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Unlike thioredoxins, glutaredoxins are involved in iron-sulfur cluster assembly and in reduction of specific disulfides (i.e. protein-glutathione adducts), and thus they are also important redox regulators of chloroplast metabolism. Using GFP fusion, AtGrxC5 isoform, present exclusively in Brassicaceae, was shown to be localized in chloroplasts. A comparison of the biochemical, structural, and spectroscopic properties of Arabidopsis GrxC5 (WCSYC active site) with poplar GrxS12 (WCSYS active site), a chloroplastic paralog, indicated that, contrary to the solely apomonomeric GrxS12 isoform, AtGrxC5 exists as two forms when expressed in Escherichia coli. The monomeric apoprotein possesses deglutathionylation activity mediating the recycling of plastidial methionine sulfoxide reductase B1 and peroxiredoxin IIE, whereas the dimeric holoprotein incorporates a [2Fe-2S] cluster. Site-directed mutagenesis experiments and resolution of the x-ray crystal structure of AtGrxC5 in its holoform revealed that, although not involved in its ligation, the presence of the second active site cysteine (Cys32) is required for cluster formation. In addition, thiol titrations, fluorescence measurements, and mass spectrometry analyses showed that, despite the presence of a dithiol active site, AtGrxC5 does not form any inter- or intramolecular disulfide bond and that its activity exclusively relies on a monothiol mechanism.

Glutaredoxins (Grxs) are thiol-disulfide oxidoreductases present in most prokaryotic and eukaryotic organisms and belonging to the thioredoxin (Trx) superfamily (1, 2). At the structural level, proteins of the Trx superfamily share the so-called Trx fold, which consists of four-stranded β-sheets surrounded by at least three α-helices (3). Using a CXXC or CXXS active site motif, Grxs are involved in the reduction of glutathionylated proteins. The regeneration of an active reduced form usually relies on glutathione (GSH), but some specific members such as human Grx2, Escherichia coli Grx4, and Chlamydomonas reinhardtii Grx3 are also or uniquely regenerated by thioredoxin reductases (4–6). This variation correlates with the type of Grx considered and depends on their redox potential and catalytic mechanism. Indeed, whereas classical Grxs have midpoint redox potentials between −170 and −230 mV (pH 7.0) (7, 8), Chlamydomonas Grx3 forms during its catalytic cycle an atypical intramolecular disulfide between the first active site cysteine and a second cysteine located in the C-terminal part of the protein (6). The more negative redox potential (−323 mV at pH 7.9) observed for this protein most likely explains its ability to receive electrons from ferredoxin:thioredoxin reductase but not from glutathione (6).

The initial Grx classification in non-plant organisms was first based on the active site sequences and resulted in defining two Grx types containing either a dithiol (CP(Y/F)C) or a monothiol (usually CGFS) active site motif (9). However, with the identification of new Grx sequences with more variable active site sequences, this classification has become insufficient. In particular, photosynthetic organisms possess a larger variety of Grxs with about 30 genes compared with two to seven in other prokaryotes or eukaryotes (10). An in-depth phylogenetic analysis of Grxs in photosynthetic organisms indicates that they are grouped into four classes (11). Class I includes “classical” Grxs with CPYC, CGYC, CPFC, or CSY(C/S) active sites and is sub-

4 The abbreviations used are: Grx, glutaredoxin; GSH, reduced glutathione; Trx, thioredoxin; At, A. thaliana; Pt, poplar; HED, 2-hydroxyethyl disulfide; CFP, cyan fluorescent protein; DTTred, reduced DTT; DTTox, oxidized DTT; GSSG, oxidized glutathione; DHA, dehydroascorbate; Prx, peroxiredoxin; Cr, C. reinhardtii.
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divided into five subgroups called GrxC1, -C2, -C3, -C4 and -C5/S12. Class II contains Grxs with a conserved CGFS active site and is divided into four subgroups called GrxS14, -S15, -S16, and -S17. In addition, class III, which contains Grxs with a CCXX active site motif, is found in terrestrial plants, and class IV, which consists of multidomain proteins containing in their N-terminal part a Grx module, is present in eukaryotic photosynthetic organisms (11).

Through their disulfide reductase activity, class I Grxs are involved in the stress response, catalyzing the reduction of dehydroascorbate (12), and the regeneration of type II peroxiredoxins (13) and 1-Cys methionine sulfoxide reductase B1 (14, 15). These regeneration mechanisms likely occur via a glutathionylation step. Although Trxs have been reported to catalyze deglutathionylation in some organisms (16), this reaction is generally considered to be specifically catalyzed by Grxs (17). Protein deglutathionylation can proceed via a monothiol or a dithiol pathway depending on the involvement of one or two cysteines in the catalytic mechanism (6, 18, 19).

Besides its disulfide reductase activity, a novel function assigned to some bacterial, yeast, human, and plant class II Grxs is their involvement in the iron-sulfur (Fe-S) cluster assembly machinery most likely through their capacity to incorporate labile Fe-S clusters and transfer them to acceptor proteins (20–22). On the other hand, specific class I Grxs such as human Grx2 and plant GrxC1 also have the capacity to incorporate Fe-S clusters that are more stable than those found in class II Grxs (8, 23). Based on the observation that oxidized glutathione promotes cluster disassembly in human Grx2, it has been hypothesized that under defined redox conditions the release of Grxs in an active form might serve as a redox sensor in human cells (23).

The determination of the three-dimensional structure of poplar GrxS12 and human Grx2 indicated that the [2Fe-2S] cluster was ligated into a homodimer by the N-terminal active site cysteine of two monomers and two glutathione molecules (8, 24, 25). A similar ligation was observed for E. coli Grx4, a class II Grx, but the different orientation of the two monomers was proposed to be responsible for the differential cluster lability (26). The capacity of class I Grxs to bind an Fe-S cluster has been studied in detail by site-directed mutagenesis, and it was suggested that the nature of the residues adjacent to the first active site Cys determined this capacity (8, 27). In particular, for Grxs with a YCPFC or YCPYC active site, the replacement of the proline by a glycine, simulating GrxC1 (YCGYC), was sufficient to allow the incorporation of such a cluster (8). In addition, it has been hypothesized that the Trp residue adjacent to the catalytic cysteine residue in GrxS12 (WCSYS active site) prevented cluster incorporation because changing it into a Tyr allowed cluster incorporation (27).

