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Localization of *S*-nitrosoglutathione and expression of *S*-nitrosoglutathione reductase in pea plants under cadmium stress*

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Abstract

***S*-nitrosoglutathione (GSNO) is considered a natural nitric oxide ($NO^{\cdot}$) reservoir and a reactive nitrogen intermediate in animal cells, but little is known about this molecule and its metabolism in plant systems. In this work, using pea plants as a model system, the presence of GSNO in collenchyma cells was demonstrated by an immunohistochemical method. When pea plants were grown with a toxic Cd concentration (50 μM) the content of GSNO in collenchyma cells was drastically reduced. Determination of the nitric oxide ($NO^{\cdot}$) and gluthathione contents in leaves by confocal laser scanning microscopy and HPLC, respectively, showed a marked decrease of both compounds in plants treated with cadmium. The analysis of the *S*-nitrosoglutathione reductase (GSNOR) activity and its transcript expression in leaves showed a reduction of 31% by cadmium. These results indicate that GSNO is associated with a specific plant cell type, and this metabolite and its related catabolic activity, GSNOR, are both down-regulated under Cd stress.**

Key words: Abiotic stress, collenchyma, formaldehyde dehydrogenase, nitric oxide, reactive nitrogen species, RNS, *S*-nitrosoglutathione, *S*-nitrosoglutathione reductase, signalling.

Introduction

In animal cells, it is well established that nitric oxide (NO) is generated from L-arginine by a family of enzymes called nitric oxide synthases (NOSs) (Alderton *et al*., 2001), and NO plays a wide range of important functions, such as vasodilatation, relaxation of muscles, neurotransmission, neuromediation, and host defence reactions (Moncada *et al*., 1991; Ignarro, 2002). In plants, research on NO has gained considerable attention in recent years due to the discovery of its function in plant growth and development and as a key signalling molecule in different intracellular processes (Lamattina *et al*., 2003, Neill *et al*., 2003; del Río *et al*., 2004; Lamotte *et al*., 2005).

However, in plants, there is little information on the enzymatic source of endogenous NO during normal development of plants and on the loci where NO is generated. Nitric oxide can be produced in plants by non-enzymatic and enzymatic systems (for a review, see del Río *et al*., 2004). The enzyme nitrate reductase is a well-established generator of NO (Dean and Harper, 1988; Yamasaki *et al*., 1999; Rockel *et al*., 2002) although this enzyme does not produce NO from L-arginine and, therefore, it cannot be considered as a characteristic NOS activity. In the past decade, many plant biologists searched diligently for an NO-generating enzyme similar to the nitric oxide synthases (NOSs) identified in mammalian systems (Wendehenne

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Abbreviations: CLSM, confocal laser scanning microscopy; NO, nitric oxide; ROS, reactive oxygen species; RNS, reactive nitrogen species; DAF-2 DA, 4,5-diaminofluorescein diacetate; GSNO, *S*-nitrosoglutathione.

et al., 2001; Corpas *et al*., 2004*a*), and there has been an increasing number of reports showing the presence of nitric oxide synthase-like activities in plants (for a review, see del Río *et al*., 2004). Recently, the presence of a constitutive arginine-dependent NOS activity in roots, stems, and leaves of pea plants was demonstrated (Corpas *et al*., 2006). At a subcellular level, a NOS activity sensitive to characteristic inhibitors of animal NOS and with the same cofactor requirements (NADPH, BH_4, FAD, FMN, calcium, and calmodulin) has been identified in peroxisomes from pea leaves (Barroso *et al*., 1999; Corpas *et al*., 2004*b*). On the other hand, a gene of a plant protein, AtNOS1, which produces NO from L-arginine, has been identified in *Arabidopsis*, although this NOS activity does not depend on BH_4, FAD, and FMN as cofactors (Guo *et al*., 2003; Guo and Crawford, 2005). To the best of our knowledge, peroxisomes and mitochondria, are the only plant cell organelles where the arginine-dependent NO generation has been demonstrated (Corpas *et al*., 2004*b*; Guo and Crawford, 2005).

In animal cells, knowledge on the physiological and metabolic functions of NO and other reactive nitrogen species (RNS) has experienced a significant advance in recent years. As a result, researchers in plant biology have started to pay attention to the interactions reported in animal cells between NO and molecules like thiols, amino acids, and proteins (Alvarez and Radi, 2003). Some important physiological functions of nitric oxide (NO) are performed through the so-called *S*-nitrosothiols (SNOs) which are formed by reaction of NO with protein or non-protein sulphydryl-containing compounds (Hogg, 2000; Foster *et al*., 2003). *S*-nitrosothiols perform important biological reactions, including nitric oxide release, transnitrosation, *S*-thiolation, as well as direct actions (Hogg, 2000; Stamler *et al*., 2001). There is considerable evidence in animal cells indicating that *S*-nitrosylation of cysteine thiols of proteins is an important redox-based post-translational modification (Stamler *et al*., 2001; Foster *et al*., 2003).

