AtGRXcp, an Arabidopsis chloroplastic glutaredoxin, is critical for protection against protein oxidative damage

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Glutaredoxins (Grxs) are ubiquitous small heat-stable disulfide oxidoreductases and members of the thioredoxin (Trx) fold protein family. In bacterial, yeast, and mammalian cells, Grxs appear to be involved in maintaining cellular redox homeostasis. However, in plants, the physiological roles of Grxs have not been fully characterized. Recently, an emerging subgroup of Grxs with one cysteine residue in the putative active motif (monothiol Grxs) has been identified but not well characterized. Here we demonstrate that a plant protein, AtGRXcp, is a chloroplast-localized monothiol Grx with high similarity to yeast Grx5. In yeast expression assays, AtGRXcp localized to the mitochondria and suppressed the sensitivity of yeast grx5 cells to H2O2 and protein oxidation. AtGRXcp expression can also suppress iron accumulation and partially rescue the lysine auxotrophy of yeast grx5 cells. Analysis of the conserved monothiol motif suggests that the cysteine residue affects AtGRXcp expression and stability. In planta, AtGRXcp expression was elevated in young cotyledons, green tissues and vascular bundles. Analysis of atgrxcp plants demonstrated defects in early seedling growth under oxidative stresses. In addition, atgrxcp lines displayed increased protein carbonylation within chloroplasts. Thus, this work describes the initial functional characterization of a plant monothiol Grx, and suggests a conserved biological function in protecting cells against protein oxidative damage.

Reactive oxygen species (ROS) can be formed as by-products in all oxygenic organisms during aerobic metabolism (1). In higher plants, chloroplasts and mitochondria are two major organelles that contribute to production of reactive oxygen species during photosynthesis and carbon metabolism (2-3). In addition, plants actively generate ROS as signals in response to environmental stresses (3-6). However, because of the cytotoxic and extremely reactive nature of ROS, they can cause wide-ranging damage to macromolecules (1, 7-9). To overcome such oxidative damage and control signaling events, plants have orchestrated an elaborate antioxidant network (4).

Of those antioxidant systems, the physiological roles of thioredoxins have been intensively studied (10), while those of Grxs have not been fully defined (11-12). Grxs are ubiquitous small heat-stable disulfide oxidoreductases which are conserved in both prokaryotes and eukaryotes (11, 13). Through an active motif, namely the conserved “CPYC” sequence (a dithiol Grx), they catalyze the reduction of protein disulfides and of glutathione (GSH)-protein mixed disulfides via a dithiol or
monothiol mechanism (14-15). In bacterial, yeast, and mammalian cells, dithiol Grxs appear to be involved in many cellular processes and play an important role in protecting cells against oxidative stresses (16-18).

Besides the dithiol Grxs, a new group of monothiol Grxs has recently been identified in yeast (Grx3, 4, and 5) and bacteria (Grx4) that have a single cysteine residue in the putative active motif (19-20). Yeast Grx5 encodes a mitochondrial monothiol Grx which is required for biogenesis of iron-sulfur clusters, whereas Grx3 and Grx4 function in detoxification of cytotoxin and cell proliferation in yeast (21-23). Interestingly, bacterial Grx4, unlike other previously characterized Grxs, can serve as a substrate for thioredoxin reductase instead of NADPH/glutathione reductase (20), suggesting that those monothiol Grxs have distinct functions. This group of monothiol Grxs is also conserved across organisms and has now been identified in malarial parasites, plants, zebrafish, mice, and humans (24-27). Recent studies also indicate that those Grxs contain a newly identified PICOT-HD (protein kinase C-interacting cousin of thioredoxin homology domain) in their carboxyl-terminal regions (24,28). However, ascertaining a unifying function of PICOT-HD Grxs has been problematic.

Photosynthetic organisms, particularly higher plants, have a large Grx gene family; however, until recently, only a few plant Grxs have been studied (12, 29-30). In the Arabidopsis genome, there are at least 31 ORFs coding for putative Grxs, which can be divided into three major classes that include the aforementioned monothiol Grxs (12,30). Genetic analysis of a CC type Grx, ROXY1, indicates an important role of this protein in floral petal development (31). In a previous study, we used a yeast functional screen, to identify a PICOT-HD containing protein that was able to activate Arabidopsis CAX1 Ca^{2+}/H^+ antiport activity in a yeast expression system; this gene was originally termed CXIP1(26). Here we determine the subcellular location of this first cloned plant PICOT-HD containing protein and reclassify the gene as AtGRXcp. We functionally characterize the protein, and perform initial structure-function studies using yeast expression assays. We go on to isolate AtGRXcp knockout mutants and describe the phenotypes of these plants at the whole-plant and biochemical levels. For the first time, we demonstrate a role for a plant monothiol Grx.

