

Proteomic Analysis of Early-Responsive Redox-Sensitive Proteins in *Arabidopsis*

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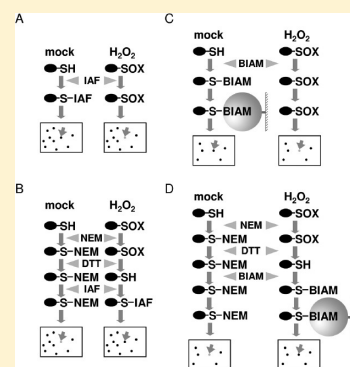
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S Supporting Information

ABSTRACT: Regulation of protein function through oxidative modification has emerged as an important molecular mechanism modulating various biological processes. Here, we report a proteomic study of redox-sensitive proteins in *Arabidopsis* cells subjected to H₂O₂ treatment. Four gel-based approaches were employed, leading to the identification of four partially overlapping sets of proteins whose thiols underwent oxidative modification in the H₂O₂-treated cells. Using a method based on differential labeling of thiols followed by immunoprecipitation and Western blotting, five of the six selected putative redox-sensitive proteins were confirmed to undergo oxidative modification following the oxidant treatment in *Arabidopsis* leaves. Another method, which is based on differential labeling of thiols coupled with protein electrophoretic mobility shift assay, was adopted to reveal that one of the H₂O₂-sensitive proteins, a homologue of cytokine-induced apoptosis inhibitor 1 (AtCIAPIN1), also underwent oxidative modification in *Arabidopsis* leaves after treatments with salicylic acid or the peptide elicitor flg22, two inducers of defense signaling. The redox-sensitive proteins identified from the proteomic study are involved in various biological processes such as metabolism, the antioxidant system, protein biosynthesis and processing, and cytoskeleton organization. The identification of novel redox-sensitive proteins will be helpful toward understanding of cellular components or pathways previously unknown to be redox-regulated.

KEYWORDS: redox proteomics, oxidative stress, hydrogen peroxide, AtCIAPIN1, salicylic acid, flg22, *Arabidopsis*



INTRODUCTION

Rapid generation of reactive oxygen species (ROS) is one of the cellular physiological events that occur in plants during exposure to various environmental stresses.^{1–4} Excessive accumulation of ROS can cause damages to cellular molecules such as proteins, nucleic acids and lipids.⁴ To counteract oxidative stress, plants evolved complex antioxidant systems.⁵ On the other hand, ROS also functions as key signaling molecules in physiological responses and development.^{1,3,5}

Mitochondria, chloroplasts, and microbodies are considered as major sources of ROS production, and cellular ROS levels are determined by their production and scavenging systems.^{3,5} During the plant hypersensitive response against avirulent pathogens, host cells actively produce high levels of ROS.¹ This oxidative burst is contributed mainly by plasma membrane-bound NADPH oxidases.^{1,6} Pathogen infection also triggers production of nitric oxide (NO) which coordinates with ROS to regulate hypersensitive cell death and defense gene induction.^{2,7,8}

Redox signals are largely sensed by redox-sensitive proteins which can undergo different forms of oxidative modifications.^{4,9,10} Some modifications are irreversible such as oxidation of cysteine thiols to sulfonic acids (P-SO₃H) and nitration of tyrosine and

tryptophan. Others are reversible such as oxidation of thiols to sulfenic acid (P-SOH), S-nitrosylation, and intra- or intermolecular disulfide bond formation. Irreversible protein oxidation often leads to permanent loss of function, whereas reversible oxidation can protect the protein from irreversible oxidation and/or act as a molecular switch for regulation of protein function.

Cysteine thiols in many proteins are highly redox-sensitive and their redox states often determine protein activities and functions. Several proteomic approaches have been developed for detection and identification of oxidant-sensitive thiol proteins.^{9–11} Generally, these methods are based on differential labeling of reduced versus oxidized thiols with specific thiol-reactive reagents. Following thiol labeling, proteins are separated by two-dimensional gel electrophoresis (2-DE), and differentially labeled proteins are identified by mass spectrometry. In addition to the gel-based methods, a modified isotope coded affinity tag technology (OxICAT) has been developed to identify redox-sensitive

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proteins in *E. coli*¹² and also in yeast.¹³ Fu et al. compared the merits and limitations of ICAT and DIGE in identifying redox-sensitive proteins in heart tissues and found that the two methods are complementary.¹⁴ Recently, a method termed isotopic tandem orthogonal proteolysis activity based protein profiling (isoTOP-ABPP) was developed and used to quantitatively profile the intrinsic reactivity of cysteine residues in human cells.¹⁵

Proteomic approaches have been used to detect and identify oxidant-sensitive proteins in plants (reviewed in refs 10,16). These reports include detection and identification of glutathionylated *Arabidopsis* proteins under *in vivo* or *in vitro* experimental conditions.^{17,18} A method based on thiol affinity chromatography was used to identify 65 *Arabidopsis* proteins that contain disulfide bonds under normal conditions,¹⁹ although the study did not reveal whether these disulfides are redox-regulated under an environmental stress. Hancock et al. identified 5 *Arabidopsis* proteins that became oxidized following the treatment of *Arabidopsis* suspension cells with 10 mM H₂O₂.²⁰ Alvarez et al. identified 22 proteins in *Arabidopsis* with altered redox states and/or abundance in response to methyl jasmonate treatment.²¹ Proteomic approaches have also been employed for identification of a large number of proteins from several plant species that are potential targets of thioredoxins (Trxs).^{22–31} In addition, using a biotin switch labeling method, a number of S-nitrosylated *Arabidopsis* proteins have been identified following treatments with the NO donor S-nitrosoglutathione or gaseous NO³² and during the hypersensitive response.³³

Here, we report the identification of proteins whose thiols underwent oxidative modifications in *Arabidopsis* cells following a 10 min treatment with a sublethal dose of H₂O₂. A majority of them have not been previously reported to undergo oxidative modifications under redox-perturbing conditions. We also established two methods for confirmation and detailed analysis of *in vivo* redox states of individual proteins in *Arabidopsis* plants challenged with H₂O₂ or two chemical inducers of the plant defense response. These results indicate that the *Arabidopsis* cells undergo rapid cellular reprogramming through oxidative modification of proteins involved in multiple cellular processes.

MATERIALS AND METHODS

Arabidopsis T87 Cell Culture and Treatments

Arabidopsis thaliana (Columbia ecotype) suspension cell line T87 was kindly provided by Dr. Kevin Lutke (Donald Danforth Plant Science Center, St. Louis, MO). The T87 cell culture was maintained in 50 mL liquid medium (1/2 MS salts, 3% sucrose, 0.2 g/L KH₂PO₄, 0.2 mg/L 2,4-D, and B5 vitamins) in 250 mL flasks by gentle agitation (50 rpm) in dark at 25 °C. Cells were subcultured weekly by transferring 3 mL culture to a new flask with 50 mL culture medium.

For the oxidant treatment of T87 cells, a 0.5 M H₂O₂ stock solution was added to the cell culture (3 days after subculturing) to a final concentration of 5 mM. For control samples, the same amount of H₂O was added to the culture cells. The cells were harvested 10 min after H₂O₂ treatment by filtering through a glass vacuum filter (VWR, West Chester, PA) and immediately frozen in liquid nitrogen for further analysis.

Protein Extraction and Labeling

All steps of protein sample preparation were carried out under reduced light. All buffers were degassed before use.