A close paralog of GrxS12 having a WCSYC active site and named GrxC5 is present only in Brassicaceae. Here we have investigated the structure-function relationship of Arabidopsis thaliana GrxC5 (AtGrxC5) and compared it to poplar GrxS12 (PtGrxS12). The putative role of this Grx in plastids of Brassicaceae is discussed in the light of other functions identified so far for other Grx members.

EXPERIMENTAL PROCEDURES

Materials—G25 columns were purchased from GE Healthcare. 2-Hydroxyethyl disulfide (HED) and 5,5'-dithiobis-2-nitrobenzoic acid were from Acros Organics and Pierce, respectively. All other reagents were from Sigma.

Cloning and Construction of AtGrxC5 and PtGrxS12 Mutants by Site-directed Mutagenesis—The open reading frame sequence encoding A. thaliana GrxC5 (At4g28730) was amplified from leaf cDNA using AtGrxC5 forward and reverse primers (supplemental Table 1) and cloned into pET3d. The amplified sequence encodes a protein deprived of the first 63 amino acids corresponding to the putative targeting sequence and in which a methionine and an alanine have been added during cloning. The protein thus starts with the N-terminal sequence 1MASFGSRM8 and ends with 98GKNGQS113 at the C-terminus. Using two complementary mutagenic primers, the four cysteines of AtGrxC5 were individually substituted into serines. The mutated proteins are called AtGrxC5 C29S, C32S, C80S, and C87S. Various combinations of cysteine substitutions by serine were also introduced in GrxC5 (C29S/C32S and C80S/C87S). In addition, to mimic the active site of AtGrxC5, a PtGrxS12 S32C variant was produced with the serine in position 32 modified into a cysteine. The primers are listed in supplemental Table 1.

Expression and Purification of Recombinant Proteins—For protein production, the E. coli BL21(DE3) strain, containing the pSBET plasmid, was co-transformed with the different recombinant plasmids (28). Cultures were successively amplified up to 2.4 liters in LB medium supplemented with ampicillin and kanamycin (50 μg/ml) at 37 °C. Protein expression was induced at exponential phase by adding 100 μM isopropyl β-D-thiogalactopyranoside for 4 h at 37 °C. The cultures were then centrifuged for 15 min at 4,400 × g. The pellets were resuspended in 30 ml of Tris-NaCl (30 mM Tris-HCl, pH 8.0, 200 mM NaCl) buffer with 1 mM GSH, and the cells were stored at −20 °C.

Cell lysis was performed by sonication (3 × 1 min with intervals of 1 min), and the soluble and insoluble fractions were separated by centrifugation for 30 min at 27,000 × g. The soluble part was then fractionated with ammonium sulfate in two steps, and the protein fraction precipitating between 40 and 80% saturation contained the recombinant protein as estimated by 15% SDS-PAGE. AtGrxC5 WT, C80S, and C87S and poplar GrxS12 S32C were purified by size exclusion chromatography after loading on an ACA44 (5 × 75-cm) column equilibrated in Tris-NaCl buffer usually supplemented with 100 μM GSH. However, some purifications were achieved without GSH. The fractions containing the protein were pooled, dialyzed by ultrafiltration to remove NaCl, and loaded onto a DEAE (diethylaminoethyl)-cellulose column (Sigma) equilibrated in a 30 mM Tris-HCl pH 8.0 buffer. The recombinant proteins passed through the DEAE column, were concentrated by ultrafiltration under nitrogen pressure (Amicon, YM10 membrane), and were stored in the same buffer at −20 °C.

Because they were mainly insoluble in the above described culture conditions, the culture and purification conditions of AtGrxC5 C29S and C32S were slightly modified following a
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Procedure described previously (29). Protein purity was checked by SDS-PAGE, and protein concentrations were determined spectrophotometrically using a molar extinction coefficient at 280 nm of 8,730 M⁻¹ cm⁻¹ for GrxC5 WT; 8,605 M⁻¹ cm⁻¹ for GrxC5 C295S, C32S, C80S, C87S, C295/C32S, and C80S/C87S; and 10,095 M⁻¹ cm⁻¹ for PtGrxS12 S32C. PtGrxC1, PtGrxC3, PtGrxS12, PtTrxh1, AtMsrB1, and AtPrxIIE were purified as described previously (8, 14, 27, 30, 31).

**Transcript Analysis**—Four to 5-week-old plants were used for the analysis of transcript levels of the four plastidial Grxs (GrxC5, -S12, -S14, and -S16). *A. thaliana* ecotype Columbia was grown on soil under controlled conditions (10 h of light at 100 μmol quanta m⁻² s⁻¹ and 23°C and 14 h of darkness at 18°C; 50% relative humidity). Four hours after the onset of light, selected stress treatments were started. Cold stress was performed at 4°C, and salt stress was applied by extensively watering the pots with 200 ml of 150 and 300 mM NaCl solution, respectively. A similar osmotic treatment was performed with 15% (w/v) PEG-5000 (same osmolarity as 150 mM NaCl). Complete rosettes of control and treated plants were harvested after 15-min or longer period at 25°C before desalting on G25 columns pre-equilibrated with 30 mM Tris-HCl, pH 8.0, 1 mM EDTA buffer contained 150 μM NADPH, 0.5 units of glutathione reductase from bakers’ yeast, 2 mM GSH, 0.7 mM HED, and 400 nm Grx except for AtGrxC5 C32S (200 nm Grx). The dehydroascorbate reductase activity was measured using a similar method except that the test was performed in 100 mM phosphate pH 7.0 buffer with 1 mM DHA and 2 μM Grx (1 μM for AtGrxC5 C32S). The reaction was started by adding Grx after a 2-min preincubation. Grx activity was determined by subtracting the spontaneous reduction rate observed in the absence of Grx. The activity was expressed as nmol of NADPH oxidized/nmol of Grx/min using a molar extinction coefficient of 6,220 M⁻¹ cm⁻¹ at 340 nm for NADPH. Three independent experiments were performed at each substrate concentration, and the apparent kcat and Km values were calculated by non-linear regression using the program GraphPad Prism 4.

