RESEARCH ARTICLE

Proteomic analysis of S-nitrosylated proteins in *Arabidopsis thaliana* undergoing hypersensitive response

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Nitric oxide (NO) has a fundamental role in the plant hypersensitive disease resistance response (HR), and S-nitrosylation is emerging as an important mechanism for the transduction of its bioactivity. A key step toward elucidating the mechanisms by which NO functions during the HR is the identification of the proteins that are subjected to this PTM. By using a proteomic approach involving 2-DE and MS we characterized, for the first time, changes in S-nitrosylated proteins in *Arabidopsis thaliana* undergoing HR. The 16 S-nitrosylated proteins identified are mostly enzymes serving intermediary metabolism, signaling and antioxidant defense. The study of the effects of S-nitrosylation on the activity of the identified proteins and its role during the execution of the disease resistance response will help to understand S-nitrosylation function and significance in plants.

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1 Introduction

Growing evidence suggests that nitric oxide (NO) is a central molecule in several physiological functions of the plant, ranging from development to defense responses. Plants use NO as a signaling molecule in pathways comparable to those of mammals, suggesting the existence of many commonalities between the action of NO in plants and animals.

One of the best characterized functions of NO refers to its role in plant defense against pathogen attack, in particular in the establishment of the hypersensitive reaction (HR; [1, 2]). HR is a form of programed cell death that contributes to plant resistance by restricting the invading pathogen at the infection site and shows some regulatory and mechanistic features characteristic of apoptosis in animal cells like membrane dysfunction, vacuolization of the cytoplasm, chromatin condensation, and endonucleolytic cleavage of DNA [3]. Moreover, the HR determines the activation of a salicylic acid-dependent systemic response in the uninfected tissue, known as systemic acquired resistance (SAR) that limits subsequent infection by a broad range of pathogens over several weeks [4]. It is now clear that NO plays a key role during plant–pathogen interaction by modulating the HR, SAR, and defense gene induction [5]. Therefore, insights on how NO functions at the molecular and subcellular levels are critical to understand how it exerts its roles.

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Abbreviations: GAPDH, glyceraldehyde-3-phosphate dehydrogenase; **GSNO**, S-nitrosoglutathione; **HR**, hypersensitive reaction; **JA**, jasmonic acid; **MDH**, malate dehydrogenase; **MDHAR**, monodehydroascorbate reductase; **NO**, nitric oxide; **PGK**, phosphoglycerate kinase; **PRK**, phosphoribulokinase

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NO can perform important posttranslational protein modifications through S-nitrosylation [6], the formation of S-nitrosothiols by covalent addition to cysteine residues of a NO moiety. Because of their reactivity with intracellular reducing agents such as ascorbic acid or glutathione, the half-life of S-nitrosothiols is tightly regulated by the redox state of the cell and can be very brief, making protein S-nitrosylation a highly sensitive regulation mechanism that allow cells to flexibly and precisely adapt protein function in response to environmental signals [7]. Many examples of protein S-nitrosylation and functional consequences of this modification have been reported in animal systems. Regulation of procaspase-3 activation is just one example of how S-nitrosylation can regulate fundamental processes such as apoptosis [7] by switching protein activities on/off [6].

Emerging evidence indicates that S-nitrosothiol turnover may be part of the regulation of S-nitrosylation in addition to contributing to the regulation of NO biosynthesis. In animals, S-nitrosylation of the antioxidant tripeptide glutathione that produces S-nitrosoglutathione (GSNO) is proposed as possible reservoirs of NO bioactivity [8]. An enzyme that metabolizes GSNO, namely GSNO reductase, has been shown to control intracellular levels of GSNO and S-nitrosylated proteins in yeast and mice [9]. Recently, a protein possessing GSNO reductase activity has been found in *Arabidopsis thaliana* [10, 11], and transgenic plants with altered levels of *AtGSNOR1* has provided first insights into its function in controlling cellular S-nitrosothiol levels under regular metabolic conditions as well as during plant pathogen interactions [11, 12]. Since the loss of AtGSNOR1 function compromises plant defense responses to pathogenic infections [12], it is likely that S-nitrosylated molecules are involved in modulating signaling pathways in plants. Knowledge on S-nitrosylation target proteins in plants is scarce. Recently, a proteomic approach led to the identification of 63 proteins from cell culture extracts treated with GSNO and 52 proteins from leaves treated with NO, therefore confirming the existence of targets for S-nitrosylation in *A. thaliana* [13]. Experimental evidence of regulation by S-nitrosylation has been only described for three plant proteins [14–16], and very little is known about the extent and physiological function of S-nitrosylation in plants [5]. To get insight into the regulatory function of NO during the hypersensitive disease resistance response we characterized, for the first time, changes in S-nitrosylated proteins in *A. thaliana* after challenge with an incompatible bacterial pathogen.

2 Materials and methods

2.1 Growth conditions and plant material

A. thaliana ecotype Col-0 plants were grown in soil-culture in a light/dark cycle of 10/14 h (22/20°C) at light intensities of 120 μmol·quanta $m^{-2} \cdot s^{-1}$ with 50% relative humidity.