**EXPERIMENTAL PROCEDURES**

**Isolation of AtGRXcp null alleles**-To isolate atgrxcp alleles, two T-DNA insertional mutant lines were obtained from the SALK T-DNA collection (32). Homozygous plants from each T3 generation were obtained by PCR screening using AtGRXcp-specific and T-DNA border primers. An AtGRXcp reverse primer, 5’-GGG CCG GAT CCT CGA GTC AAG AGC ACA TAG CTT TCT C -3’ and a T-DNA left border primer, 5’- GCG TGG ACC GCT TGC TGC A -3’, were used to screen for the atgrxcp1 allele. The AtGRXcp reverse primer and a T-DNA right border primer, 5’- TGG GAA AAC CTG GCG TTA CCC AAC TTA AT-3’, were used to screen for the atgrxcp2 allele. An AtGRXcp forward primer, 5’-GCG AAG CTT ATA AGT TTT AAT CGT TTA TGG GGT-3’ and the AtGRXcp reverse primer were used to amplify the wild type AtGRXcp gene. The location of the T-DNA insertion was determined by sequencing the PCR product. Both atgrxcp alleles were backcrossed to their respective parental plants to remove any potential unlinked mutations.

**Plant Growth Conditions**-Arabidopsis wild-type (ecotype Columbia, Col-0) and atgrxcp mutant seeds were surface-sterilized, germinated and grown on one-half strength Murashige and Skoog (MS) medium (33) solidified with 0.8%
agar and the same media supplemented with various concentrations of H\textsubscript{2}O\textsubscript{2}. Iron sufficient and deficient media were made following published protocol (34).

**DNA Constructs and Site-Directed Mutagenesis**

Yeast Grx5 was amplified by PCR using a forward primer: 5'-GCC GGA TCC ATG TTT CTC CCA AAA TTC AAT-3' and a reverse primer: 5'-CCG GAG CTC TCA ACG ATC TTT GGT TTC TTC-3' and the PCR products were cloned into pGEM-T Easy (Promega, Madison, WI). The full-length cDNA of AtGRXcp was isolated through a yeast functional screen and originally termed CXIP1 (C\textsubscript{A}X\textsubscript{I} interacting protein 1; 26). AtGRXcp was predicted to have a 63 amino acid signal peptide by analysis with the Chloro P (version 1.1) program (http://www.cbs.dtu.dk/services/ChloroP/). To remove this N-terminal signal peptide, a truncated form of AtGRXcp was amplified by PCR using a forward primer: 5'-GGG CTC GAG AGA TCT GCG ATG GCG TCG GCT CTT ACG CCG-3' and the reverse primer: 5'-CCG GAG CTC TCA ACG ATC TTT GGT TTC TTC-3' and the PCR products were cloned into pGEM-T Easy. Site-directed mutagenesis was performed as described previously (35). A forward primer: 5'-GAA TCC CGT CTC CCC ATG GCT GGA TTC TCC AAC ACT GTG GTT CAG ATT TTG-3' and a reverse primer CGFS: 5'-GAA TTC CGT CTC CAT GGG GAA GTC TCT CGT TCC TTC C-3' were used for creating the C97A mutation. A forward primer: 5'-GAA TCC CGT CTC CCG ATG TGT GGA GCA TCC AAC ACT GTG GTT CAG ATT TTG-3' and the reverse primer CGFS were used for creating the F99A mutation. The fidelity of all clones was confirmed by sequencing.

**Yeast strains, expression constructs, and growth assays**

Saccharomyces cerevisiae wild type strain CML235 (MAT\textsubscript{a} ura3-52 leu2\textsubscript{Δ}1 his3\textsubscript{Δ}200), grx5 (MAT\textsubscript{a} ura3-52 leu2\textsubscript{Δ}1 his3\textsubscript{Δ}200 grx5::kanMX4) were gifted from Dr. Enrique Herrero (Universitat de Lleida, Lleida, Spain) and used in all yeast experiments. Yeast Grx5 and AtGRXcp and its variants were cloned into pIUGpd (36). Yeast cells were transformed by using the LiOAc method (37). All yeast strains were assayed on YPD medium (yeast peptone dextrose, rich media) with or without various concentrations of H\textsubscript{2}O\textsubscript{2}, and SC medium plus six amino acids or five amino acids without Lysine (21, 26).

**Localization of AtGRXcp-GFP fusions in yeast and plant cells**

Full-length and truncated AtGRXcp and its variants were fused to the N-terminus of green fluorescent protein (GFP) using a procedure described previously (38). The GFP constructs were subcloned into yeast and plant expression vectors as described previously (38). The subcellular localization of the fused proteins was imaged in comparison with labeled organelle markers—chloroplasts, mitochondria, Golgi, and peroxisomes—as described previously (38). A peroxisome-targeted DsRed, DsRed-per, was constructed by adding the plant peroxisome-targeting signal, KS\textsubscript{RM}, to the end of DsRed (39). The fluorescence signals were detected at 510 nm (excitation at 488nm) for GFP, at 582 nm (excitation at 543nm) for DsRed and at 660nm (excitation at 633nm) for chlorophyll using Leica TCS SP2 AOBS confocal laser scanning microscope. The fluorescence intensities were quantified by using the LCS software.