**Insulin Reduction**—Insulin reduction was monitored spectrophotometrically at 30°C using 2 μM Trx or 10 μM Grx and a procedure described previously (29).

**Glutaredoxin-dependent Peroxidase Activity of PrxIIE**—Rates of peroxide reduction by PrxIIE were determined in an assay coupled with the glutaredoxin system by monitoring NADPH oxidation following absorbance at 340 nm. The assay typically contained 50 μM t-butyl hydroperoxide in 100 mM HEPES pH 8.0 buffer, 2 mM EDTA, 1 mM GSH, 150 μM NADPH, 2 μM PrxIIE, and from 0.1 to 6 μM Grx. The comparative mutant analysis was then performed with 1 μM Grx.

**Methionine Sulfoxide Reductase Activity Assay**—The MsrB1 activity was measured by following NADPH oxidation at 340 nm in the presence of a Grx reducing system at 25°C. A 500-μl reaction mixture in 30 mM Tris-HCl, pH 8.0, contained 200 μM NADPH, 0.5 units of glutathione reductase from bakers’ yeast, 10 mM GSH, 2 mM N-acetylmethionine sulfoxide, 2.5 μM MsrB1, and 25 μM AtGrxC5 corresponding to a saturating concentration. MsrB1 activity was calculated as described previously (14).

**Analytical and Spectroscopic Methods**—Analytical gel filtration analyses were performed with an ÄKTA Avant system (GE Healthcare). 150 μl at about 100 μM apo- and holoforms were separately loaded on a Superdex 75 HR 10/30 column (GE Healthcare) equilibrated with 50 mM Tris-HCl, pH 8.0, 150 mM NaCl and calibrated with gel filtration standards (Bio-Rad). Elution profiles were simultaneously recorded at 280 and 430 nm with a flow rate of 0.5 ml min⁻¹.

Protein concentrations were determined by using BSA as a standard using the Bio-Rad DC protein assay in conjunction with the microscale modified procedure of Brown et al. (34). Iron concentrations were determined colorimetrically by using bathophenanthroline under reducing conditions after digestion of the protein in 0.8% K2MnO4, 0.2 M HCl (35). Fe-S cluster reconstitution experiments in AtGrxC5 C32S were carried out under anaerobic conditions following a procedure described...
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previously (20). UV-visible absorption and CD spectra were recorded at room temperature using a Shimadzu UV-3101PC spectrophotometer and Jasco J-715 spectropolarimeter, respectively. Resonance Raman spectra were recorded as described previously (36) using an Instruments SA Ramonar U1000 spectrometer coupled with a Coherent Sabre argon ion laser with 20-μl frozen droplets of sample mounted on the cold finger of an Air Products Displex Model CSA-202E closed cycle refrigerator.

Crystallization, Data Collection, Structure Determination, and Crystallographic Refinement—Crystallization experiments were performed by the microbatch under oil (paraffin) method at 4 °C under aerobic conditions. Drops were prepared by mixing 1.5 μl of protein solution with an equal volume of crystallization solution. Solutions of apo- and holoproteins had concentrations of 8.9 and 16.7 mg·ml⁻¹, respectively. Suitable crystals for x-ray diffraction were obtained without optimization using crystallization screen kits. The apoform crystallized in 1.6 M ammonium sulfate and 500 mM lithium chloride (Jena Bioscience, JBS 5-B4), whereas brownish crystals were obtained for the holoform by using 0.1 M HEPES, pH 7.5 and 70% 2-methyl-2,4-pentanediol solution (Hampton Research crystal screen 2-35). X-ray diffraction experiments were performed at 100 K with holoform crystals flash cooled directly from the drop and apoform crystals and soaked briefly in crystallization solution supplemented with 20% (v/v) glycerol. Apo- and holoform data sets were collected on beamlines BM30A and ID23-1 at the European Synchrotron Radiation Facility (Grenoble, France), respectively. The data were indexed and processed using XDS (37) and scaled and merged with SCALA from the CCP4 program package (38, 39). Both structures were solved by molecular replacement with MOLREP (40) using the poplar GrxS12 coordinates (Protein Data Bank code 3FZ9). The models were refined using REFMAC version 5.4 (41) interspersed with manual inspection and corrections using COOT (42). In the final rounds of apoform refinement, anisotropic B-factors were included for selected atoms. The validation of both crystal structures was performed with MolProbity (43). All figures were prepared with PyMOL. All statistics are available in Table 1.

RESULTS

AtGrxC5, the Fourth Plastidial Grx of A. thaliana—The A. thaliana Grx family is composed of 33 members, which are predicted to be distributed in different subcellular compartments (11). GrxS12, -S14, and -S16 in poplar as well as GrxS14 in A. thaliana (previously referred to as AtGRXcp) have already been confirmed as being located in plastids by GFP fusion (20, 27, 44). From localization prediction programs, only three additional A. thaliana Grxs exhibit a potential plastidial targeting sequence, namely AtGrxC5 (class I), AtGrxC10, and AtGrxS13 (class III) (10, 11). It is important to note that GrxC5 and GrxS12, which form a specific subgroup of class I Grxs, display about 70% identity when considering the mature form of the proteins. The phylogenetic analysis further indicated that whereas all plants analyzed possess a GrxS12 isoform only species of the Brassicaceae family possess both isoforms (supplemental Fig. 1).

