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S-Nitrosylated proteins in pea (*Pisum sativum* L.) leaf peroxisomes: changes under abiotic stress

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Abstract

Peroxisomes, single-membrane-bounded organelles with essentially oxidative metabolism, are key in plant responses to abiotic and biotic stresses. Recently, the presence of nitric oxide (NO) described in peroxisomes opened the possibility of new cellular functions, as NO regulates diverse biological processes by directly modifying proteins. However, this mechanism has not yet been analysed in peroxisomes. This study assessed the presence of S-nitrosylation in pea-leaf peroxisomes, purified S-nitrosylated peroxisome proteins by immunoprecipitation, and identified the purified proteins by two different mass-spectrometry techniques (matrix-assisted laser desorption/ionization tandem time-of-flight and two-dimensional nano-liquid chromatography coupled to ion-trap tandem mass spectrometry). Six peroxisomal proteins were identified as putative targets of S-nitrosylation involved in photorespiration, β-oxidation, and reactive oxygen species detoxification. The activity of three of these proteins (catalase, glycolate oxidase, and malate dehydrogenase) is inhibited by NO donors. NO metabolism/S-nitrosylation and peroxisomes were analysed under two different types of abiotic stress, i.e. cadmium and 2,4-dichlorophenoxy acetic acid (2,4-D). Both types of stress reduced NO production in pea plants, and an increase in S-nitrosylation was observed in pea extracts under 2,4-D treatment while no total changes were observed in peroxisomes. However, the S-nitrosylation levels of catalase and glycolate oxidase changed under cadmium and 2,4-D treatments, suggesting that this post-translational modification could be involved in the regulation of H_2O_2 level under abiotic stress.

Key words: Abiotic stress, cadmium, 2, 4-dichlorophenoxy acetic acid, nitric oxide, S-nitrosylation, peroxisomes.

Introduction

Peroxisomes are organelles that have an essentially oxidative type of metabolism (Huang *et al.*, 1983; Fahimi and Sies, 1987; del Río *et al.*, 2006). The important role of plant peroxisomes in a variety of metabolic reactions, including photorespiration, fatty acid β-oxidation, the glyoxylate cycle, and in the biosynthesis of plant hormones such as auxin and jasmonic acid are well known (del Río *et al.*, 2009). Recent proteomic studies have identified new proteins that appear to have novel metabolic and regulatory functions of peroxisomes, such as the production of compatible osmosolute Gly betaine and the degradation of branched amino acids (Reumann *et al.*, 2004). Furthermore, novel regulatory proteins in peroxisomes, such as heat-shock proteins, kinases, and

phosphatases have been reported (Hayashi and Nishimura, 2006; Reumann *et al.*, 2007).

Peroxisomes are also essential in plant responses to abiotic and biotic stress (Romero-Puertas *et al.*, 1999; Koh *et al.*, 2005; Rodríguez-Serrano *et al.*, 2009), increasing even during different stress conditions such as xenobiotics, ozone, cadmium, H_2O_2, and light (del Río *et al.*, 2009). In the last two decades, plant peroxisomes have been shown to produce reactive oxygen species (ROS) such as hydrogen peroxide (H_2O_2) and the superoxide radical (O_2^-) as a consequence of their normal metabolism (reviewed by del Río, 2011). In addition, a complex battery of antioxidative systems had been described, including catalase (CAT), superoxide dismutase

(Sandalio and del Río, 1987; del Río *et al.*, 2002), and the ascorbate–glutathione cycle (Jimenez *et al.*, 1997; Mittova *et al.*, 2004; Kuzniak and Sklodowska, 2005; Romero-Puertas *et al.*, 2006). Recently, the presence of an L-arginine-dependent nitric oxide synthase as well as nitric oxide (NO) in plant peroxisomes have also been shown (Barroso *et al.*, 1999; Corpas *et al.*, 2004). In plants, NO is a key signalling molecule involved in several physiological processes from the development to the defence response to both biotic and abiotic stress (Delledonne *et al.*, 2004; Neill *et al.*, 2008). The occurrence of NO in peroxisomes adds new cellular functions to these organelles related to oxygen and nitrogen reactive species (ROS/RNS). No function of NO in peroxisomes, however, has been discovered so far.

The way by which NO undertakes a plethora of physiological functions is still largely unknown. Some time ago in animal tissues, and very recently in plant tissues, it was shown that NO regulates diverse biological processes by directly modifying proteins. In fact, NO and RNS can oxidize, nitrate, or nitrosylate proteins. S-Nitrosylation refers to the binding of a NO group to a cysteine residue and it plays a significant role in NO-mediated signalling (Stamler *et al.*, 2001). Recent evidence suggests that many proteins are S-nitrosylated in plants under physiological or stress conditions, leading to the first insights into S-nitrosylation-dependent regulation of protein function (Lindermayr and Durner, 2009). Although the mechanism remains unknown, several peroxisomal proteins have been shown to be regulated by NO. Thus, the activity and protein level of glutathione peroxidase is down-regulated by NO, whereas the activity of the peroxisomal H_2O_2-producing β-oxidation is stimulated by NO (Dobashi *et al.*, 1997). Furthermore, in a reversible way, NO inhibits the activities of tobacco CAT and ascorbate peroxidase, whereas both are irreversibly inhibited by peroxynitrite (Clark *et al.*, 2000).

In the present work, the occurrence of S-nitrosylation in plant peroxisomes is assessed. Six peroxisomal proteins are identified as a target for S-nitrosylation, using proteomic techniques (involving a specific biotin-switch method for detecting and purifying S-nitrosylated proteins) together with two other different mass spectrometry techniques: matrix-assisted laser desorption/ionization tandem time-of-flight (MALDI TOF/TOF) and two-dimensional nano-liquid chromatography coupled to two-dimensional ion-trap tandem mass spectrometry (2D-nLC-MS/MS). Additionally, the impact of S-nitrosylation on the functionality of three key peroxisomal proteins, CAT, glycolate oxidase (GOX), and malate dehydrogenase (MDH) has been analysed. Finally, the pattern of CAT and GOX S-nitrosylation under two different types of abiotic stress, metal toxicity by cadmium and treatment with the herbicide 2,4-dichlorophenoxy acetic acid (2,4-D) has also been studied.

Materials and methods

Plant material and growth conditions

Pea (*Pisum sativum* L. cv. Lincoln) plants were obtained from Royal Sluis (Enkhuizen, Holland). Plants were grown in greenhouse in aerated full-nutrient medium under optimum conditions for 14 d as indicated elsewhere (Sandalio *et al.*, 2001). Then, the medium either remained unsupplemented (control plants) or was supplemented with 50 μM $CdCl_2$ (cadmium-treated plants), and the plants were grown for 14 d. For 2,4-D treatment, plants were grown for 25 d and then herbicide was applied by spraying the leaves with 22.6 mM 2,4-D. The leaves were collected after 3 d of treatment. The concentrations of cadmium and 2,4-D were optimized in previous work to show that both of the selected concentrations induced an oxidative stress response in pea leaves (Sandalio *et al.*, 2001; Romero-Puertas *et al.*, 2004).

Purification of peroxisomes

Peroxisomes were purified from pea leaves by differential and sucrose density-gradient centrifugation (35–60%; w/w) and were recovered from gradients by puncture with a syringe as described by López-Huertas *et al.* (1995). Peroxisomes were broken by osmotic shock and sucrose was diluted (around 100 times) in 25 mM HEPES buffer (containing 1 mM EDTA and 0.1 mM neocuproine; pH 7.7). Finally, peroxisomes were concentrated by centrifugation (Amicon Ultra, Millipore).

Detection of S-nitrosylated proteins

This study used the biotin-switch method, which converts S-nitrosylated groups into biotinylated groups (Jaffrey *et al.*, 2001) to detect S-nitrosylated proteins, and Western blotting in leaves and peroxisomes with slight modifications. Pea leaves were homogenized in MAE buffer (25 mM HEPES, 1 mM EDTA, 0.1 mM neocuproine, 0.2% Triton X-100; pH 7.7) containing complete protease inhibitor cocktail (Sigma, St. Louis, MO, USA). The extract was centrifuged at 4 °C for 30 min, and the protein concentration in the supernatant was measured by the Bradford assay (Bio-Rad, Hercules, CA, USA). When necessary, homogenates or peroxisomal proteins were pre-treated with the NO donors S-nitroso-*N*-acetylpenicillamine (SNAP, 1 mM, Calbiochem) and S-nitrosoglutathione (GSNO, 1 mM, Calbiochem) and the glutathionylating agent glutathione disulphide (GSSG, 1 mM, Sigma) in the dark at room temperature for 30 min with regular vortexing. Treatment with the reducing agent *tris*(2-carboxyethyl)phosphine (TCEP, 10 mM, Sigma) was carried out for 1 h under the same conditions. Reagents were removed by precipitation twice with two volumes of cold acetone and proteins were assayed by the biotin-switch method. Extracts were incubated with 20 mM methylmethanethiosulphonate and 2.5% sodium dodecyl sulphate (SDS) at 50 °C for 30 min with frequent vortexing to block free Cys. Methylmethanethiosulphonate was removed by protein precipitation with two volumes of cold acetone, proteins were resuspended in 0.1 ml RB buffer (25 mM HEPES, 1 mM EDTA, and 1% SDS, pH 7.7) per 1 mg protein. After the addition of 1 mM HPDP-biotin (Pierce, Rockford, IL, USA) and 1 mM ascorbic acid, the mixture was incubated for 1 h at room temperature in the dark with intermittent vortexing. S-Nitrosylated proteins were detected with an anti-biotin antibody (1:10,000, Sigma).