**AtGRXcp::GUS transgenic plants**

A 397 bp DNA sequence upstream of ATG of AtGRXcp ORF was amplified from genomic DNA by using the following primer sets: Forward primer: 5'-GGC AAG CTT ATA AGT TTT AAT CGT TTA TGG GGT-3', and Reverse primer: 5'-GCC TCT AGA TTT TGA CGA CTT TTA GAT TTG GAA-3'. The PCR fragment was cloned into pBI121 to replace the 35S promoter, resulting in plasmid pAtGRXcp::GUS. Agrobacterium transformation of Arabidopsis plants was performed as described previously (40). More than 50 T2 generation plants were selected for Kan resistance.

**Protein oxidation analysis**

Carbonyl assays for analysis of oxidized proteins in both yeast and...
plant cells were performed as previously described (19, 41, 42). Yeast cultures of the strains (CML235 and grx5) expressing vector, Grx5, and AtGRXcp were grown in YPD media overnight at 30°C. Half of the culture of each strain was subjected to treatment with 5mM H2O2 for 1hr. Total proteins were extracted from cells with and without treatment. Western blot analysis was used to determine carbonyl group content. Arabidopsis chloroplasts were isolated from photosynthetic tissues of six-week-old flowering wild type, atgrxcp and AtGRXcp-overexpressing plants (43). The oxidized proteins were detected by protein gel blotting using anti-dinitrophenylhydrazone (DNP) antibody (42; Bethyl Lab, Montgomery, TX).

Measurement of iron concentration-Yeast strains (CML235 and grx5) expressing vector, Grx5, and AtGRXcp were grown in 50 ml of selection media (-Ura) overnight at 30°C. Each yeast culture was inoculated into 300 ml of YPD media and grown until an OD600 of 1.0 was reached. Yeast cells were harvested and washed and dried at 70°C and subjected to Inductively Coupled Plasma spectrometry (ICP) analysis (44). To determine the soluble iron concentration, cells were sonicated and broken with a Sonic Dismembrator (Fisher Scientific, Pittsburgh, PA, USA) and the intracellular iron content was examined with a QuantiChron™ Iron Assay Kit (BioAssay Systems, Hayward, CA, USA).

RESULTS

AtGRXcp is a member of the monothiol glutaredoxins-CXIP1 (CAX Interacting Protein 1: accession no. AY157988) was originally identified based on its function in a yeast assay (26); however, we propose that CXIP1 should be reclassified as AtGRXcp. Our computational analysis revealed that AtGRXcp is similar to yeast monothiol Grxs (Grx3, 4, and 5), bacterial Grx4, PfGLP-1 from a malarial parasite, and both zebrafish and mice Grx5 (Fig.1). This group of monothiol Grxs also contains a PICOT-HD, which is conserved in PICOTs from mammalian cells and plants (28). Several Grxs, like yeast Grx 3 and 4, and human PICOT, have an N-terminal extension; however, AtGRXcp, similar to the bacterial Grx4, both yeast and zebrafish Grx5, does not contain the N-terminal extension (Fig.1; 19-20, 27). In addition, our analysis suggests that in higher plants, these monothiol Grxs exist in both monocots and dicots (Fig.1). In Arabidopsis, there are four members of these Grxs (Fig.1). A dithiol Grx has two cysteine residues in the active motif that are able to catalyze protein disulfides and GSH-protein mixed disulfides (11, 17-18); however, the subfamily of Grxs has only one conserved cysteine residue in the putative active motif, termed “CGFS” (Fig.1). These observations indicate that monothiol Grxs are also conserved throughout prokaryotes and eukaryotes (Fig.1). Based on the sequence analysis of those Grxs, we conclude that AtGRXcp is the first cloned plant monothiol Grx.

AtGRXcp is a chloroplast-localized monothiol Grx-Monothiol Grxs have a diverse subcellular distribution in multiple organisms. For example, yeast Grx5 is mitochondria localized (21), Grx3 is targeting to nuclei (45), while a human PICOT is located in the cytosol (24). In order to gain insight into the function of AtGRXcp, we fused it with green fluorescent protein (GFP) at its C-terminus and transiently expressed this fusion protein in tobacco leaf cells. Using various organelle markers, AtGRXcp-GFP was shown to clearly target to chloroplasts, rather than mitochondria, Golgi, or peroxisomes in mesophyll cells (Fig. 2A). Analysis of the AtGRXcp sequence predicts that a 63 amino acid signal peptide is present at the N-terminus (data not shown). To experimentally verify this, we removed this putative signal peptide and fused the truncated AtGRXcp (AtGRXcpΔ63) with GFP for transient expression in tobacco cells. As shown in Fig.2B, AtGRXcpΔ63-GFP no longer targeted to chloroplasts and instead was dispersed throughout the cytosol and nuclei, similar to observations with
free GFP (Fig.2B, data not shown).