For the direct 5-(iodoacetamido)fluorescein (IAF) labeling method and the blocking-IAF labeling method, 500 mg cells were ground in the homogenization buffer [20 mM Tris-HCl (pH 8.0), 5 mM EDTA, 0.5% Triton X-100, 100 mM NaCl and 1% protease inhibitor cocktail (Sigma, St. Louis, MO)] supplemented either with 200 μ M IAF (Molecular Probe Inc., Eugene, OR) (the direct IAF labeling method), or 10 mM N-ethylmaleimide (NEM) (the blocking-IAF labeling method). The homogenate was incubated on ice for 30 min and centrifuged for 45 min at 12000 \times g at 4 °C. The supernatant was mixed with an equal volume of ice-cold phenol (Tris-buffered, pH 6.4–6.8) and centrifuged at 12000 \times g for 15 min at 4 °C to separate phenol and aqueous phases. The upper aqueous phase was removed leaving the interface intact, and the phenol phase was extracted twice with 50 mM Tris-HCl, pH 8.0 and then mixed with 5 volumes of cold 0.1 M ammonium acetate in methanol and left at –20 °C overnight to precipitate proteins. After centrifugation at 12000 \times g for 15 min, the protein pellet was washed five times with 1 mL methanol and air-dried for 10 min in a fume hood. For the direct IAF labeling method, the pellet was resuspended in the rehydration buffer (7 M urea, 2 M thiourea, 4% CHAPS, 20 mM DTT and 0.5% IPG buffer) (GE Healthcare). After centrifugation at 12000 \times g for 3 min, the supernatant was transferred to a new tube, and the protein concentration was determined with the CB-X Protein Assay kit (G-Biosciences, St. Louis, MO). For the blocking-IAF labeling method, the pellet was resuspended in the reduction buffer (50 mM Tris-HCl, pH 8.0, 7 M urea, 2 M thiourea, 1% CHAPS and 1 mM DTT). After sitting at room temperature for 30 min, IAF was added to a final concentration of 200 μ M and the mixture was incubated at room temperature in dark for 30 min. The reaction was stopped by adding 5 volumes of methanol and free IAF was removed by precipitation with centrifugation. The pellet was resuspended in the rehydration buffer and protein concentration was determined as above.

For the direct N-(biotinoyl)-N'-(iodoacetyl)-ethylenediamine (BIAM) tagging method, cells were broken in the homogenization buffer (described above) supplemented with 100 μ M BIAM (Invitrogen, Carlsbad, California). The protein homogenate was incubated at room temperature for 10 min and centrifuged at 12000 \times g for 10 min. Supernatant (2.5 mL) was taken and passed through a PD-10 desalting column (GE Healthcare) equilibrated with the homogenization buffer to remove free BIAM. BIAM-tagged proteins in the sample were then immunoprecipitated with 300 μ L NeutrAvidin agarose resin (Thermo Scientific, Rockford, IL). Proteins were eluted from the resin by 6 M guanidine-HCl, pH 1.5 and purified with Perfect-FOCUS (G-Biosciences). The protein pellet was resuspended in the rehydration buffer and protein concentration was determined as above.

For the blocking-BIAM tagging method, cells were broken in the homogenization buffer supplemented with 10 mM NEM. The protein homogenate was incubated at room temperature for 15 min and then centrifuged at 12000 \times g for 10 min. The supernatant was taken and cysteine was added to a final concentration of 10 mM to eliminate remaining NEM. After incubation at room temperature for 10 min, DTT was added to a final concentration of 1 mM and the sample was kept at room temperature for another 10 min. The samples were cleaned by passing through PD-10 desalting columns (GE Healthcare) equilibrated with the homogenization buffer. After that, 100 μ M BIAM was added and the samples were further processed as described in the direct BIAM tagging method.

Two-dimensional Gel Electrophoresis (2-DE)

Protein samples (200 μ g) were mixed with the rehydration buffer to bring the volume to 180 μ L, and the samples were then applied to 11 cm IPG strips (pH 4–7, Bio-Rad, Hercules, CA). The IPG strips were rehydrated overnight at room temperature. Isoelectric focusing (IEF) was carried out on a Bio-Rad PROTEAN IEF Cell at 20 °C with a maximum current of 50 μ A/strip and the following settings: 250 V for 30 min, 500 V for 1 h, a gradient increase to 8000 V in 2.5 h, and remaining at 8000 V until reaching 35000 Vh. The strips were then transferred to 8–16% Criterion Precast Gels (Bio-Rad) for the second dimension electrophoresis using Criterion Cell system (Bio-Rad). SDS-PAGE was run at 60 V for 15 min and then 200 V until the bromophenol blue dye reached the gel end.

Protein Image Scanning

The gels were scanned using a Typhoon 9410 scanner (GE Healthcare). Gels with IAF-labeled samples were scanned using 488 nm laser and a 520 nm band-pass emission filter. Postelectrophoretic staining with the SYPRO Ruby (Bio-Rad) fluorescent dye was performed according to the manufacturer's instructions and subsequent scanning was performing using 488 nm laser and 610 nm band-pass emission filter. All gels were scanned at 100 μ m pixel size, and the photomultiplier tube was set to ensure maximum pixel intensity between 40000 and 80000 to avoid saturation.

Two-dimensional Gel Image Analysis

The 2-D gel images were analyzed using the Progenesis SameSpots software version 2.0 as described in the user's instruction (Nonlinear Dynamics, Durham, NC). Briefly, a control sample was chosen as a reference image, and the software will automatically generate alignment vectors between the reference image and the other samples. After alignment, the images of three replicate samples for the same treatment were grouped together and the aligned images were analyzed for spot volume quantification and volume ratio normalization of different samples in the same treatment group. Statistical, quantitative, and qualitative analysis sets were created between the control group and each treated group. Protein spots with more than 2 fold increase or decrease in the IAF labeling intensities (for the IAF labeling methods) or SYPRO Ruby labeling intensities (for the BIAM tagging methods) with a *p* value smaller than 0.05 (Student's *t* test) between the H₂O₂-treated samples and control samples were picked for identification.

Spot Picking and Mass Spectrometry

A spot picking list generated from Phoretix 2D Evolution gel analysis software (Nonlinear Dynamics Ltd.) was exported to Gelpix (Genetix Inc., Boston, MA). The excised spots were then digested with trypsin as previously described.²¹ Protein digests were subjected to nano-LC–ESI–MS/MS analysis. Nano-LC was performed with a nanoLC-2D (Eksigent, Dublin, Ireland) equipped with a capillary trap LC Packings PepMap (DIONEX, Sunnyvale, CA) and LC Packings C18 Pep Map 100 (75 μ m, 15 cm) connected to the MS. Peptides (5 μ L injections) were desalted for 10 min with a flow rate 5 μ L/min of 90.5% solvent A (0.1% formic acid in Milli-Q water). Peptides were then resolved on a gradient from 9.5 to 35% solvent B (0.1% formic acid in ACN) for 4 min, from 35 to 45% B for 31 min, and from 45 to 90.5% B over the final 6 min at 200 nL/min flow rate.

The MS analysis was performed on an ABI QSTAR XL (AB Sciex, Foster City, CA) hybrid QTOF MS/MS mass spectrometer

equipped with a nanoelectrospray source (Protana XYZ manipulator). Positive mode nanoelectrospray was generated from fused-silica PicoTip emitters with a 10 μ m aperture (New Objective, Woburn, MA) at 2.5 kV. The *m/z* response of the instrument was calibrated daily with manufacturer standards. TOF mass and product ion spectra were acquired using information dependent data acquisition (IDA) in Analyst QS v1.1 with the following parameters: mass ranges for TOF MS and MS/MS were *m/z* 300–2000 and 70–2000, respectively. Every second, a TOF MS precursor ion spectrum was accumulated, followed by three product ion spectra, each for 3 s. The switching from TOF MS to MS/MS is triggered by the mass range of peptides (*m/z* 300–2000), precursor charge state (2–4) and ion intensity (>50 counts). The DP, DP2, and FP settings were 60, 10, and 230, respectively, and rolling collision energy was used.

Protein Database Search

The peptide tandem mass spectra were processed using Analyst QS software v1.1 (AB Sciex) and searched against the NCBI nr_20081206 database (7463447 entries) using an in-house version of MASCOT v2.20 (Matrix Science Inc., Boston, MA). The following parameters were selected: tryptic peptides with ≤ 1 missed cleavage site; precursor and MS/MS fragment ion mass tolerance of 0.8 and 0.8 Da, respectively; variable carbamidomethylation and fluorescein modification of cysteines for direct IAF labeling method; variable carbamidomethylation, *N*-ethylmaleimide and fluorescein modification of cysteines for blocking-IAF labeling method; variable carbamidomethylation and IED-biotin modification of cysteines for direct BIAM tagging method; variable carbamidomethylation, *N*-ethylmaleimide and IED-biotin modification of cysteines for blocking-BIAM tagging method; and variable oxidation of methionine for all methods. The Mascot results were imported into Scaffold 3.0 (ProteomeSoftware, Portland, OR) and protein identification based on the following criteria were reported: protein probability $\geq 99\%$ with at least 2 peptide with peptide probability $\geq 95\%$.