Purification of peroxisome S-nitrosylated proteins

Peroxisome S-nitrosylated proteins were purified by immunoprecipitation with anti-biotin antibody. Samples were prepared with a slight modification of the biotin-switch method (Jaffrey *et al.*, 2001). In brief, 5 mg peroxisomes were incubated with 1 mM GSNO in the dark for 30 min with repeated vortexing. Samples treated with 20 mM dithiothreitol (DTT) were also kept for 1 h under the same conditions as a negative control. Peroxisomal proteins were recovered by precipitating with eight volumes of acetone for 20 min at –20 °C to remove excess GSNO/DTT and were assayed with the biotin-switch method as described before. Biotinylated proteins were purified by anti-biotin-Immobilized Protein A (IPA) as described elsewhere (Perazzolli *et al.*, 2004). Briefly, biotinylated proteins were purified by immunoprecipitation overnight at 4 °C with 15 μl IPA

(Pierce)/mg of protein, preincubated with 2 μl of anti-biotin antibody (Sigma). Beads were washed three times with phosphate-buffered saline. Bound proteins were eluted with 10 mM DTT in SDS-PAGE solubilization buffer, separated using 12% polyacrylimide gel, and visualized by Brilliant Blue-G Colloidal Stain (Sigma). Protein bands were analysed by MALDI TOF/TOF. Alternatively, purified proteins were analysed directly by 2D-nLC-MS/MS. When indicated, immunopurified proteins were transferred to a polyvinylidene fluoride (PVDF) membrane (Millipore, Bedford, MA, USA) to detect CAT and GOX proteins with their specific antibodies.

In-gel protein digestion and sample preparation

Bands of interest from Coomassie-stained gels were excised manually, deposited in 96-well plates, and processed automatically in a Proteineer DP (Bruker Daltonics, Bremen, Germany). The digestion protocol was based on Shevchenko et al. (1996) with minor amendments: gel plugs were washed first with 50 mM ammonium bicarbonate and second with acetonitrile (ACN) prior to reduction with 10 mM DTT in 25 mM ammonium bicarbonate solution; and alkylation was carried out with 55 mM iodoacetamide in 50 mM ammonium bicarbonate solution. Gel pieces were then rinsed first with 50 mM ammonium bicarbonate and second with ACN, and were dried under a stream of nitrogen. Modified porcine trypsin (sequencing grade, Promega, Madison WI, USA), at a final concentration of 16 ng/μl in 25% ACN/50 mM ammonium bicarbonate solution, was added and the digestion took place at 37 °C for 6 h. The reaction was stopped by adding 0.5% trifluoroacetic acid (TFA) for peptide extraction. The eluted peptides were dried by speed-vacuum centrifugation and were resuspended in 4 μl of MALDI solution (70% ACN/0.1% TFA aqueous solution). A 0.8 μl aliquot of each peptide mixture was deposited onto a 386-well OptiTOF plate (Applied Biosystems, Framingham, MA, USA) and allowed to dry at room temperature. A 0.8 μl aliquot of matrix solution (3 mg/ml α-cyano-4-hydroxycinnamic acid in MALDI solution) was deposited onto the dried digest and allowed to dry at room temperature.

MALDI peptide mass fingerprinting and MS/MS analysis

For MALDI TOF/TOF analysis, samples were automatically acquired in an ABI 4800 MALDI TOF/TOF mass spectrometer (Applied Biosystems) in positive ion reflector mode (the ion acceleration voltage was 25 kV to MS acquisition and 1 kV to MS/MS) and obtained spectra were stored into ABI 4000 Series Explorer Spot Set Manager. Peptide mass fingerprinting (PMF) and MS/MS fragment ion spectra were smoothed and corrected to zero baseline using routines embedded in ABI 4000 Series Explorer software v3.6. Each PMF spectrum was internally calibrated with mass signals of trypsin autolysis ions to reach a typical mass measurement accuracy of <25 ppm. Known trypsin and keratin mass signals, as well as potential sodium and potassium adducts (+21 Da and +39 Da) were removed from the peak list.

Two-dimensional nano-liquid chromatography and ion-trap tandem mass spectrometry

Alternatively, for 2D-nLC-MS/MS analysis the tryptic peptide mixtures were directly injected onto a strong cationic exchange micro-precolumn (500 mm ID615 mm BioX-SCX, LC Packings, Amsterdam, The Netherlands) with a flow rate of 30 ml/min as a first-dimension separation. Peptides were eluted from the column as fractions by injecting three salt steps of increasing concentration of ammonium acetate (10, 100, and 2000 mM). Each three fractions together with non-retained fraction was on line injected onto a C-18 reversed-phase micro-column (300 mm ID65 mm PepMap, LC Packings) to remove salts, and the peptides were analysed in a continuous ACN gradient consisting of 0–50% B in 45 min and 50–90% B in 1 min (B = 95% ACN, 0.5% acetic acid in water) on a C-18 reversed-phase self-packing nanocolumn (100 mm ID615 cm Discovery BIO Wide pore, Supelco, Bellefonte,

PA, USA). A flow rate of about 300 nl/min was used to elute peptides from reversed-phase nanocolumn to a PicoTip emitter nano-spray needle (New Objective, Woburn, MA, USA) for real-time ionization and peptide fragmentation on an Esquire HCT ion-trap mass spectrometer (Bruker Daltonics). Every 1 s, the instrument cycled through the acquisition of a full-scan mass spectrum and one MS/MS spectrum. A 4 Da window (precursor m/z 62), an MS/MS fragmentation amplitude of 0.80 V, and a dynamic exclusion time of 0.30 min were used for peptide fragmentation. 2D-nLC-MS/MS was automatically performed on an advanced micro-column switching device (Switchos) coupled to an auto sampler (Famos) and a nano-gradient generator (Ultimate nano-HPLC, all from LC Packings). The software Hystar 2.3 was used to control whole analytical process.

Database analysis

To submit the combined PMF and MS/MS data to MASCOT version 2.2.04 software (Matrix Science, London, UK), GPS Explorer version 4.9 was used to search in a non-redundant NCBI protein database (NCBInr 20090406, http://blast.ncbi.nlm.nih.gov/Blast.cgi; 8,198,267 sequences, 2,824,199,726 residues). The following search parameters were used: enzyme, trypsin; allowed missed cleavages, 1; carbamidomethyl cysteine as fixed modification by the treatment with iodoacetamide; variable modifications, oxidation of methionine; mass tolerance for precursors was set to ± 50 ppm and for MS/MS fragment ions to ± 0.3 Da. The confidence interval for protein identification was set to ≥95% ($P < 0.05$) and only peptides with an individual ion score above the identity threshold were considered correctly identified.

Western blot analysis

Protein samples were separated on 12% polyacrylimide and transferred onto PVDF membranes (Sambrook and Russell, 2001). The membrane was stained with Ponceau red to check equivalency of protein loading. For detection of CAT and GOX, antibodies were produced against specific CAT and GOX peptides heterologously expressed in Escherichia coli (Sigma-Genosys) at 1:2000 dilution. Anti-biotin antibody (Sigma) was used at 1:10,000 dilution. Probing and detection of immunocomplexes were performed as described for the SuperSignal West Pico detection system (Pierce).

NO detection by confocal laser scanning fluorescence microscopy

For NO detection, pea-leaf segments (10 mm²) were incubated for 1 h at 25 °C in the dark with 10 μM 4,5-diaminoflorescein diacetate (DAF-2DA, Calbiochem; excitation 495 and emission 515) in 10 mM TRIS-HCl (pH 7.4), as described before (Rodríguez-Serrano et al., 2006).

Electron microscopy and immunolabelling

The samples were processed basically as described elsewhere (Sandalio et al., 1997). Specific polyclonal antibodies for GSNO and glutathione (GSH) were obtained from Calbiochem and Millipore Corporation, respectively. Immunolabelling was carried out by using goat anti-rabbit IgG conjugated to 15 nm gold particles (Sandalio et al., 1997). To avoid cross reactions, primary antibodies were used at minimal concentration for immunodetection and the pre-immune serum was used as a reaction control.