The N-terminal targeting sequences of the six putative plastidial Grxs of A. thaliana were fused in-frame to the green fluorescent protein variants CFP and YFP. The constructs were used to transform A. thaliana mesophyll protoplasts. Similarly to poplar isoforms, AtGrxS12, -S14 and -S16 are clearly located in plastids as indicated by the superimposition of the YFP or CFP signal with the chlorophyll autofluorescence in the merged images (Fig. 1). Concerning AtGrxC5, the reporter protein is also directed to the chloroplast with some dots of stronger signal within the organelle. Although the fluorescence signal for AtGrxS13 is more diffuse than for AtGrxC10, both proteins likely have a cytosolic localization at least under these experimental conditions. Therefore, these two Grxs were excluded from further analysis.

Transcript Analysis of Plastidial Grxs under Environmental Constraints—The transcript expression of AtGrxC5, -S12, -S14, and -S16 was measured using semiquantitative RT-PCR in leaves of plants grown under control and stress conditions, namely cold treatment at 4 °C, salt stress administered by watering with 150 and 300 mM NaCl solutions, and osmotic stress induced by 15% (w/v) PEG-5000 for 24 h, respectively. Under these stress conditions, the plants developed a pronounced stress phenotype (45). The signals were detected after 34 PCR cycles for GrxC5 and GrxS12, 32 cycles for GrxS14, and 30 cycles for GrxS16 (Fig. 2A), indicating that, in these growth conditions, GrxS16 transcripts are the most abundant within the plastidial Grx group in rosettes. Regarding environmental constraints, a significant response of the grx transcript level could not be observed except for a slight down-regulation under extreme salinity for AtGrxS14 and an up-regulation of AtGrxC5 in plants subjected to a cold treatment (Fig. 2B). Likewise only moderate

![table](http://www.jbc.org/)

**Table 1**

Statistics of x-ray diffraction data collection and model refinement

|          | Apoform | Holoform |
|----------|---------|----------|
| Space group | P2₁,2,2 | R3       |
| Cell dimensions a, b, c (Å) | 63.5, 39.0, 37.6 | 111.7, 111.7, 58.5 |
| Resolution (Å) | 33.25-1.2 (1.26-1.20) | 27.99-2.4 (2.53-2.4) |
| Rmerge (%) | 15.03 (18.6) | 19.1 (24.3) |
| Rfree (%) | 17.58 (24.1) | 23.8 (28) |
| n residues | Chain A: 100 (6–105) | 103 (3–105) |
| n ligands | 1 GSH, 3 SO₄²⁻, 1 GSH, 1 [Fe₂-2S]³⁻ |
| n water molecules | 108 | 29 |
| n atoms | 978 | 1,662 |
| Isotropic | 122 | 1,662 |
| Anisotropic | 857 | 0 |
| Average B-factor (Å²) | 18.5 | 57.6 |
| Ramachandran statistics (%) | Residues in preferred regions: 100 | 97 |
| Residues in allowed regions | 100 | 3 |
| Outlier residues | 0 | 0 |
| r.m.s. deviations | Bond length (Å) | 0.025 | 0.021 |
| Bond angle (%) | 2.209 | 1.999 |

* Values in parentheses are for highest resolution shell.

* Rfree was determined from all the reflections (working set + test set), whereas Rmerge corresponds to a subset of 5% of reflections (test set).

* Root mean square.

![image](http://www.jbc.org/)
responses to diverse environmental stress conditions were found when analyzing the Affymetrix GeneChip data available via the Genevestigator tool (supplemental Table 2).

AtGrxC5 Exists in Two Forms upon Expression in E. coli—To characterize the structure-function relationship of AtGrxC5, the mature form of the protein was produced in E. coli as a recombinant protein by removing the N-terminal 63 residues that constitute the putative targeting sequence. The final product comprises 113 amino acids with a predicted molecular mass of 12,482 Da. As observed for PtGrxC1, lysed bacterial cells had a slight but visible brownish color (8). Similarly, following gel filtration, the protein separated into two peaks, an apoform with an apparent size corresponding to a monomer and a holoform most likely containing an Fe-S center and with an apparent exclusion volume corresponding to a dimer (supplemental Fig. 2). This result was unexpected as AtGrxC5 possesses the active site Trp residue, which was thought previously to prevent the incorporation of an Fe-S cluster in the close paralog PtGrxS12 (27).

The nature of the Fe-S center in holoGrxC5 was initially assessed using a combination of analytical and spectroscopic techniques. Iron and protein analyses of the holoform indicated 1.0 ± 0.2 iron/protein monomer consistent with one [2Fe-2S] cluster per dimer. This conclusion was unambiguously confirmed by the nearly identical UV-visible absorption/CD and resonance Raman spectra compared with those established for the structurally characterized PtGrxC1 (Fig. 3). Moreover, the close similarity of the spectroscopic properties of the [2Fe-2S]²⁺ centers in AtGrxC5 and PtGrxC1 indicates analogous cluster coordination involving cysteinyl sulfur ligands from two GSHs and two Grxs. We have shown earlier by analyzing UV-visible absorption/CD and resonance Raman spectra that the Grx conformations are clearly distinct in class I and II Grxs, which both bind [2Fe-2S] clusters (8, 20, 26).

To determine how similar the ligation of the [2Fe-2S] cluster is to the one in PtGrxC1, the four cysteine residues of AtGrxC5 in positions 29, 32, 80, and 87 were substituted by serine residues. The substitution of either Cys80 or Cys87 did not prevent the incorporation of the Fe-S cluster. In contrast, the single substitution of Cys29 or Cys32 produced proteins that were devoid of the cluster, raising the question of the role of the second active site cysteine for cluster ligation or stability. Interestingly, a S32C variant of PtGrxS12, mimicking AtGrxC5 active site sequence, incorporated an Fe-S cluster with a UV-visible absorption spectrum similar to AtGrxC5 (supplemental Fig. 3). However, both the spectroscopic signature and the three-dimensional structure of holoGrxC5 (see below) showed that Cys32 is not a direct ligand for the Fe-S cluster. Next we wanted to determine whether the second cysteine is essential for cluster integration or only for its stability as observed previously for PtGrxC1. A PtGrxC1 C34S variant, although still unstable, was able to incorporate the Fe-S cluster, demonstrating that the second cysteine is not absolutely required for cluster incorporation.

