing 50 μg ml⁻¹ kanamycin was inoculated with 20 ml of a non-induced overnight bacterial culture and was grown to optical density (OD₆₀₀) 0-6 at 37 °C. Expression of recombinant protein was induced by adding L-rhamnose monohydrate to a final concentration of 0.2 % (w/v) at 30 °C. After 3 h, the cell pellet obtained by centrifugation (Supra 22k, Hanil, Korea) at 4000 g for 20 min was resuspended in lysis buffer (50 mM Na₂HPO₄, 500 mM NaCl, pH 8-0) and stored at −20 °C until used. For protein purification, the cell suspension was sonicated (VCX500, Sonics, USA) at an amplitude of 23 % on ice for 15 min intermittently. The cell debris was removed by spinning at 7000 g for 20 min at 4 °C. The soluble crude protein extract was loaded onto a nickel nitритriacetate agarose column (Pepton, Daejeon, Korea), previously equilibrated with five column volumes of phosphate-buffered saline (PBS). The
protein was eluted with 150 mM imidazole in PBS and the protein-containing fraction was pooled and dialysed three times against 10 mM Tris–HCl (pH 7.4) for 3 h. Protein concentrations were determined with the Bio-Rad protein assay using bovine serum albumin as a reference.

**Size exclusion chromatography and polyacrylamide gel electrophoresis**

Protein size determination and purification of 2-Cys Prx A and its mutants was performed by fast protein liquid chromatography (FPLC; AKTA, Amersham Biosciences, Uppsala, Sweden) using a Superdex 200 HR 10/300 GL column (Amersham Biosciences) following a method described earlier (An et al., 2011a). The column was equilibrated and run with 50 mM Tris–HCl (pH 8.0) buffer containing 100 mM NaCl (0.5 ml min⁻¹). Fractions (F1, F2 and F3) with the desired protein were pooled, concentrated and used for further analysis. Sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) under reducing or non-reducing conditions and native PAGE were performed as described previously (Moon et al., 2005).

**Measurement of hydrophobicity**

The hydrophobicity of 2-Cys Prx A and its mutants was measured by examining the binding of 1,1′-bi(4-anilino)naphthalene-5,5′-disulphonic acid (bis-ANS; Invitrogen Corporation, Carlsbad, CA, USA) with protein in an assay mixture of 10 μM bis-ANS and 50 μM protein in 50 mM HEPES buffer (pH 8.0). Bis-ANS fluorescence excitation was set as 380 nm and emission was scanned between 400 and 600 nm using an Infinite M200 (Tecan Group, Manndorf, Switzerland) as described earlier (Sharma et al., 1998).

**Transmission electron microscopy and image processing**

Transmission electron microscopy (TEM) and image analysis were performed following the method described by Lee et al. (2009). Briefly, different molecular weight fractions of 2-Cys Prx A were applied to glow-discharged carbon-coated copper grids. After allowing the protein to absorb for 1–2 min, the grids were rinsed with deionized water and stained with 2 % uranyl acetate. For metal shadowing, proteins were mixed with an equal volume of glycerol and solution was sprayed onto freshly cleaved mica. Grids were examined in a Technai G2 Spirit Twin TEM (FEI, USA) operated at 120 kV at a magnification of 51 k. The electron microscopy methods used were as described by Burgess et al. (2004).

**Circular dichroism spectroscopy**

Circular dichroism (CD) spectral analysis of 10 μM purified proteins of WT 2-Cys Prx A and its mutants in 100 mM potassium phosphate buffer (pH 8.0) and 5.0 mM NaCl was performed with a spectropolarimeter (J-715; Jasco, UK) using a modification of Reed’s method (Reed and Reed, 1997). All spectra were collected and averaged at least three times from independent experiments.