2.2 "Biotin switch"

To detect S-nitrosylated proteins in plants, we adopted the biotin-switch method [17], with slight modification. *A. thaliana* leaves were homogenized in MAE buffer (25 mM HEPES, 1 mM EDTA, 0.2% Triton X-100, pH 7.7) containing complete protease inhibitor cocktail (Sigma, St. Louis). The extract was centrifuged at 4°C for 30 min, and the protein concentration in the supernatant was measured using a Bradford assay (BioRad, Hercules, CA). When necessary, proteins were incubated with the S-nitrosylating agent GSNO (200 μM), GSH (200 μM), or GSSG (200 μM) in the dark at room temperature for 30 min with regular vortexing. Treatment with the reducing agent DTT (20 mM) was carried out for 1 h under the same conditions. Reagents were then removed by two precipitations with two volumes of cold acetone or by gel filtration through a MicroBioSpin 6 (BioRad).

For the experiments with pathogens, *A. thaliana* leaves were infiltrated with *Pseudomonas syringae* pv. *tomato* (Pst) or with *Pst* carrying the *avrB* avirulence gene (Pst avrB). Leaf extracts were obtained at the indicated times postinfiltration. Next, 600 μg of protein extracts were incubated with 20 mM methyl-methanethiosulfate (MMTS) and 2.5% SDS at 50°C for 30 min with frequent vortexing to block free cysteines. Excess MMTS was removed by precipitation with two volumes of cold acetone, and proteins were resuspended in 0.1 mL RB buffer (25 mM HEPES, 1 mM EDTA, and 1% SDS, pH 7.7) *per* milligram of protein. After the addition of 1 mM HPDP-biotin (Pierce, Rockford, IL) and 1 mM ascorbic acid, the mixture was incubated 1 h at room temperature in the dark with intermittent vortexing. Proteins were then subjected to Western blot analysis using an antibiotin antibody.

2.3 Purification of S-nitrosylated proteins

Biotinylation of S-nitrosylated proteins was carried out as described above with slight modifications. For Western blot analysis the starting material was 1–2 mg of protein. For proteomic analysis, the starting material was 4 mg of protein when the gels were stained with Sypro Ruby and 25 mg of protein when stained with CBB. In addition, protein incubation with HPDP-biotin was extended to 90 min, HPDP-biotin was removed by protein precipitation with two volumes of cold acetone, and proteins were resuspended in 0.1 mL of RB buffer (25 mM HEPES, 1 mM EDTA, and 1% SDS, pH 7.7) *per* milligram of protein and 2 volumes of neutralization buffer (25 mM HEPES, 1 mM EDTA, 100 mM NaCl, and 0.5% Triton X-100, pH 7.7). A total of 30 μL of neutravidin-agarose *per* milligram of protein were added and incubated 1 h at RT and then overnight at 4°C. The matrix was washed extensively with 10 volumes of washing buffer (600 mM NaCl in neutralization buffer) and bound proteins were eluted with 150 mM β-mercaptoethanol in neutralization buffer.

2.4 2-DE and gel staining

Immediately after affinity separation by the biotin-switch method, purified proteins were suspended in a buffer containing 7 M urea, 2 M thiourea, 3% w/v CHAPS, 1% v/v carrier ampholytes (pH 3–10), 40 mM Tris, 5 mM TBP (as reducing agent), and stored at room temperature for 90 min. Samples were then mixed with 20 mM iodoacetamide (IAA), incubated for alkylation at room temperature for 90 min [18] and loaded onto a 17 cm IEF strip, pH 4–7. The IPG strips were rehydrated with the proteins containing traces of bromophenol blue for monitoring the electrophoretic run. IEF was performed with a low initial voltage and then by applying a voltage gradient up to 10 000 V with a limiting current of 50 μA/strip. The total product time × voltage applied was 75 000 V·h for each strip and the temperature was set at 20°C. The strips were then equilibrated with an SDS denaturing solution containing 6 M urea, 2% SDS, 20% glycerol, 0.375 M Tris-HCl (pH 8.8), and loaded onto the second dimension using 0.5% agarose solubilized in cathode buffer (192 mM glycine, 0.1% SDS, Tris to pH 8.3) for the interfacing, and gels with a porosity gradient from 10 to 18% w/v acryl/bisacrylamide and 1.5 mm thickness. The second dimension run was performed by using a PROTEAN II xl Multi-Cell (BioRad) set at 11°C and 10 mA/gel until the end of the run. The gels where then stained for Sypro Ruby and for CBB as described [19, 20]. Samples were digitized with a Versa Doc Scanner (BioRad,).

2.5 Protein pattern and statistical analysis

The digitized images were acquired with the software PDQuest 6.2 (BioRad). A standard gel was generated out of the image with the highest spot number. Spot quantities of all gels were normalized by removing nonexpression-related variations in spot intensity; the raw quantity of each spot in a gel was divided by the total quantity of all the spots in that gel and included in the standard. The final synthetic image was a Gaussian scan image that contained all the Gaussian spots with a defined volume and quality. All subsequent spot matching and analysis steps in the PDQuest software were performed on Gaussian spots. The results were evaluated in terms of spot OD. Statistical analysis (Student's *t*-test) *via* PDQuest allowed the study of proteins that were significantly ($p<0.05$) increased or decreased in the three sets of samples.

