The hydrogen peroxide-sensitive proteome of the chloroplast in vitro and in vivo

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INTRODUCTION

Chloroplasts are essential organelles in plant cells with a wide range of metabolic functions. The redox cascades of the light-driven photosynthetic electron transport chain provide the driving force for metabolism, but they also conditionally generate oxidizing power in the form of reactive oxygen species (ROS). ROS are produced as by-products of oxygen metabolism, which can be harmful but also useful. ROS are essential for plant physiological processes such as stress response, developmental regulation, and programmed cell death. They are formed from molecular oxygen during cellular metabolism and accumulate under various stresses causing serious redox imbalances. Many proteomics studies aiming to identify proteins sensitive to H2O2 used concentrations that were above the physiological range. Here the chloroplast proteins were subjected to partial oxidation by exogenous addition of H2O2 equivalent to 10% of available protein thiols which allowed for the identification of the primary targets of oxidation. The chosen redox proteomic approach employed differential labeling of non-oxidized and oxidized thiols using sequential alkylation with N-ethylmaleimide and biotin maleimide. The resulting oxidation targets were analyzed using 2-DE and MALDI-TOF mass spectrometry for the identification of the primary targets of oxidation. In conclusion, the presented approach enabled the identification of early targets of H2O2 oxidation within the cellular proteome under physiological experimental conditions.

Keywords: chloroplast proteome, hydrogen peroxide, methyl viologen, ribulose-bisphosphate carboxylase, redox regulation

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Hydrogen peroxide (H2O2) evolves during cellular metabolism and accumulates with environmental changes (Scheibe and Dietz, 2012). Cysteine (Cys) residues in proteins harbor thiol side chains that are highly reactive toward oxidants and can undergo various redox-based modifications. The oxidation of sensitive Cys may cause intra- or intermolecular disulfides. The conformational changes often regulate the activity and protect the critical thiols against irreversible oxidation (Brandes et al., 2009; König et al., 2012). Reactive thiols can form higher oxidation states like sulfenic acid (SOH) and sulfinic acid (SO2H), which are reversed by thiol-specific cellular reductants like glutathione, thioredoxin, or sulfiredoxin (Liu et al., 2006). Hyperoxidation describes two forms of Cys oxidation, namely the SO2H and SO3H states, the latter one being irreversible to our present-day knowledge. Cys can also form mixed-disulfides which enhances the plant tolerance to environmental stresses.

Because of their central role in plant cell signaling, chloroplasts are also considered to function as sensors of environmental fluctuations. According to this scenario, the redox status of chloroplasts is crucial in biological stress response and helps the plant to cope with environmental changes (Scheibe and Dietz, 2012). Cysteine (Cys) residues in proteins harbor thiol side chains that are highly reactive toward oxidants and can undergo various redox-based modifications. The oxidation of sensitive Cys may cause intra- or intermolecular disulfides. The conformational changes often regulate the activity and protect the critical thiols against irreversible oxidation (Brandes et al., 2009; König et al., 2012). Reactive thiols can form higher oxidation states like sulfenic acid (SOH) and sulfinic acid (SO2H), which are reversed by thiol-specific cellular reductants like glutathione, thioredoxin, or sulfiredoxin (Liu et al., 2006). Hyperoxidation describes two forms of Cys oxidation, namely the SO2H and sulfinic acid (SO2H) states, the latter one being irreversible to our present-day knowledge. Cys can also form mixed-disulfides which enhances the plant tolerance to environmental stresses.
with glutathione (glutathionylation) or is S-nitrosylated. Both modifications receive increasing attention as important redox regulatory mechanism in biology (Meyr and Chock, 2012). Through these different post-translational modifications Cys appears to be involved in virtually all cellular activities, including immediate metabolic regulation and control of transcriptional and translational activities in development and defense.

Analytical methods have been developed to detect reversible thiol oxidation and they employ combinations of labeling and blocking strategies. The challenge is to identify a few oxidized disulphides among the mass of reduced thiols of a healthy cell. Generally this method includes a saturating blockage of free thiols with thiol reactive reagents, followed by reduction of the disulfides (Muthuramalingam et al., 2010). Subsequently, the newly exposed thiol groups from the reduction step are labeled with detectable thiol-specific reagents. Depending on the specificty of the thiol reductant, this method can be used to identify all reversible thiol modifications (Leichert and Jakob, 2004). Novel labeling techniques based on isotope-coded affinity tags (ICAT) enable quantification of differential protein expression (Bishtaraman et al., 2004). The combination of labeling strategies and advanced proteomic methodologies led to the identification of redox proteins which are regulated by thiol-disulfide transitions (Motohashi et al., 2001; Buchanan and Balmer, 2005; Bousher et al., 2005; Bartsch et al., 2008; Stroher and Dietz, 2008). Often these studies employ rather extreme oxidizing condition. Thus a major open issue concerns the question as to which of the redox-regulated proteins are the primary targets of oxidation and how an initial redox imbalance is sensed in the cells.

Hydrogen peroxide \((\text{H}_2\text{O}_2)\) is a by-product of normal metabolism and has a sufficient half-life to allow its spreading throughout the entire cell (Bhattachrjee, 2005). \(\text{H}_2\text{O}_2\) is involved in a number of signaling cascades (Neill et al., 2002) and also in programmed cell death in plants (Levine et al., 1994). A recent study has shown that aquaporins facilitate the movement of \(\text{H}_2\text{O}_2\) across the membrane (Bienert et al., 2007). As thiols play major roles in ROS-mediated signaling pathways, identification of thiols that are most sensitive to \(\text{H}_2\text{O}_2\) will help to understand the redox-signaling pathways. Several proteomics studies have addressed the effects of \(\text{H}_2\text{O}_2\) treatment of seedlings, roots and shoot on proteome composition and carbonylation state of proteins (Tanou et al., 2010; Barba-Espin et al., 2011; Zhou et al., 2011). These studies provide important insight into non-redox effects of \(\text{H}_2\text{O}_2\) stress and downstream events of \(\text{H}_2\text{O}_2\)-dependent signaling in plants.