Investigation of *in vivo* Redox State of Individual Proteins by Differential Labeling of Thiols Coupled with Immunoprecipitation and Western Blotting

For each gene under investigation, the genomic sequence from ~ 2 kb upstream of its start codon to the codon before stop codon was cloned into pCR-Blunt II-TOPO (Invitrogen) and then subcloned into the binary vector pBAR-FLAG to fuse in frame with the FLAG tag at its C-terminus. *Agrobacterium* carrying the resulting construct was used to transform *Arabidopsis*. Stable transgenic *Arabidopsis* lines were generated that express each putative redox-sensitive protein fused with the FLAG tag driven by its native promoter. Three week-old plants of the transgenic lines (grown at 22 °C with 50% humidity under 9 h light/15 h dark photoperiod and at a light intensity of 125 mol m⁻² s⁻¹) were used for analysis.

For AtCIAPIN1, eEF1 α and AtPTP1, transgenic plants were vacuum-infiltrated with 5 mM H₂O₂ or water. Ten minutes later, 0.1 g leaves were collected and total proteins were extracted by homogenization in the extraction buffer (20 mM Tris-HCl, pH 8.0, 5 mM EDTA, 0.5% Triton X-100, 100 mM NaCl, 1% protease inhibitor cocktail) supplemented with 100 μ M BIAM. After incubation at room temperature for 10 min, the samples were centrifuged at 12000 $\times g$ for 10 min. Supernatant was taken and excessive BIAM was removed by Zeba Spin Desalting Columns (Thermo Scientific) pre-equilibrated with the extraction buffer. FLAG-tagged protein was then affinity-purified by

anti-FLAG M2 Affinity Gel (Sigma) according to the manufacturer's instructions. Immunoprecipitated protein was separated by SDS-PAGE and immunoblotted with either anti-FLAG M2-Peroxidase (HRP) antibody (Sigma) or Horseradish Peroxidase-Conjugated Streptavidin (Thermo Scientific).

For AtNAP1;1, AtPDIL1-1 and 14-3-3 λ , after the oxidant treatment of leaves as described earlier, total protein was extracted in the extraction buffer supplemented with 5 mM iodoacetamide (IAM). After incubation at room temperature for 10 min and centrifugation at $12000 \times g$ for 10 min, the supernatant was taken and DTT was added to a final concentration of 7.5 mM. After incubation at room temperature for 15 min, the samples were cleaned by passing through Zeba Spin Desalting Columns. The samples were further processed as described for AtCIAPIN1 and eEF1 α .

Protein Electrophoretic Mobility Shift Assay (PEMSA) for Analysis of *in vivo* Redox States of AtCIAPIN1

Stable transgenic plants expressing AtCIAPIN1-FLAG driven by its native promoter were vacuum-infiltrated with either water, 5 mM H₂O₂, 10 μ M flg22, or 250 μ M salicylic acid (SA). Leaves were harvested 10 min after H₂O₂ treatment or 30 min after all other three treatments. PEMSAs were performed as described previously³⁴ with modifications. Briefly, leaf samples were homogenized in the urea buffer [8 M urea, 100 mM Tris (pH 8.2), 1 mM EDTA] supplemented with 30 mM iodoacetic acid (IAA). After 15 min at 37 °C, the extracts were centrifuged at $12000 \times g$ for 10 min. Supernatant was taken and free IAA was removed by acetone precipitation of proteins. The protein pellet was resuspended in the urea buffer containing 3.5 mM DTT and incubated at 37 °C for 30 min. After that, IAM was added to a final concentration of 10 mM and samples were incubated at 37 °C for 15 min. Protein samples were then separated by urea-polyacrylamide gel electrophoresis, transferred onto nitrocellulose membrane, and immunoblotted with the anti-FLAG M2 HRP-conjugated antibody (Sigma). Mobility standards were prepared by treating total leaf proteins extracted in the urea buffer with 3.5 mM DTT at 37 °C for 30 min followed by labeling all free thiols with either 30 mM IAA or 10 mM IAM at 37 °C for 15 min, respectively.

RESULTS AND DISCUSSION

Methods for Differential Labeling of Reduced versus Oxidized Protein Thiols

We used two distinct thiol labeling approaches (IAF labeling and BIAM tagging) for detection of redox-sensitive proteins. For each approach, two methods (the direct labeling method and the blocking-labeling method) were employed. The procedures are schematically represented in Figure 1. For the direct IAF labeling method (Figure 1A), proteins are extracted from oxidant-treated and control cells and free thiols are directly labeled with the fluorescent probe 5-(iodoacetamido)fluorescein (IAF). After that, proteins are separated by 2-DE and the IAF labeling patterns are imaged using Typhoon 9410. If a particular protein becomes oxidized in the cells following oxidant treatment, the protein spot will show a reduced labeling intensity compared with the corresponding spot in the control sample. The direct labeling method is relatively straightforward. However, thiol/disulfide exchange may occur during protein extraction.⁹ An alternative method (Figure 1B) is to freeze the thiol/disulfide states of proteins by first extracting proteins in the presence of a thiol alkylation reagent such as *N*-ethylmaleimide (NEM) which

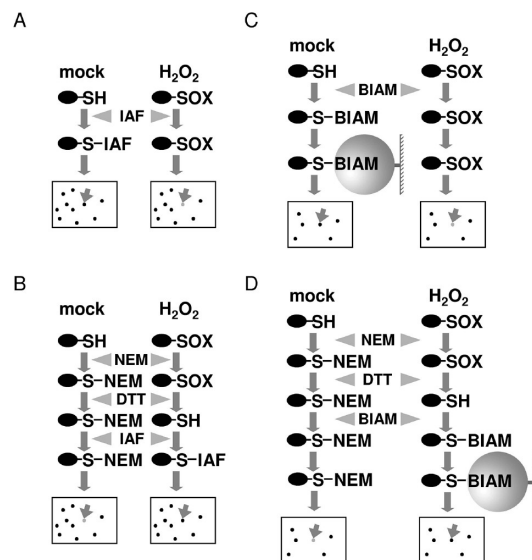


Figure 1. Schematic drawing of the proteomics methods used in this study. (A) Direct IAF labeling method. (B) Blocking-IAF labeling method. (C) Direct BIAM tagging method. (D) Blocking-BIAM tagging method. –SH, a reduced (free) thiol; –SOX, an oxidized thiol.

reacts and blocks free thiols. Oxidized thiols present in the samples are then reduced with DTT and the newly generated free thiols are labeled with IAF. In this blocking-IAF labeling method, if a protein becomes oxidized in the oxidant-treated cells, it is expected to display an increased labeling intensity in the treated sample compared with that in the control sample. The blocking-IAF labeling method could only detect reversibly oxidized (DTT-reducible) proteins.

One drawback of the IAF labeling methods is the limited resolution and sensitivity of 2-DE for resolving complex total proteins from plant tissues. Hence, we developed another labeling approach, BIAM tagging, in an attempt to at least partially overcome this drawback (Figure 1C,D). In this approach, IAF is replaced by *N*-(biotinoyl)-*N'*-(iodoacetyl)-ethylenediamine (BIAM) which is also thiol-reactive and contains a biotin moiety. Following direct BIAM tagging or blocking-BIAM tagging, tagged proteins are affinity-purified using NeutrAvidin agarose resin, separated by 2-DE, and stained with SYPRO Ruby. In this approach, only proteins that contain reduced thiols (in the direct BIAM tagging method) or reversibly oxidized thiols (in the blocking-BIAM tagging method) in original tissue samples can be affinity-purified. As a result, the complexity of protein samples subjected to 2-DE is reduced.