2.6 In-Gel digestion, MS and database searching

Spots of interest were excised from gels using a razor blade and subjected to in-gel trypsin digestion according to [21] with minor modifications. The gel pieces were swollen in a digestion buffer containing 50 mM NH_4HCO_3 and 12.5 ng/μL of trypsin (modified porcine trypsin, sequencing grade, Promega, Madison, WI) in an ice bath. After 30 min the supernatant was removed and discarded, 20 μL of 50 mM NH_4HCO_3 were added to the gel pieces and digestion allowed to proceed at 37°C overnight. After digestion, the remaining supernatant was removed and stored at −20°C until use. Peptides were then desalted and concentrated according to [22]. Homemade 5 mm nanocolumns were packed with POROS R2 chromatographic resin (PerSeptive Biosystems, Framingham, MA) in a constricted GELoader tip (Eppendorf, Hamburg, Germany). A syringe was used to force liquid through the columns by applying a gentle air pressure. The columns were equilibrated with 20 μL of 5% formic acid (FA) and the analyte solutions were added. The columns were washed with 20 μL of 5% FA and the bound peptides subsequently eluted directly onto the MALDI target with 0.5 μL of CHCA solution (5 μg/μL in ACN, 0.1% TFA, 70:30 v/v). Samples were analyzed on an Applied Biosystems 4700 Proteomics Analyzer MALDI-TOF/TOF (Applied Biosystems, Framingham, MA, USA). Positively charged ions were analyzed in reflectron mode and the collision gas used for fragmentation was atmospheric air. The MoverZ software (Proteometrics, Winnipeg, Canada) and the Data Explorer (Applied Biosystems) were used to analyze spectra. The spectra were calibrated using trypsin autolysis products (m/z 842.504 and 2211.109) as internal standards. In a few cases this was not possible and external calibration was carried out using peaks from the tryptic digest of β-lactoglobulin. Protein identification was performed by searching in the National Center for Biotechnology Information nonredundant database (NCBInr) using MASCOT program (http://www.matrixscience.com). The following parameters were used for database searches: all entries, peptide tolerance at 70 ppm, MS/MS tolerance at 0.5 Da, carbamidomethylation of cysteine (fixed modification), methionine oxidation (variable modifications). For positive identification, the score of the result of ($-10 \times \log(p)$) had to be over the significance threshold level ($p<0.05$).

2.7 Western blot analysis

Immunoblotting was performed using standard protocols [23]. Protein samples were loaded in 12% SDS-PAGE and transferred onto PVDF (Millipore, Bedford, MA, USA) membranes. The membrane was stained with Ponceau red to check for equivalency in protein loading. For detection of PrxII E, antibodies were produced against proteins heterologously expressed in *Escherichia coli* [24] at a 1:2000 dilution. For detection of monodehydroascorbate reductase (MDHAR), polyclonal antibodies against cucumber MDAR [25] were used at a 1:2000 dilution. Antibiotin antibody was used at 1:10 000 dilution. Probing and detection of immunocomplexes were performed as described for the SuperSignal West Pico detection system (Pierce, Rockford, IL).

3 Results

3.1 Detection of S-nitrosylated proteins in *A. thaliana* leaf extracts

To detect S-nitrosylated proteins we used the biotin-switch method, which biotinylates S-nitrosylated cysteines [17]. We first tested the method to detect S-nitrosylated proteins in plants, as recently reported [13]. Protein extracts from *A. thaliana* leaves were incubated with either the trans-nitrosylating agent GSNO to induce S-nitrosylation or with the reducing agent DTT to remove the SNO bonds as a negative control, subjected to the biotin switch and then to Western blot analysis (Fig. 1). Several bands were detected in non-treated extracts, indicating the presence of endogenously S-nitrosylated proteins. The signal was significantly increased in GSNO-treated extracts and strongly reduced in DTT-treated extracts, as expected. Endogenously biotinylated proteins were not detected, and the antibiotin antibody showed no nonspecific crossreaction with unlabeled proteins, since no signal was detected in vehicle (minus biotin) treated samples (Fig. 1).

It has been suggested that the biotin-switch method may also detect S-glutathionylated proteins [26]. However, treatments with reduced and oxidized glutathione (GSH, GSSG) did not show any differences with respect to untreated extracts (data not shown). Therefore, the method was considered suitable for studying the pattern of S-nitrosylated proteins in *A. thaliana* during the HR.

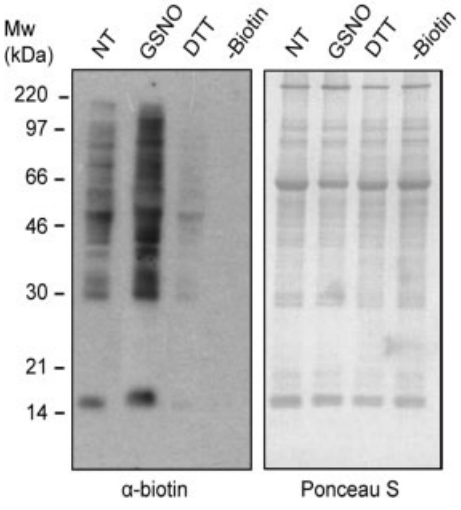

Figure 1. Detection of S-nitrosylated proteins in *A. thaliana* leaf extracts. Protein extracts (200 μg) from *A. thaliana* leaves not treated (NT) or treated with 1 mM GSNO or 20 mM DTT were subjected to the biotin-switch assay, separated by SDS-PAGE and immunoblotted with an antibiotin antibody. The absence of endogenously biotinylated proteins was verified by loading a sample not subjected to the biotin-switch (-Biotin). Protein loading was verified by Ponceau staining of the membrane. The experiment was repeated three times with similar results.