If little is known in plant systems on the enzymatic source(s) of NO, much less information is available on *S*-nitrosothiols and their metabolism. The thiol tripeptide, γ-glutamyl cysteinyl glycine (glutathione, GSH) is one of the major low-molecular-weight soluble antioxidants of plant cells and is involved in the antioxidative ascorbate–glutathione cycle (Noctor and Foyer, 1998; Foyer, 2001). But GSH also has important functions apart from the antioxidative system, including the detoxification of xenobiotics and heavy metals (Steffens, 1990) and involvement in signal transduction processes (Puppo *et al*., 2005). The *S*-nitrosylation reaction of $NO^{\cdot}$ with GSH to form *S*-nitrosoglutathione (GSNO) has a significant physiological relevance in plants since GSNO is thought to function as a mobile reservoir of NO bioactivity (Durner *et al*., 1999; Díaz *et al*., 2003), as reported in animals (Stamler *et al*., 2001). Recently, total *S*-nitrosothiol (SNO) levels were measured in *Arabidopsis* plants that were altered in GSNOR activity (Feechan *et al*., 2005) and, as far as is known, no more experimental evidence has been obtained on the presence of GSNO in plant cells.

In recent years, the glutathione-dependent enzyme formaldehyde dehydrogenase (FALDH; EC 1.2.1.1) has been demonstrated to have GSNO reductase activity in bacteria, yeast, and mammals (Liu *et al*., 2001) and to be involved in the mechanism of protein *S*-nitrosation in mammalian cells (Haqqani *et al*., 2003). The enzyme GSNO reductase (GSNOR) catalyses the NADH-dependent reduction of GSNO to GSSG and NH_3 (Liu *et al*., 2001; Lamotte *et al*., 2005). In plants, glutathione-dependent formaldehyde dehydrogenase has been found very active in the reduction of *S*-nitrosoglutathione (GSNO) in *Arabidopsis* (Sakamoto *et al*., 2002; Achkor *et al*., 2003) and tobacco (Díaz *et al*., 2003). In tobacco plants, the gene coding for this enzyme is modulated in response to wounding, jasmonic acid, and salicylic acid (Díaz *et al*., 2003). The glutathione-dependent formaldehyde dehydrogenase, also denominated class III alcohol dehydrogenase, has been purified and characterized in pea seeds (Uotila and Koivusalo, 1979; Shafqat *et al*., 1996) and *Arabidopsis* plants (Martínez *et al*., 1996).

Cadmium is a toxic trace pollutant for humans, animals, and plants (Wagner, 1993; He *et al*., 2005). Different metabolic processes such as photosynthesis and respiration are affected by Cd (Romero-Puertas *et al*., 2004). In pea plants grown with 50 μM $CdCl_2$ this metal produced a significant inhibition of growth, as well as a reduction in the transpiration and photosynthesis rate, chlorophyll content, and disturbances in the nutrient status of pea plants (Sandalio *et al*., 2001). Moreover, Cd produced disturbances in plant antioxidant defences and was demonstrated to induce oxidative stress in pea plants (Romero-Puertas *et al*., 2004).

In this work, using pea plants as a model system and employing biochemical, molecular, and cellular approaches, the presence and tissue distribution of GSNO in leaves was studied, and this *S*-nitrosothiol and its catabolic activity, GSNO reductase, were found to be down-regulated under Cd stress conditions.

Materials and methods

Plant material and growth conditions

Pea (*Pisum sativum* L., cv Lincoln) seeds were obtained from Royal Sluis (Enkhuizen, Holland). Plants were grown in the greenhouse in aerated full-nutrient media under optimum conditions for 14 d (Sandalio *et al*., 2001). The media were then either unsupplemented (control plants) or were supplemented with 50 μM $CdCl_2$, and plants were grown for a further 14 d.

Preparation of leaf extracts

All operations were carried out at 0–4 °C. Leaves were ground using a mortar and pestle in a solution containing 0.1 M TRIS–HCl pH 7.5,

2 mM DTT, 0.1 mM EDTA, 0.2% (v/v) Triton-X-100, and 10% (v/v) glycerol. Homogenates were centrifuged at 27 000 *g* for 25 min. Then, the supernatants were passed through Sephadex G-25 gel filtration columns (NAP-10 from Amersham) which were equilibrated and eluted with the same buffer before the enzymatic assays.