Establishment of Experimental Conditions for Induction of Oxidative Stress in *Arabidopsis* Cell Cultures

We aimed to identify proteins that undergo oxidative modification in *Arabidopsis* suspension cells following treatment with hydrogen peroxide (H₂O₂). *Arabidopsis* suspension cells offer an advantage over whole plant tissues for achieving more uniform exposure of all cells to the oxidant, which increases the sensitivity for detecting oxidatively modified proteins. It has been reported that no substantial cell death was caused by treatment with H₂O₂ at a concentration as high as 10 mM for 6 h³⁵ or even at 88 mM for 16 h.³⁶

In this study, *Arabidopsis* suspension cells were treated with 5 mM H₂O₂ to induce oxidative stress. The direct IAF labeling

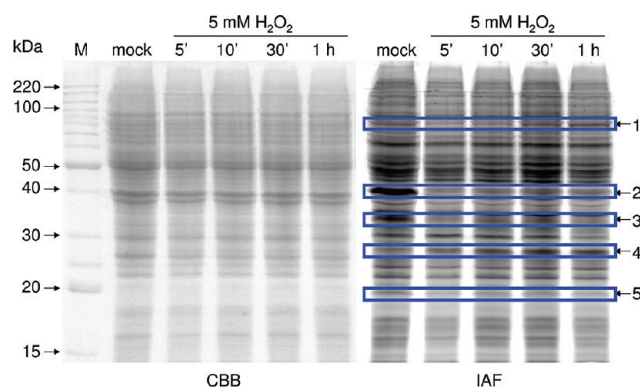


Figure 2. Effect of H_2O_2 treatment on protein oxidation. *Arabidopsis* suspension cells were treated with 5 mM H_2O_2 for different time periods (as indicated). For each sample, 150 μg protein was labeled with IAF and separated by discontinuous SDS-PAGE with a 12% separating gel and a 4% stacking gel. The IAF labeling pattern (right panel) was scanned by Typhoon 9410. Total proteins were visualized by Commassie Brilliant Blue (CBB) staining (left panel). The arrows point to proteins that displayed altered IAF labeling intensities after H_2O_2 treatment. Mock: cells were treated with H_2O for 10 min.

method was used to evaluate whether the oxidant treatment leads to detectable changes in protein redox state in the suspension cells. At different time points following the 5 mM H_2O_2 treatment, cells were harvested and free thiols were labeled with IAF during protein extraction. Proteins were then separated by one-dimensional SDS-PAGE (1-DE). As shown in Figure 2, multiple bands showed altered IAF labeling intensities within 5 min of the treatment (marked by arrows), indicating that some proteins in the suspension cells were oxidized (with decreased IAF labeling intensities) or reduced (indicated by Arrow 4 with an elevated IAF labeling intensity). The most striking change came from a 40 kDa band (indicated by Arrow 2), whose IAF labeling intensity significantly diminished during the 5 min–1 h treatment. Since we are interested in early responsive redox-sensitive proteins, in our subsequent redox proteomics experiments, cells were harvested after being treated with 5 mM H_2O_2 for 10 min. The short oxidant treatment was also intended to minimize the chance of false positives resulting from changes in abundance of a thiol-containing protein, which is more likely to occur after a longer period of the oxidant treatment.

Detection and Identification of H_2O_2 -Sensitive Thiol-Containing Proteins in *Arabidopsis* Cell Cultures

Three biological replicates were included for each proteomic method for detection and identification of proteins that underwent oxidative modifications in *Arabidopsis* suspension cells following the oxidant treatment. After IAF labeling of thiol-containing proteins or affinity-purification of BIAM-tagged proteins, protein samples were separated by 2-DE. For the IAF-labeled samples, the IAF labeling patterns of gels were imaged. Then the gels were stained with SYPRO Ruby which helps to locate IAF-labeled protein spots for spot picking. For the BIAM tagging methods, the gels were stained with SYPRO Ruby to detect differentially tagged proteins.

Protein spots with an IAF labeling intensity (for the IAF labeling methods) or SYPRO Ruby staining intensity (for the BIAM tagging methods) differing by more than 2-fold (with a p value of <0.05) as determined by the Progenesis SameSpots software were marked for identification by mass spectrometry

(MS) (shown as circled spots in Figure 3). In direct labeling methods, gel images from control and oxidant-treated samples were pseudocolored as red and green, respectively (Figure 3A, C). In blocking-labeling methods, gel images from control and oxidant-treated samples were pseudocolored as green and red, respectively (Figure 3B,D). Gel images from control and oxidant-treated samples were then superimposed. The spots in pink represent proteins that were in more oxidized states in the H_2O_2 -treated samples than in the control samples whereas the spots in green were in more reduced states in the treated samples. The spots that appear black were proteins with relatively similar labeling intensities between control and treated samples.

Some IAF-labeled spots could not be detected by SYPRO Ruby staining, suggesting that they were at very low abundance and generally could not be identified due to the sensitivity limitation of the mass spectrometer utilized for identification. Such spots were marked by yellow circles in Figure 3. In total, 27 spots from direct IAF labeling, 22 from blocking-IAF labeling, 15 from direct BIAM tagging, and 19 from blocking-BIAM tagging yielded positive identification. In total, 84 proteins were identified as putative oxidant-sensitive proteins (Table 1, Table S1, S2, Supporting Information). Among them, 13 proteins were identified by more than one method.

One common problem with gel-based proteomics methods is that the limited resolution of 2-DE might lead to false positives. Multiple protein IDs were obtained from some of the protein spots particularly in the gel areas where proteins were not well separated. These spots might contain protein(s) that were not differentially labeled but have similar molecular weights and isoelectric points to redox-sensitive proteins. Among proteins identified in this study, two nonthiol-containing proteins were found. One is AT1G16470, identified from Spot 18 in the blocking-IAF labeling method. This spot was also identified as a cytosolic triose phosphate isomerase which was reported to be S-nitrosylated in the hypersensitive response.³³ The other is AT1G09760, identified from both Spot 8 in the direct IAF labeling method and Spot 19 in the blocking-IAF labeling method. These two spots also contained peptides from AtPDX1.1 and AtPDX1.3, respectively. Such nonthiol-containing false positive proteins were not found with the BIAM tagging methods.

Some spots were assigned with multiple proteins that share very high sequence similarities due to the fact that a peptide might match to more than one protein. For instance, Spot 17 and 18 in Figure 3A were identified as translation elongation factor 1 α (eEF1 α), which is encoded by 4 distinct genes (At1g07920, At1g07930, At1g07940 and At5g60390). However, these 4 genes encode proteins with identical amino acid sequences.

Oxidative Modification of Identified Proteins in *Arabidopsis* Plants upon H_2O_2 Treatment

To make a rough assessment of the reliability of the proteomics methods, six putative redox-sensitive proteins were chosen and their *in vivo* redox state under oxidant treatment were individually investigated in *Arabidopsis* plants. These proteins include eEF1 α and protein tyrosine phosphatase 1 (AtPTP1) identified from the direct IAF labeling method, AtCIAPIN1 from the direct BIAM tagging method, nucleosome assembly protein 1;1 (AtNAP1;1) and 14-3-3 λ from the blocking-IAF labeling method, and protein disulfide isomerase like 1-1 (AtPDIL1-1) from the blocking-BIAM tagging method.