In this context, the present study focuses on the identification of chloroplast stroma proteins which are most sensitive to \(\text{H}_2\text{O}_2\). Arabidopsis thaliana stroma proteins were subjected to partial oxidation by exogenous addition of limited amounts of \(\text{H}_2\text{O}_2\) in order to observe the global response of the chloroplast redox network to an oxidizing stimulus. Initial targets of oxidation were identified by mass spectrometry (MS). In order to confirm the response of identified proteins to oxidation in vivo, proteins were subjected to methyl viologen (MV) treatment. MV is a redox-active herbicide that accepts electrons at the photosystem I site and produces superoxide \((\text{O}_2^-)\). The resuspended chloroplasts were loaded on top of a Percoll step gradient consisting of layers with 40 and 80% Percoll medium containing 0.02 g Ficoll and 0.1 g FEG. The gradient was centrifuged at 3000 rpm for 30 min without brakes. Intact chloroplasts were collected from the interphase between the Percoll layers and washed twice by spinning at 3000 rpm for 2 min. The stroma proteins were extracted following lysis and Rhodamine was partially removed according to Stroher and Dietz (2008). The purity of stromal protein preparation was verified by using organelle specific enzymatic and antibody assays (Figure 1). The cytosolic marker enzyme UDP-glucose pyrophosphorylase (UGPase) activity was measured according to Zrenner et al. (1993). Using UDP-glucose and pyrophosphate as substrates, Glc-1-P was converted to glucose-6-phosphate (Glc-6-P) which was quantified by coupling to NADP+- reduction by Glc-6-P dehydrogenase. Mitochondrial type II peroxiredoxin F (AtPrxII F) was used as a marker for mitochondrial contaminations. Equal amounts of total plant and stromal proteins (25 μg) were loaded and resolved on SDS-PAGE gels. Western blot analysis with antibodies raised against heterologous expressed AtPrxII F was performed as described in Finkemeier et al. (2005).

DTNB-based QUANTIFICATION OF THIOL GROUPS
Total sulfhydryl contents of stroma proteins were determined as described by Tietze (1969). Proteins were precipitated in 3% trichloroacetic acid (TCA) and recovered after a brief centrifugation. To expose buried thiol groups the resulting pellet was dissolved in denaturing buffer containing 100 mM Tris-HCl (pH 8.0), 6 M guanidinium HCl or 1% SDS. The free thiol groups were quantified spectrophotometrically at 412 nm using 6 mM 5,5-dithiobis(2-nitrobenzoic acid, DTNB) as substrate.

SAMPLE PREPARATION (IN VITRO AND IN VIVO OXIDATION TREATMENT)
Partial oxidation in vitro was performed in 20 mM Tris-HCl (pH 7.8) buffer by adding \(\text{H}_2\text{O}_2\) in varying stoichiometric quantities equivalent to 1, 2.5, 5, and 10% of the protein thiol content.
All buffers were depleted from dissolved O$_2$ by bubbling with nitrogen gas to maintain the oxygen content at less than 0.4%. Excess DTT was removed by repeated TCA precipitation. The labeling of the reductively purified proteins was achieved with 25 mM biotin maleimide in the dark for 90 min under constant shaking. Excess labeling reagent was removed either by TCA precipitation or with PD-10 desalting columns (GE Healthcare) depending on the downstream processing.

To monitor the in vivo redox status of proteins, proteins from MV-treated plants were extracted in the presence of 100 mM NEM. Further, the proteins were reduced with DTT and subsequently labeled with biotin maleimide as described above. Biotinylated proteins were separated either by one- or two-dimensional gel electrophoresis (2DE) and subsequently transferred onto nitrocellulose membrane using the semidyblot Fastblot B4 (Whatman/Biometra, Germany). After a blocking step with 1% fish gelatin (Sigma, Germany) for 2 h at RT, the membrane was probed with anti-biotin antibody (Clone BN-34 from Sigma–Aldrich, St. Louis, USA). Then the membrane was incubated for 1 h with horseradish peroxidase-conjugated anti-mouse antibody (Sigma–Aldrich, St. Louis, USA) and developed using the enhanced chemiluminescence method (Thermo Scientific, Germany).

**DETECTION OF REDOX-REGULATED PROTEINS VIA A SEQUENTIAL LABELING STRATEGY**

All buffers were depleted from dissolved O$_2$ by bubbling with argon gas at room temperature (RT). The stroma protein fraction was completely reduced in the presence of 25 mM dithiothreitol (DTT). The reaction was performed inside a closed microaerobic chamber continuously flushed with nitrogen gas produced in a nitrogen generator to maintain the oxygen content at less than 0.4%. Excess DTT was removed by desalting using 10 ml desalting columns. The proteins in 20 mM Tris–HCl (pH 7.8) buffer with about 100 μM thiols were treated with H$_2$O$_2$ (about 10 μM) for 5 min with gentle shaking to identify the initial targets of oxidation. Remaining cysteinyi thiols were alkylated using 100 mM N-ethylmaleimide (NEM) in darkness for 1 h to prevent oxidation. Excess NEM was removed by TCA precipitation according to Muthuramalingam et al. (2010). The washed precipitate was solubilized in denaturing buffer containing 200 mM Bis–Tris (pH 6.5), 6 M urea, 0.3% (w/v) SDS and 10 mM EDTA supplemented with 100 mM DTT to allow full reduction of oxidized thiols. Excess DTT was removed by repeated TCA precipitation. The labeling of the reductively purified proteins was achieved with 25 mM biotin maleimide in the dark for 90 min under constant shaking. Excess labeling reagent was removed either by TCA precipitation or with PD-10 desalting columns (GE Healthcare) depending on the downstream processing.