AtGRXcp suppresses the sensitivity of a yeast grx5 mutant to H₂O₂- Yeast grx5 cells are growth impaired in minimal media and sensitive to oxidative stresses (19, 21). To examine if AtGRXcp could restore Grx5 function and suppress the sensitivity of grx5 cells to an external oxidant, H₂O₂, we expressed vector control, AtGRXcp, and yeast endogenous Grx5 in grx5 cells. All yeast strains grew normally in YPD liquid media (rich media) after 48hr growth (Fig. 3A). While vector-expressing grx5 cells were growth impaired in the medium with 3mM H₂O₂, both AtGRXcp- and Grx5-expressing grx5 cells grew in a similar manner to wild type cells (Fig.3A). These observations suggest that AtGRXcp is able to suppress the sensitivity of grx5 to oxidative stress.

Given that AtGRXcp localized to chloroplasts, which are specific to plants (Fig.2) and yeast Grx5 is a mitochondrial Grx (21), the suppression of grx5 phenotypes mandates that the subcellular localization of AtGRXcp in yeast cells differs from that seen in plants. To investigate this, AtGRXcp-GFP was expressed in yeast cells. Yeast growth assays revealed that AtGRXcp-GFP was functional and could suppress the sensitivity of grx5 cells to H₂O₂ (Fig.3B). Through immunolabeling studies, AtGRXcp-GFP localized to mitochondria in yeast cells, while a truncated AtGRXcpΔ63-GFP was unable to target to this organelle (Fig.3C). Targeting of AtGRXcp to mitochondria was essential for the function of this protein in yeast as both AtGRXcpΔ63 and AtGRXcpΔ63-GFP were unable to suppress the sensitivity of grx5 cells to H₂O₂ (Fig.3 A and 3B). AtGRXcp is able to protect cells against protein oxidation and rescue the lysine auxotrophy of a yeast grx5 mutant - In grx5 cells, total protein carbonyl content is significantly increased under oxidative stress (Fig.4A), suggesting that yeast Grx5 plays a vital role in directly protecting enzymes from oxidative damages (19, 21).

In order to determine if AtGRXcp could directly reduce protein carbonylation in the grx5 cells, we performed western blot analysis of total proteins isolated from vector-, AtGRXcp- and Grx5-expressing grx5 cells grown in H₂O₂-containing YPD media. Oxidized protein content in AtGRXcp-expressing cells was reduced compared with the vector-expressing cells, but similar to Grx5-expressing cells (Fig.4A). These results demonstrate that AtGRXcp can protect cells against protein oxidative damages.

Yeast Grx5 is a mitochondrial Grx required for the maturation of Fe-S clusters (21, 46). Deletion of Grx5 results in inactivation of the mitochondrial Fe-S enzyme homoaconitase, which is involved in lysine synthesis (21, 27). Previous work details that grx5 cells fail to grow on lysine deficient media (21; Fig.4B). Expression of AtGRXcp rescued the lysine auxotrophy of grx5 cells, although AtGRXcp suppression was less efficient in comparison with Grx5-expressing grx5 cells (Fig.4B). These results again indicate that AtGRXcp can partially restore Grx5 function in yeast cells.

AtGRXcp suppresses iron accumulation in grx5 cells - Previous studies in both yeast and zebrafish indicate that deletion of Grx5 disrupts iron-sulfur cluster maturation and, as a consequence, iron homeostasis, which resulted in the increased levels of intracellular iron (21, 27). This iron accumulation results in the sensitivity of grx5 cells to external oxidants, such as H₂O₂, because of iron-mediated ROS formation (19, 21). We have demonstrated that AtGRXcp protected yeast cells against oxidative damage (Figs. 3A, 3B, and 4A). To further delineate the potential function of AtGRXcp, we assayed the iron content in wild type and grx5 cells expressing vector, Grx5, and AtGRXcp. As shown in Fig.4, C and D, AtGRXcp- and Grx5-expressing cells were both able to suppress the iron accumulation (as measured at the whole cell and intracellular levels).