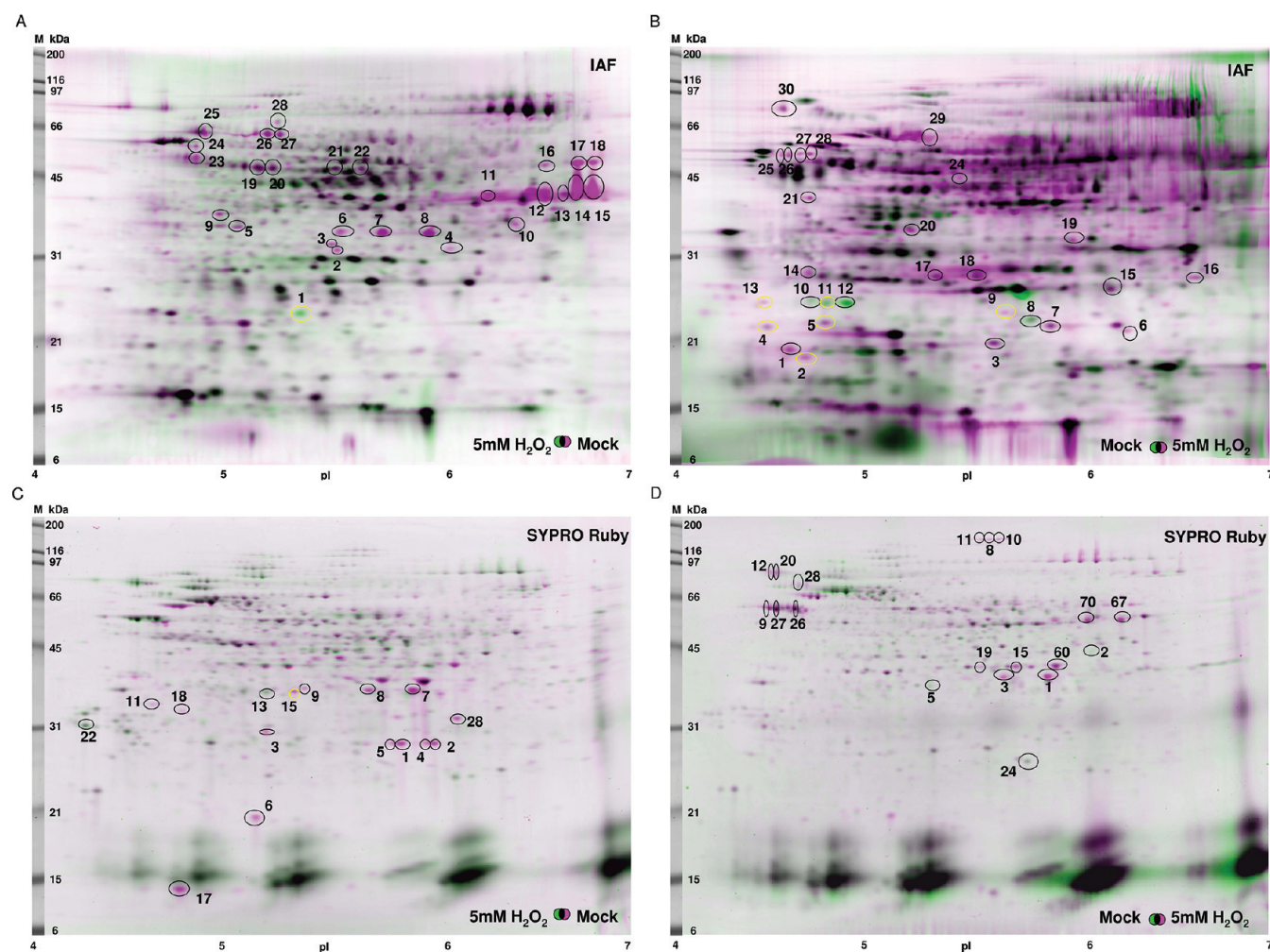


Figure 3. Detection of redox-sensitive proteins upon H_2O_2 treatment. IAF labeling images (for the IAF labeling methods) or SYPRO Ruby stained images (for the BIAM tagging methods) from oxidant-treated samples and control samples were pseudocolored and superimposed. Differentially labeled spots (marked by circles in black or yellow) were numbered and excised for MS identification. The MW markers and pI markers are indicated. The yellow circled spots were not identified by MS analysis while the black circled spots yielded positive identification. The images are based on the results from (A) direct IAF labeling, (B) blocking-IAF labeling, (C) direct BIAM tagging, and (D) blocking-BIAM tagging. For each method, three biological replicates were conducted and a representative superimposed image is presented.

Stable transgenic *Arabidopsis* lines expressing each of the six proteins fused with the FLAG tag driven by its native promoter were generated. Soil-grown plant leaves were vacuum-infiltrated with either 5 mM H_2O_2 or water (as a control) and harvested after 10 min of the treatment. To determine the redox state of the proteins previously identified from the direct labeling methods, total protein was extracted in the BIAM-containing buffer to tag free thiols. The FLAG-tagged protein was affinity-purified with anti-FLAG M2 affinity gel and detected by immunoblotting with horseradish peroxidase-conjugated streptavidin to determine the amount of BIAM attached to the recombinant protein. The total amount of the recombinant proteins was determined by immunoblotting with the anti-FLAG antibody. If a protein was oxidatively modified by the treatment, we would expect reduced BIAM tagging of this protein. Indeed, as shown in Figure 4, the amount of BIAM-tagged recombinant AtCIAPIN1, eEF1 α and AtPTP1 was greatly reduced in the H_2O_2 -treated samples. For the proteins identified from the blocking methods, free thiols were first alkylated by IAM and samples were then treated with DTT followed by BIAM labeling to tag thiols that were originally

in a reversibly oxidized state. Therefore, enhanced BIAM tagging of a protein would be expected if it underwent oxidation during the treatment, which was indeed the case for AtNAP1;1 and AtPDIL1-1 (Figure 4). However, we failed to detect BIAM tagging of the recombinant 14-3-3 λ in either control or treated samples in our experimental conditions, suggesting that 14-3-3 λ may be unsusceptible to BIAM labeling. It is also possible that 14-3-3 λ constitutively exists in its reduced state during the H_2O_2 treatment and thus represents a false positive from the redox proteomics experiment. Another possibility is that 14-3-3 λ might indeed undergo redox changes in the oxidant-treated suspension cells, but such a change did not occur in the oxidant-treated leaves.

Although it is impractical to individually verify the redox states of all 84 putative redox-sensitive proteins identified from the redox proteomics approaches, the result that 5 of the 6 selected proteins were confirmed to undergo oxidative modification in the oxidant-treated leaves indicates that at least a majority of the putative redox-sensitive proteins identified in this study are likely real positives.