Enzyme assay and protein determination

GSNOR activity was assayed spectrophotometrically at 25 °C by monitoring the oxidation of NADH at 340 nm as described by Sakamoto *et al*. (2002). The leaf extracts were incubated in an assay mixture containing 20 mM TRIS–HCl (pH 8.0), 0.2 mM NADH, and 0.5 mM EDTA, and the reaction was started by adding GSNO (Calbiochem) to the mixture at a final concentration of 400 μM. The activity was expressed as nmol NADH consumed min^{-1} mg^{-1} protein (ε_{340}=6.22 mM^{-1} cm^{-1}). Protein concentration was assayed according to Bradford (1976) using bovine serum albumin as standard.

Native polyacrylamide gel electrophoresis was performed using 6% acrylamide gels in TRIS-boric-EDTA buffer, pH 8, as described by Laemmli (1970). Staining for GSNOR activity was performed using a modification of the method reported by Seymour and Lazarus (1989). Gels were soaked in 0.1 M sodium phosphate, pH 7.4, containing 2 mM NADH, for 15 min, in an ice-bath. Excess buffer was drained and gels were covered with filter paper strips soaked in freshly prepared 3 mM GSNO. After 10 min, the filter paper was removed and gels were exposed to ultraviolet light and analysed for the disappearance of the NADH fluorescence, indicating GSNOR activity (Fernández *et al*., 2003).

Quantification of glutathione

The concentration of reduced glutathione was determined according to Jiménez *et al*. (1997) in leaf extracts obtained in 6% (v/v) perchloric acid, by measuring the reaction of iodoacetic acid with SH-groups to produce *S*-carboxymethyl derivatives. Amine groups were then derivatized with the Sanger reagent to produce 2,4-dinitrophenyl derivatives which were detected by HPLC using a 3-aminopropyl column.

RNA isolation and cloning of a pea GSNOR cDNA

Total RNA was isolated from pea leaves with the Trizol Reagent Kit (Gibco BRL) as described in the manufacturer's manual. RNA was quantified spectrophotometrically. One μg of total RNA was used as a template for the reverse transcriptase (RT) reaction. It was added to a mixture containing 5 mM $MgCl_2$, 1 mM dNTPs, 0.5 μg polydT_{23} primer, 1× RT-Buffer, 20 U Rnasin ribonuclease inhibitor, and 20 U AMV reverse transcriptase (FIZZYMES). The reaction was carried out at 42 °C for 40 min, followed by a 5 min step at 98 °C, and then by cooling to 4 °C. Using the protein sequence of the pea glutathione-dependent formaldehyde dehydrogenase (accession number P80572), oligonucleotides were designed for conserved regions (Table 1) and by RT-PCR a partial cDNA of 257 bp was obtained (accession no. DQ084382).

The polymerase chain reaction (PCR) was as follows: 1 μl of the produced cDNA diluted 1/20 was added to 250 μM dNTPs, 1.5 mM $MgCl_2$, 1× PCR buffer, 1.25 U of Hot Start *Taq* polymerase (Eppendorf) and 0.5 μM of each primer (Table 1) in a final volume of 20 μl. Reactions were carried out in a Master Cycler (Eppendorf). A first step of 2 min at 94 °C was followed by 30 cycles of 1 min at 94 °C, 1 min at 55 °C, and 1 min at 65 °C with a final extension of 10 min at 65 °C. Amplified PCR products were detected after electrophoresis in 1% agarose gels stained with ethidium bromide. The PCR reaction was loaded on a 1% agarose gel and the visualized bands were cut and extracted from the gel (Qiaex II gel extraction kit, Qiagen). The purified fragments were cloned into the pGEM-T easy vector (Promega) and sequenced.

Table 1. *Oligonucleotides used for GSNOR cloning and quantitative PCR analysis*

f and r correspond to forward and reverse oligonuclotides, respectively. For the degenerated oligonucleotides the letters mean: M=A,C; R=A,G; K=G,T.