**Molecular modelling**

Homologues of 2-Cys Prx A were identified by performing sequence database searches using standard tools, such as PSI-BLAST and blastp. Homology modelling was conducted to
construct the 3-D structure of 2-Cys Prx A, Haem-binding protein 23 (HBP23)/Prx I from Rattus norvegicus (PDB ID: 2Z9S) (Matsumura et al., 2008) was selected as a structural template to construct the reduced decamer. A 3-D model of 2-Cys Prx A was constructed using the MODELLER module in Discovery Studio (DS) 3.5 (Marti-Renom et al., 2000; Eswar et al., 2006). The structures were validated with the PROCHECK (Laskowski et al., 1993) program and ProSA-web (Wiederstein and Sippl, 2007) to evaluate stereochemical properties. Energy minimization was performed for refinement of the seven systems (WT and six mutants) using the Smart Minimizer algorithm implemented in DS 3.5. The active-site Cys 119 (Cp) in each subunit was oxidized to Cys sulphenic acid (Cp-SOH) using the Vienna-PTM webserver (Margreitter et al., 2013) for all systems. In total, seven different MD simulations were performed using the GROMACS program (version 4.5.3) (Berendsen et al., 1995; Van der Spoel et al., 2010) with the GROMOS54A7 force field (Schmid et al., 2011). The modified structures were immersed in an orthorhombic water box (1·2 nm thickness) and the net charge was neutralized by the addition of Na+ counter ions. The long-range electrostatic interactions were calculated by the particle mesh Ewald (PME) method (Darden et al., 1993). Constant pressure and temperature for the whole system (1 bar and 300 K) were achieved with a Parrinello-Rahman barostat (Parrinello and Rahman, 1981) and V-rescale thermostat (Bussi et al., 2007). Production runs (10 ns) were performed under periodic boundary conditions with an NPT ensemble. The time step for the simulations was set to 2 fs and the coordinate data were written to the file every 10 ps. All analyses of MD simulations were carried out with GROMACS and DS 3.5 software.

Statistical analyses

The relative peroxidase and chaperone activities of various fractions of the purified 2-Cys Prx A and of the mutated and wild 2-Cys Prx A proteins were statistically analysed by one-way ANOVA (SPSS version 16.0), followed by a multiple comparison of the mean values (presented in Figs 3 and 4) by Duncan’s test (P = 0.05). The mean and standard error were calculated from triplicates (n = 3) from three independent experiments.

RESULTS

2-Cys Prx A functions as both a peroxidase and a molecular chaperone

Figure 1A shows the peroxidase activity of 2-Cys Prx A measured in terms of NADPH oxidation. Curves of NADPH are shown for different concentrations of 2-Cys Prx A protein. Recombinant protein was incubated in 50 mM HEPES (pH 8.0) containing 0·3 mM NADPH, 1·0 mM yeast thioredoxin and 5·0 mM yeast thioredoxin reductase at 30 °C for 5 min, followed by addition of 1·0 mM H2O2. Enzyme activity was recorded by measuring the change in absorbance at 340 nm for 10 min. The control shows the thermal aggregation of MDH without 2-Cys Prx A. Holdase chaperone function of WT-2-Cys Prx A was measured using MDH as substrate. Thermal aggregation curves are shown for 1:1 and 1:3 molar ratios between MDH and 2-Cys Prx A. (C) Holdase chaperone activity was measured using G6PDH as substrate. The cysteine-free form of G6PDH was denatured in guanidine–HCl and subsequently refolded in renaturation buffer in the presence of increasing concentrations of WT 2-Cys Prx A (1, 3, 5 and 10 μM). GroEL indicates the activity of a standard chaperonin of E. coli and SP indicates the spontaneous reactivation of G6PDH without 2-Cys Prx A. Values are mean of three replicates (n = 3) with s.e. <3·0 % (not shown).

2-Cys Prx A exists in multiple oligomeric forms

Based on the analysis of molecular size of the purified fraction of 2-Cys Prx A by size exclusion chromatography (SEC),...
three distinct forms – F1, F2 and F3 – were identified (Fig. 2A). Subsequent native PAGE analysis showed that fraction F1, being an HMW complex, could not be resolved and thus was retained almost at the top of the separating gel (Fig. 2B). Fraction F2, an LMW complex, was present as an oligomeric complex of various sizes, whereas F3 exhibited a dimeric form (Fig. 2B). However, all three fractions showed a single band in SDS-PAGE analysis, with an apparent molecular weight of ∼22 kDa (Fig. 2B, lower panel), suggesting that the 2-Cys Prx A protein forms a homo-oligomeric complex. A third, faint band appeared in the native gel of the dimeric form of F3 (Fig. 2B); the reason for this was unknown, but might be related to the presence of reduced, oxidized or over-oxidized forms of protein together in this fraction.