Table 1. List of the Redox-Sensitive Proteins Identified in This Study^a

gene locus	protein function	number of peptides matched ^b	coverage (%) ^b	method ^c
Primary metabolism				
AT1G64190	6-phosphogluconate dehydrogenase	9	23.0	D/I
AT1G77120	alcohol dehydrogenase 1 (ADH1)	2	8.2	B/B
AT3G48000	aldehyde dehydrogenase 2B4 (ALDH2B4)	7	15.0	B/B
AT3G17940	aldose 1-epimerase, putative	5	16.0	D/I
AT1G14810	aspartate semialdehyde dehydrogenase	4	15.0	D/B
AT3G55440	cytosolic triose phosphate isomerase	10	42.0	B/I
AT1G74030	enolase 1 (ENO1)	4	8.6	D/I
AT2G36530	enolase 2 (ENO2)	3	9.7	D/I
AT3G52930	fructose-bisphosphate aldolase, putative	11	49.0	D/I
AT3G04120	GAPDH C subunit 1 (GAPC-1)	8	24.0	D/I
AT1G13440	GAPDH C subunit 2 (GAPC-2)	4	13.0	D/I
AT1G53240	malate dehydrogenase (NAD), mitochondrial	3	12.0	D/I
AT5G43330	malate dehydrogenase, cytosolic, putative	2	5.4	D/I
AT1G79550	phosphoglycerate kinase (PGK)	6	20.0	B/I
AT5G50850	pyruvate dehydrogenase	2	6.1	D/B
AT2G19940	semialdehyde dehydrogenase family protein	4	12.0	D/I
AT1G01800	short-chain dehydrogenase/reductase family protein	7	35.0	D/B
Antioxidant system				
AT3G11630	2-Cys peroxiredoxin (2-Cys PrxA)	4	22.0	B/I
AT1G07890	ascorbate peroxidase (APX1)	2	9.6	B/I D/B
AT1G75270	dehydroascorbate reductase 2 (DHAR2)	2	9.9	B/B
AT5G42980	encodes a cytosolic thioredoxin	4	40.0	D/B
AT3G10920	manganese superoxide dismutase (MSD1)	2	9.5	B/I
AT1G65980	thioredoxin-dependent peroxidase 1 (TPX1)	2	6.2	D/B
Translational and post-translational control				
translation				
AT3G09200	60S acidic ribosomal protein P0	2	10.0	D/I
AT1G07920	elongation factor 1-alpha	6	19.0	D/I
AT5G60390	elongation factor 1-alpha	6	19.0	D/I
AT1G07940	elongation factor 1-alpha	6	19.0	D/I
AT1G07930	elongation factor 1-alpha	6	19.0	D/I
AT5G19510	elongation factor 1B alpha-subunit 2 (eEF1Balpha2)	4	26.0	D/B
AT4G11120	translation elongation factor Ts (EF-Ts)	3	7.8	D/B
protein folding				
AT2G04030	a chloroplast-targeted 90-kDa heat shock protein	3	4.7	B/B
AT1G21750	protein disulfide isomerase-like protein (AtPDIL1-1)	14	29.0	B/B
AT1G77510	protein disulfide isomerase-like protein (AtPDIL1-2)	10	30.0	B/B
AT3G54960	protein disulfide isomerase-like protein (AtPDIL1-3)	10	21.0	B/B
AT5G60640	protein disulfide isomerase-like protein (AtPDIL1-4)	10	17.0	B/I B/B
AT3G16110	protein disulfide isomerase-like protein (AtPDIL1-6)	5	9.7	D/I B/B
AT2G47470	protein disulfide isomerase-like protein (AtPDIL2-1)	5	16.0	D/B B/B
AT1G56340	calreticulin1 (CRT1)	2	4.5	B/I
AT2G28000	chaperonin-60 alpha (CPN60A)	15	30.0	D/I B/I B/B
AT3G56070	cytosolic cyclophilin (CYP2)	4	24.0	B/I
AT1G60420	DC1 domain-containing protein/PDI-like protein	10	19.0	D/I
AT3G12580	heat shock protein 70(HSP70)	15	29.0	D/I
AT5G28540	luminal-binding protein 1(BIP1)	18	32.0	D/I
AT4G37910	mitochondrial heat shock protein 70-1 (MTHSC70-1)	24	44.0	D/I B/I
AT4G26110	nucleosome assembly protein 1;1 (NAP1;1)	3	8.6	B/I
AT3G62030	rotamase/CYP 4	2	8.1	B/I

Table 1. Continued

gene locus	protein function	number of peptides matched ^b	coverage (%) ^b	method ^c
protein degradation				
AT5G35590	20S proteasome alpha subunit A1 (PAA1)	2	9.8	D/B
AT1G16470	20S proteasome alpha subunit B1 (PAB1)	5	20.0	B/I
AT2G27020	20S proteasome alpha subunit G1 (PAG1)	2	8.4	D/B
AT3G26340	20S proteasome beta subunit E, putative	2	9.5	B/I
AT5G42790	26S proteasome alpha subunit F1 (PAF1)	6	25.0	D/I B/I
AT4G12060	double Clp-N motif protein	2	9.1	B/I
AT4G20850	Tripeptidyl Peptidase II	10	7.6	B/B
AT2G17190	ubiquitin family protein	4	9.3	B/I
post-translational modifications				
AT1G50370	phosphoprotein phosphatase	2	6.6	D/I
AT5G53140	protein phosphatase 2C, putative (PP2C)	4	9.0	B/I
AT1G71860	protein tyrosine phosphatase 1 (PTP1)	4	13.0	D/I
Cytoskeleton				
AT5G09810	actin 7 (ACT7)	10	31.0	D/I B/I
AT4G14960	tubulin alpha-6 chain (TUA6)	11	36.0	D/I
AT5G62690	tubulin beta-2 (TUB2)	5	11.0	D/I
Others				
AT5G10450	14-3-3 lambda	4	21.0	B/I
AT4G37000	accelerated cell death 2 (ACD2)	4	14.0	D/I
AT1G12910	anthocyanin1 (AtAN11)	3	8.4	B/I
AT5G10920	argininosuccinate lyase (AtArgH)	5	8.5	D/I
AT4G24830	arginosuccinate synthase family protein	11	25.0	D/I
AT5G18400	AtCIAPIN1	3	11.0	D/B
AT1G78900	catalytic subunit A of the vacuolar ATP synthase	19	35.0	D/I B/I
AT1G13870	deformed roots and leaves 1 (DRL1)	2	7.9	D/B
AT3G25530	gamma-hydroxybutyrate dehydrogenase	2	7.6	D/I
AT4G34540	isoflavone reductase family protein	2	6.9	D/I
AT3G07720	kelch repeat-containing protein	2	6.7	D/I
AT5G48480	lactoylglutathione lyase	4	27.0	B/I
AT3G44300	nitrilase 2 (NIT2)	5	15.0	D/B B/B
AT4G08790	nitrilase, putative	2	6.8	D/I
AT1G63000	nucleotide-rhamnose synthase/epimerase-reductase	4	14.0	D/I D/B
AT3G62530	PBS lyase, HEAT-like repeat-containing protein	3	13.0	B/I
AT3G07090	PPPDE putative thiol peptidase family protein	3	13.0	D/I
AT4G14000	Putative methyltransferase family protein	4	19.0	B/I
AT2G38230	pyridoxine biosynthesis 1.1 (ATPDX1.1)	5	18.0	D/I
AT5G01410	pyridoxine biosynthesis 1.3 (ATPDX1.3)	3	11.0	B/I
AT1G07750	RmlC-like cupins superfamily protein	4	14.0	D/B B/B
AT2G41530	S-formylglutathione hydrolase	2	7.4	D/I
AT1G09760	U2 small nuclear ribonucleoprotein A	4	20.0	D/I B/I
AT4G21580	zinc-binding dehydrogenase family protein	6	20.0	D/I

^a More detailed information on protein identification and properties are listed in Tables S1 and S2 (Supporting Information). ^b For proteins identified from multiple spots, derived from the spot yielding most numbers of uniquely matched peptides. ^c The method by which the protein was identified: D/I, direct IAF labeling. B/I, blocking-IAF labeling. D/B, direct BIAM tagging. B/B, blocking-BIAM tagging.

AtCIAPIN1 Alters its Redox State in Response to the Treatment with Flg22 or SA

Rapid production of ROS is usually associated with the plant defense response. It is interesting to investigate whether the H₂O₂-sensitive proteins identified are also sensitive to redox-perturbing chemicals such as flg22 and SA. Flg22 is a peptide derived from the bacterial flagellin which triggers the innate

immune response.³⁷ Flg22 has been found to induce ROS production catalyzed by the NADPH oxidase AtrbohD.³⁸ SA is a key regulator of the plant defense response which causes perturbation of cellular redox homeostasis.³⁹

A modified Protein Electrophoretic Mobility Shift Assay (PEMSA)³⁴ method was used to analyze the redox profiles of AtCIAPIN1 which contains 11 cysteine residues. Soil-grown

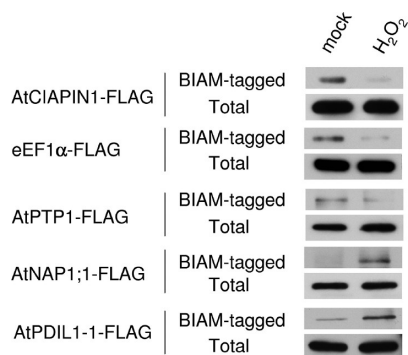


Figure 4. Oxidative modification of identified proteins in planta upon H_2O_2 treatment. Transgenic plants expressing the protein of interest fused with the FLAG tag were vacuum infiltrated with either water (mock) or 5 mM H_2O_2 . For analysis of AtCIAPIN1, eEF1 α , and AtPTP1, free thiols in the total protein were labeled with BIAM during protein extraction. For analysis of AtNAP1;1 and AtPDIL1-1, free thiols in the samples were first alkylated by IAM. Samples were then treated with DTT and newly generated free thiols were labeled by BIAM. After that, FLAG-tagged protein from each sample was affinity purified, separated by SDS-PAGE, and detected by HRP-Conjugated Streptavidin (to determine the amount of BIAM attached to the FLAG-tagged protein) or by the anti-FLAG M2 antibody (to determine the amount of the total recombinant protein).