Enzyme assays

S-nitrosoglutathione reductase (GSNOR) activity was assayed in solution according to Sakamoto et al., (2002). Commercial purified proteins (CAT, EC 1.11.1.6; GOX, EC 1.1.3.1; and MDH, EC 1.1.1.37; Sigma) were incubated with two different NO donors, GSNO and diethylenetriamine (DETA) NONOate, at concentrations 0–1000 μM in the dark for 20 min. When indicated, after 1 mM GSNO and DETA NONOate treatment, proteins were

incubated with DTT (10 mM) in the dark for 40 min. Pea extracts were incubated with 1 mM GSNO or SNAP for 45 min and activities were measured. GOX activity was assayed spectrophotometrically according to Kerr and Groves (1975), CAT activity was determined as described before (Aebi, 1984), and MDH activity was analysed as previously reported (Walk and Hock, 1977).

Results

NO production and GSNOR activity under 2,4-D treatment

To check possible changes in NO metabolism which could affect S-nitrosylation pattern in pea leaf during abiotic stress, NO production was first studied under 2,4-D treatment (22.6 mM). The herbicide reduced NO accumulation in pea leaves as indicated by DAF-2DA fluorescence (Fig. 1A,B,D). In control plants, the main NO production was located in vascular tissue from the midrib (Fig. 1A) and was eliminated by 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-l-oxyl-3-oxide (Fig. 1C). Also, a reduction in NO production was observed previously under cadmium treatment (Barroso *et al.*, 2006; Rodríguez-Serrano *et al.*, 2009). NO can react with GSH, giving rise to GSNO, which is considered to be a NO reservoir (Liu *et al.*, 2001). GSNOR metabolizes GSNO, an enzyme that regulates internal levels of nitrosothiols. Therefore, this study analysed the effect of 2,4-D on this activity and found that GSNOR was induced by 2,4-D treatment (Fig. 2). GSNOR activity was, however, reduced under cadmium treatment, as shown before (Barroso *et al.*, 2006).

S-nitrosylated proteins in pea-leaf extracts under abiotic stress

The biotin-switch assay (Jaffrey *et al.*, 2001) that converts S-nitrosylated cysteine into biotinylated cysteine was first
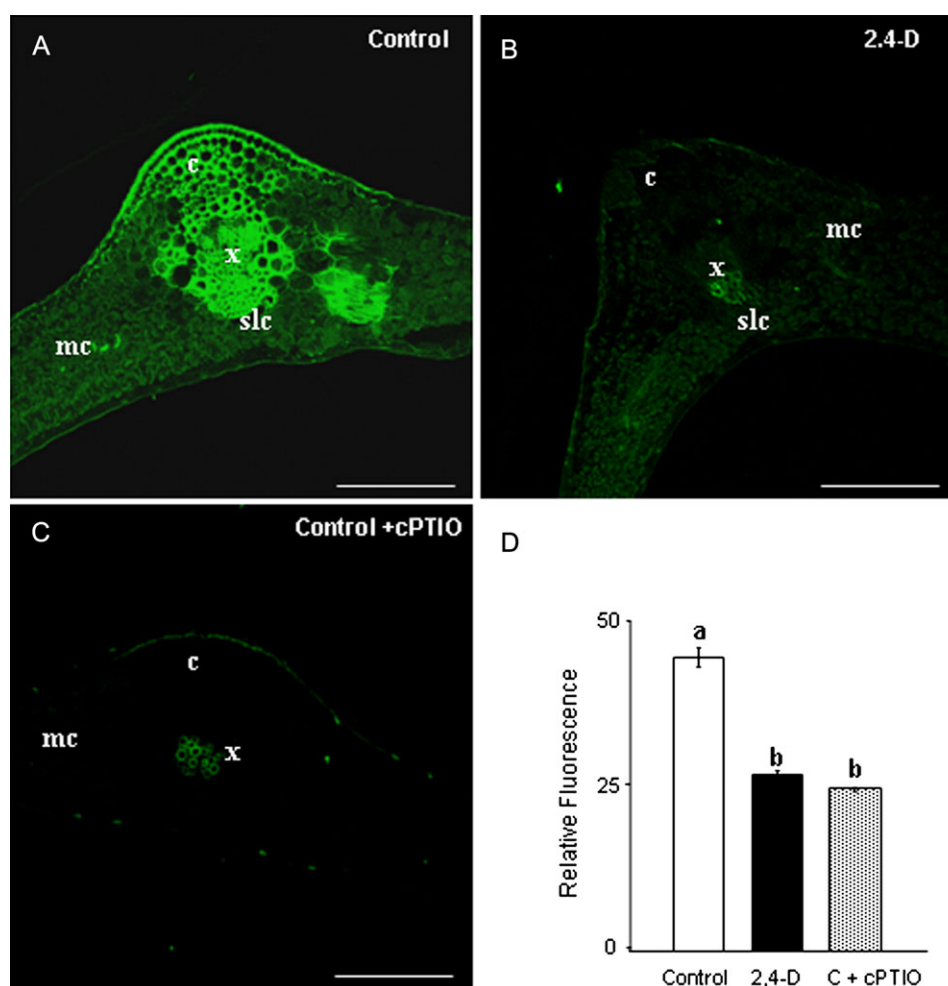
Fig. 1. Nitric oxide (NO) production in pea leaves by confocal laser scanning fluorescence microscopy, after treatment with 22.6 mM 2,4-dichlorophenoxy acetic acid (2,4-D). (A–C) Maximum projections of several optical sections collected by confocal microscopy showing fluorescence due to 4,5-diaminoflorescein diacetate (excitation at 495 nm, emission at 520 nm) in leaf cross-sections of (A) control and (B) 2,4-D-treated plants; (C) negative control, leaves from control plants incubated with 300 μM carboxy 2-phenyl-4,4,5,5-tetramethylimidazoline-1-oxyl 3-oxide (cPTIO), which acts as NO scavenger. (D) Histogram showing relative fluorescence intensities corresponding to NO quantified in arbitrary units using LCS Lite software from Leica. Different letters indicate significant difference ($P < 0.01$) according to Student's t-test. These results are representative of at least three independent experiments. c, collenchyma; mc, mesophyll cells; scl, sclerenchyma; x, xylem vessels. Bars, 250 μm.
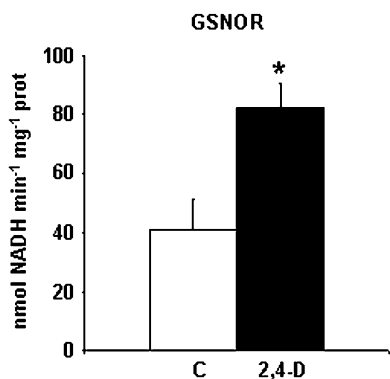
Fig. 2. Effect of 2,4-dichlorophenoxy acetic acid (2,4-D) on the activity of S-nitrosoglutathione reductase (GSNOR) in pea leaves. Each column represents the mean ± SE of three independent experiments. *, Differences were significant at $P < 0.01$ according to Student's t-test.

evaluated in pea-leaf extracts, for which no previous description is available. Pea-leaf extracts were incubated with the NO donors SNAP or GSNO to induce S-nitrosylation, with GSSG as the glutathionylating agent, or with the reducing agent TCEP as a negative control, subjected to the biotin-switch method, separated by SDS-PAGE, and immunoblotted into a PVDF membrane. Some endogenous S-nitrosylated proteins can be detected in control (non-treated) extracts. Meanwhile, the signals corresponding to S-nitrosylated proteins increased in both extracts treated with a NO donor, SNAP or GSNO, although the signal was higher with the *trans*-nitrosylating agent GSNO. A similar signal was detected in non-treated extracts, in proteins pre-incubated with glutathionylating agent GSSG, showing that the biotin-switch method does not detect glutathionylation (Fig. 3). Incubation with TCEP, which reduces all cysteine, eliminated the signal, demonstrating the specificity of the method. Based on these results, this method was considered suitable for studying S-nitrosylation in the pea and for analysing the S-nitrosylation pattern during abiotic stress. S-Nitrosylation in pea leaves was studied under cadmium (50 µM) and 2,4-D (22.6 mM) treatments. Both treatments altered NO metabolism in pea plants, although no data on S-nitrosylation has been previously provided. No differences in S-nitrosylation were found in pea-leaf extracts from cadmium-treated plants with respect to control (Fig. 4A), whereas an increase in this post-translational modification was found in plants after 2,4-D treatment (Fig. 4B).