**STREPTAVIDIN AFFINITY PURIFICATION**

The protein samples were desalted to remove labeling solution. This was needed since denaturing reagents used for solubilizing TCA-precipitated protein pellets inhibited binding of biotinylated polypeptides to the streptavidin column. Biotinylated proteins were enriched using streptavidin agarose. The biotinylated sample was incubated with streptavidin agarose equilibrated with phosphate buffered saline (PBS) buffer, pH 7.4, at 4°C with constant shaking overnight. Nonspecifically bound proteins were washed with 1× PBS until the absorbance at 280 nm reached zero. Proteins were incubated with elution buffer containing 1% SDS, 30 mM biotin (pH 12) for 15 min at RT, followed by heating at 96°C for 15 min.

**IDENTIFICATION OF PROTEINS USING MALDI MS ANALYSIS**

Proteins purified by streptavidin agarose were resolved by one-dimensional SDS-PAGE and stained with silver nitrate according to Blum et al. (1987). Spots of interest were excised from the gel and placed in U-shaped microtiter plate wells (Greiner Bio-one, Germany). To remove the silver, the excised gel spots were de-stained using Farmer’s reducing reagent containing 30 mM potassium ferricyanide (III) and 100 mM sodium thiosulfate. Then the gel spots were washed several times with ultrapure H$_2$O$_2$ until the gel slices became transparent. Protein spots were washed twice with 30% (v/v) acetonitrile in 0.1 M ammonium hydrogen carbonate and subsequently dried in the speedvac. The gel slices were rehydrated in the presence of 0.01 μg trypsin/μl (Promega, Mannheim, Germany) at RT for 30 min followed by overnight incubation at 37°C according to manufacturer’s protocol. The gel spots were collected and digested using the above protocol. The resulting tryptic peptides were analyzed by MALDI MS/MS. The MS spectra were acquired using a Bruker Daltonics microflex MALDI mass spectrometer with a nitrogen laser, operated in linear mode. The laser wavelength was 337 nm, and the laser energy was set at 3000 mJ/cm$^2$. The mass range was set from 500 to 3000 Da. The spectra were searched against the Swiss-Prot database using the software Mascot (Matrix Science, UK) with the following parameters: mass tolerance of 100 ppm, maximum missed cleavages of 1, and tryptic cleavage with trypsin specificity.

**FIGURE 1** Estimation of cross-contamination of purified chloroplast fraction. (A) Western blot analysis of stroma and plant extract proteins using specific antibody against AtPrxII F (Finkemeier et al., 2005). Equal amounts of protein (25 μg) were loaded in each lane. AtPrxII F was detected as monomer around 21 kDa and was only found in total leaf extract. (B) Activity of UGPase as cytosolic marker enzyme, represented as rate of NADPH$^+$H$^+$ formation in plant extract and stroma fraction. The assay was performed three times. Data express means ± SD.
slices were vacuum dried, and the peptides were extracted with 50% acetonitrile and 0.1% trifluoroacetic acid for MS analysis. Acquisition of peptide mass fingerprint data and corresponding LIFT spectra was performed using an ultrafleXtreme matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) device (Bruker Daltonics, Bremen, Germany) equipped with a Smartbeam-II laser with a repetition rate of 1000 Hz. The spectra were calibrated using external calibration and subsequent internal mass correction. For databank searching, Biotools 3.2 software (Bruker Daltonics) with the implemented MASCOFT search engine (Matrix Science) was used, searching for A. thaliana in the non-redundant National Center for Biotechnology Information database (26/07/2010, 56802 sequences). Search parameters were as follows: monoisotopic mass accuracy; 50 ppm tolerance; fragment tolerance of 0.3 Da; missed cleavages 1; and the allowed variable modifications were oxidation (Met), propionamide (Cys), and carbamidomethyl (Cys). Proteins were identified from all three independent experiments applying MASCOFT significance scores of 60 (protein level) and 32 (peptide level). Proteins found in just one of the experiments are indicated in table legends (Tables 1–3).

BIOTIN QUANTIFICATION ASSAY

The extent of biotinylation was quantified using the HABA-avidin assay developed by Green (1975). HABA forms a red complex with avidin that can be monitored spectrophotometrically at 500 nm. Due to its higher affinity, biotin displaces HABA, accompanied by the decrease in absorbance at 500 nm. The biotinylated protein samples were desalted to remove the excess of biotin maleimide reagent before performing the assay. The assay mixture consisted of 1× PBS buffer containing HABA-avidin reagent (Sigma, Germany) and 10 μg of biotinylated protein sample. After 2 min incubation, the absorbance at 500 nm was recorded. The change in absorbance at 500 nm is proportional to the amount of biotin in the assay. A standard curve was generated using free biotin and used to estimate the number of moles of biotin incorporated after biotinylating the protein.

RESULTS

PURITY OF THE CHLOROPLAST FRACTION

Chloroplasts were isolated and lysed to obtain a stromal protein fraction which was checked for contaminations by other cellular constituents. Type II peroxiredoxin F (AtPrxII F) was used as a marker for mitochondrial contamination using Western blot analysis. In total plant protein extract AtPrxII F was detected at the expected size of about 21 kDa, while it was absent in the stromal fraction (Figure 1A). As shown in Figure 1B the plant protein extract exhibited high rates of nicotinamide adenine dinucleotide phosphate (NADPH) formation at 340 nm, representative for high UGPase activity, while its enzymatic activity in the stromal fraction was minimal with less than 6% relative contamination.