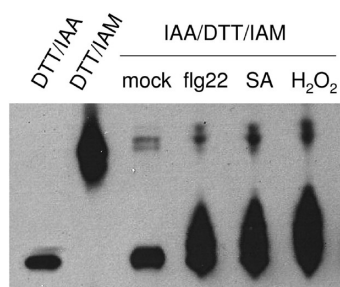


Figure 5. AtCIAPIN1-FLAG underwent oxidative modification in leaves upon flg22, SA and H_2O_2 treatment. Lane 1 and lane 2 are mobility standards corresponding to fully reduced and fully oxidized AtCIAPIN1-FLAG protein, respectively. They were prepared by treating total leaf protein extracts with DTT followed by labeling all free thiols with either IAA (lane 1) or IAM (lane 2). For the other samples, total proteins were extracted in the buffer containing IAA to carboxymethylate free thiols. The samples were then treated with DTT followed by amidomethylating newly generated thiols with IAM. The protein samples were then separated by urea-PAGE and AtCIAPIN1-FLAG protein was detected by immunoblotting with the anti-FLAG M2 antibody.

transgenic plants expressing AtCIAPIN1-FLAG were vacuum infiltrated with either water, flg22, SA or H_2O_2 . Total protein was extracted and free thiols were carboxymethylated by iodoacetic acid (IAA). Thiols that were in a reversibly oxidized state in the samples were reduced by DTT and amidomethylated by iodoacetamide (IAM). The IAM adducts are neutral while the ionized IAA adducts of free thiols lead to faster protein migration toward the anode during urea-PAGE. Therefore, AtCIAPIN1 proteins with more oxidized thiols in the original leaf samples tend to migrate more slowly than those with more free thiols. As shown in Figure 5, in the control sample (lane 3), the large majority of the AtCIAPIN1-FLAG protein had the similar mobility to the

AtCIAPIN1-FLAG whose thiols were all labeled by IAA (lane 1), suggesting that they were in a reduced state. However, the samples from the leaves treated with flg22, SA, or H_2O_2 all resulted in multiple bands with slower mobility than that in lane 1. These bands represent AtCIAPIN1-FLAG with different numbers of oxidized thiols. This result indicates that flg22 and SA, like H_2O_2 , lead to the oxidative modification of AtCIAPIN1 presumably through alteration of cellular redox homeostasis.

Categorization of Proteins Identified as Redox-sensitive Proteins

Our study led to the identification of 84 putative redox-sensitive proteins (Table 1 and Table S1, Supporting Information). All peptides identified from the MS analysis are listed in Table S2 (Supporting Information). These proteins can be classified into multiple functional groups, as discussed below.

Metabolism. Seventeen of the redox-sensitive proteins are enzymes involved in primary metabolic processes. Strikingly, at least 8 of them are involved in glycolysis, including 2 cytosolic GAPDHs (GapC1 and GapC2), triose phosphate isomerase, phosphoglycerate kinase (PGK), aldose 1-epimerase, aldolase, and 2 enolases. Several of them or their paralogs have been identified as S-nitrosylated proteins in *Arabidopsis*.³² It has been reported that in an *in vitro* assay, GAPDH activity can be inhibited by oxidation or nitrosylation and restored by reductant treatment.²⁰ Inactivation of glycolysis enzymes likely acts to redirect the metabolic flux from glycolysis to the pentose phosphate pathway (PPP) to generate NADPH, the reducing power for cellular antioxidant systems. Such a mechanism was reported during the oxidative stress in yeast.^{40,41}

It has been found that in response to H_2O_2 stress, yeast GAPDH becomes oxidized and mediates phosphorelay from the Mak2/3 sensor kinase to the Mcs response regulator to activate a MAPK cascade.⁴² More recently, S-nitrosylated GAPDH was found to bind to an E3-ubiquitin-ligase and then translocates to the nucleus to initiate a cell death cascade through activation of p53.⁴³ It is intriguing to speculate that in addition to modulating the glycolytic pathway, oxidative modifications of GAPDH may play a role in other signaling pathways in plants.

Our results also suggest the presence of redox control on the metabolic processes downstream of glycolysis. There are two pathways for the conversion of pyruvate to acetyl-CoA. This reaction can be catalyzed by pyruvate dehydrogenase (PDH) under normal conditions. During ethanolic fermentation, pyruvate is converted by pyruvate decarboxylase (PDC) to acetaldehyde. The detoxification of acetaldehyde by aldehyde dehydrogenases (ALDH) produces acetate, which can then be used by acetyl-CoA synthetase (ACS) to synthesize acetyl-CoA. This PDC-ALDH-ACS pathway, also termed the PDH bypass, has been reported in yeast⁴⁴ and recently also in *Arabidopsis*.⁴⁵ In our results, the ALDH was found to be oxidized upon H_2O_2 treatment while the PDH became more reduced. Although further studies are needed to evaluate potential redox regulation of ALDH and PDH activity, it is possible that the two pathways are synchronously and probably differentially regulated under oxidative stress.

Antioxidant System. Six well-known proteins of the antioxidant system were identified, including dehydroascorbate reductase 2 (DHAR2) which serves to maintain a reduced ascorbate pool through oxidation of reduced GSH to glutathione disulfide, thioredoxin-dependent peroxidase 1 (TPX1), a cytosolic thioredoxin, a mitochondrial manganese superoxide dismutase

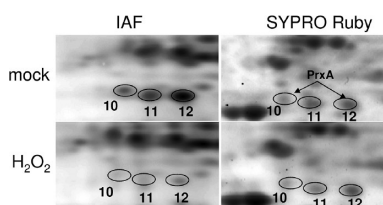


Figure 6. Comparison of the IAF-labeled protein spots and SYPRO Ruby-stained protein spots from a portion of 2-D gels from the blocking-IAF labeling method. The numbered protein spots correspond to those in Figure 3B. Note the reduced IAF labeling of Spots 10, 11, and 12 in the H_2O_2 -treated sample compared to that in the mock-treated sample. Spot 10 and Spot 12 were identified as PrxA. Staining of the gels with SYPRO Ruby revealed that the abundance of these proteins was not changed by the oxidant treatment.

MSD1, the cytosolic L-ascorbate peroxidase APX1, and the chloroplast 2-cysteine peroxiredoxin PrxA.

A cluster of three protein spots (Spot 10, 11, and 12 in Figure 3B) detected from the blocking-IAF labeling method showed a reduction in IAF labeling in oxidant-treated samples compared to the control samples, suggesting that these proteins might become more reduced following the H_2O_2 treatment. We were not able to identify Spot 11. However, the two other spots were found to be the same protein, a 2-Cys peroxiredoxin (PrxA), which is localized in chloroplasts and protects photosynthetic machinery from oxidative damage.^{46,47} Peroxiredoxins reduce hydrogen peroxide and alkyl hydroperoxides and therefore modulates peroxide levels. During the reaction, its active site cysteine (the peroxidatic cysteine) is oxidized to a sulfenic acid intermediate which forms an intermolecular disulfide with a second cysteine on the other identical substrate. The disulfide can then be reduced to thiol by glutathione or thioredoxin. There are several possibilities which lead to its reduced IAF labeling in the H_2O_2 -treated cells. One possibility is that the oxidant treatment might cause a rapid drop in the PrxA protein level, which can be ruled out since PrxA protein levels were found to be similar in the control samples and the H_2O_2 -treated samples based on SYPRO Ruby staining (Figure 6). The second possibility is that thiols of some PrxA proteins might be in an oxidized state under normal conditions. The oxidant treatment might activate the antioxidant system, which leads to temporary reduction of the oxidized thiol by such as thioredoxin or glutathione in a manner similar to reduction of NPR1.⁴⁸

Protein Synthesis, Folding, Modification, and Degradation. Regulation of protein synthesis and post-translational modifications allows cells to obtain a rapid response to an environmental signal. Not surprisingly, a large group of the oxidant sensitive proteins listed in Table 1 are involved in protein synthesis, folding, modification, and degradation.

eEF1 α was identified from the direct IAF labeling method and was confirmed to undergo oxidation in the oxidant-treated leaves (Figure 4). Inhibition of protein synthesis in response to oxidative stress has been reported in mammalian cells and in yeast, mediated by phosphorylation of the elongation initiation factor eIF2 α .^{49,50} Our result suggests another level of redox regulation of protein synthesis through regulation of translational elongation by oxidative modification of eEF1 α . However, the consequence of eEF1 α oxidation under oxidative stress remains to be defined.