Detection and identification of S-nitrosylated proteins in pea-leaf peroxisomes

NO has been shown to be produced in pea peroxisomes and these organelles have been involved in stress/signalling related to oxygen and nitrogen species in response to abiotic stress (del Río, 2011), although no information about S-nitrosylation is available to date. First, by immunodetection, electron microscopy was used to check the presence of GSH in peroxisomes that, together with NO, could give rise to GSNO, a well-known S-nitrosylating agent. GSH has
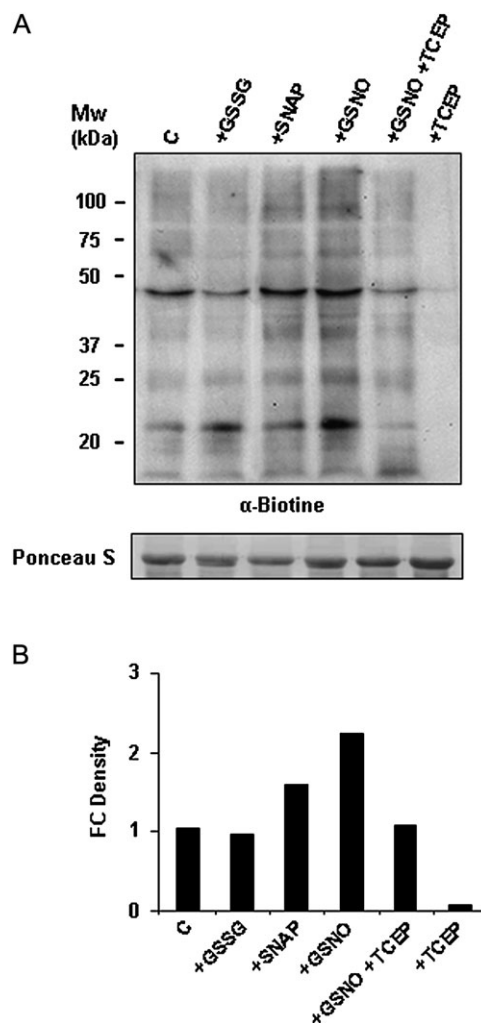
Fig. 3. Detection of S-nitrosylated proteins in pea-leaf (*Pisum sativum* L.) extracts. (A) Protein extracts (150 µg) from pea leaves were not treated (control, C) or treated with 1 mM glutathione disulphide (GSSG), 1 mM S-nitroso-N-acetylpenicillamine (SNAP), 1 mM S-nitrosoglutathione (GSNO), or 10 mM *tris*(2-carboxyethyl)-phosphine (TCEP) previously treated or not with GSNO and were subjected to the biotin-switch assay, separated by SDS-PAGE, and immunoblotted with an anti-biotin antibody. Protein loading was verified by Ponceau staining. (B) Histogram showing relative quantification of the Western blot showed in (A). The intensity of bands was quantified using Quantity One Software (version 4.6.2). Band intensity was expressed as fold-change (FC) density. Each band density was first normalized by dividing it by the density of the Ponceau band in the same lane. Then, the relative increase or decrease in density was calculated by dividing the normalized band density of the problem sample by the normalized band density of the control sample. The results are representative of three different Western blots assayed.

been previously described in peroxisomes by biochemical techniques (Jimenez *et al.*, 1997) and by electron microscopy (Zechmann *et al.*, 2008). In addition to GSH, the current study detected GSNO in peroxisomes under physiological conditions (Fig. 5B,C). The presence of GSNO suggests that S-nitrosylation could occur in peroxisomes.
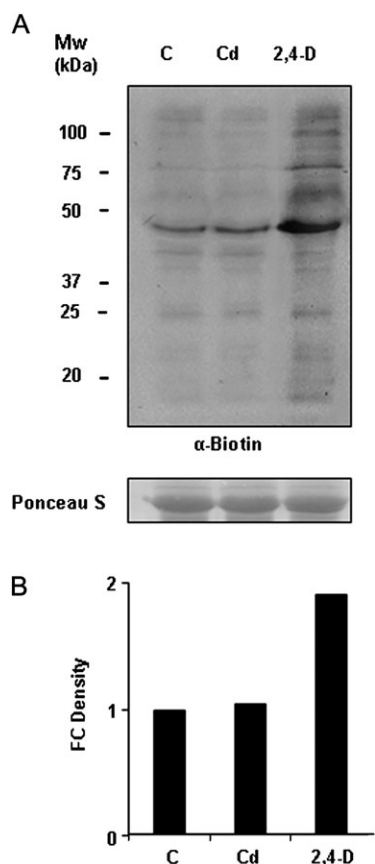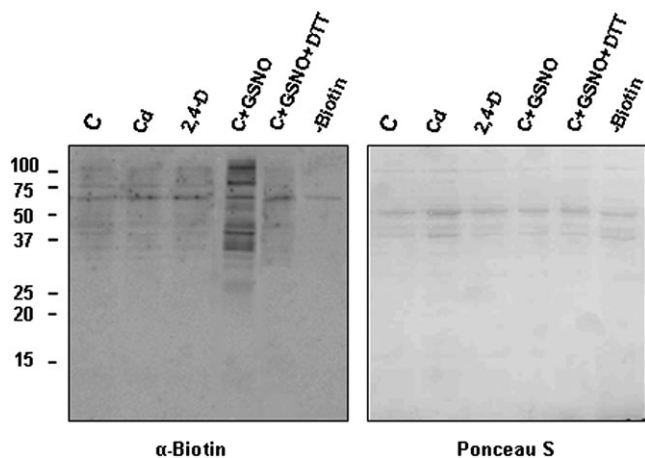
Fig. 4. Detection of S-nitrosylated proteins in pea-leaf (*Pisum sativum* L.) extracts under abiotic stress. Protein extracts (150 µg) from pea leaves were not treated (control, C) or treated with 50 µM cadmium or 22.6 mM 2,4-dichlorophenoxy acetic acid (2,4-D) and were subjected to the biotin-switch assay, separated by SDS-PAGE, and immunoblotted with an anti-biotin antibody. Protein loading was verified by Ponceau staining. B, Histogram showing relative quantification of Western blot showed in (A). The intensity of bands was quantified as described in the legend for Fig. 3. The results are representative of four different Western blots assayed.

Also, GSNO labelling was observed to be located in the chloroplast (Fig. 5), but also located mainly in the collenchyma cell wall and xylem (data not shown).

To study the pattern of S-nitrosylated proteins in peroxisomes, purified peroxisomes were treated with GSNO (1 mM) and S-nitrosylation was further evaluated by the biotin-switch assay. The analysis by immunoblots using anti-biotin antibodies showed a band of about 60 kDa in the absence of biotin, which probably corresponds to an endogenous biotinylated protein. Incubation with GSNO consistently intensified and increased the number of bands observed related to control peroxisomes, although some endogenous S-nitrosylated proteins were present under physiological conditions (Fig. 6). As the S-nitrosylation signal under physiological conditions was weak, the biotin-switch method after treating peroxisomal proteins with GSNO was used to identify S-nitrosylated proteins in pea-leaf peroxisomes. Additionally, peroxisomes were treated with DTT as a negative control. To identify S-nitrosylated
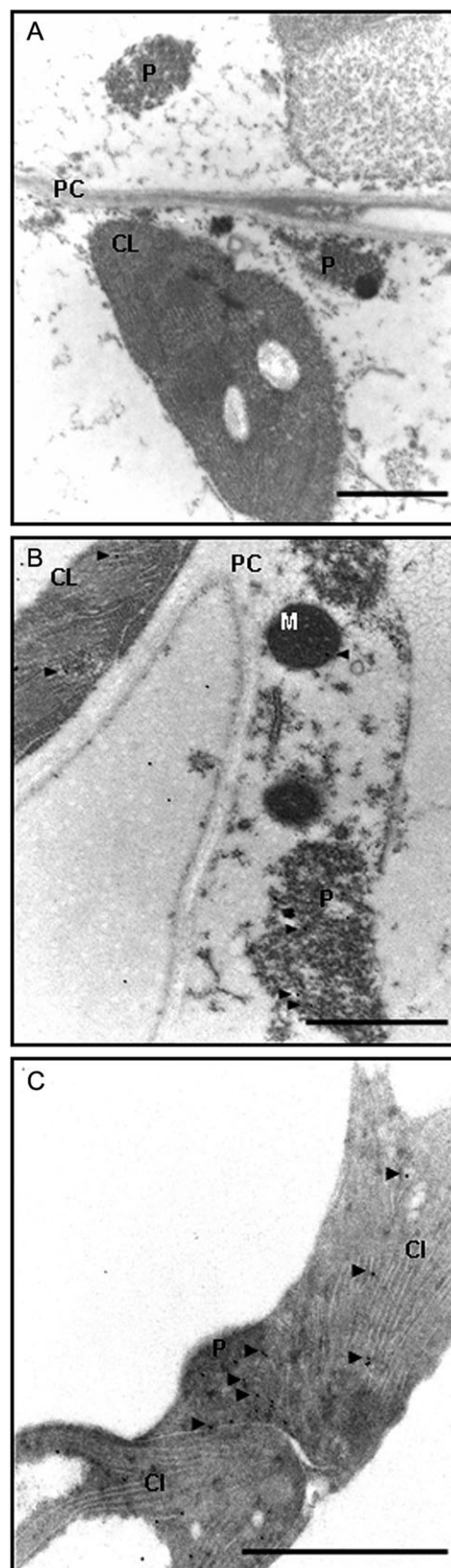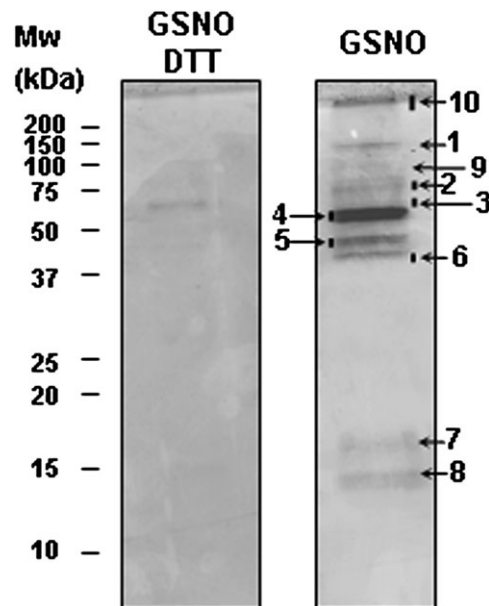