FIGURE 1. YFP/CFP localization of putative plastidial Grxs in A. thaliana mesophyll protoplasts. The sequences coding for the N-terminal extensions of AtGrxC5, AtGrxC10, AtGrxS12, AtGrxS14, AtGrxS15, and AtGrxS16 were fused in-frame with the CFP/YFP coding sequence, mesophyll protoplasts were transfected, and accumulation of the fluorescent reporter was monitored as described under “Experimental Procedures.” The scale bars correspond to 20 μm.

FIGURE 2. Transcript analysis of plastidial Grxs in rosettes of A. thaliana wild type plants. A, the abundance of Grx transcripts was semiquantified by RT-PCR. Stars indicate the number of cycles selected for the analysis of transcript abundance in response to the indicated stress conditions depicted in B. The pictures are from two independent experiments with two to three replicates with similar results.
able to bind an Fe-S cluster, contained a far less stable Fe-S cluster with only 0.19 iron/monomer (8). However, no Fe-S cluster could be reconstituted in AtGrx5 C32S under anaerobic conditions following a standard procedure using glutathione and catalytic amounts of E. coli IscS, indicating that, in this case, the cysteine is indispensable (data not shown).

**Determination of AtGrx5 Redox State**—The presence of four cysteine residues led us to investigate the redox state of the protein to better understand the catalytic mechanism utilized by the apoform of Grx5. Whereas Cys29, Cys32, and Cys87 are conserved in many class I Grxs, Cys80 is not conserved even in other plant Grx5 isoforms. Analysis by mass spectrometry of AtGrx5 WT (purified or not in the presence of GSH) revealed the presence of three protein peaks with molecular masses of 12,348.2, 12,654.4, and 12,961.2 Da (data not shown). Although the intensity of the different peaks varied slightly, similar results were obtained by treating prereduced AtGrx5 with GSSG. The peak at 12,654.4 Da was generally predominant. The first peak is consistent with a protein where the methionine is cleaved. The other peaks, corresponding to mass increments of 306.2 and 613 Da, are consistent with proteins also deprived of the methionine but containing one or two glutathione adducts. After reduction with DTT, a single protein peak with a molecular mass of 12,350 Da was observed, further supporting that the protein is glutathionylated. The same three protein peaks were obtained for AtGrx5 C80S, indicating that Cys80 is not a glutathionylation site. Concerning the AtGrx5 C87S mutant, we detected two protein peaks corresponding to a reduced protein and to an oxidized protein with only one glutathione adduct, indicating that Cys87 is most likely one of the glutathionylation sites; the other site is either Cys29 or Cys32. In the case of the AtGrx5 C32S mutant, only two protein peaks corresponding to oxidized proteins with one or two glutathione adducts were detected, a result consistent with Cys29 and Cys87 being the two modified cysteines. For AtGrx5 C29S, the protein is so unstable that we were not able to obtain reliable results confirming the loss of one glutathione adduct.

As AtGrx5 possesses a single Trp residue as well as two Tyr residues very close to the active site at least in the primary sequence (IYSKTCWCSYCT), the redox state of the apoform was also assessed by measuring its intrinsic fluorescence properties under reducing (GSH or DTTred) or oxidizing (GSSG or DTTox) conditions. Reduced AtGrx5 displayed an emission spectrum with two peaks at approximately 300 and 350 nm characteristic of a signal originating from Tyr and Trp residues, respectively (Fig. 4) (11). Addition of 1 mM GSSG but not DTTox led to the quenching of the fluorescence signal, whereas the subsequent addition of DTTred restored the initial spectrum (Fig. 4 and data not shown). Because similar results were obtained for all monocysteine mutants except AtGrx5 C29S and because DTTox had no effect on the AtGrx5 redox state, the fluorescence quenching can be attributed to the glutathionylation of Cys29.

**Reducase Activity and Catalytic Mechanism of AtGrx5**—To clarify the catalytic mechanism used by AtGrx5 and to...
compare its efficiency with that of PtGrxS12, we first measured the activity of all protein variants using the conventional DHA and HED assays at a fixed enzyme concentration by following NADPH oxidation in the presence of a glutathione recycling system. In both assays, we obtained very similar results: AtGrxC5 C29S was inactive, whereas AtGrxC5 C80S and AtGrxC5 C87S appeared to be as efficient as the WT protein, and AtGrxC5 C32S was nearly 2 times more efficient (Fig. 5, A and B). Strikingly, the AtGrxC5 C32S mutant, mimicking the GrxS12 active site, was as efficient as PtGrxS12, and PtGrxS12 C32S, a protein variant mimicking the GrxC5 active site, had a lower efficiency comparable with AtGrxC5 WT (Fig. 5, A and B). These results indicate that Cys32 is not essential for the reaction mechanism but that it influences protein reactivity.