TOTAL PROTEIN THIOL DETERMINATION

The present study aimed to identify the primary protein targets of H2O2 oxidation in the chloroplast in vitro and in vivo. Percoll-purified intact chloroplasts were lysed, and the stroma

| No. | Protein | Accession number | MM (kDa) | Cys number | Functional role |
|-----|---------|-----------------|----------|------------|----------------|
| 1   | Fd-GOGAT | AT5G04140       | 165.3    | 24         | Nitrogen assimilation |
| 2   | Transketolase | AT3G60750   | 73.1     | 8          | Photosynthesis |
| 3   | Rubisco large subunit | ATCC00490 | 52.9     | 9          | Photosynthesis |
| 4   | Fructose-bisphosphate aldolase-2 | AT4G38970 | 37.9     | 2          | Photosynthesis |
| 5   | GAPDH subunit B | AT1G42970 | 39.9     | 7          | Photosynthesis |
| 6   | GAPDH subunit A-2 | AT1G28900 | 377.5    | 5          | Photosynthesis |
| 7   | Ferredoxin-NADP+ oxidoreductase 1 | AT5G66180 | 35.2     | 5          | Photosynthesis |
| 8   | Carbonic anhydrase 1 | AT3G15050 | 25.6     | 6          | Photosynthesis |
| 9   | 2-Cys peroxiredoxin | AT3G1630 | 22.4     | 2          | Detoxification |
| 10  | Cyclophilin 20-D | AT3G62030 | 19.9     | 4          | Protein folding |
| 11  | Rubisco small subunit | AT1G67090 | 14.7     | 4          | Photosynthesis |
| 12* | Phosphoribulokinase | AT1G20660 | 39.2     | 4          | Photosynthesis |
| 13* | STN7 kinase | AT1G88830 | 58.5     | 5          | Serine/threonine kinase |
| 14* | Glutamine synthetase 2 | AT5G35630 | 42.5     | 6          | Ammonia assimilation cycle |
| 15* | ATP synthase beta subunit | ATC000480 | 52.5     | 1          | ATP synthase |
| 16* | HCF 136 | ATG523120 | 35.8     | 0          | PSII stability complex |
| 17* | DRT 112 | AT1G23430 | 10.5     | 1          | Electron carrier |

Each protein is annotated by its name, accession number, molecular weight (without predicted transit peptide), and the biological function. The number of cysteines theoretically present in the mature protein is given. The table compiles the results from three independent experiments. Proteins 1–11 were identified in each experiment. Asterisk ‘*’ denotes that these proteins were identified in single experiments where 10% molar equivalence to protein thiols corresponded to 15 μM H2O2 concentration.
Table 2 | Identification of cysteines modified upon oxidation.

| Protein identified          | Peptide sequence                  | Predicted mass | Observed mass in H$_2$O$_2$ treated |
|----------------------------|-----------------------------------|----------------|-------------------------------------|
|                            |                                   | NEM            | Biotin-mal                          |
| Rubisco large subunit      | AVYEC*LR                          | 978            | 1304.5                              |
|                            | VALEAC*VQAR                       | 1184.2         | 1510.7                              |
| Fd-GOGAT                   | IC*NQDR                           | 843.8          | 1170.3                              |
|                            | FCGGMLSAKR                        | 1424.5         | 1751                                |
|                            | ALYLYEADAQVR                      | 1697.0         | 2024.2                              |
|                            | GQVPEATVGCTLYQGATGQFA             | 2910.1         | 3234.2                              |
| Femtoxidih-NADP+ reductase 1| C*LLINTK                          | 815.9          | 1142.4                              |
|                            | TVSLCKV                           | 873.9          | 1200.4                              |
| GAPDH subunit B            | GIVC*NFCLDLK                      | 1304.5         | 1631                                |
| Rubisco small subunit      | LPLFCCDADULK                      | 1616.6         | 1943.3                              |
|                            | OVC*ISAYKPPSFTSG                  | 2011.2         | 2337.7                              |
|                            | WIPC*VFEFLQGFGYR                  | 2149.3         | 2475.8                              |

Each entry indicates the peptide sequence and the predicted mass in the presence of the alkylating agents either NEM (±1 Da) or biotin maleimide (±45.1 Da). The mass observed in MALDI-TOF/TOFS of the H$_2$O$_2$-treated sample is shown. Mass of NEM-labeled Cys matches to experimental mass value suggesting that these Cys are not modified during oxidation. C* marks Cys in the peptide sequence which is preferentially labeled with biotin maleimide, since the predicted mass matches to the measured value. Here, the predicted mass of biotin maleimide-labeled peptide was not found in the corresponding control sample. These data suggest that the approach used can identify proteins sensitive to oxidation and in addition localizes the Cys modification.

Protein fraction was recovered by centrifugation. Total thiol contents of stroma protein extract was determined using the DTNB assay in order to adjust the amount of H$_2$O$_2$ to be added for oxidation to 10% of total protein thiols. Protein thiols were quantified under reducing and denaturing conditions to obtain an average amount of thiols to be used as a conversion factor for future experiments. Low molecular weight thiols metabolites such as glutathione were removed by TCA precipitation, followed by a centrifugation. Both denaturing methods, namely guanidinium hydrochloride- and SDS-treatment gave a highly similar result of an average amount of thiols to be used as a conversion factor for future experiments. Under optimal conditions for photosynthesis, H$_2$O$_2$ concentrations are considered to be below 1 μM. H$_2$O$_2$ accumulates under stress.