3.2 Proteomic analysis of S-nitrosylated proteins during the HR

Infiltration of leaves from *A. thaliana* Col-0, which contain the *RPM1* resistance gene, with *P. syringae* pv. *tomato* carrying the *avrB* avirulence gene (Pst avrB), leads to rapid accumulation of ROS and NO with subsequent HR cell death [27]. Although Western blot analysis from 1-D protein separation using an antibiotin antibody did not show significant differences in the pattern of S-nitrosylated proteins from *A. thaliana* leaves challenged with the avirulent pathogen and subjected to the biotin-switch assay, a clear increase in the signal during the progression of the HR was observed (Fig. 2). We therefore combined the biotin-switch method with a proteomic approach. S-nitrosylated proteins extracted from *A. thaliana* leaves undergoing HR were subjected to the biotin-switch method, purified by affinity chromatography on a neutravidin-agarose matrix and then separated by 2-DE. Four biological replicates were carried out at each time point (0, 4, and 8 h postinfection). More than 100 proteins were detected in the pH range between 4 and 7, and were included in the analysis. Twelve proteins showed differences in 0 *versus* 4 h, 14 proteins in 4 *versus* 8 h, and 4 proteins in 0 *versus* 8 h. Avoiding redundancy, 18 spots showed an intensity variation greater than two-fold ($p<0.05$) during the defense response (Fig. 3). To characterize the S-nitrosylated proteins of interest, additional 2-D gels were produced starting from a larger amount of material. Selected spots stained with CBB were excised from gels, analyzed by MALDI-TOF/TOF MS and unambiguously identified as members of different protein families including stress-related proteins, redox-related proteins, signaling-regulating proteins and metabolic enzymes

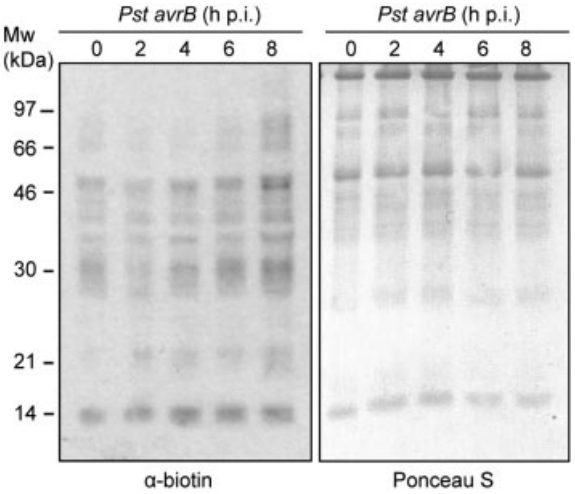

Figure 2. Protein S-nitrosylation in *A. thaliana* undergoing HR. Protein extracts from *A. thaliana* leaves at indicated times post-infiltration with 10^8 cfu/mL avirulent *P. syringae* (Pst avrB) were subjected to the biotin-switch method. Proteins were then separated by SDS-PAGE and immunoblotted with an antibiotin antibody. The experiment was repeated two times with similar results.