To further confirm the oligomeric status of 2-Cys Prx A, peak SEC fractions were examined by TEM. The electron micrograph of negatively-stained 2-Cys Prx A showed three different configurations. Fraction F1, corresponding to the HMW complex, was observed as spherical-shaped particles with a diameter of ∼20 nm (Fig. 2C, E), whereas fraction F2 (LMW) was observed as a ring-shaped structure with size ranging from 14 to 16 nm (Fig. 2D–F). However, the protein in fraction F3 did not form any regular structure and therefore no image was taken.

**Dual functions of 2-Cys Prx A are associated with its quaternary structure**

The dual functions of a Prx are closely associated with its oligomeric status; hence, this relationship was explored by examining the peroxidase and molecular chaperone activities of the various fractions of 2-Cys Prx A protein obtained by SEC. The HMW complexes (F1) showed 4- to 5-fold higher holdase chaperone activity (Fig. 3B) but lower peroxidase activity (75 %) than that of total protein (Fig. 3A). In contrast, the F3 fraction exhibited ∼1.5-fold higher peroxidase activity but 50 % lower holdase chaperone activity compared with total protein (Fig. 3A, B). The three fractions showed foldase chaperone activity similar to that of total protein (Fig. 3C). These results confirmed that the dual functions of 2-Cys Prx A are determined by its oligomeric status. Multimerization of 2-Cys Prx A enhanced its holdase chaperone activity, whereas dissociation promoted its peroxidase functions. Foldase chaperone activity appears to be associated with the concentration of 2-Cys Prx A rather than its conformational state.

**Substitution with additional Cys residues in 2-Cys Prx A enhances its dual functions**

In the present work, several mutants of 2-Cys Prx A were generated by replacing Ser with Cys in its crucial z-helix region. Among these mutants, S150C, S180C and S182C showed 3-, 2- and 2.7-fold, respectively, higher peroxidase activity compared with WT (Fig. 4A). Similarly, the holdase chaperone activity of S127C, S131C, S150C and S182C was 4-, 3.5-, 4- and 3.1-fold higher than that of WT (Fig. 4B). All the mutants except S127C showed similar or relatively better foldase chaperone (up to ∼1.3-fold) activity compared with WT (Fig. 4C). Interestingly, S150C showed an increase in all three activities, suggesting that substitution of Cys for Ser at position 150 can play an important role in enhancing peroxidase and molecular chaperone functions.

To further investigate the structural changes in 2-Cys Prx A after addition of Cys, WT 2-Cys Prx A and mutants were analysed by SEC. The SEC results showed that WT and the mutants formed three different types of quaternary structure (Fig. 4D). S150C predominantly formed HMW and LMW complexes whereas S180C produced a dimeric form (Fig. 4D). S127C and S182C showed a pattern similar to that of WT. S180C was present mainly as a dimeric form, whereas S131C, and S127C, having a higher proportion of HMW complex, showed higher holdase chaperone activity. Mutants with a dimeric form showed higher peroxidase activity, but higher holdase chaperone activity was seen in the mutants with a higher proportion of HMW.
complexes, indicating a relation between structure and function in these mutants. However, S150C, exceptionally, showed higher peroxidase and chaperone activity.