Effect of H$_2$O$_2$-mediated oxidation on stroma thiol proteins

Stroma proteins were subjected to H$_2$O$_2$ oxidation, subsequently reduced and labeled with biotin maleimide. Oxidation was performed by adding H$_2$O$_2$ at amounts of varying stoichiometry relative to protein thiol contents (1, 2.5, 5, and 10%) which corresponded to 1 to 10 μM concentration. Under optimal conditions for photosynthesis, H$_2$O$_2$ concentrations are considered to be below 1 μM. H$_2$O$_2$ accumulates under stress. 10 μM H$_2$O$_2$ inhibits the Calvin cycle in isolated chloroplast by half (Kaiser, 1976; Asada, 1999; Polle, 2001). Remaining free thiols were blocked with NEM followed by reduction of reversibly oxidized proteins with DTT. The newly recovered thiol groups were then labeled with biotin maleimide. Hence biotinylated proteins corresponded to Cys-containing proteins that had been reversibly oxidized by the added H$_2$O$_2$. The increase in biotin label corresponding to increased oxidation is shown in Figure 3A. In the control reaction, the proteins were reduced and directly blocked with NEM without exposure to H$_2$O$_2$. Complete reduction and immediate blocking of free thiols in the control sample resulted in only minor incorporation of biotin maleimide into proteins (Figure 3A; lane 1). The labeling degree increased with increasing H$_2$O$_2$ concentrations.

The amount of biotin maleimide in the labeled samples was quantified with the HABA-avidin assay. Equal amounts of protein from different H$_2$O$_2$-treated and control samples were mixed with HABA-avidin reagent. The assay displayed a decrease in the absorbance that is proportional to the amount of biotin maleimide present in the sample. The degree of biotinylation was calculated as
### Table 3 | Oxidation-susceptible proteins in *A. thaliana* treated with methyl viologen, subjected to differential labeling and identified by MS.

| No. | Protein name | Accession number | Molecular weight (kDa) | Number of Cys | Localization | Function |
|-----|--------------|------------------|------------------------|---------------|-------------|----------|
| 1   | Rubisco large subunit | ATCG00490 | 52.9 | 9 | Chloroplast | Photosynthesis |
| 2   | Myrosinase | AT5G26000 | 61.1 | 9 | Vacuole | Defense |
| 3   | Chaperonin 60 subunit alpha 1 | AT2G28000 | 62.1 | 3 | Chloroplast | Chaperonin |
| 4   | Fructose-bisphosphate aldolase-2 | AT4G38790 | 43.0 | 2 | Chloroplast | Photosynthesis |
| 5   | 2-Cys peroxiredoxin | AT3G11630 | 29.1 | 2 | Chloroplast | Detoxification |
| 6   | Sedoheptulose-1,7-bisphosphatase | AT3G55605 | 42.4 | 7 | Chloroplast | Photosynthesis |
| 7   | Annexin D1 | AT1G35700 | 36.2 | 2 | Cytoplasm/Membranes | Detoxification |
| 8   | PS II oxygen-evolving complex 1 (PsbO-1) | AT5G66570 | 35.1 | 4 | Chloroplast | Photosynthesis |
| 9   | PS II oxygen-evolving complex 23K protein (PsbP-1) | AT1G06680 | 26.1 | 3 | Chloroplast | Photosynthesis |
| 10  | Glutathione S-transferase F2 (GSTF2) | AT4G22520 | 24.1 | 4 | Chloroplast | Photosynthesis |
| 11  | Glutathione S-transferase TAu19 (AOSST19) | AT1G78390 | 25.7 | 1 | Cytoplasm | Detoxification |
| 12  | Glutathione S-transferase TAuv20 (AOSST20) | AT1G78730 | 25.0 | 2 | Cytoplasm | Detoxification |
| 13  | Putative plastocyanin (DRT112) | AT1G20340 | 170 | 1 | Chloroplast | Photosynthesis |
| 14  | PS II oxygen-evolving enhancer (PSBQ-2) | AT4G01580 | 24.6 | 0 | Chloroplast | Photosynthesis |
| 15  | PS II oxygen-evolving enhancer (PSBQ-1) | AT4G21280 | 23.9 | 0 | Chloroplast | Photosynthesis |
| 16  | Rubisco small subunit | AT1G21700 | 20.2 | 5 | Chloroplast | Photosynthesis |
| 17  | Thioredoxin M1 | AT1G02680 | 19.7 | 4 | Chloroplast | Photosynthesis |
| 18  | Putative peroxisomal IS-2-hydroxy-acid oxidase 2 | AT3G14420 | 40.3 | 1 | Peroxisome | Oxidoreductase |
| 19  | NAADP-binding Rossmann-fold-containing protein | AT2G37660 | 34.9 | 2 | Chloroplast | Oxidoreductase |
| 20  | Glutathione S-transferase DHAR1 | AT1G19570 | 23.6 | 2 | Unclear | Detoxification |
| 21  | Ferredoxin-NADP(+) oxidoreductase 1 (FRN1) | AT5G66190 | 40.3 | 6 | Chloroplast | Photosynthesis |
| 22  | Kinesin-5 | AT4G05190 | 89.2 | 11 | Cytoplasm | Microtubule motor |
| 23  | Nucleoside diphosphate kinase 2 | AT5G66720 | 25.6 | 5 | Chloroplats | Metabolism |
| 24  | Unknown protein (partial) | AT4G21280 | 7.0 | 4 | Probable peroxin | |

Each protein is annotated by its name, accession number, molecular weight, subcellular localization, and biological function as given in the plant protein database (PPDB). The number of cysteines theoretically present in the mature protein is given. The table compiles the results from three independent experiments. Proteins 3, 7, 12, 18, 20, 22, 23, and 24 were significantly identified in one experiment only.

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micromol biotin-labeled thiol per gram protein. As expected the increasing amounts of H$_2$O$_2$ and subsequent reduction allowed the incorporation of more biotin, representing the extent of thiol oxidations (Figure 3B).