Structural and functional analysis of the
conserved “CGFS” motif-Monothiol Grxs contain a conserved putative active motif “CGFS” (Fig. 1; 47). To determine the importance of the “CGFS” domain for AtGRXcp function, we made two single-amino acid mutants (Cys97 to Ala97 change was termed AtGRXcp-AGFS and Phe99 to Ala99 change was termed AtGRXcp-CGAS). We performed the aforementioned liquid growth assays to examine if these mutations in the conserved motif would alter AtGRXcp function. Yeast cells (grx5) expressing AtGRXcp-AGFS (AtGRXcp-AGFS-GFP) or AtGRXcp-CGAS (AtGRXcp-CGAS-GFP) grew similarly in YPD liquid media in comparison with vector- and AtGRXcp-expressing grx5 cells (Fig. 5A; data not shown). However, under oxidative stress, AtGRXcp-CGAS- (AtGRXcp-CGAS-GFP-) and AtGRXcp-expressing grx5 cells grew significantly better than vector-expressing grx5 cells, whereas AtGRXcp-AGFS- (AtGRXcp-AGFS-GFP-) expressing grx5 cells grew at a slower rate than vector-expressing cells (Fig. 5A; data not shown). To determine if these amino acid changes (Cys97 to Ala97 and Phe99 to Ala99) affects protein expression and stability, we performed western blots to detect AtGRXcp-GFP and the variants among yeast total proteins. Indeed, AtGRXcp-AGFS-GFP was rarely detectable, while AtGRXcp-CGAS-GFP levels were similar to wild type AtGRXcp (Fig. 5B). These findings suggest that the Cys97 variant alters the protein expression and the half-life of AtGRXcp similarly in both yeast and plant cells.

AtGRXcp is expressed in cotyledon, leaves, vascular tissues and flowers—Previously, RNA blot analysis indicated that AtGRXcp is ubiquitously expressed in Arabidopsis plants with high levels of AtGRXcp mRNA accumulating in green leaves (26). To further determine spatial and temporal AtGRXcp expression, the 397 bp AtGRXcp promoter was cloned and transcriptionally fused with the β-glucuronidase gene (GUS) and then transformed into Arabidopsis plants. More than 50 independent transgenic lines were generated. Preliminary GUS staining indicated that all transgenic lines harboring AtGRXcp::GUS had similar expression patterns (data not shown). AtGRXcp::GUS was highly expressed in the young cotyledons at 3 days after germination (Fig. 6A). In addition, AtGRXcp::GUS was detected in the green tissues (leaves), vascular bundles, roots, stems, and flowers (Fig. 6, B, C, D and E).

AtGRXcp is critical for early seedling growth—To gain insight into the biological function of AtGRXcp in planta, we analyzed two Salk T-DNA insertional lines (Salk_125903 and Salk_056587; 32) carrying a T-DNA in AtGRXcp (Fig. 7A). To confirm the presence of the T-DNA in atgrxcp plants, we used AtGRXcp-specific and T-DNA-specific primers to PCR-screen T3 progeny and obtained two homozygous lines, termed atgrxcp1 and atgrxcp2 (Fig. 7A). Sequence analysis of the T-DNA flanking regions revealed that in atgrxcp1 the T-DNA is located in the middle of AtGRXcp at position Met-71, and in atgrxcp2 the T-DNA is inserted at position Ile-12 (Fig. 7A). Both T-DNA insertions disrupted AtGRXcp expression as determined by RNA gel blot analysis (Fig. 7B).

Both atgrxcp1 and atgrxcp2 failed to
display visible defects in seed germination and early growth in normal nutrient media in comparison with wild type (Fig. 8). Given that AtGRXcp is able to restore yeast Grx5 function (Figs. 3, 4, and 5), whose mutation causes iron accumulation (21), we were interested in determining if an AtGRXcp deletion would affect the iron sensitivity of seedlings. Both wild type and atgrxcp seeds were germinated and tested on both iron-sufficient and iron-deficient media. No significant difference was seen between wild type and atgrxcp mutants. However, when wild type and atgrxcp seeds were germinated and grown on ½ MS media containing 3mM H2O2, young seedlings of atgrxcp grew at a reduced rate as measured by alterations in primary root growth (Fig. 8).

Given that AtGRXcp is localized to chloroplasts, which are major sites of ROS production, we hypothesize that AtGRXcp functions in protecting against protein oxidation in chloroplasts. To test this, we isolated total chloroplast proteins from wild type, atgrxcp, and AtGRXcp-GFP-overexpressing plants and detected carbonyl content of the protein samples. As shown in Fig. 7C, the protein carbonylation for extracts from atgrxcp alleles was higher than that observed for extracts from wild type and AtGRXcp-GFP-overexpressing plants (Fig. 7C, data not shown).

DISCUSSION

ROS-mediated protein oxidation in chloroplasts impairs both photosynthesis and metabolic enzyme activities (1, 10). Plants have evolved a sophisticated network to scavenge ROS (2–4). Evidence presented here indicates that plant Grx coding sequences are similar to those found in other species; and, as we detail in this study, functional analysis also justifies the reclassification of CXIP1 to AtGRXcp (Fig. 1; 26). Our findings reveal that the first characterized plant Grx, AtGRXcp, is a functional monothiol Grx localized to chloroplasts (Fig. 2). We also demonstrate that loss of AtGRXcp in Arabidopsis leads to protein oxidation in chloroplasts and seedlings sensitive to external oxidants, such as H2O2 (Figs. 7 and 8), implicating a critical role of AtGRXcp in regulating redox state in chloroplasts.