Ser → Cys substitution in 2-Cys Prx A leads to increased surface hydrophobicity and a change in structure

The interaction of non-native substrates with 2-Cys Prx A and its Cys-substituted mutants was confirmed using the fluorescent compound bis-ANS, which binds to hydrophobic patches of aminoacyl residues (Sharma et al., 1998). Most 2-Cys Prx A mutants showed a significant increase in fluorescence intensity compared with WT, the maximum increase being shown by S150C (Fig. 5A). This suggests that Cys substitution led to an increase in exposure of hydrophobic patches of 2-Cys Prx A. However, S219C showed a decrease in hydrophobicity. These proteins showed a similar pattern of increase in their holdase chaperone activity (Fig. 4B). S150C, S131C, S182C and S127C, with relatively high increases in hydrophobicity, also showed better holdase chaperone activity. The increase in surface hydrophobicity is closely related to increased chaperone activity of proteins. These results suggest that additional Cys residues lead to exposure of more hydrophobic patches in 2-Cys Prx A mutants and in turn enhance holdase chaperone activity.

The influence of the replacement of Ser with Cys on the secondary structure of the protein was determined using UV–CD analysis. This analysis revealed the secondary structure of WT as 27.4 % α-helix, 51.2 % β-sheet and 24.4 % random coil without turn (Fig. 5B). The structures of S127C, S182C and S219C were almost similar to that of WT. However, in S150C the proportion of α-helix was markedly increased (to 71.2 %), with a concomitant decrease in the proportion of β-sheet (to 28.8 %). Interestingly, S131C and S180C proteins had completely lost their random coil segment.

Molecular modelling for the construction of 3-D structure of 2-Cys Prx A and correlation of structural differences with functional changes

A detailed atomistic molecular modelling study was undertaken to investigate the structure of 2-Cys Prx A and to find out why mutation leads to changes in structure and function. First, the 3-D structure of 2-Cys Prx A was constructed based on HBP23/Prx I from R. norvegicus (PDB ID: 2Z9S) (Matsumura et al., 2008) (Fig. 6). The sequence of the 2-Cys Prx A protein shared ~41.6 % sequence identity and 56.9 % sequence similarity with the template. The proportion of residues of 2-Cys Prx A in the most favoured regions was 91.2 %. The Z-score for WT 2-Cys Prx A (~6.83) was within the range of scores typically found for experimentally determined native proteins of similar size. Subsequently, C\textsubscript{\textgamma} residues in the validated structures were modified to Cys sulphenic acid (C\textsubscript{\textgamma}-SOH), considering the intermediate oxidation state. Six mutants (mutated from Ser to Cys at residues 127, 131, 150, 180, 182 and 219) were generated. The formation of inter-disulphide bonds in WT 2-Cys Prx A and six mutant systems were generated by linking the Cys residues and then conducting energy minimization.

In order to investigate the dynamic behaviour of these seven systems, 10-ns MD simulations were carried out with these four modified structures containing Cys sulphenic acid. The closest snapshot to an average structure over the last 1 ns of the trajectory was selected as a representative structure. The snapshots of WT 2-Cys Prx A (9680 ps), S127C (9130 ps), S131C (9560 ps), S150C (9600 ps), S180C (9570 ps), S182C (9070 ps) and S219C (9980 ps) were used to compare the protein structures between WT and mutant systems. S219C was not included in this study due to low chaperone and peroxidase activities.
Peroxiredoxin exists as an obligate dimer that can form intermolecular disulphide bonds or remain in reduced form; the reduced and hyperoxidized dimeric structures have a strong tendency to form decamers or dodecamers (Barranco-Medina et al., 2009). Previous studies have suggested that the high molecular weight of typical 2-Cys Prx is related to chaperone activity, and hyperoxidation will induce oligomerization from the LMW to the HMW form, which assumes a chaperone function (Lim et al., 2008). The reduced form of 2-Cys Prx is fully folded in the HMW oligomerization process. Partial unfolding of the structure by movement of helix \( \alpha_2 \) is needed to form the \( C_p \)-loop, to enable the peroxide substrate to oxidize the catalytic

![Fig. 4](https://academic.oup.com/aob/article-abstract/116/4/713/94153)