Two-dimensional gel electrophoresis was performed in order to get insight into the complexity of the H$_2$O$_2$-mediated oxidative changes. One hundred micrograms of biotin maleimide-labeled control and H$_2$O$_2$-treated proteins were separated by 2DE and subsequently visualized by silver staining (Figure 4A). The corresponding Western blot membranes probed with anti-biotin antibody are shown in Figure 4B. The patterns of biotinylated polypeptides differed strongly between control and treated samples, while the patterns from the silver-stained gel detecting total proteins revealed a similar spot pattern despite the fact that apparently slightly more protein had been solubilized in the H$_2$O$_2$-treated sample. The direct comparison of the blots and the silver-stained gels for identifying and excising the proteins of interest appeared unreliable due to the expected background of unlabeled polypeptides. Therefore the biotinylated proteins were further enriched by purification via streptavidin agarose column chromatography.

**Purification and Identification of Biotinylated Proteins Following H$_2$O$_2$-Mediated Oxidation**

Biotin-labeled control and H$_2$O$_2$-treated samples were purified by streptavidin agarose chromatography to separate the H$_2$O$_2$-sensitive biotinylated proteins from the complex protein mixture. Affinity-purified proteins were precipitated with TCA (10% w/v) to remove excess biotin from elution. The protein samples were resolved by one-dimensional SDS-PAGE analysis and visualized by silver staining (Figure 5A). Both control and H$_2$O$_2$-treated proteins exhibited a similar pattern on the gel, which is explained by loading equal protein amounts. However, immuno-reactive signals only appeared on the blot from H$_2$O$_2$-treated samples, confirming that the biotin labeling was linked to protein oxidation by H$_2$O$_2$ (Figure 5B). To identify proteins containing redox sensitive Cys thiols the indicated gel sections were excised from the gel. Results of MALDI-TOF/MS analysis are summarized in
FIGURE 2 | Overview of the procedure for labeling reversibly oxidized cysteines and test experiment with 2-Cys Prx. (A) Workflow used for identifying protein targets sensitive to low H2O2 concentrations consisted of five steps: step 1 includes complete reduction of protein thiols using the artificial reductant DTT. Step 2: After desalting to remove excess DTT, proteins were exposed to H2O2 for 5 min. In step 3, free thiol groups were blocked using NEM under denaturing conditions. Reversibly oxidized thiols were reduced with DTT in step 4. The newly exposed thiol groups were labeled using the thiol specific reagent biotin maleimide in step 5. As a control reaction, proteins were not subjected to oxidation (step 2) while all other steps were done as described above. From steps 1 to 3, the samples were treated inside the microaerobic chamber with <0.4% O2. Subsequent steps were performed in ambient air. (B) Test of experimental workflow with 2-Cys Prx. Each subunit of the 2-Cys Prx dimer has two cysteines. Biotin labeling of reduced 2-Cys Prx (lane 1); oxidized (lane 2); reduced and blocked with NEM (lane 3), and reduced, oxidized and re-reduced sample (lane 4). On the left is the Ponceau S-stained membrane which shows equal protein amounts loaded. The corresponding blot developed with antibody against biotin is shown on the right and is representative for three experiments.

Table 1. In total 17 proteins were identified from affinity purified H2O2-treated samples. All identified proteins are located to the chloroplast. Five proteins function in the Calvin cycle (protein # 2, 5, 6, 11, and 12). The other identified proteins have various functions, such as nitrogen assimilation (proteins #1 and 14), adenosine triphosphate (ATP) synthesis (protein #15) and electron transport (protein #17) among others. The analysed amino acid composition of these proteins revealed that except for the photosystem II (PSII) stability/assembly factor HCF136 (protein #16) one or more Cys are present in all identified proteins. To confirm oxidation-mediated Cys modification in H2O2-treated samples, the mass lists of unmatched peptides were compared with the predicted mass of in silico trypsin-digested and biotinylated peptides (Table 2). This approach allowed us to confirm two peptides of the large subunit (LSU) and small subunit (SSU) of RuBisCO single peptides of ferredoxin-dependent glutamate synthase (Fd-GOGAT), subunit B of GAPDH and ferredoxin-NADP oxidoreductase (FNR).

PURIFICATION AND IDENTIFICATION OF BIOTINYLATED PROTEINS FOLLOWING MV-MEDIATED OXIDATION

To determine whether and which proteins are oxidized in vivo, 6 week old plants were sprayed with MV that induces photo-oxidative stress. The MV treatment of plants revealed a slight increase in biotinylated proteins after different times (Figure 6A). Under oxidizing conditions the catalytic Cys of 2-Cys Prx form an intermolecular disulfide bridge, and it runs as dimer at about 43 kDa, whereas the fully reduced form runs as monomer of...
After 30 min of MV treatment 2-Cys Prx was fully oxidized as control and MV-treated plants. Proteins that were differentially nase, and fructose-bisphosphate aldolase-2 were found both in (protein #20). Some target proteins such as RubisCO, myrosi-tein #2), the peroxisome (protein #18) and the mitochondrion cytoplasm (protein #7, 10, 11, 12, and 22), the vacuole (pro-tein #19) were located to the chloroplast, while some are located to the