A large number of Grxs have been identified in various species based solely on genome analysis (Fig. 1). In plants, several members of these proteins are predicted to target to and function in plastids/chloroplasts (30); however, little is known about their physiological roles. Both plant AtGRXcp and zebrafish Grx5 are able to suppress yeast grx5 mutant phenotypes (Figs. 3, 4, and 5; 27), suggesting that the biological function of this group of monothiol glutaredoxins is evolutionarily conserved. Interestingly, AtGRXcp-GFP, like Grx5, localized to the mitochondria when expressed in yeast cells (Fig. 3C); however, AtGRXcp localized to chloroplasts in plant cells (Fig. 2). The dual-targets of AtGRXcp have been observed for other plant and yeast proteins (45, 48). For example, the plant phosphate transporter, Ph2,1, localizes to the chloroplast envelope in plants, but to mitochondria when expressed in yeast (48). The nuclear localized yeast Grx3 has been shown to be able to compensate for Grx5 when localized to the mitochondria (45). Mitochondrial localization of AtGRXcp in yeast is necessary for its function as evidenced by the fact that when the 63 amino acid signal peptide of AtGRXcp was removed, the truncated form of AtGRXcp was unable to target mitochondria and suppress the sensitivity of grx5 cells to H2O2 (Fig. 3).

The ability of AtGRXcp to partially restore the Fe-S enzyme activities and suppress iron accumulation in yeast grx5 cells suggests that AtGRXcp may be required for biogenesis of iron-sulfur (Fe-S) clusters and/or involved in the regulation of iron homeostasis in chloroplasts. In
plants, Fe-S clusters have an important role in the light-harvesting photosystem I (PSI) and the cytochrome b6/f complex for electron transport (49). Chloroplasts/plastids also contain many Fe-S proteins, such as a 2Fe-2S ferredoxin and 4Fe-4S ferredoxin-thioredoxin reductase (FTR) (50). Recent studies indicate that Arabidopsis proteins directly and indirectly linked to chloroplast activities exhibit more sensitivity to oxidative damage (9). There is a possibility that AtGRXcp protects protein oxidative damage via modulating iron homeostasis to control iron-generated oxygen radicals within chloroplasts. Support for this model was observed in AtGRXcp-expressing yeast cells. Apparently, AtGRXcp attenuates protein oxidation by reducing intracellular iron levels (Fig.4, C and D). Furthermore, in plants, proteins from chloroplasts of atgrxcp plants were subjected to increased carbonylation, an indicator of increased oxidative damage to proteins (Fig. 7C). It is also possible that AtGRXcp suppression of yeast grx5 mutant phenotypes was due to activation of the ROS scavenging system (Figs.3 and 4). In support of this hypothesis, recent studies indicate that monothiol Grxs can modulate cellular signaling events through protein-protein interactions mediated by PICOT-HD (23, 26, 51). Future work will be necessary to determine the target(s) of AtGRXcp and understand the complexity of ROS regulation in planta.

AtGRXcp contains the putative active motif, “CGFS” (Fig. 1), and substitution of the conserved cysteine residue (Cys97) with alanine caused a deceased expression and shorter half-life of AtGRXcp in both yeast and plant cells (Fig.5, B, C and D). In contrast, mutation of both the conserved Cys60 and the non-conserved Cys117 in yeast Grx5 and the conserved Cys30 in bacterial Grx4 did not significantly affect protein stability (20, 47, 52). These findings suggest that this particular cysteine residue (Cys97) may play a unique role in the plant AtGRXcp. In addition, our results suggest that the conserved phenylalanine residue (Phe62) is not essential for the function of AtGRXcp (Fig.6). In this case, our findings are similar to that observed with the Phe62 variants in yeast Grx5 (47). Recently, the resolved three-dimensional solution structure of the bacterial Grx4 reveals that this monothiol Grx has a unique structure which is significantly different from the dithiol Grxs (53). AtGRXcp consists of four cysteine residues, the conserved Cys97 and the non-conserved Cys151, which are located in the conserved GRX region (Fig.1), and two additional cysteine residues (Cys62 and Cys172) that are not conserved in the monothiol Grxs (Fig.1, 26). Future work will need to be directed at the various roles these cysteine residues may have in disulfide formation, electron donation, and redox-catalysis.

We have already pursued several avenues to further discern the biochemical property of AtGRXcp. Initial analysis indicated that AtGRXcp, like yeast Grx5 and bacterial Grx4, was not active in either the insulin or the β-hydroxyethyl disulfide (HED) assays (data not shown; 20, 52). We certainly envision AtGRXcp playing a myriad of roles in planta; however, studies directed at clarifying other AtGRXcp functions require additional inquiry.