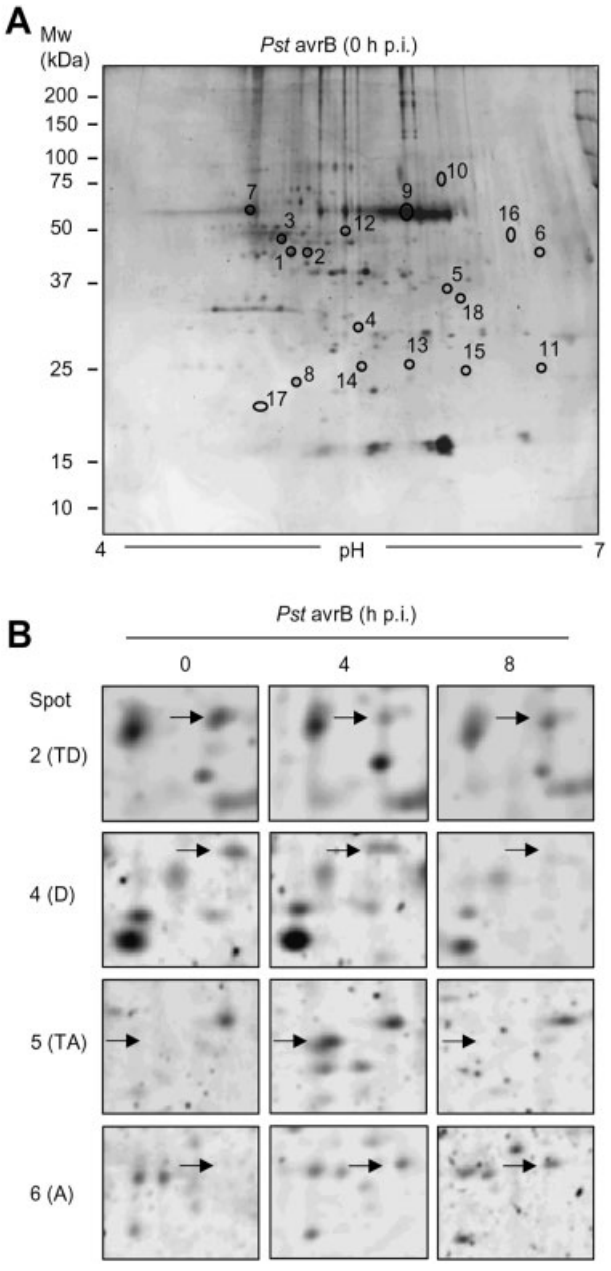

Figure 3. Identification of differentially S-nitrosylated proteins in *A. thaliana* undergoing HR. Protein extracts from *A. thaliana* leaves 0, 4, and 8 h postinfiltration with 10^8 cfu/mL avirulent *P. syringae* (Pst avrB) were subjected to the biotin-switch method. S-nitrosylated proteins were purified by affinity chromatography on a neutravidin-agarose matrix, separated by 2-DE, stained with Sypro Ruby and subjected to digital fluorescent image analysis. For each condition, four replicas of the entire process were performed. (A) The protein spots revealing statistically significant changes in density between treatments and time points are indicated on a 2-D gel representative of time 0 h. (B) Abundance of selected S-nitrosylated proteins representative of the different S-nitrosylation patterns observed. T, D, and A indicate a transient, disappearing or appearing S-nitrosylation pattern. Numeric labeling of the spots corresponds to the number assigned in Table 1.

(Fig. 4 and Table 1). Their S-nitrosylation pattern during the progression of the HR is reported in Table 2. About 50% of the proteins identified have previously been reported as being subject to S-nitrosylation, thus supporting the reliability of our results (Table 2).

To verify the reliability of the proteomic analysis, we assessed differential S-nitrosylation of type II peroxiredoxin E (PrxII E) and monodehydroascorbate reductase (MDHAR), two antioxidant defense enzymes involved in ROS scavenging that have not been previously detected as being S-nitrosylated in plants. Protein extracts from *A. thaliana* leaves challenged with Pst avrB or Pst were subjected to the biotin-switch method and purified on neutravidin-agarose. Western blot analysis with antiPrxII E and antiMDHAR antibodies confirmed the S-nitrosylation of the two proteins at 4 and 8 h after incompatible pathogen challenge but not during the compatible interaction (Fig. 5A), therefore confirming that S-nitrosylation of the identified proteins is a specific response. To determine whether this observation reflected an increase in S-nitrosylation of previously existing protein or an increase

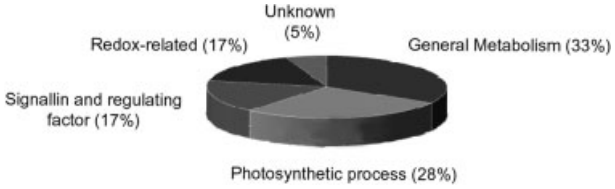

Figure 4. Functional categorization of differential S-nitrosylated proteins identified in *A. thaliana* undergoing HR.

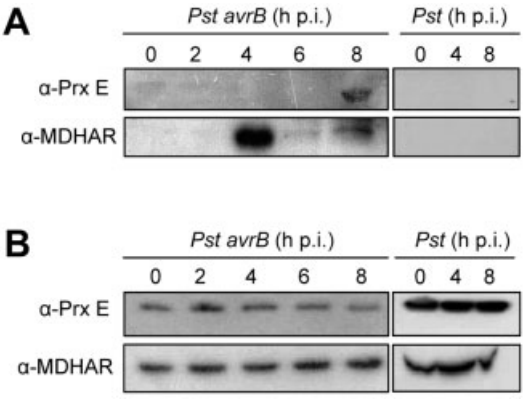

Figure 5. S-nitrosylation pattern of PrxII E and MDHAR following pathogen challenge. (A), Protein extracts from *A. thaliana* leaves at indicated times after infiltration with 10^8 cfu/mL avirulent (Pst avrB) or virulent (Pst) *P. syringae* were subjected to the biotin-switch method. S-nitrosylated proteins were then purified by affinity chromatography on a neutravidin–agarose matrix and subjected to Western blot analysis with the indicated antibodies. (B) Variation in PrxII E and MDHAR protein accumulation during the HR was monitored by Western blot analysis of protein extracts as described above not subjected to the biotin switch. The experiments were repeated three times with similar results.