Following these experiments, we determined the catalytic parameters of AtGrxC5 WT and all monocysteinic mutants in steady-state conditions (Table 2). During the initial preincubation time of the reaction mixture, HED and GSH give rise to a glutathionylated β-mercaptoethanol mixed disulfide (46). The kinetic analysis revealed an apparent $K_m$ value of 0.20 ± 0.02 mM for glutathionylated β-mercaptoethanol and a $k_{cat}$ of 1.21 ± 0.03 s$^{-1}$. These values are similar to those reported for human Grx2, which contains an SCSYC active site, but are lower by a factor 10–20 when compared with other class I Grxs including plant GrxC1 and -C4 (5). Concerning DHA reductase activity, the results provided an apparent $K_m$ value of 0.21 ± 0.03 mM for

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5 J. Couturier and N. Rouhier, unpublished data.
DHA and a $k_{cat}$ of 0.23 ± 0.01 s$^{-1}$ (Table 2). These values are in the same order as those obtained for PtGrxS12 and CrGrx1 (6, 27). The apparent $K_m$ value for GSH was determined by varying the amount of GSH in the DHA assay. AtGrxC5 WT possesses an apparent $K_m$ value of 3.6 ± 0.8 mM for GSH similar to the one obtained for PtGrxS12 but also for other plant and non-plant Grxs in particular human Grx2 (5, 27). AtGrxC5 C80S and C87S possess catalytic parameters very similar to those of AtGrxC5 WT with only small variations observed in the affinity for glutathionylated β-mercaptoethanol. On the other hand, the AtGrxC5 C32S mutant displayed a better catalytic efficiency (about 2 times) compared with AtGrxC5 WT both in HED and DHA assays, originating from a better turnover number (Table 2).

In a second series of experiments, we used the classical insulin reduction assay to assess the disulfide reductase activity of AtGrxC5 (47). In the presence of DTT, AtGrxC5 and PtGrxC3, another dithiol Grx used for comparison, were able to reduce insulin but, as expected, with a lower efficiency than poplar Trxl (Fig. 5C). In alternative experiments, we measured the capacity of Grxs to reduce insulin by using an NADPH/glutathione thione reductase/GSH regeneration system (Fig. 5D). The ability of AtGrxC5 to reduce insulin was not improved compared with the DTT-based assay (Fig. 5D). Regarding the mutated proteins, as already observed for DHA and HED assays, AtGrxC5 C80S and AtGrxC5 C87S appeared almost as efficient as the WT protein, and AtGrxC5 C32S and PtGrxS12 S32C enzymatic activities were similar to PtGrxS12 WT and AtGrxC5 WT, respectively (Fig. 5D). Although delayed in time, AtGrxC5 C29S also surprisingly exhibited the capacity to slightly reduce insulin compared with the GSH alone control, possibly indicating some reactivity of the remaining cysteines toward insulin (Fig. 5D).

Next the capacity of AtGrxC5 to reduce putative physiological target proteins was evaluated by measuring the Grx-mediated recycling of MsrB1 and PrxIIE, two plastidial enzymes, which are efficiently reduced by GrxS12 (14, 48). Hence, as already observed for previous activity tests, AtGrxC5 C80S was as efficient as AtGrxC5 WT for the regeneration of MsrB1, whereas AtGrxC5 C32S mutant was slightly more efficient (Fig. 6A). On the contrary, AtGrxC5 C87S mutant was significantly less efficient as already observed for PtGrxS12 C87S mutant (Fig. 6A) (14). Moreover, MsrB1 possesses an apparent $K_m$ value of 2.3 ± 0.2 μM for AtGrxC5 WT (Fig. 6B), a value comparable with that obtained for PtGrxS12 (14). Strictly similar results were obtained with AtPrxIIE (supplemental Fig. 4). These results are consistent with the previously described regeneration mechanism of these enzymes by class I Grxs (GrxC5 or -S12) through a monothiol mechanism (14), i.e. (i) the reduction of the sulfenic acid by GSH followed by (ii) the deglutathionylation of the glutathione adduct by the nucleophilic attack of AtGrxC5 catalytic cysteine and (iii) the glutathione-mediated reduction of glutathionylated AtGrxC5.

**Structures of GrxC5 Apo- and Holoforms and Quality of Models**—The crystallographic structures of apo- (GrxC5apo) and holofoms (GrxC5holo) of AtGrxC5 were solved to the resolution limits of 1.2 and 2.4 Å, respectively. AtGrxC5 in the apoform crystallizes in the space group P2₁2₁2 with one glutathionylated polypeptide chain in the asymmetric unit, whereas AtGrxC5 in the holoform crystallizes in the space group R3, and the asymmetric unit consists of two monomers and two glutathione molecules ligating one [2Fe-2S] cluster. AtGrxC5 adopts
the classical Grx/Trx fold consisting of a four-stranded mixed β-sheet flanked by five α-helices (see nomenclature in Fig. 7). The crystal structures of AtGrxC5 and poplar GrxS12 are quite similar (root mean square deviation, 0.48 Å). The major difference between the two proteins concerns the presence of two additional cysteines (Cys32 and Cys80) in AtGrxC5. The residue Cys80, which is replaced by a Gly in PtGrxS12, is located in the loop between α3 and α4, and its lateral chain does not form any noticeable interaction. Cys32 is replaced by a serine in PtGrxS12. Both lateral chains exhibit different rotamers (Table 3). In GrxC5apo, the thiol group of Cys32 is in the vicinity of the catalytic cysteine (Cys29) and interacts with the backbone NH group of Lys26, whereas in PtGrxS12, the side chain of Ser32 points toward the hydroxyl group of Ser25.

In GrxC5apo, the glutathione molecule is covalently bound to Cys29, and its binding mode is identical to that of PtGrxS12 and very similar to that of other known glutathionylated Grx structures (25, 49–52). (i) The carboxylate of the glycinyl residue forms a salt bridge with the side chain of His72 from the loop α2–β3. (ii) The NH and carbonyl groups of the cysteinyl residue are hydrogen-bonded to the main chain of Val74, a generally conserved residue that precedes the cis-Pro75 found in Trx superfamily members. (iii) The carboxylate of the γ-glutamate interacts with both NH groups of Cys87 and Thr88 from helix α3. The high resolution of the electron density maps allowed us to observe two conformations for the lateral chain of Thr88. Only in conformer m (χ2 = 12°) is the hydroxyl group within hydrogen bond distance from the γ-Glu carboxylate. The other possible hydrogen bond donors and acceptors of the GSH moiety interact with water molecules or symmetry-related molecules.