Name	Oligonucleotide sequence (5′ to 3′)	Product size (bp)
cDNA cloning		
f2-GSNOR	GGC AAG GAT CCT GAR GGT CT	300
r1-GSNOR	GRA ACA CCR GTK CCA AGM AG	
Q-PCR		
f4-GSNOR	CCACGAAGCTGCAGGGATT GTTGAAAGTG	169
r4-GSNOR	TAACCCCAACACCAGTGGCA GCACGAAC	
RNA 18S-f1	TTT GAT GGT ACC TGC TAC TCG GAT AAC C	274
RNA 18S-r1	CTC TCC GGA ATC GAA CCC TAA TTC TCC	

Real-time quantitative PCR

The real-time quantitative PCR was performed in 20 μl of reaction mixture, composed of 1 μl of different cDNAs and master mix IQ™ SYBR® Green Supermix with a final concentration of 0.5 U of hot-start iTaq™ DNA Polymerase (Bio-Rad), 20 mM KCl, 16 mM TRIS–HCl, pH 8.4, 0.16 mM each dNTPs, 2.4 mM $MgCl_2$, 0.5 μM gene-specific primers (Table 1), and SYBR Green I, 8 nM fluorescein, using a iCycler iQ system (Bio-Rad). Amplifications were performed under the following conditions: initial polymerase activation: 95 °C, 4 min; then 30 cycles of 30 s at 95 °C, 30 s at 60 °C, and 1 min at 72 °C.

The specific primers for the pea GSNO reductase (Table 1) were designed to anneal at different exons at distances large enough to avoid the appearance of false positive bands caused by co-amplification of contaminating DNA based on the partial cDNA previously obtained, using the RNA 18S as an internal control.

$NO^{\cdot}$ detection by confocal laser scanning microscopy

Pea leaf segments of approximately 25 mm^2 were incubated for 1 h at 25 °C, in darkness, with 10 μM 4,5-diaminoflorescein diacetate (DAF-2 DA, Calbiochem) prepared in 10 mM TRIS–HCl (pH 7.4) (Corpas *et al*., 2004*b*), this probe being highly specific for NO (Nakatsubo *et al*., 1998). They were then washed twice in the same buffer for 15 min each. After washing, leaf sections were embedded in a mixture of 15% acrylamide-bisacrylamide stock solution as described elsewhere (Peinado *et al*., 2000), and 100 μm-thick sections, as indicated by the vibratome scale, were cut under 10 mM phosphate-buffered saline (PBS). Sections were then soaked in glycerol:PBS (containing azide) (1:1; v:v) and mounted in the same medium for examination with a confocal laser scanning microscope system (Leica TCS SL), using standard filters and collection modalities for DAF-2 green fluorescence (excitation 495 nm; emission 515 nm) and chlorophyll autofluorescence (chlorophyll *a* and *b*, excitation 429 and 450 nm, respectively; emission 650 nm and 670 nm, respectively) as orange. Background staining, routinely negligible, was controlled with leaf sections unstained.

Cellular localization of GSNO by immunofluorescence microscopy

Pea leaves were cut into 4–5 mm pieces and fixed in 4% (w/v) *p*-formaldehyde in 0.1 M phosphate buffer, pH 7.4 (PB), for 3 h at room temperature. They were then cryoprotected by immersion in 30% (w/v) sucrose in PB overnight at 4 °C. Serial sections, 60 μm thick, were obtained by means of a cryostat (2800 Frigocut E,

Reichert-Jung, Vienna, Austria). Free floating sections were incubated at room temperature overnight with a commercial rat antibody against *S*-nitrosoglutathione (Calbiochem, Cat. no. 487932) diluted 1:2500 in 5 mM TRIS-buffer, pH 7.2, containing 0.9% (w/v) NaCl, 0.05% (w/v) sodium azide, 0.1% (w/v) bovine serum albumin, and 0.1% (v/v) Triton X-100 (TBSA-BSAT). After several washes with TBSA-BSAT, sections were incubated with biotinylated goat anti-rat IgG (Amersham, Buckinghamshire, UK), diluted 1:1000 in TBSA-BSAT, for 1 h at room temperature. Sections were then washed again and incubated with Cy2-streptavidin (Amersham) and diluted 1:1000 in TBSA-BSAT at room temperature for 1.5 h. The sections were mounted on glass slides with PBS-glycerol (v:v; 1:1), covered with a cover slip, and observed using either a fluorescence microscope or a confocal laser scanning microscope with standard filters for Cy2 (excitation 495 nm; emission, 515 nm). Controls for background staining, usually negligible, were performed by replacing the corresponding primary antiserum by preimmune serum in adjacent sections or without primary antiserum.