Fig. 5. Subcellular localization of S-nitrosoglutathione (GSNO) and glutathione (GSH) in pea leaves. (A) Pre-immune serum control. (B) Pea-leaf cells with anti-GSNO 1:250. (C) Pea-leaf cells with anti-GSH 1:250. Immunogold labelling of GSH and GSNO are indicated by arrowheads. CL, chloroplast; M, mitochondrion; P, peroxisome; PC, cell wall. Bars, 1 µm.

Fig. 6. Detection of S-nitrosylated proteins in pea-leaf (*Pisum sativum* L.) peroxisomes. Peroxisomal proteins (250 µg) from pea plants were not treated (control, C), treated with 50 µM cadmium or 22.6 mM 2,4-dichlorophenoxy acetic acid (2,4-D), or incubated with 1 mM S-nitrosoglutathione (C+GSNO) or with 20 mM dithiothreitol previously incubated with GSNO (C+GSNO+DTT) and were subjected to the biotin-switch assay, separated by SDS-PAGE, and immunoblotted with an anti-biotin antibody. Protein loading was verified by Ponceau staining of the membrane. The experiment was repeated three times with similar results.

Fig. 7. S-Nitrosylated proteins of peroxisomes from pea leaves (*Pisum sativum* L.). Peroxisomal proteins (5 mg) were treated with 1 mM S-nitrosoglutathione (GSNO) and then with 20 mM dithiothreitol (DTT) or not and were subjected to the biotin-switch assay. Biotinylated proteins were purified by immunoprecipitation with an anti-biotin antibody. Eluates were separated by SDS-PAGE and stained by Coomassie blue. The protein bands were identified by matrix-assisted laser desorption/ionization tandem time-of-flight (Table 1).

proteins after the biotin-switch method, the biotinylated proteins were purified by immunoprecipitation with anti-biotin antibody-IPA while two other methods were used to identify the proteins. First, in the proteomic approach, 2D gels were made but there were difficulties in purifying adequate quantities of S-nitrosylated protein for identification by mass spectrometry. Next, samples were loaded on a 1D SDS-PAGE gel, which showed 10 protein bands stained with Coomassie blue, whereas just one band was slightly visible in the GSNO plus DTT-derived eluate (Fig. 7). Thus, with Coomassie staining, this study found bands >100 kDa (band 1), a band of about 75 kDa (band 2), another of 50–75 kDa (band 4), and two bands 37–50 kDa (bands 5 and 6). These presumably correspond to bands observed at these molecular weights by Western blotting (Fig. 6). In the Coomassie gel, the bands corresponding to 25–37 kDa observed in the Western blot were not detected, probably due to the low quantity of these proteins.

The Coomassie bands were digested with trypsin and the resulting peptides were subjected to MALDI TOF/TOF analysis (Table 1). To identify low-abundance peroxisomal targets of S-nitrosylation, GSNO-treated proteins were labelled with biotin, immunopurified as described above, and the eluates were subjected to 2D-nLC-MS/MS analysis. In this way, an additional peroxisomal protein could be identified as candidate for S-nitrosylation (Table 1). In total, this study identified 13 proteins having a significant score (score > 50). Two proteins, corresponding to bands 2 and 9, were albumin and immunoglobulin, respectively. Neither protein was a peroxisomal protein but both were involved in the process of obtaining peroxisomes and immunopurified S-nitrosylated proteins. Another protein was

a chloroplast one, i.e. Rubisco, which has been shown to be inhibited by S-nitrosylation (Abat *et al.*, 2008), and four proteins were mitochondrial protein contaminations. The mitochondrial proteins found as a target for S-nitrosylation were subunits of the glycine decarboxylase complex, important for the photorespiratory C_2 cycle in plants, which was previously found to be a putative target of S-nitrosylation (Palmieri *et al.*, 2010), a finding that strengthens the reliability of the current results. Finally, six peroxisomal proteins were identified as putative targets of S-nitrosylation (Table 1). Four of these, hydroxypyruvate reductase, glycolate oxidase, serine-glyoxylate aminotransferase, and aminotransferase 1, are enzymes that participate in photorespiration, the key physiological process involving peroxisomes. Another enzyme is CAT, one of the main enzymes involved in ROS detoxification. Finally, MDH has also been identified as one of the putative S-nitrosylated peroxisomal proteins. This enzyme, involved in fatty acid β-oxidation in germinating seeds (Pracharoenwattana *et al.*, 2007), is also involved in supplying NADH to hydroxypyruvate reductase in the photorespiration pathway (Reumann and Weber, 2006).

Regulation of selected proteins activity by NO

Approximately 90% of NO-Cys sites have both positively and negatively charged residues within 8 Å (Marino and Gladyshev, 2010) and therefore this study checked this feature for CAT and GOX. Although cytoplasmic MDH (Cys 154) lacks the acid–base motif, it seems that a His at 7.9 Å from the sulphur group of Cys is sufficient for direct

Table 1. Identified S-nitrosylated proteins from pea-leaf peroxisomes

Peroxisomes treated with S-nitrosoglutathione were subjected to the biotin-switch method and analysed by matrix-assisted laser desorption/ionization tandem time-of-flight and two-dimensional nano-liquid chromatography coupled to ion-trap tandem mass spectrometry (filled circle).

Protein name[a]	Accession number	Mol mass (M_r)/ Cal pI	Identified peptides		Cited as S-nitrosylated
			Sequence and score[b]	Sequence coverage (%)	
Catalase (CAT1) (3, 4, 10)	gil115705	57594 / 6.72	K.GFFEVTHDISHLTCADFLR.A (126) + 3	>42	Foster and Stamler (2004);[e] Rhee *et al.* (2004);[e] Palmieri *et al.* (2010)
Aminotransferase 1 (5)	gil18032028	44275 / 8.13	K.LGSGVAAASAYLQNNIPLIPSR.I (144)	16	
Serine-glyoxylate aminotransferase (5)	gil115477148	44466 / 8.19	R.NHLFVPGPVNIPDQVIR.A (116)	10	
Glycolate oxidase (GOX1) (6)	gil167961875	40825 / 9.16	R.IAVQSGAAGIIVSNHGAR.Q (136) + 1	42	Abat *et al.* (2008)
Hydroxypyruvate reductase (6)	gil223528624	42192 / 6.97	K.MNLIYFDLYQATR.L (63) + 1	23	
Malate dehydrogenase, glyoxysomal precursor (●)	gil3183079	37362 / 6.9	R.LLGVTTLDVVR.A (41)	25	
Glycine dehydrogenase (1, 10)[c]	gil121083	115411 / 7.17	R.ESPYLTHPIFNTYQTEHELLR.Y (140) + 4	38	Palmieri *et al.* (2010)
Chain A, dihydrolipoamide dehydrogenase (of Gly decarboxylase) (3)[c]	gil9955321	49998 / 6.06	K.LTVEPSAGGEQTIIEADVVLVSAGR.T (103)	34	Foster and Stamler (2004);[e] Rhee et al. (2004)e
Aminomethyltransferase (5)[c]	gil3915699	44656 / 8.79	R.VLDINGSQCFLTR.T (95) + 2	45	Palmieri *et al.* (2010)
H protein (7)[c]	gil737595	13979 / 4.57	K.IKPTSPDELESLLGAK.E (59) + 1	19	Palmieri *et al.* (2010)
RuBisCO large subunit (3, 4, 10)[d]	gil113374116	49312 / 6.46	K.TFQGPPHGIQVER.D (79) + 1	35	Lindermayr *et al.* (2005); Abat *et al.* (2008); Abat and Deswal (2009)
RuBisCO small subunit (8)[d]	gil132097	20402 / 9.24	K.ELDEVVAAYPQAFVR.I (125) + 5	69	Abat *et al.* (2008)