Table 1. Proteins and peptide sequences identified from selected spots by MALDI-TOF/TOF MS

Spot No.	Identified protein (A. thaliana)	Peptide sequence (ion score)
1	PRK precursor	LTSVFGGAAKPPK (65)
		FYGEVTQQMLK (52)
		KPDFDAFIDPQK (100)
		ILVIEGLHPM(ox)FDER (73)
2		ANDFDLM(ox)YEQVK (69)
		ILVIEGLHPM(ox)FDER (39)
		FYGEVTQQM(ox)LK (56)
		IRDLYEQLIANK (52)
3	Putative PGK	ADLNVPLDDNQTITDDTR (148)
		FSLAPLVPR (60)
		LASLADLYVNDAFGTAHR (99)
		ELDYLVGAVSNPK (106)
		RPFAAIVGGSK (41)
		IVPASGIEDGWM(ox)GLDIGPDSIK (57)
4	Cytosolic triosephosphate isomerase	FFVGGNWK (54)
		VAYALAQGLK (80)
		VIACVGETLEER (68)
		AILNESSEFVGDK (85)
		SDFFVAAQNCWVK (48)
		VASPAQAQEVHDELRK (60)
		VTNWSNVVIAYEPVWAIGTGK (69)
		ELGGQADVDGFLVGGASLKPEFIDIIK(120)
5	Putative mitochondrial NAD-dependent MDH	ALEGADLVIIPAGVPR (49)
		SEVVGYMGDDNLAK (37)
6	Putative RNA binding protein	EGHQVTLFTR (56)
		EAEEVEPILEALPK (107)
		AGGFPEPEIVHYNPK (93)
		QLPGESDQDFADFSSK (112)
	GAPDH (NADP+) (phosphorylating)	TFAEEVNAAFR (54)
		MASVTFSVPK (71)
		VPTPNVSVVDLVVQVSK (67)
7	Large subunit of ribulose 1,5-bisphosphate carboxylase/oxygenase	DTDILAAFR (42)
		VALEACVQAR (37)
		DNGLLLHIHR (33)
		DLAVEGNEIIR (47)
		TFQGPPHGIQVER (79)
		EITFNFPTIDKLDGQE (62)
8		LTYYTPEYETK (39)
		LTYYTPEYETKDTDILAAFR (95)
		DTDILAAFR (55)
		ALAALRLEDLR (48)
		TFQGPPHGIQVER (69)
9		DTDILAAFR (65)
		VALEACVQAR (62)
		DLAVEGNEIIR (69)
		FLFCAEAIYK (80)
		EITFNFPTIDK (43)
		TFQGPPHGIQVER (102)
		YGRPLLGCTIKPK (39)
		WSPELAAACEVWK (78)
		GHYLNATAGTCEEMIK (42)
		EITFNFPTIDKLDGQE (73)
		GGLDFTKDDENVNSQPFMR (88)
		ELGVPIVMHDYLTGGFTANTSLSHYCR (110)

Table 1. Continued

Spot No.	Identified protein (A. thaliana)	Peptide sequence (ion score)
10		DTDILAAFR (56)
		VALEACVQAR (41)
		DLAVEGNEIIR (59)
		ALAALRLEDLR (50)
		TFQGPPHGIQVER (64)
		NEGRDLAVEGNEIIR (53)
		LEGDRESTLGFVDLLR (37)
		GGLDFTKDDENVNSQPFM(ox)R (45)
11	Oxygen-evolving enhancer protein 3 precursor-like protein	VGGPPLPSGGLPGTDNSDQAR (149)
		FYIQPLSPTEAAAR (112)
		LFQTIDNLDYAAR (113)
	Photo system II oxygen-evolving complex protein 3-like	FYLQPLPPTEAAAR (97)
		DIINVKPLIDR (61)
12	Putative translation elongation factor EF-Tu precursor, chloroplast	VGETVDLVGLR (59)
		KYDEIDAAPEER (57)
		ILDEALAGDNVGLLLR (137)
		HSPFFAGYRPQFYM(ox)R (44)
		IYELMDAVDDYIPIPQR (73)
		IYELM(ox)DAVDDYIPIPQR (39)
13	nClpP1	EYGLIDGVIM(ox)NPLK (58)
		INQDTDRDFFM(ox)SAK (40)
		EAKEYGLIDGVIM(ox)NPLK (60)
		ANLNGYLAYHTGQSLEK (52)
14	AOC	ALSQNGNIENPRPSK (36)
		VQELSVYEINELDR (61)
		VQELSVYEINELDRHSPK (42)
15	Germin-like protein	AAVTPAFAPAYAGINGLGVSLAR (57)
		GPQSPSGYSCK (35)
16	Putative MDHAR protein	EAVAPYERPALSK (52)
		GYLFPEGAAR (72)
		GADSKNILYLR (71)
		NILYLR (38)
		AAEGGAAVEEYDYLPFFYSR (66)
		FGAYWVQGGK (40)ARPSAESLDELVK (33)
17	Peroxiredoxin II E	RYAILADDGVVK (42)
		TILFAVPGAFTPTCSQK (113)
		VLNLEEGGAFTNSSAEDM(ox)LK (132)
18	At5g35100	IFPGQYFLAGR (56)
		QFNDFAEFLGDER (44)
		VFLDFSLCPTYFR (42)

in protein synthesis, protein extracts from *A. thaliana* leaves challenged with Pst avrB were immunoblotted and analyzed with the antiPrxII E and antiMDHAR antibodies. The abundance of the two proteins did not change, indicating S-nitrosylation of the constitutive pools of PrxII E and MDHAR during the HR (Fig. 5B).