Recent reports have shown that Arabidopsis has high steady-state levels of protein carbonylation, particularly chloroplastic proteins, during vegetative growth (9, 54). AtGRXcp is highly expressed in young cotyledons, green leaves, and the vasculature of roots (Fig. 6, A, B, and C), implicating that AtGRXcp may be critical for protecting chloroplasts/plastids against oxidative damage during early growth and development. In agreement with this, atgrxcp mutant roots are more sensitive to external oxidants (Fig. 8). It is possible that plastidic AtGRXcp could be involved in the glutathiolation/deglutathiolation of proteins through coupling with GSH and GSH reductase (GR) in chloroplasts/plastids, which is required...
for early seedling growth (55). This hypothesis is reinforced by results which suggest changes in glutathione redox state controlled via GR activities have an important role in cell differentiation, root growth, and plant development (56, 57). In addition, a CC type Grx, ROXY1, appears to play a role in post-translational regulation of floral identity gene products that are required for floral petal development (31).

Although atgrxcp plants did not display any altered sensitivity to iron imbalance at the whole plant level (data not shown), this may be due to tight regulation of iron uptake (34) or iron-related phenotypes being masked by functional redundancy among Grxs (12, 30). The interplay among Grxs and various antioxidant systems will also be addressed in future studies.

The characterization of AtGRXcp reported here is particularly noteworthy in that Grxs have not been previously functionally characterized. AtGRXcp appears to be evolutionarily conserved across taxa and the capability of AtGRXcp to rescue yeast Grx5 deficiency phenotypes suggests a conserved biochemical mechanism among monothiol Grxs. The AtGRXcp deficient plant lines demonstrate that this protein plays a critical role in protecting protein oxidation during stress conditions. Given that there are at least 31 genes in the Arabidopsis genome coding glutaredoxins, the characterization of AtGRXcp is an important first step toward understanding how this large gene family functions in adapting plants to external stresses.

**REFERENCE**

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FOOTNOTES
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The abbreviations used are: PICOT-HD, protein kinase C interacting cousin of thioredoxin homology domain; Grx, glutaredoxin; Trx, thioredoxin; GSH, glutathione; GFP, green fluorescent protein; DsRed, red fluorescent protein; ORF, open reading frame; H2O2, hydrogen peroxide; DNPH, dinitrophenylhydrazone; CaMV, cauliflower mosaic virus.

FIGURE LEGENDS
Fig.1. Sequence analyses of monothiol Grxs. Alignment of monothiol Grx sequences was performed with ClustalW software (http://www.ebi.ac.uk/clustalw/). The conserved putative monothiol active motif “CGFS” are indicated by asterisks. AtGRXcp (AY157988), AtGRX2 (AY157989), AtGRX3 (ADD17344), AtGRX4 (BAB02297), EcGrx4 (P37010), DrGrx5 (AAZ30729), GaGRX (Tc29218), Hspicot (AAF28844), HsGrx5 (AAH47680), Mmpicot (AAF28842), MmGrx5 (Q80Y14), MtGRX (Tc102010), OsGRX1 (AAO20065), OsGRX2 (ABA96598), OsGRX3 (AAM93692), OsGRX4 (BAB62565), OsGRX5 (BAB91855), OsGRX6 (BAD87472), OsGRX7 (BAD68123), PfGLP-1 (CAB38997), ScGrx3 (Q03835), ScGrx4 (P32642), ScGrx5 (Q02784), and SyneGrx (AAD19873), TaGRX (Tc254245), XtGrx (AAH75374). At, Arabidopsis thaliana; Dr, Danio rerio; Ec, Escherichia coli; Ga, Gossypium arboreum; Hs, Homo sapiens; Mm, Mus musculus; Mt, Medicago truncatula; Os, Oryza sativa; Pf, Plasmodium falciparum; Sc, Saccharomyces cerevisiae; Syne, Synechocystis; Ta, Triticum aestivum; Xt, Xenopus tropicalis.

Fig.2. Subcellular localization of AtGRXcp-GFP in plant cells. A, AtGRXcp-GFP is localized to chloroplasts in tobacco cells. Left panels display the transient expression of AtGRXcp-GFP in tobacco cells; Central panels display fluorescence from individually-labeled markers for plant organelles or fluorescing chloroplasts; Right panels show the merged images. B, A truncated AtGRXcp-GFP fusion is not targeting to chloroplasts. Scale bars=25µm.

Fig.3. AtGRXcp is able to suppress the sensitivity of grx5 cells to H2O2. A and B, Vectors-expressing wild
type cells, and vector-, AtGRXcp-, AtGRXcp-GFP, AtGRXcpΔ63-, AtGRXcpΔ63-GFP, and Grx5-expressing grx5 cells were grown in YPD liquid media and the same media supplemented with 3.0 mM H2O2. Cell density was measured at OD600 after 48hr growth at 30°C. Shown are two representative experiments from four independent experiments conducted. The bars indicate the standard error (n=8). C, Subcellular localization of AtGRXcp-GFP (Top panel) and AtGRXcpΔ63-GFP (bottom panel) in yeast cells. Scale bars=10µm.