[a] Comassie bands are indicated in brackets.
[b] The best-matching peptide identifying the protein is given, with the score in parentheses. If there were further peptides found, the number of the peptides is given as an additional number.
[c] Protein localized in mitochondria
[d] Protein localized in chloroplasts
[e] In animal tissue or microbes.

nitrosylation of this Cys (Marino and Gladyshev, 2010). The structural model for AtGOX1 (http://swissmodel.expasy.org) gave insight into the spatial disposition of Cys residue in this enzyme and an acid–base motif was also found within 8 Å from the sulphur group of the Cys (Supplementary Fig. S1, available at *JXB* online). This reinforces the idea that this protein is a promising target for S-nitrosylation. All six cysteine residues that are present in CAT1 are free amino acids and therefore all are possible targets for NO. Using the computational prediction of the protein S-nitrosylation program (Xue *et al.*, 2010), it was found that Cys-230 has a high probability of being the Cys that is potentially S-nitrosylated. The structural model for CAT1 from *Arabidopsis thaliana* (http://swissmodel.expasy.org) enabled the finding an acid–base motif within 8 Å from the sulphur group of this Cys (Supplementary Fig. S2), supporting the data gathered by

this program. To verify the effects of S-nitrosylation on CAT, GOX, and MDH activities, their function was monitored after treatment with two different NO donors, GSNO and DETA NONOate (Fig. 8). Significant inhibition of these enzyme activities was found after incubation with both NO donors in a concentration-dependent manner, up to 25–30% of the total activity when treated with 1 mM GSNO or DETA NONOate, compared with untreated proteins. This effect could be counteracted by the thiol-specific reductant DTT (Fig. 8). To certify the effect of S-nitrosylation on pea CAT and GOX activities, pea extracts were incubated with the NO donors GSNO and SNAP, showing a similar reduction (Fig. 9). As MDH presents more than one isoform located in different compartments, pea-leaf peroxisomes were incubated with the NO donors, achieving a reduction in MDH activity as well (Fig. 9).
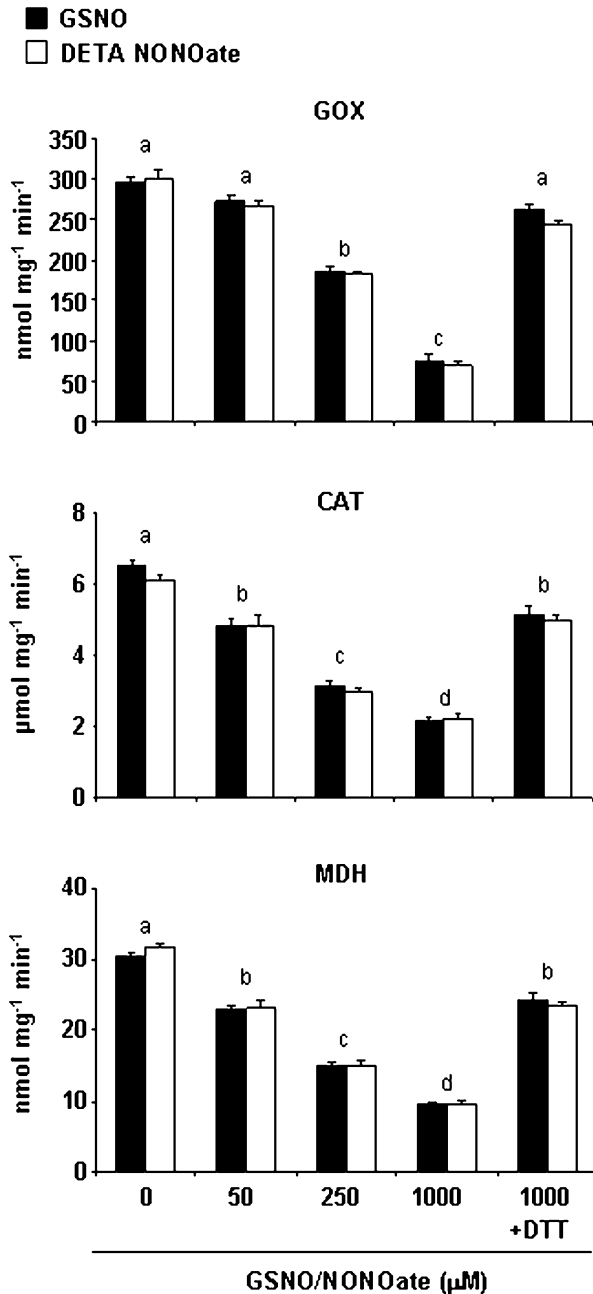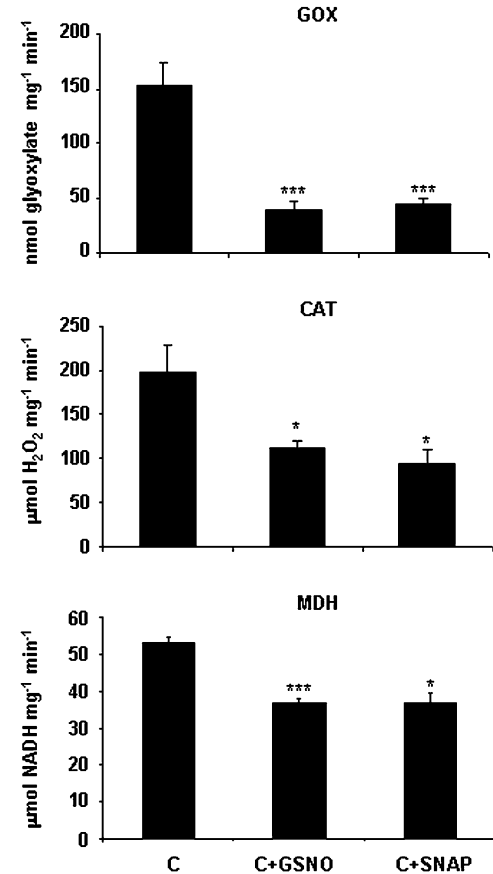
Fig. 8. Effect of NO on glycolate oxidase (GOX), catalase (CAT), and malate dehydrogenase (MDH) activities. Commercial proteins were preincubated with different concentrations (0–1000 μM) of GSNO (black bars) or diethylenetriamine (DETA) NONOate (white bars) for 30 min at room temperature and then enzyme activities were determined (Materials and methods). Incubation with 10 mM dithiothreitol (DTT) after 1000 μM GSNO or DETA NONOate restored enzyme activities. For each concentration, measurements were made in triplicate. Different letters indicate significant differences ($P < 0.001$ for GOX and MDH and $P < 0.01$ for CAT), as determined by Student's t-test.

S-nitrosylated proteins in pea-leaf peroxisomes under abiotic stress

The peroxisomal targets for S-nitrosylation are key enzymes involved in important metabolic processes, so this study

Fig. 9. Effect of NO on glycolate oxidase (GOX), catalase (CAT), and malate dehydrogenase (MDH) activities in pea leaves (*Pisum sativum* L.). Pea extracts for measuring CAT and GOX activity and pea peroxisomes for MDH activity were preincubated with 1 mM S-nitrosoglutathione (GSNO) and 1 mM S-nitroso-N-acetylpenicill-amine (SNAP) for 45 min at room temperature and then enzyme activities were determined (Materials and methods). Three different extracts were used for each measurement. Asterisk indicates significant differences (*, $P < 0.05$; ***, $P < 0.001$) according to Student's t-test.