4 Discussion

The recent demonstration that S-nitrosothiols play a central role in the regulation of multiple modes of plant disease resistance [12] prompted us to monitor changes in the S-nitrosylated proteome during the hypersensitive disease resistance response. Direct detection of this modification has been hampered by its liability, especially evident in the breakage of the nitrosothiol during MALDI analysis [28]. The biotin-switch method [17] overcomes this limitation by replacing S-nitrosylation with the more stable biotinylation. We coupled the biotin-switch method with affinity purification of biotinylated proteins, followed by 2-DE, to analyze the protein S-nitrosylation pattern of *A. thaliana* subjected to a pathogen challenge *in vivo*. In our analysis we detected 18 spots showing statistically significant changes in density

Table 2. Proteins identified as differentially S-nitrosylated in *A. thaliana* undergoing HR

Tendency	Identified protein (*A. thaliana*)	Acc. no. (NCBInr)	Mw Calc p*I*	Id. pept./seq. cov. (%)	Cited as S-nitrosylated
TD	PRK precursor	gi|23197622	44721 5.71	18/60%	–
D	Putative PGK	gi|17065610	50195 5.91	18/47%	*Lindermayr *et al.*, 2005 [13]
D	Cytosolic triosephosphate isomerase	gi|7076787	27380 5.39	14/57%	*Lindermayr *et al.*, 2005 [13]
TA	Putative mitochondrial NAD-dependent MDH	gi|12642848	36010 8.54	5/23%	*Foster and Stamler, 2005 [34]
A	Putative RNA binding protein	gi|3850621	42303 7.71	18/43%	
A	GAPDH (NADP+) (phosphorylating)	gi|1402885	42766 7.62	16/42%	*Lindermayr *et al.*, 2005 [13]
TD TA	Large subunit of ribulose 1,5-bisphosphate carboxylase/oxygenase	gi|5881702	53435 5.88	25/48%;10/ 26%;33/56% 18/38%	Lindermayr *et al.*, 2005 [13]
TA	Oxygen-evolving enhancer protein 3 precursor-like protein	gi|21593587	24628 9.72	8/40%	
TA	Photosystem II oxygen-evolving complex protein 3-like	gi|21593902	23781 9.64	10/48%	
A	Putative translation elongation factor EF-Tu precursor, chloroplast	gi|24030503	51883 5.84	19/44%	*Rhee *et al.*, 2005 [42]
TA	nClpP1	gi|5360579	32506 8.35	8/30%	–
TA	AOC	gi|34391982	27732 6.90	6/33%	–
TA	Germin-like protein	gi|1755188	19382 5.86	3/28%	–
A	Putative MDHAR protein	gi|22022508	46629 6.41	19/50%	–
A	Peroxiredoxin II E	gi|15231718	24783 9.12	12/49%	*Lindermayr *et al.*, 2005 [13]
TA	At5g35100	gi|51536584	30771 6.82	8/30%	–

Previous reports of the proteins (or related *) as S-nitrosylated are presented. (T, D, A) indicate a transient, disappearing, or appearing S-nitrosylation pattern during the defense response, respectively. Acc. no., accession number; Mw, molecular weight; p*I*, isoelectric point; Id. pept., number of identified peptides; seq. cov., sequence coverage.

during progression of the hypersensitive response to Pst avrB infection. The analysis of these spots by MALDI-TOF/TOF MS resulted in the identification of 16 proteins, which belong to diverse classes such as stress-related proteins, signaling proteins, or metabolic enzymes.

The identification of enzymes from the glycolysis, oxidative phosphorylation, and the pentose phosphate pathway as targets of S-nitrosylation in *A. thaliana* plants undergoing HR suggests that NO can modulate the concentration of metabolites and the balance of energy status. Indeed, of the five enzymes involved in general metabolism, three belong to glycolysis pathway: phosphoglycerate kinase (PGK), triosephosphate isomerase, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH). All of them have previously been described as candidates for S-nitrosylation in *A. thaliana* treated with an exogenous NO donor [13]. Interestingly, triosephosphate isomerase is known to be S-glutathionylated during oxidative stress without any dramatic effect on its activity [29] although the *A. thaliana* recombinant protein is reversibly inactivated by oxidized glutathione [30]. GAPDH is a target of S-nitrosylation in animal and plant tissues, and its NO-dependent reversible inhibition of enzymatic activity is a clear example of how this modification may affect protein function by inactivation of the cysteine residue in the protein active center [13, 31]. In addition to its well known glycolytic functions, GAPDH is involved in nuclear events such as transcription, RNA transport or DNA replication [32]. Recent works have shown that GADPH influences Siah1-dependent

cytotoxicity by moving to the nucleus during apoptosis. The S-nitrosylation of the enzyme would increase GADPH binding to Siah1, stabilizing it to enhance the degradation of nuclear proteins, which leads to cell death [33]. If GADPH is involved through S-nitrosylation in the hypersensitive response-related cell death cascade in plants is still unknown.