In the dimeric GrxC5holo, each iron atom of the Fe-S cluster is tetracoordinated by sulfur atoms: two from the cluster, one from the catalytic Cys29, and one from glutathione. A comparative structural analysis of GrxC5apo and GrxC5holo illustrates the conformational changes upon Fe-S cluster binding. All the lateral chains of the residues of the 28WCSYC32 signature but those of the cysteines adopt different rotamers in the apo- and holoforms with Trp28 exhibiting flipped rotamers (Table 3). The side chain is located on the molecular surface, partially buried by a symmetry-related molecule in the apoform. Previ-
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ous analyses of the PtGrxS12 crystal structure led to the conclusion that Trp28 cannot adopt this p-90 conformation in the dimer because of steric conflicts (27). In GrxC5_holo, the Trp28 rotamer observed is p90 where the NH group of the pyrrole moiety points toward the Fe-S cluster, whereas the phenyl ring is solvent-exposed (Fig. 7). The two residues Tyr30 and Ser31 also present in the signature of human (hGrx2) undergo the same conformational rearrangement upon Fe-S cluster binding in both enzymes (Table 3) (25). Gln62 is the last residue that attracted our attention. Indeed, in GrxC5_apo, the lateral chain participates in the stabilization of the loop αγ–β5 containing the TVP75 motif involved in GSH binding, whereas it adopts another rotamer in GrxC5_holo, interacting with the GSH C-terminal carboxylate group, which also undergoes a conformational change. When GrxC5_apo and GrxC5_holo are superposed, one noticeable difference is the backward movement of the loops α2–β5 and β6–αγ of the N-terminal part of the helix αγ and of the glutathione molecule to accommodate the Fe-S cluster and to preserve their interactions (Fig. 7). In addition to its interaction with the carboxamide of Gln62, the GSH C-terminal group is involved in a salt bridge to Lys36. The rest of the new GSH/protein interactions are quaternary in nature, involving hydrogen bond donors or acceptors of the 28WCSYC32 signature of monomer B for the GSH molecule of monomer A. These interactions are similar to those observed in the crystal structure of the holoform of hGrx2 (25).

**DISCUSSION**

*GrxC5 Is the Fourth Plastidial Grx in A. thaliana*—Trxs and Grxs are key players of the chloroplast redox network. Although considerable knowledge has been accumulated on chloroplastic Trx isoforms (53), the investigation of the roles of chloroplastic Grxs still lags behind. Hence, the characterization of AtGrxC5 is part of a more comprehensive work aimed at deciphering whether Grxs have specialized or redundant functions in plants and particularly in plastids.

The phylogenetic analysis of the GrxC5/S12 subgroup indicates that this subgroup is specific to land plants and that the GrxC5 isoforms are restricted to a specific plant family, the Brassicaceae, likely indicating that a single gene has been duplicated in the last common ancestor of this family. The fact that both genes have been retained suggested that they have developed specialized functions. At the transcript level, although AtGrxS12 shows slightly more expression than AtGrxC5 in rosettes, the expression of these two genes is relatively unaffected in *A. thaliana* leaves subjected to environmental constraints. Thus, despite having similar subcellular localization and transcript expression pattern, the replacement of a single amino acid in the AtGrxC5 active site cysteine conferred it specific properties.

Based on these results and on the previous characterization of GrxS12, GrxS14, and GrxS16, a model summarizing their mode of reduction and possible functions in the plastid has been established (Fig. 8). Importantly, GrxC5, GrxS14, and GrxS16 could exist as both apo- and holoforms, which exhibit different functionalities. From the literature on plant Grxs, it is likely that all proteins, except GrxS16, possess the capacity to deglutathionylate protein substrates, making them potential central components regulating many plastidial metabolic pathways as several glutathionylated proteins have been identified in chloroplasts (6, 54, 55). However, this reaction is likely to be preferentially carried out by class I Grxs as only GrxC5 and -S12 have the capacity to efficiently regenerate target proteins, which uses only one cysteine for catalysis, such as PrxIIE and MsrB1 (14, 48). Another major difference is that the reductase activity of GrxC5 and -S12 is dependent on GSH, whereas by analogy to the work achieved with *C. reinhardtii* Grx3, GrxS14 and presumably GrxS16 may be reduced by ferredoxin-thioredoxin reductase but not glutathione (6).

Regarding the role of the holoforms, it has been suggested from experimental data or by analogy to results obtained in yeast that class II Grxs (GrxS14 and -S16), which incorporate air-labile and transferable clusters, play a role in Fe-S cluster assembly machineries or in iron sensing (56). On the contrary, AtGrxC5, which binds a more stable Fe-S cluster that cannot be transferred for example to an apoferredoxin (data not shown), should play a different role. As initially suggested for human Grx2, it could act as a redox sensor, responding to oxidative conditions (23).

**Minimum Requirement for Fe-S Cluster Assembly in Grxs**—The discovery that AtGrxC5 can assemble an Fe-S cluster was initially unexpected. Our results demonstrate that, among the two additional candidate cysteines present in AtGrxC5 compared with PtGrxS12, Cys32 determines the ability of GrxC5 to incorporate an Fe-S cluster and also influences protein activity, whereas Cys80 has no impact on both properties. Previous characterization of PtGrxS12 led us to conclude that the presence of a Trp residue in the active site signature prevented the incorporation of an Fe-S cluster as its replacement by a Tyr residue was sufficient to allow Fe-S center assembly (27). The present mutagenesis experiments conducted on both paralogs and their structural interpretation pointed to the importance of Cys32, which is indispensable for AtGrxC5 to integrate a cluster, despite the presence of the *a priori* unfavorable Trp28. Interestingly, the comparison of the crystallographic structures of PtGrxS12 and GrxC5_apo with GrxC5_holo showed that Trp28 adopts a different conformation in GrxC5_holo with the hydrophobic part of its side chain being strikingly solvent-exposed. The beneficial effect of Cys32 on Fe-S cluster incorporation is likely attributed to its ability to form a hydrogen bond with the main chain of Lys62 (d(S–HN) = 3.46 Å where d is the bond distance), which would stabilize the loop containing residues 26–32 essential for Fe-S cluster binding. A serine at position 32 adopting the same conformation as Cys32 could not stabilize this loop because d(O–HN) would exceed 3.5 Å. This observation is consistent with the results obtained for the PtGrxC1 C34S mutant (*10*YCGYS*14* active site) that incorporates a much more labile cluster than the WT. In this case, however, we believe that the mutant kept the ability to integrate a cluster due to the presence of Tyr30, which participates in GSH stabilization.