investigated their putative post-translational alteration in response to abiotic stress. Western blot analysis using an anti-biotin antibody showed no differences in the global pattern of S-nitrosylated proteins on peroxisomes from pea plants treated with cadmium or 2,4-D assayed with the biotin-switch method (Fig. 6). To gain further insight into the proteins identified previously, pea plants were treated with cadmium or 2,4-D, S-nitrosylated peroxisomal proteins were purified and blotted and specific antibodies used against CAT and GOX were used (Fig. 10). Both proteins were found to be S-nitrosylated under physiological conditions. A reduction of S-nitrosylated CAT under cadmium treatment was observed, while no differences were found under the 2,4-D treatment with respect to non-treated plants (Fig. 10A). Similar changes occurred in the total amount of this protein under both types of stress (Fig. 10B). Although an increase in total GOX protein occurred under cadmium treatment, S-nitrosylated GOX nearly disappeared under metal stress (Fig. 10). Under 2,4-D treatment,
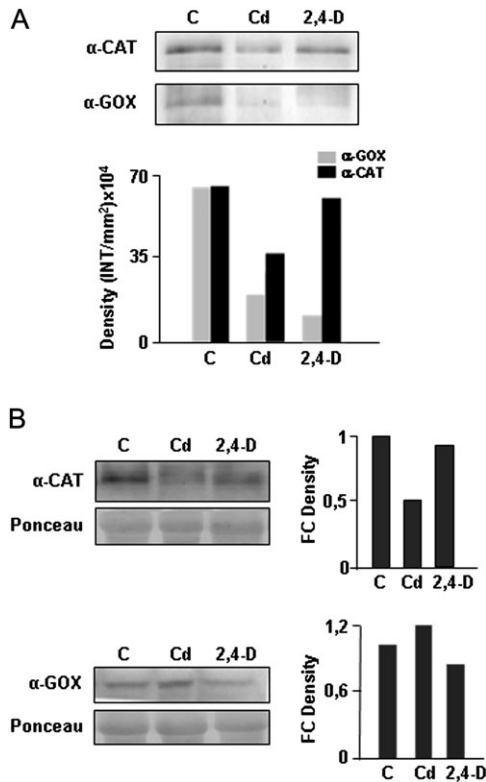
Fig. 10. S-Nitrosylation level of catalase (CAT) and glycolate oxidase (GOX) during abiotic stress. (A) Peroxisomal proteins from pea plants were not treated (control, C) or treated with cadmium and 2,4-dichlorophenoxy acetic acid (2,4-D) and were subjected to the biotin-switch assay. S-Nitrosylated proteins were immuno-purified with anti-biotin antibody and subjected to Western blot analysis with anti-CAT and anti-GOX antibodies. (B) Variation in CAT and GOX protein accumulation during abiotic stress was monitored by Western blot analysis of peroxisomal proteins, as described above and not subjected to the biotin-switch method. The intensity of bands was quantified using Quantity One version 4.6.2 software. Band intensity was expressed as density in (A) and as fold-change (FC) density in (B) as described in the legend for Fig. 3. The figure is representative of two independent experiments.

a slight reduction in total GOX was noted (Fig. 10B) and S-nitrosylation level of the protein practically vanished (Fig. 10A).

Discussion

In recent years, few proteomic studies have been made to identify targets for protein S-nitrosylation in plants. In *A. thaliana* cell-culture extracts treated with GSNO and in NO-treated plants, more than 100 proteins have been identified (Lindermayr *et al.*, 2005) and 16 proteins have been found to be differentially S-nitrosylated in *A. thaliana* plants under a hypersensitive response (Romero-Puertas *et al.*, 2008). Additionally, 19 and 20 proteins were identified in *Kalanchoe pinnata* and *Brassica juncea*, respectively, some being modulated by low temperature in *Brassica* (Abat *et al.*, 2008; Abat and Deswal, 2009). The identification of the candidates was the starting point for the functional and biochemical characterization of S-nitrosylation in plants. Thus, different physiological processes, such as ethylene biosynthesis, defence response against both abiotic and biotic stress, antioxidant defence, proteolitic activity, DNA binding, photosynthesis, and the mitochondrial photorespiratory pathway has been shown to be regulated by S-nitrosylation of key enzymes involved in these processes (Perazzolli *et al.*, 2004; Lindermayr *et al.*, 2006; Belenghi *et al.*, 2007; Romero-Puertas *et al.*, 2007; Serpa *et al.*, 2007; Abat *et al.*, 2008; Tada *et al.*, 2008; Wang *et al.*, 2008; Palmieri *et al.*, 2010). However, it should be borne in mind that S-nitrosylation leads to changes not only in protein activity but also in protein–protein interactions and/or subcellular localization (Benhar and Stamler, 2005), and therefore spatiotemporal distribution of S-nitrosylated proteins are key components for S-nitrosylation significance. This finding prompted this study to monitor the identification of the S-nitrosylated proteome of peroxisomes and its changes under abiotic stress.

Here, this study shows the presence of GSNO in peroxisomes by immunoelectron microscopy, suggesting that S-nitrosylation could occur in these organelles. An S-nitrosylation-dependent signal was also observed in pea peroxisomes by the biotin-switch method, although this signal was weak probably due to lability of this modification and the long process required to obtain the organelles. Peroxisomal proteins are, however, active and able to be S-nitrosylated after GSNO treatment despite the osmotic shock and dilution of peroxisomes. To identify the S-nitrosoproteome of peroxisomes, they were treated with GSNO, an effective *trans*-nitrosylating agent that increases S-nitrosylated proteins in the peroxisome. The biotin-switch method (Jaffrey *et al.*, 2001) was used, coupled with affinity purification of biotinylated proteins that have previously been used in different plant species (Lindermayr *et al.*, 2005; Abat *et al.*, 2008; Romero-Puertas *et al.*, 2008) and even in organelles such as the mitochondria (Foster and Stamler, 2004; Palmieri *et al.*, 2010). The analysis of Coomassie bands found by MALDI TOF/TOF mass spectrometry and the direct analysis of the eluates by 2D-nLC-MS/MS resulted in the identification of six proteins belonging to peroxisomal metabolism related to ROS detoxification, β-oxidation, and photorespiration.

One of the main metabolic processes responsible for generation of H_2O_2 in peroxisomes is β-oxidation of fatty acids, exclusive of peroxisomes in plants and with several signalling molecules derived from it. In fact, jasmonic acid and its derivatives, salicylic acid and indolacetic acid, are generated from β-oxidation in peroxisomes (Nyathi and Baker, 2006). Additionally, β-oxidation of fatty acid converts the seed-lipid reserve into sugars to be used for germination and plant growth (Huang *et al.*, 1983; Baker and Graham, 2002). The current analysis revealed that NAD-dependent MDH, the glyoxysomal precursor enzyme (peroxisomal MDH) that serves to reoxidize NADH produced from fatty acid β-oxidation (Pracharoenwattana

et al., 2007), appears as a target of S-nitrosylation in pea-leaf peroxisomes. The mitochondrial MDH isoform has also been considered a candidate for S-nitrosylation in rat-liver mitochondrial extracts exposed to GSNO (Foster and Stamler, 2004) and in *Arabidopsis* leaves undergoing a hypersensitive response (Romero-Puertas *et al.*, 2008). The effect of this modification on the activity of the protein, however, has not been studied up to now, although it is well known that the oxidation of -SH groups inactivates the enzyme (Varrone *et al.*, 1970). The current study shows that different NO donors severely inhibit MDH activity in a concentration-dependent manner, and further studies are necessary to determine whether this post-translational modification could affect the NAD/NADH ratio in peroxisome and sugar bioavailability in the cell where MDH is involved. A key antioxidant enzyme such as CAT has been found to be one of the targets of S-nitrosylation in peroxisomes, which has been reported to undergo S-nitrosylation in the analysis of mitochondrial S-nitrosoproteomes in both animal and plant tissue. These mitochondrial fractions, however, seemed to be contaminated with peroxisomes (Foster and Stamler, 2004; Palmieri *et al.*, 2010). Interestingly, CAT has not been described as a target for S-nitrosylation in proteomic studies from plant tissues, indicating that an analysis of isolated organelles can contribute to finding new targets that could go unnoticed in total extracts. It has been shown that S-nitrosylation inhibits the activity of a member of the peroxiredoxin family, peroxiredoxin II E (Romero-Puertas *et al.*, 2007), which is located in chloroplast and reduces H_2O_2 and thereby protects cells from oxidative damage (Dietz, 2003). To test the functional effect of S-nitrosylation on CAT activity, this study incubated commercial enzyme and pea extracts with different NO donors and found an inhibition in activity. The results suggest that NO could regulate the antioxidant defence system and H_2O_2 levels in peroxisomes through S-nitrosylation. This effect is of particular interest because both molecules, NO and H_2O_2, have emerged as key players in plant-cell signalling, particularly under various types of stress. Starting from the same quantity of proteins, this study observed that under cadmium stress both the protein content and the S-nitrosylation level of CAT decreased with respect to non-treated plants. Apparently, the reduction observed in CAT activity in a previous work could be due to the reduction in protein content (Sandalio *et al.*, 2001; Romero-Puertas *et al.*, 2007) and not to changes in post-translational modifications or oxidation (Romero-Puertas *et al.*, 2002). No changes were found in the content of CAT and S-nitrosylated CAT under 2,4-D treatment with respect to the control plants. In fact, CAT was not affected by the herbicide 2,4-D, either in its activity or its transcript levels (Pazmiño *et al.*, 2011). It appears that ascorbate peroxidase could be the main enzyme involved in detoxifying H_2O_2 during the treatment with the herbicide (Pazmiño *et al.*, 2011).