Mitochondrial ATP synthesis *via* oxidative phosphorylation is fuelled by NADH generated during glycolysis. Our analysis revealed that the mitochondrial NAD-dependent malate dehydrogenase (MDH), enzyme that take part in the malate–aspartate shuttle and involved in ATP generation through NADH, changes its S-nitrosylation pattern during progression of the HR. MDH has also been found candidate for S-nitrosylation in rat liver mitochondrial extracts exposed to GSNO [34]. Although the effect of this modification on the activity of the protein have not been studied yet, it is well known that the oxidation of the –SH groups is known to inactivate the enzyme [35].

Finally, this work has shown for the first time the S-nitrosylation of phosphoribulokinase (PRK), a unique enzyme in the reductive pentose phosphate pathway which primary functions are generate reducing equivalents (NADPH) and provide the cell with ribose-5-phosphate (R5P) for the synthesis of the nucleotides and nucleic acids. PRK activity depends on thioredoxin-mediated reduction [36], and alkylation and site-directed mutagenesis have previously revealed the existence of a regulatory disulfide bridge [37, 38].

A second group of proteins found to be differentially S-nitrosylated after pathogen challenge includes several enzymes involved in the photosynthetic process, suggesting NO-mediated regulation of the cellular energy transduction system. The most relevant was Rubisco, a key enzyme of the Calvin–Benson cycle, which is regulated by light in a redox-dependent manner [39]. Its large subunit was previously found to be S-nitrosylated in *A. thaliana* plants treated with exogenous NO donors [13]. *A. thaliana* Rubisco presents a Cys residue adjacent to the active site that has been suggested to play a role in the activity and degradation of the protein [40]. Therefore, it is likely that S-nitrosylation of the protein regulates its activity and probably its turnover. We also identified two proteins of photosystem II (oxygen-evolving enhancer protein 3 precursor-like protein and photosystem II oxygen-evolving complex protein 3-like), which had previously been proposed to be NO targets [41].

A third group of proteins show homology to signaling and regulating factors. These are the elongation factor EF-Tu, the protease ClpP1, and the allene oxide cyclase (AOC). The identification of ClpP1 and AOC as targets of S-nitrosylation is described here for the first time, whereas EF-Tu was previously reported as S-nitrosylated in *Mycobacterium tuberculosis* treated with bactericidal concentrations of reactive nitrogen species [42]. Other elongation and initiation factors had been identified in *A. thaliana* treated with NO or NO donors [13] suggesting that NO, hence presumably S-nitrosylation, could be involved in the regulation of protein synthesis and turnover. Consistent with this idea is the transient S-nitrosylation of the serine-protease ClpP1 observed during the HR. This protein belongs to the Clp protease family [43], which appears to be involved in the degradation of thylakoid membrane proteins [44, 45].

AOC catalyzes the stereospecific cyclation of an unstable allene oxide to the ultimate precursor of jasmonic acid (JA; [46]) and seems to be the preferential target in the regulation of JA biosynthetic capacity [47]. Since plant responses to many biotic and abiotic stresses are orchestrated locally and systemically by signaling molecules known as the jasmonates [48], S-nitrosylation and putative regulation of AOC could link NO to JA biosynthesis. Further studies are now needed to investigate possible crosstalk between NO and JA-dependent signaling pathways, especially during defense response.

The last group of proteins is represented by redox-related proteins or proteins related to the plant antioxidant system. The three proteins involved in the antioxidant machinery are of particular interest because at the onset of the HR the relative rates of NO and ROS, in particular O_2^- and H_2O_2, are critical in channeling NO through the cell death program. The first is a germin-like protein, a member of a large family of proteins that based on their expression pattern and oxalate oxidase activity are suggested to function both in cell expansion during development and in resistance to stress, especially pathogen infection [49]. The second is the monodehydroascorbate reductase (MDHAR), an FAD enzyme that catalyzes the reduction of MDA radical to ascorbate using NAD(P)H as an electron donor. MDHAR is part of the ascorbate-glutathione enzymatic antioxidant cycle and its activity is inhibited by thiol-modifying reagents [50] which suggest that S-nitrosylation could be involved in the regulation of enzymatic activity. The third is a type II peroxiredoxin, PrxII E, a protein that reduces hydrogen peroxide and alkyl hydroperoxides [24]. Although S-nitrosylated PrxII E was observed for the first time in this work, a few other peroxiredoxins had previously been detected as possible S-nitrosylation target not only in *A. thaliana* treated plants [13], but also in endothelial cells [51]. We have investigated the molecular mechanism for S-nitrosylation of PrxII E and found that it inhibits both the peroxidase and the peroxynitrite reductase activities of PrxII E, thus revealing a novel regulatory mechanism for peroxiredoxins [52]. This suggests that NO may regulate the effects of its own radicals through S-nitrosylation of crucial components of the antioxidant defense system that function as common triggers for ROS and NO-mediated signaling events. The full range of biological functions for these molecules remains to be cataloged, and determining the ways in which they interact still has to be fully elucidated.

In sum, this work has identified S-nitrosylated proteins in *A. thaliana* plants during the disease resistance response, paving the way to further studies on the effects of S-nitrosylation on the activity of the identified proteins, and its role during the progression of hypersensitive response that will help to understand S-nitrosylation function and significance in plants.

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