Fig.4 AtGRXcp can suppress yeast grx5 phenotypes. A, Protein carbonyl contents were analyzed by western blotting with anti-DNP antibody (1:1000). A parallel run stained with Coomassie brilliant blue is shown in the bottom panel. Total proteins were extracted from vector-expressing wild type strain (lane 1 and 5); vector- (lane 2 and 6), Grx5- (lane 3 and 7), AtGRXcp- (lane 4 and 8) expressing grx5 cells. B, AtGRXcp partially rescues the lysine auxotrophy of yeast grx5 mutant. grx5 cells expressing vector, Grx5, and AtGRXcp were assayed on SC medium with or without lysine. The photographs were taken after 3 day growth at 30°C. C, whole cell iron contents were measured by ICP. All results shown here are the means of three independent experiments, and the bars indicate the standard errors. D, intracellular iron levels were measured by a Quantichrom™ Iron Assay Kit. Shown is one representative experiment of four independent experiments. The bars represent standard errors (n=3).

Fig.5. Effect of mutations of the monothiol motif on protein expression and half-life of AtGRXcp. A, Vector-, AtGRXcp-GFP-, AtGRXcp-AGFS-GFP-, and AtGRXcp-CGAS-GFP-expressing grx5 cells were assayed as indicated in Fig.3. B, The expression of AtGRXcp and mutant variants in yeast was analyzed by western blotting with anti-GFP antibody (1:1500). The bottom panel displays the protein loading stained with Coomassie brilliant blue. Total proteins were isolated from vector- (Lane 1), AtGRXcp-GFP-(Lane2), AtGRXcp-AGFS-GFP-(Lane3), and AtGRXcp-CGAS-GFP-(Lane4) expressing grx5 cells. C, Transient expression of AtGRXcp-AGFS-GFP and AtGRXcp-CGAS-GFP in tobacco cells at 5 hr post bombardment. Scale bars=20µm (top panel) or 25µm (bottom panel). D, The fluorescent intensities of AtGRXcp-GFP, AtGRXcp-AGFS-GFP, and AtGRXcp-CGAS-GFP in tobacco cells were quantified at 5hr and 24 hr post bombardment. Error bars represent the standard error (n>100).

Fig.6. Histochemical analyses of Arabidopsis plants expressing an AtGRXcp promoter::GUS fusion. A, GUS staining in cotyledons in 3 day-old developing seedlings. Scale=50µm. B, GUS staining in rosette leaf. Scale bar=2mm. C, GUS staining in primary and lateral roots. Scale bar= 200µm. D, GUS staining in vascular bundle in stems. Scale bar= 1mm. E, GUS staining in flowers. Scale bar=2mm.

Fig.7. AtGRXcp deficiency in Arabidopsis plants results in protein oxidative damage. A, Genomic structure of AtGRXcp showing the sites of T-DNA insertions. B, RNA gel blotting analysis of total RNA isolated from wild type (Col-0, lane1), atgrxcp 1(lane2), atgrxcp 2 (lane3), and 35S-AtGRXcp-GFP (lane4) plants, demonstrating the absences of AtGRXcp mRNA in both atgrxcp plants. The 35S-AtGRXcp-GFP transgenic plants contain both endogenous AtGRXcp and transgene AtGRXcp-GFP mRNAs. C, Protein oxidation in chloroplasts was analyzed by western blotting as indicated in Fig.4. Lane1: wild type; Lane2: atgrxcp 2; Lane3: 35S-AtGRXcp-GFP.

Fig.8 AtGRXcp knockout mutants are sensitive to external oxidants. Wild type (Col-0) and atgrxcp allele seeds were germinated and grown on ½ MS and the same media supplemented with 3 mM H2O2 for ten days. A, Shown is one representative experiment of three independent experiments. B, root growth measurement. The length of primary roots of seedlings was measured after 10 days of growth on the medium with or without H2O2. Error bars represent the standard error (n>50).
A

Cell density (OD_{600})

- Wild type/vector
- Δgrx5/vector
- Δgrx5/Grx5
- Δgrx5/AtGRXcp
- Δgrx5/AtGRXcpΔ63

YPD vs YPD+3mM H$_2$O$_2$

B

Cell density (OD_{600})

- Δgrx5/vector
- Δgrx5/AtGRXcp-GFP
- Δgrx5/AtGRXcpΔ63-GFP

YPD vs YPD+3mM H$_2$O$_2$

C

AtGRXcp-GFP
- Mito-trackII
- Merged

AtGRXcpΔ63-GFP
- Bright field
- Merged
AtGRXcp, an Arabidopsis chloroplastic glutaredoxin, is critical for protection against protein oxidative amage


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