Fig. 4. (A) Relative peroxidase activities of Ser → Cys substituted 2-Cys Prx A mutants. The activities of the 2-Cys Prx A mutants were compared with that of WT 2-Cys Prx A, set at 100 %. (B, C) Relative holdase (B) and foldase (C) chaperone activities of point-mutated 2-Cys Prx As. Activities of 2-Cys Prx A mutants were compared with that of WT 2-Cys Prx A, set at 100 %. (D) Structural analyses of 2-Cys Prx A mutants by SEC. The six mutants of 2-Cys Prx A proteins (S127C-2-Cys Prx A, S131C-2-Cys Prx A, S150C-2-Cys Prx A, S180C-2-Cys Prx A, S182C-2-Cys Prx A and S219C-2-Cys Prx A) were separated by SEC and compared with WT 2-Cys Prx A. Proteins were divided into three fractions (F1, F2 and F3) by SEC, as shown in Fig. 2A. Bars marked with different letters are significantly different from each other \( (P < 0.05) \). Each data point is the mean ± s.e. of three replicates \( (n = 3) \) from three independent experiments.

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site (Cp) (Barranco-Medina et al., 2009). In other words, unfolding the $\alpha_2$ helix will convert Prx to an oxidized decamer, which can increase the chaperone activity of Prx. Hence, $\alpha_2$ helix structures of WT and three mutants (S127C, S131C and S150C) showing high chaperone activity were examined (Fig. 7). Residues 127 and 131 were located in the $\alpha_2$ helix and residue 150 in the dimer–dimer interface but very close to the active site Cys119, located in the $\alpha_2$ helix (Cys119–Lys135) (Fig. 7A). The reduced form of WT had a fully folded $\alpha_2$ helix, but the $\alpha_2$ helix of three mutants had a partial unfolding structure (Fig. 7B–D). Mutant S127C, which had the highest chaperone activity, was unfolded more completely than the others, which is in accordance with the experimental observation. It is evident that changes in the chaperone activity of 2-Cys Prx A could be induced by unfolding the $\alpha_2$ helix.

Earlier research suggested that the peroxidase activity of Prxs can be regulated by the formation of reduced decamer (Barranco-Medina et al., 2009). Hence, the $\alpha_2$ helix and dimer–dimer interface in the decamer were examined for changes in the peroxidase activity of WT and three mutants. The locations of three mutation sites were identified using the 3-D structure of the constructed 2-Cys Prx A. Residues of Ser150, Ser180 and Ser182 were located in dimer–dimer interface regions (Fig. 8A). In these three mutants (S150C, S180C, and S182C), mutation positions moved closer to the adjacent dimer (Fig. 8B–D), which can easily form a stable reduced decamer to increase peroxidase activity. Mutants S127C and S131C were located away from the dimer–dimer interface. Therefore, it can be postulated that the distance between mutation residues in each dimer is related to changes in peroxidase activity. On the other hand, S180C and S182C had a fully folded $\alpha_2$ helix like that of WT 2-Cys Prx A (Fig. 9) and had high peroxidase and low chaperone activity.

Typical 2-Cys Prxs can regulate H$_2$O$_2$-mediated cell signalling. In this process, the Prx is inactivated by the hyperoxidation of an active site of a Cys residue to Cys sulphinic acid. Sulphiredoxin (Srx) can restore peroxidase activity and terminates the signal. Srx binds to the C-terminal of Prx (Jönsson et al., 2008), and the C-term of 2-Cys Prx A was therefore studied for peroxidase activity change. A difference was observed in the C-term loop region (Asp$^{258}$–Pro$^{256}$) near the C-term helix. The C-term loops of S180C and S182C were more flexible.
than that of WT. On the contrary, S127C and S131C had a more stable C-term loop than WT. To demonstrate this difference using quantitative data, root mean square fluctuations (RMSF) were calculated for the six systems (except for S219C). In the presence of the inter-disulphide bond, the C-term loop was more flexible than in other cases (Table 1). From the RMSF analysis of the C-term loop region, we clearly observed that the average RMSF values of the regions in the inter-disulphide bond-containing systems (S150C, S180C and S182C) were relatively greater than the values of other systems. This indicates that the inter-disulphide bond made the C-term loop flexible, so that it could easily bind to Srx. Thus, the flexible C-term loop may be a reason for the changes in peroxidase activity of 2-Cys Prx A.