**DISCUSSION**

In order to identify stroma protein targets sensitive to oxidation by H$_2$O$_2$, this work adopted a strategy similar to the "biotin switch" method used to detect post-translational S-nitrosylation or glutathionylation (Jaffrey and Snyder, 2001; Lind et al., 2002). The present study relies on differential labeling of reduced thiols and formerly oxidized thiols, using two different alkylation reagents (NEM and biotin maleimide) of distinct molecular mass, which eased the preferential identification of the H$_2$O$_2$-sensitive proteins. Often redox proteomic studies identify redox-sensitive proteins by direct labeling of proteins during cell extraction, possibly leading to Cys oxidation during the lysis and labeling steps causing false positive results. The cross contamination assays indicate that the isolated chloroplast fractions were highly pure since the mitochondrial protein AtPrxII F was below detection limit (Figure 1A) and the cytosolic UGPase constituted less than 6% on a protein basis (Figure 1B). In the in vitro part of this work, the stroma proteins were completely reduced and then oxidized with low amounts of H$_2$O$_2$ to isolate only the most oxidant-sensitive protein thiols. It has been suggested that about 4 μmol H$_2$O$_2$ m$^{-2}$ of leaf area is formed in the chloro-plast during photosynthesis under normal conditions (Foyer and Noctor, 2003). This value corresponds to about 300 μmol H$_2$O$_2$ L$^{-1}$ stroma produced every second, assuming a leaf chlorophyll content of 900 mg m$^{-2}$ and a stroma volume of 40 μL mg$^{-1}$ chlorophyll. The H$_2$O$_2$-detoxification capacity of the chloro-plast ascorbate- and peroxiredoxin-dependent water-water cycles is high, and models predict that resting H$_2$O$_2$ concentrations are low as long as reductants are available (Polle, 2001). Here a low H$_2$O$_2$ concentration of about 10 μM equivalent to 10% of total protein thiols was used to identify primary targets of oxidation and can be considered to represent physiologically relevant conditions. In previous studies much higher concen-trations of oxidants ranging from 1 mM up to 10 mM in non-stoichiometric amounts have been used in plant and animal redox proteomic studies. The results from such studies should be considered with care, since the employed concentrations are
FIGURE 4 | H₂O₂-mediated oxidation of stroma proteins. (A) Two-dimensional map of the A. thaliana stroma proteins after differential labeling. Separation was performed on 18 cm Immobiline DryStrip (pH 3–10 NL) and by 12% SDS-PAGE as second-dimension. Silver-stained gels of stroma proteins (100 μg) that remained untreated (left) and were treated with H₂O₂ at about 10 μM for 5 min (right) are presented. (B) Two-dimensional Western blot map after decoration with anti-biotin antibody. Control and H₂O₂-treated samples of the respective gels are shown. Oxidized protein thiols were labeled with biotin maleimide as described. The figure is representative of three independent experiments. Molecular weight markers, in kDa, are indicated on the left.

often outside the reasonable physiological range (Kim et al., 2000; Baty et al., 2002; Marx et al., 2003; Sethuraman et al., 2004; Winger et al., 2007).

The degree of labeling with biotin-maleimide increased in the H₂O₂-treated sample indicating the occurrence of oxidation-mediated Cys modification in our experiments (Figure 3A). Two-dimensional-immunoblots provided insight into the response of the H₂O₂-mediated oxidative changes. Linking our approach with MALDI-TOF analysis enabled the identification of proteins which are most sensitive to oxidation. Most proteins identified possess oxidation-susceptible Cys and are known to undergo dithiol-disulfide exchange reactions as reported in previous studies (Meyer et al., 2005; Ströher and Dietz, 2008; Lindahl and Kieselbach, 2009). All the identified proteins had functional annotations. Based on their function they can be generally categorized as enzymes involved in carbohydrate metabolism, photosynthesis, redox homeostasis, and nitrogen assimilation (Table 1).

FUNCTIONAL CLASSIFICATION OF IDENTIFIED PROTEINS
Enzymes such as phosphoribulokinase, GAPDH, and transketolase were identified as primary targets responding to reduction or oxidation of regulatory thiols. They were previously reported to be redox-regulated targets of Trx (Motohashi et al., 2001; Marchand et al., 2004). Rubisco was identified as one of the primary targets of oxidation. Rubisco plays a major role in photosynthesis, hence it is regulated by several mechanisms and redox-dependent modulation is one of them. Based on our MALDI-MS data analysis (Table 2) two Cys of Rubisco LSU C192 and C427 are predicted to be oxidized. In Chlamydomonas reinhardtii C192 was previously shown to be inactivated by arsenite (Moreno and Spreitzer, 1999). However, site-directed mutagenesis suggested that...
C192 lacks a role in disulphide-mediated inactivation. Rather C449 and 459 are assumed to act as redox buffer to maintain a suitable redox state of the chloroplast in the presence of transiently increased ROS release. Rubisco SSU was identified as thoredoxin target by several studies (Motohashi et al., 2001; Balmer et al., 2003). In this work C41 and 117 were found to be modified by biotin maleimide due to oxidation, although the distance between both Cys is too far from each other (11–25 Å) to form a disulfide bond (Taylor and Andersson, 1997). But these Cys might be a target for SOH oxidation or mixed-disulfide formation and function as oxidant sensor. In Chlamydomonas, Zaffagnini et al. (2012) recently reported 225 glutathionylated proteins, among them many Calvin cycle enzymes, indicating a role of glutathionylation in protecting and regulating chloroplast carbohydrate metabolism. We can exclude glutathionylation, since the proteins were desalted following the reduction with DTT (see Materials and Methods). This step also eliminated any glutathione from the extracts.

In higher plant chloroplasts, Fd-GOGAT is a major enzyme for glutamate synthase, involved in the conversion of glutamine and 2-oxoglutarate to glutamate. Thioredoxin-mediated redox regulation of Fd-GOGAT was addressed in vitro by several studies (Lichter and Häberlein, 1998; Motohashi et al., 2001; Balmer et al., 2003). The amino acid sequence of the protein shows 24 Cys residues in the mature form, three of which were alkylated with NEM (Table 2). FNR catalyzes the electron transfer between ferredoxin and NADPH producing reducing equivalents for chloroplast metabolism and thus represents a crucial enzyme for various pathways requiring reductants. The predicted mass of a peptide containing a Cys residue matched the measured mass value (±0.5 Da) suggesting, that this particular Cys is sensitive to oxidation (Table 2). GAPDH is subjected to post-translational modifications which involve thiol-disulfide transitions of regulatory Cys, complex formation with ribulose-5-phosphate kinase and a regulatory protein named CP12 (Scheibe et al., 2002). This mechanism allows the coordination of GAPDH redox regulation with availability of its substrate 1,3-bisphosphoglycerate.