It bears mentioning that among the six peroxisomal proteins that were identified as targets of S-nitrosylation, four of them (five if MDH is included), were proteins of the

photorespiration cycle, which is the main physiological process of peroxisomes. This metabolic pathway involves chloroplast, mitochondria, and peroxisomes, with more than 16 enzymes and at least six membrane channels distributed in the three organelles (Douce and Heldt, 2000; Reumann and Weber, 2006). It seems that photorespiration is a wasteful process: in this pathway, O_2, ATP, and reducing equivalents are used for CO_2, NH_3, and H_2O_2 production (Buchanan *et al.*, 2000). Although the functions of photorespiration are not clear, it is well accepted that this pathway affects a wide range of processes, such as carbon metabolism, nitrogen assimilation, bioenergetics, photosystem-II function, and respiration, and it is a critical source of H_2O_2 in photosynthetic cells (Foyer and Noctor, 2009). It seems that NO is able to modulate photorespiratory metabolism as the two enzymes involved in the mitochondrion step, glycine decarboxylase and serine hydroxymethyltransferase, which are responsible for the conversion of Gly to Ser, are targets of S-nitrosylation (Palmieri *et al.*, 2010). Also, the current results show that all of the enzymes involved in the peroxisomal photorespiration steps are targets of S-nitrosylation as well. The key enzyme of the photorespiratory cycle in peroxisomes is GOX, which oxidizes glycolate, transfers the electrons to O_2, and generates the product glyoxylate and H_2O_2 (Reumann, 2002). The current study has shown that GOX is inhibited by NO donors and this protein has been found to be S-nitrosylated under physiological conditions. These results suggest that NO could regulate H_2O_2 levels under physiological conditions, through S-nitrosylation by not only regulating the antioxidant defence system but also the H_2O_2-producing enzymes. Furthermore, this regulation could change, depending on the need to control the H_2O_2 level in the plants during stress conditions. In previous studies, an increase in H_2O_2 production in pea plants treated with cadmium has been shown (Romero-Puertas *et al.*, 2004) probably due to the greater GOX activity under the metal stress (Romero-Puertas *et al.*, 1999). Thus, the current study has shown that GOX is S-nitrosylated under physiological conditions while the S-nitrosylated protein almost disappears under cadmium stress, although an increase in GOX protein content is observed. This result agrees with the intensification of GOX activity found previously (Romero-Puertas *et al.*, 1999), which was probably due to not only a slight increase in protein content but also a post-translational regulation through the reduction of S-nitrosylation of the enzyme. On the other hand, the reduction on S-nitrosylation of GOX in pea plants treated with 2,4-D (this work) is not correlated with the reduction of GOX activity observed after herbicide treatment (Pazmiño *et al.*, 2011). This could be due to the reduction in the protein content observed with 2,4-D treatment. It has been shown that there is a reduction in the phosphorylation of GOX during 2,4-D treatment, which could be also responsible for the observed decrease in GOX activity (Pazmiño, 2009).

Although both abiotic stresses, cadmium (Barroso *et al.* 2006; Rodríguez-Serrano *et al.* 2009) and 2,4-D, reduced

NO in pea plants, this study found no differences in the S-nitrosylation of proteins under cadmium treatment but an increase in the signal in the presence of the herbicide. This divergence is probably due to differences in NO metabolism under both types of stress. More intense GSNOR activity was found during 2,4-D treatment, while a reduction in this activity has previously been reported with the metal (Barroso *et al.*, 2006). While the current results appear to contradict the findings reported in *Arabidopsis* mutants over-expressing *AtGSNOR* (obtained by expression of transgenes or T-DNA insertion mutants), which showed a lowering of total S-nitrosylation levels, no data on NO production, which could influence S-nitrosylation levels as well, are reported in these plants (Feechan *et al.*, 2005; Rusterucci *et al.*, 2007). Additionally, the methods used in these studies to analyse total SNOs in the plants are not the same as those used in the present work. In addition, this study examined the response of pea plants under specific stress conditions, while over-expression of a gene such as GSNOR could give rise to some pleiotropic effect involving hormone or nitrate impairment (Feechan *et al.*, 2005; Lee *et al.*, 2008) and cannot be compared with the response to a specific stress. In fact, it has been shown that GSNOR activity is necessary for the acclimation of plants to high temperatures and for normal development and fertility under optimal growth conditions (Lee *et al.*, 2008). GSNOR controls both NO availability and intracellular S-nitrosylation levels, affecting protein S-nitrosylation and important metabolic pathways such as disease resistance (Liu *et al.*, 2001; Feechan *et al.*, 2005; Rusterucci *et al.*, 2007). The current results suggest that this activity could be involved in cadmium and 2,4-D response as well.

In conclusion, six peroxisomal proteins have been identified as the target of S-nitrosylation and the activities of three proteins have been shown to be affected by this modification. The S-nitrosylation levels of CAT and GOX, the main H_2O_2-removing and -producing enzymes in peroxisomes, have been analysed in peroxisomes from pea leaves treated with two abiotic stresses. It was shown that S-nitrosylation could regulate the peroxisomal level of key signalling molecules such as H_2O_2, as explained in the model presented in Fig. 11. Future studies on the identified proteins will help in understanding the significance of S-nitrosylation in plant peroxisomes and the peroxisomal contribution throughout plant development and during plant responses to different stress conditions. Special interest on the photorespiration pathway is needed because it appears to be regulated by S-nitrosylation. Post-translational modifications fine tune the photorespiratory enzymes, which could be a fast adaptive mechanism to prevent oxidative damage by H_2O_2 accumulation, but also could regulate the flux of the metabolites shared by different pathways in peroxisomes. In addition to S-nitrosylation, other post-translational modifications such as phosphorylation or nitration could help to regulate peroxisomal metabolism. Preliminary results according to proteomic approaches have demonstrated that CAT, GOX, and MDH are also phosphorylated (Pazmiño, 2009), pointing to the relevance of post-translational
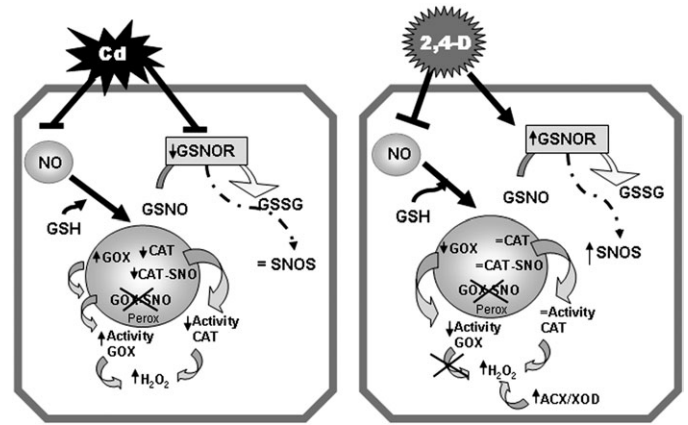
Fig. 11. Model of the effect of cadmium and 2,4-dichlorophenoxy acetic acid (2,4-D) treatments on NO metabolism. Both types of abiotic stress reduced NO production, although a differential effect was observed in GSNOR activity. An increase in S-nitrosylated proteins under 2,4-D treatment was detected while no differences were found under cadmium treatment. However, a reduction in GOX S-nitrosylation was found in peroxisomes under both treatments while CAT S-nitrosylation was reduced only under cadmium treatment. The increase of GOX protein and the reduction of the S-nitrosylation pattern of this protein, which induced GOX activity under cadmium stress, with the reduction of CAT activity could in part be responsible for the increase in H_2O_2 observed under this stress. Under 2,4-D stress, however, the reduction of GOX protein led to a reduction in its activity, although GOX S-nitrosylation practically disappeared. It seems that the increase in H_2O_2 found with the herbicide treatment was not due to GOX activity but to ACX and XOD (Pazmiño *et al.*, 2011). ACX, acyl CoA oxidase; CAT, catalase; GOX, glycolate oxidase; GSH, glutathione; GSNO, S-nitrosoglutathione; GSNOR, S-nitrosoglutathione reductase; NO, nitric oxide; SNO, S-nitrosylated; SNOS, S-nitrosylated proteins; XOD, xanthine oxidase.

regulation of photorespiration and paving the way for future studies on the relevance of post-translational modification cross-talk.

Supplementary material

Supplementary data are available at *JXB* online.
Supplementary Fig. S1. Structure of *Arabidopsis* GOX1.
Supplementary Fig. S2. Structure of *Arabidopsis* CAT1.

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