Fig. 7. Decameric structure of four systems showing a change in chaperone activity. Reduced decamer structures of (A) WT 2-Cys Prx A, (B) S127C, (C) S131C and (D) S150C. Detailed views of the dimer region are indicated by squares, showing the catalytic Cys (Cys\textsuperscript{119} and Cys\textsuperscript{241}) and mutation positions marked by CPK style. The $\alpha_2$ helix is displayed by secondary structure in organ.
DISCUSSION

The 2-Cys Prxs are ubiquitous and highly abundant proteins serving multiple functions in plant cell (Bhatt and Tripathi, 2011). The present study demonstrates the regulation of the dual functions (peroxidase and chaperone) of 2-Cys Prx 2-Cys Prx A through site-targeted replacement of Ser with Cys. For this purpose, a 2-Cys Prx A was identified from *A. thaliana*, cloned, and characterized for its structure and function. *In vitro* enzymatic analysis showed that 2-Cys Prx A, like other 2-Cys Prxs, can perform the dual functions of a Trx-dependent
peroxidase and a molecular chaperone, which is in good accordance with previous studies (An et al., 2011a). Based on their observations, An et al. (2011a) has proposed a model for the functional switching of 2-Cys Prx (PaPrx) based on reversible changes in protein structure. According to this model, 2-Cys Prx can reversibly change its protein structure from HMW complexes to LMW using two different pathways. Furthermore, it was stated that principally 2-Cys Prx exists as oligomers and HMW forms and acts as a chaperone, and subsequently Trx switches most of the HMW complexes to LMW forms to function as Trx-dependent peroxidases, depending on the redox status of the cell (An et al., 2011a). Purified 2-Cys Prx A also showed discretely sized multiple structures with a diverse range of molecular states, including HMW, LMW and dimeric structures.

2 The functional analysis of fractionated 2-Cys Prx A suggests that chaperone activity was higher than peroxidase activity in spherical-shaped HMW complexes, whereas peroxidase activity was predominant in dimeric forms. The ring-shaped LMW complexes showed dual functions as peroxidase and molecular chaperone. Oligomerization of thiol group proteins is considered to be an important mode of regulation of these dual enzymatic functions. These findings are consistent with earlier reports showing that the dual function of 2-Cys Prx is closely linked with its quaternary structure (König et al., 2013).

3 Furthermore, several mutants, viz. S127C, S131C, S150C, S180C, S182C and S219C, were generated by site-directed mutagenesis by replacing Ser with Cys in α-helix regions between two active Cys residues of 2-Cys Prx A. These variants formed three types of oligomer under normal conditions, viz. HMW complex, LMW complex and dimeric form, with variable proportions. S150C and S180C formed more HMW and LMW complexes than WT, while S180C and S219C predominantly produced dimeric forms. In general, dimeric and HMW forms possess high peroxidase and chaperone activities, respectively while LMW forms have better dual functions (Jang et al., 2004; Moon et al., 2005). This might be the reason why S150C and S182C, having higher proportions of HMW and LMW forms, showed a significant increase in peroxidase and chaperone activities compared with WT. Similarly, S180C, S182C and S219C showed high peroxidase activity because of their higher proportions of dimeric forms, whereas S127C and S131C showed high chaperone activity due to their HMW forms. Exposure of the hydrophobic domains of a protein is related to its molecular chaperone activity, as to protect target substrates against stress-induced aggregation, chaperones bind to non-native states of protein substrates through these hydrophobic interactions (Ganea, 2001; Jang et al., 2006a). Replacement of Ser with Cys in the α-helix region of 2-Cys Prx A resulted in an increase in hydrophobicity of most of mutants of could have increased their chaperone activity. However, S150C, having a low dimer peak, showed a different pattern and exhibited higher peroxidase and chaperone activities. In the light of the available data, it is difficult to account exactly for this behaviour of S150C. Residue Ser150 (in WT) lies in the dimer–dimer interface region and replacement of Ser with Cys at the 150 position moves it close to an adjacent dimer, which can easily form a stable decamer to increase peroxidase activity. König et al. (2013) has also noted variation in function in relation to variation in the conformation of 2-Cys Prx mutants with altered Cys. Further research is required using such mutants to provide detailed understanding of the relation between specific conformational states of 2-Cys Prx A and its function.
Molecular evolution has favoured multifunctional 2-Cys Prx proteins switching between peroxidase and chaperone. However, growing interest in the development of an enzyme with enhanced resistance to inactivation and aggregation (König et al., 2013) has further necessitated the manipulation in 2-Cys Prxs in order to harness their potential to greatest advantage. Several attempts have already been made to regulate and understand the structure and dual functions of 2-Cys Prx using various methods, including over-oxidation, phosphorylation, chemical modification by methylglyoxal (MGO), heat treatment, gamma radiation, electron beams and Ser → Cys substitution in the α-helix regions between two active Cys residues of a Prx (Rogalla et al., 1999; Nagaraj et al., 2003; Akhtar et al., 2004; Andrew-Aquilina et al., 2004; Jang et al., 2006b; Kanade et al., 2009; Lee et al., 2009; Park et al., 2009; An et al., 2011b, 2011c; Hong et al., 2012). However, none of these methods has been shown to be efficient in regulating and enhancing the dual functions of these proteins. The present study has demonstrated that site-directed mutagenesis to replace Ser with Cys at the desired sites between two active Cys residues is an effective means to understand the structure of 2-Cys Prx A (2-Cys Prx) and hence regulate their function.