Proof of concept is also provided by the identification of 2-Cys Prx and cytochrome 20-3. 2-Cys Prx is on the top 20 most abundant stroma proteins (Peltier et al., 2006), functions as high-affinity thiol-peroxidase (Konig et al., 2003), and undergoes large redox-dependent conformational changes linked to functional switches (Dietz, 2012). Here the recombinant 2-Cys Prx protein was a convincing system to test and validate the various steps of oxidation, blocking, labeling, and detection in the work flow (Figure 2B), and its recovery in vitro and in vivo shows also, that the early oxidizable proteins are indeed trapped with this method (Tables 1–3). Likewise cytochrome 20-3 is a known target of thiol/disulfide transition (Laxa et al., 2007) and this redox switch affects its peptidylprolyl-cis/trans isomerase activity and probably also its capability for protein/protein interactions.

**REDOX MODIFICATION OF PROTEINS UPON OXIDATIVE STRESS**

**IN VIVO**

The in vivo redox status of protein thiols was monitored using MV-mediated oxidative stress. The majority of the identified proteins are involved in antioxidant defense (Table 3). GST catalyzes hydroperoxide detoxification in the presence of glutathione. GST tau is also known to detoxify herbicides and plays a role in signal transduction (Neuhaus et al., 1997; Drigo et al., 2003). Thiol-mediated light/dark regulation of Calvin cycle enzymes is a well known process for years (Buchanan, 1980). In this line SBPase and fructose-bisphosphate aldolase were identified. SBPase is activated by disulfide reduction. The involved Cys have been identified by site-directed mutagenesis (Danford et al., 1998). Interestingly overexpression of SBPase stimulates growth during early development and under stress suggesting that SBPase activity controls fluxes in the Calvin cycle to a major extent (Leifvqvist et al., 2005; Feng et al., 2007). The sensitivity of SBPase to oxidation in vivo may provide an explanation why SBPase plays a particular role under stress with increased ROS production.
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**FIGURE 6** Biotinylated proteins from *A. thaliana* leaves after methyl viologen treatment. (A) The immunoblot shows increased labeling in protein extracts after treating plants with 50 μM MV for different time periods. Control plants (0) were sprayed with water. All samples were extracted in the presence of NEM to block free thiols, then reduced with DTT and finally labeled with biotin maleimide. (B) The same samples were probed with 2-Cys Prx antibody to determine the redox state of 2-Cys Prx. The oxidized dimer and reduced monomers are indicated.

**Table 4** Cysteines modified upon methyl viologen-mediated oxidation.

| No. | Protein name                       | Peptide sequence | Predicted mass in MV treatment | Observed mass in MV treatment |
|-----|-----------------------------------|------------------|--------------------------------|-------------------------------|
| 1   | Rubisco large subunit             | AVYEC*LR         | 978.0                          | 1304.5                        |
| 2   | Myrosinase                        | C*PSKIDVR        | 1113.5                         | 1440.0                        |
| 19  | NAD(P)-binding Rossmann-fold-containing protein | SUVSDSTSCGPSKFTGK | 1938.9                         | 2265.4                        |
| 21  | Ferredoxin-NADP(+)-oxidoreductase 1 (FNR1) | C*LUNMK         | 815.9                          | 1142.4                        |

The table shows the peptide sequence and the predicted mass in the presence of the alkylating agents either NEM or biotin maleimide. The mass observed in MALDI-TOF MS of the MV-treated sample is shown. NEM-labeled cysteine (C) mass matches to experimental mass value suggesting that these Cys are not modified during oxidation. C* denotes Cys in the peptide sequence which is labeled with biotin maleimide, since the predicted mass matched to the experimentally observed value. The underlined peptides were addressed to be modified also in in vitro oxidation treatment (refer Table 2).

Regulators of PS II oxygen-evolving complex (OEC) such as PsbO-1, PsbP-1, and PsbQ were also identified as oxidation-sensitive proteins. A recent study has proposed the presence of an intra-molecular disulfide bond in PsbO-1 and PsbP-1 using a diagonal two-dimensional gel (Ströher and Dietz, 2008). PsbQ indeed has no Cys but could be associated and coeluted with other PS II OEC proteins. Biochemical studies showed that *A. thaliana* nucleoside diphosphate kinase 2 (NDPK2) is associated with MAPK-mediated H$_2$O$_2$ signaling in plants (Moon et al., 2003). Being one of the early oxidation targets, NDPK2 thiois might play a major role in the ROS-mediated signaling pathway. The in vivo experimental strategy should be improved in the future, e.g., by partial removal of Rubisco followed by enrichment of less abundant proteins. The here applied method does not distinguish between direct oxidation by H$_2$O$_2$ and proximity-based oxidation mechanism where thiol proteins such as peroxiredoxins first react with H$_2$O$_2$ to form a SOH intermediate, which then oxidizes a thiol of another protein (König et al., 2012).

**CONCLUSION**

In conclusion, the here developed approach provides insight into early targets of oxidation by H$_2$O$_2$ within the stroma proteome under physiologically relevant conditions. This method is applicable to identify redox-dependent protein modifications in vitro and ex vivo, e.g., redox changes upon exogenous MV treatment but also in response to other stresses. The study identified expected early targets such as 2-Cys peroxiredoxin which has been classified as redox sensor and many known targets of redox regulation such as SBPase and FNR. Redox input elements, transmitters, targets, and sensors form the cellular redox regulatory
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**APPENDIX**

![Graph showing cysteine conservation](image)

**FIGURE A1** Percentage of cysteine conservation in A. thaliana Rubisco large and small subunit protein in comparison to the following organisms: Rice, maize, Physcomitrella, Chlamydomonas, Chlorella, Euglena, Synechococcus, Nostoc, Hydrogenovibrio, R. capsulatus and R. sphaeroides. Encircled are the identified redox sensitive cysteines.