From all these observations and the work published to date on other Fe-S-containing Grxs, it seems that, in addition to the
catalytic cysteine that is absolutely required for the [2Fe-2S] incorporation, there are at least three crucial residues at positions \(-1, +1, +3\) of the Cys, but they are not necessarily of the same weight (supplemental Table 3). Usually, when two residues at any of these positions have a positive effect, the protein can assemble a cluster. This is exemplified by this study where the stabilizing role of the second active site Cys in AtGrxC5, compared with PtGrxS12, could compensate for the \(a\ priori\) unfavorable effect of the Trp, which has to change its conformation without really participating in the cluster or GSH stabilization or in the dimerization.

Nevertheless, the recently identified trypanosome Grx possessing a TCPYC active site is able to incorporate an Fe-S cluster, whereas previous mutagenesis studies had suggested that the presence of the proline prevented Fe-S cluster assembly in plant and human Grxs with a YCPYC active site sequence. This indicates that an additional factor should be considered (8, 57, 58).

FIGURE 8. **Confirmed or potential roles for plastidial Grxs.** Grxs of class I are depicted in white, and those of class II are in gray. When the reduction pathway is not clearly demonstrated, broken lines are used. GrxC5, GrxS14, and GrxS16 possibly oscillate between an apomonomer and a holodimer state binding an Fe-S cluster. Fdx, ferredoxin; FNR, ferredoxin:NADPH reductase; FTR, ferredoxin-thioredoxin reductase; GR, glutathione reductase.

**Role of Second Active Site Cysteines in Dithiol Grxs**—Besides its importance for Fe-S cluster assembly in some Grxs, the role of the second active site Cys residue in the catalytic mechanism of dithiol Grxs remains an important open question. Its mutation affects Grx activity. However, whereas an increase in activity was observed for some yeast and mammalian dithiol Grxs (e.g. yeast Grx1, human Grx1 and Grx2, and pig Grx) mutated on this second active site cysteine, other studies revealed a decrease in activity as in the case of yeast Grx2 and \(E. coli\) Grx1 and Grx3 (5, 18, 19, 50, 52, 59–62). The difference observed between yeast Grx1 and Grx2 has been attributed, at least partially, to the nature of two residues (59). The replacement in yeast Grx2 of Ser by Ala at position 23 and of Gln by Glu at position 52 was proposed to stabilize the glutathionylated form compared with yeast Grx1. However, the absence of conservation of these two residues in other studied proteins prevents the generalization of this hypothesis.

Another recent analysis conducted with several dithiol Grxs has demonstrated that the second active site cysteine determined the glutathione specificity of the second step of peptide deglutathionylation (i.e. the reduction of a glutathionylated Grx by a second GSH molecule) in \(E. coli\) Grx1 but not in human and yeast Grx1 (61). Alternatively, the formation of an intramolecular disulfide bond between the two active site cysteines could constitute a sort of side reaction, which might serve as a protective mechanism for Grx thiol groups in some conditions, although it would certainly at the same time decrease the catalytic efficiency by slowing down Grx regeneration and thus its specific activity. However, this explanation is consistent only for yeast Grx1 and mammalian Grxs but not for yeast Grx2 and \(E. coli\) Grx1 and Grx3, for which, by extrapolation of the work achieved with \(E. coli\) Grx1, one can assume that the decrease in
activity observed is correlated to a change in glutathione recognition, binding, or affinity.

Our mutagenesis results and activity measurements clearly demonstrate that AtGrxC5 falls in the same group as mammalian Grxs and yeast Grx1, which might indicate that the second active site cysteine is not involved in the specificity of the second step of the deglutathionylation reaction either (this has yet to be experimentally confirmed) but that an intramolecular disulfide can be formed and can decrease protein reactivity. Contrary to this expectation, we were not able to find conditions allowing the formation of such an intramolecular disulfide. Instead, we found that both Cys29 and Cys87 can be stably, at least partially, glutathionylated. This is for instance very similar to PtGrxS12, a monothiol Grx, where Cys29 was shown to be glutathionylated and Cys87 was bound to a β-mercaptoethanol molecule resulting from an HED pretreatment (27). Overall, this indicates that AtGrxC5 likely functions through a monothiol mechanism involving only Cys29, but it does not clarify the role of Cys32.

A comparison between structures of reduced and glutathionylated forms of PtGrxC1 and human Grx2 (although GSH is not covalently linked in this case), which are both dithiol Grxs, shows that thiol groups of the active site cysteines do not adopt the same conformers in both forms (8, 24, 25). Whereas the thiol mechanism involving only Cys29, but it does not clarify the specificity of the second step of the deglutathionylation reaction either (this has yet to be experimentally confirmed) but that an intramolecular disulfide can be formed and can decrease protein reactivity. Contrary to this expectation, we were not able to find conditions allowing the formation of such an intramolecular disulfide. Instead, we found that both Cys29 and Cys87 can be stably, at least partially, glutathionylated. This is for instance very similar to PtGrxS12, a monothiol Grx, where Cys29 was shown to be glutathionylated and Cys87 was bound to a β-mercaptoethanol molecule resulting from an HED pretreatment (27). Overall, this indicates that AtGrxC5 likely functions through a monothiol mechanism involving only Cys29, but it does not clarify the role of Cys32.

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Arabidopsis Chloroplastic Glutaredoxin C5 as a Model to Explore Molecular Determinants for Iron-Sulfur Cluster Binding into Glutaredoxins

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