Previous research has demonstrated that the typical 2-Cys Prx adopts four different conformation states, which are related to the switching of its function. Dimers strongly tend to form decamers or dodecamers, while the oxidized form is preferentially regulated in 2-Cys Prx to inactivate and aggregate in vitro. Prx adopts four different conformation states, which are related (2-Cys Prx) and hence regulate their function. Various methods, including over-oxidation, phosphorylation, chemical modification by methylglyoxal (MGO), heat treatment, gamma radiation, electron beams and Ser → Cys substitution in the α-helix regions between two active Cys residues of a Prx (Rogalla et al., 1999; Nagaraj et al., 2003; Akhtar et al., 2004; Andrew-Aquilina et al., 2004; Jang et al., 2006b; Kanade et al., 2009; Lee et al., 2009; Park et al., 2009; An et al., 2011b, 2011c; Hong et al., 2012). However, none of these methods has been shown to be efficient in regulating and enhancing the dual functions of these proteins. The present study has demonstrated that site-directed mutagenesis to replace Ser with Cys at the desired sites between two active Cys residues is an effective means to understand the structure of 2-Cys Prx A (2-Cys Prx) and hence regulate their function.

Previous research has demonstrated that the typical 2-Cys Prx adopts four different conformation states, which are related to the switching of its function. Dimers strongly tend to form decamers or dodecamers, while the oxidized form is preferentially present as a dimer. It is a dynamic process. The reduced decamer has strong peroxidase activity, and decamers may associate to produce an HMW form that has chaperone activity (Barranco-Medina et al., 2009). Hence, the α helix, dimer–dimer interface and C-term loop have been examined to explain the functional changes (chaperone and peroxidase) of 2-Cys Prx A mutants. Mutants with different activities have shown different structural changes, which are in accordance with previous research. Among seven systems, only S150C showed both chaperone and peroxidase activity, which can be explained by its multiple structural changes. There is strong evidence that activity change of 2-Cys Prx A is not accidental or may unilaterally cause many factors. It is undoubtedly the results. Finally, this modelling study can provide insight into the mechanism by which these mutants change the structure and activity of the protein.

A series of molecular modelling approaches was used to understand the mechanism of the 2-Cys Prx A proteins at the molecular level and to trace the reasons why mutants showed altered activities. Seven different models were constructed by homology modelling and then the structures were modified to enhance the dual functions of these proteins. The present study can provide insight into the mechanism by which these mutants change the structure and activity of the protein.

In conclusion, our work showed that 2-Cys Prx A has dual enzymatic functions. The present findings are mainly based on in vitro analyses, but Cys mutation replacing Ser with Cys at amino acid position 150 in the α-helix of 2-Cys Prx A might be a means of developing plants that tolerate various abiotic stresses. In planta validation of these findings is the future objective of our research.

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