Among proteins in this category, 6 protein disulfide isomerase-like proteins (AtPDILs) were found. All of the 6 PDILs were

identified by the blocking methods, indicating that their oxidative modification is reversible. One of them, AtPDIL1-1, was confirmed to be oxidized in the H_2O_2 -treated leaves (Figure 4). Oxidative stress is expected to increase mis-folded proteins in the ER and thus causes ER stress, which is also called unfolded protein response (UPR). Protein disulfide isomerases (PDIs), which catalyze the formation and breakage of disulfide bonds within substrate proteins and thus facilitate their correct folding, are key protein folding catalysts activated during UPR in animals and yeast. It has been reported that the redox state of PDILs is regulated by a thiol-disulfide oxidoreductase OsEro1 in rice,⁵¹ but little is known about the influence of oxidative stress on their functions *in vivo*.

The nucleosome assembly protein 1 (AtNAP1;1) was identified from the blocking-IAF labeling method. AtNAP1;1 is a histone chaperone involved in nucleosome assembly/disassembly and regulates the expression of some genes in the nucleotide excision repair pathway.⁵² The oxidation of AtNAP1;1 may act to regulate nucleosome formation and downstream gene expression.

Besides protein folding, several proteins involved in protein degradation were identified as targets of H_2O_2 , indicating the involvement of protein degradation systems in coping with the accumulation of unfolded and mis-folded proteins under oxidative stress. Among them, the 20S proteasome alpha subunit A1, B1, G1 and F1 were also found to be S-glutathionylated upon tert-butylhydroperoxide treatment.¹⁸

Three protein phosphatases were found to be oxidant-sensitive, including two serine/threonine phosphatases and a tyrosine protein phosphatase known as AtPTP1.⁵³ It has been reported that AtPTP1 dephosphorylates MAPKs^{54,55} and is inactivated by H_2O_2 treatment *in vitro*.⁵⁵ Our result further reveals that AtPTP1 was oxidized *in vivo* in the oxidant-treated plants (Figure 4) which likely inactivates AtPTP1. Protein phosphatases are well-known targets of H_2O_2 which oxidizes their conserved catalytic cysteine residues to render them inactive.^{56,57} Inactivation of AtPTP1 as well as other protein phosphatases might serve to enhance the oxidative stress-induced MAPK cascade.^{55,58}

Cytoskeleton Proteins. Another group of the oxidant sensitive proteins are components of the cytoskeleton, including actin 7 (ACT7), tubulin alpha-6 chain (TUA6), and tubulin beta-2 (TUB2). Actin and tubulin have previously been found to be sensitive to oxidation in animals^{59,60} and *Arabidopsis*.¹⁸ In yeast, actin is considered as an oxidative stress sensor whose oxidation regulates stress-triggered cell death.⁶¹

Other Proteins. In this group of proteins, special attention was paid to AtCIAPIN1, which was identified from the direct BIAM tagging method. AtCIAPIN1 encodes a putative iron-sulfur protein homologous to human cytokine-induced apoptosis inhibitor 1 (Ciapin1),⁶² and the yeast Dre2 protein, a negative regulator of H_2O_2 -induced cell death.⁶³ In yeast, the Tah18-Dre2 complex is part of an electron transfer chain involved in an early step of cytosolic Fe-S protein biogenesis and this complex can be functionally replaced by human Ndor1-Ciapin1 complex, with Ndor1 being a human homologue of Tah18.⁶⁴ Recently, AtCIAPIN1 was identified in a yeast 2-hybrid screen to interact with ATR3, an *Arabidopsis* homologue of Tah18.⁶⁵ These results suggest that this protein complex is involved in a pathway conserved among eukaryotes. AtCIAPIN1 was confirmed to be oxidized in leaves upon H_2O_2 treatment (Figure 4). In addition, AtCIAPIN1 also underwent oxidative modification when plants were treated with flg22 and SA (Figure 5), two inducers of plant defense response. It has been reported that the levels of H_2O_2

induced by flg22 and SA generally do not exceed several μM .^{66,67} Our results indicate that AtCIAPIN1 could be oxidatively modified by low levels of endogenously produced ROS. Alternatively, AtCIAPIN1 in the SA- or flg22-treated samples might not be directly oxidized by endogenous ROS, but by another protein through thiol-disulfide exchange reaction. The exact function of AtCIAPIN1 and its homologues is still unknown, but the sensitivity of AtCIAPIN1 to even low levels of ROS and the role of its yeast homologue plays in H_2O_2 -stimulated cell death suggest that AtCIAPIN1 might be an ancient redox sensor which integrates redox signals to the process of cell death.

Four Proteomic Methods are Complementary

The direct IAF or BIAM labeling methods are less tedious than the blocking-labeling methods, but they present a higher risk of false positives due to possible thiol-disulfide exchanges occurring during protein extraction. Additionally, direct labeling can potentially identify both reversibly and irreversibly oxidized proteins whereas the blocking-labeling methods only identify reversibly oxidized proteins. The BIAM-tagging methods reduced the complexity of protein samples loaded onto 2-DE, thus reduced the chance of false positives. However, since affinity purification of BIAM tagged proteins by NeutrAvidin agarose resin requires a non-denaturing condition, proteins need to be labeled by BIAM in their native configurations. Therefore, it is possible that some redox sensitive cysteines may be inaccessible to bulky BIAM probe for steric reasons, as reported before.⁶⁸

We initially expected that many redox-sensitive proteins would be identified by more than one method. However, our results showed that each method identified a very different set of proteins. The fact that only a small portion of them were identified by more than one method might be partly explained by the following examples. Assuming that protein X contains three thiols that are present in the reduced form in the control sample and the oxidant treatment causes two of the thiols to be oxidized. Such a change could be detected by the direct IAF labeling method because the protein will show a 3 fold (3:1) IAF labeling intensity in the control sample compared to that in the treated sample. In the direct BIAM tagging method, however, this protein in both the control and treated samples will be purified by the NeutrAvidin agarose resin regardless of the number of thiols tagged by BIAM. Therefore, the change in the redox state of such a protein will not be detected by the direct BIAM tagging method. On the other hand, assuming that protein Y also contains three free thiols but only one of them becomes oxidized in the oxidant-treated sample. Such a protein will not be identified by the direct IAF labeling method in our experiment as we set 2-fold difference in IAF labeling intensity as a threshold for identifying differentially labeled proteins. However, it will possibly be identified by the blocking-IAF labeling method because one thiol in protein Y from the oxidant-treated sample will be labeled with IAF but none of the thiols in protein Y from the control sample will be labeled with IAF. The above examples illustrate that the four methods we used are complementary in identifying redox-sensitive proteins.

CONCLUDING REMARKS

In summary, through the proteomics approaches, we have identified a number of *Arabidopsis* proteins that undergo rapid oxidative modifications in *Arabidopsis* suspension-cultured cells in response to a sublethal dose of H_2O_2 treatment. Each of the four proteomics approaches has its distinct limitations and

advantages, leading to the identification of four sets of proteins with small overlaps. To evaluate the validity and physiological relevance of the proteomics approaches used, we also established two methods for detailed analysis of individual putative redox-sensitive proteins in *Arabidopsis* plants. One such protein, AtCIAPIN1, was also shown to be oxidized upon treatment with SA and Flg22, two inducers of defense response. The identification of oxidant-sensitive proteins will be helpful toward in-depth characterization of other signaling pathways previously unknown to be redox-regulated.

ASSOCIATED CONTENT

Supporting Information

Table S1. Summary of redox-sensitive proteins identified by the four proteomic methods. Table S2. The list of identified peptides from the differentially labeled proteins. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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