Results

The cellular localization of GSNO in pea leaf sections was analysed by immunofluorescence microscopy, using a commercial antibody against this molecule (Fig. 1). A strong green immunofluorescence attributable to GSNO was detected mainly in collenchyma cells (Co) of pea leaf sections from control plants (Fig. 1A), but the green fluorescence was considerably reduced in pea leaves of plants grown with cadmium (Fig. 1B). In both cases, the autofluorescence appeared as a yellowish colour in xylem (X), parenchymal (Pa), and epidermal cuticle (Ec) cells of both control (Fig. 1A) and Cd-treated plants (Fig. 1B), with only a change in Ec intensity in Cd-treated plants. Similar results were obtained when the leaf sections were observed by confocal laser scanning microscopy (data not shown). When the antibody against GSNO was omitted in the localization experiments, no immunofluorescence was detected (Fig. 1E). On the other hand, the effect of cadmium on the $NO^{\cdot}$ production in pea leaves was evaluated by confocal laser scanning microscopy (CLSM) using the fluorescent probe 4,5-diaminofluorescein diacetate (DAF-2 DA). An intense green fluorescence, characteristic of $NO^{\cdot}$, was observed in vascular tissues and was less intense in cells of the epidermis, and the palisade and spongy mesophyll (Fig. 1C). When the leaf sections were incubated without the fluorescencent probe, no fluorescence was detected (Fig. 1F). However, in leaves from plants grown with cadmium, the green fluorescence was considerably reduced (Fig. 1D).

The GSH content of pea leaves was also analysed and was found to be 49.3 ± 4.2 nmol ml^{-1} and 22.0 ± 2.0 nmol ml^{-1} in control and Cd-treated plants, respectively. This indicated that, in leaves from plants grown with 50 μM Cd^{2+}, a reduction of the GSH level of more that 50% was produced.

It has recently been described that *Arabidopsis* glutathione-dependent formaldehyde dehydrogenase has also *S*-nitrosoglutahione reductase (GSNOR) activity (Sakamoto *et al*., 2002). On the basis of this result, the possible existence of this GSNOR activity in leaves from pea plants was investigated. The specificity of the GSNOR activity was assayed by monitoring the oxidation of NADH which took place after the addition of the substrate GSNO (Fig. 2). In these conditions, a GSNOR activity of 2.67 ± 0.28 nmol NADH min^{-1} mg^{-1} protein was determined in crude extracts of pea leaves, and when plants were grown with cadmium this activity was reduced by 31% (Fig. 3A). On the other hand, analysis of leaf crude extracts by native polyacrylamide gel electrophoresis and staining for GSNOR activity showed a single band in control plants and this activity was also reduced in Cd-treated plants (Fig. 4).

To analyse the mRNA expression of the pea GSNOR by quantitative real-time PCR, it was necessary to obtain a partial cDNA of the pea GSNOR (accession number DQ084382). A cDNA was obtained which coded for a protein fragment of 85 amino acids. This cDNA showed a 92% identity with the glutathione-dependent formaldehyde dehydrogenase (which has been demonstrated to have GSNOR activity) of *Oryza sativa* (BAD21676), *Lycopersicon peruvianum* (AAX44241), and *Zea mays* (CAA71913), and a 90% identity with the enzyme of *Arabidopsis thaliana* (Q96533). With this sequence information it was posible to design specific primers (Table 1) to study the mRNA expression of GSNOR by quantitative real-time PCR. The results obtained showed that the transcripts of GSNOR were down-regulated (32%) in leaves of pea plants treated with cadmium (Fig. 3B).

Discussion

Nitric oxide has been recognized as an important molecule in many plant physiological processes and, depending on the $NO^{\cdot}$ level in the cell, this free radical can act either as a signal or a toxic molecule (Lamattina *et al*., 2003; Neill *et al*., 2003; del Río *et al*., 2004). However, in plant cells there is little information available on the presence and modulation of molecules derived from the $NO^{\cdot}$ metabolism. In animal cells, GSNO is being intensively studied since this molecule is considered a natural reservoir of $NO^{\cdot}$ (Padgett and Whorton, 1995; Steffen *et al*., 2001; He *et al*., 2004; Zhang and Hogg, 2004) and one of the most relevant compounds to perform nitrosation reactions under physiological conditions (Steffen *et al*., 2001).

Nevertheless, the presence of GSNO in plants has only been hypothesized as a molecule involved in the nitric oxide pathway (Durner and Klessig, 1999; Durner *et al*., 1999; del Río *et al*., 2003; Díaz *et al*., 2003; Lamotte *et al*., 2005; Lindermayr *et al*., 2005) but thus far there is very little experimental evidence for the presence and distribution of GSNO in plant cells. This situation contrasts with the abundant information available on the NO content in

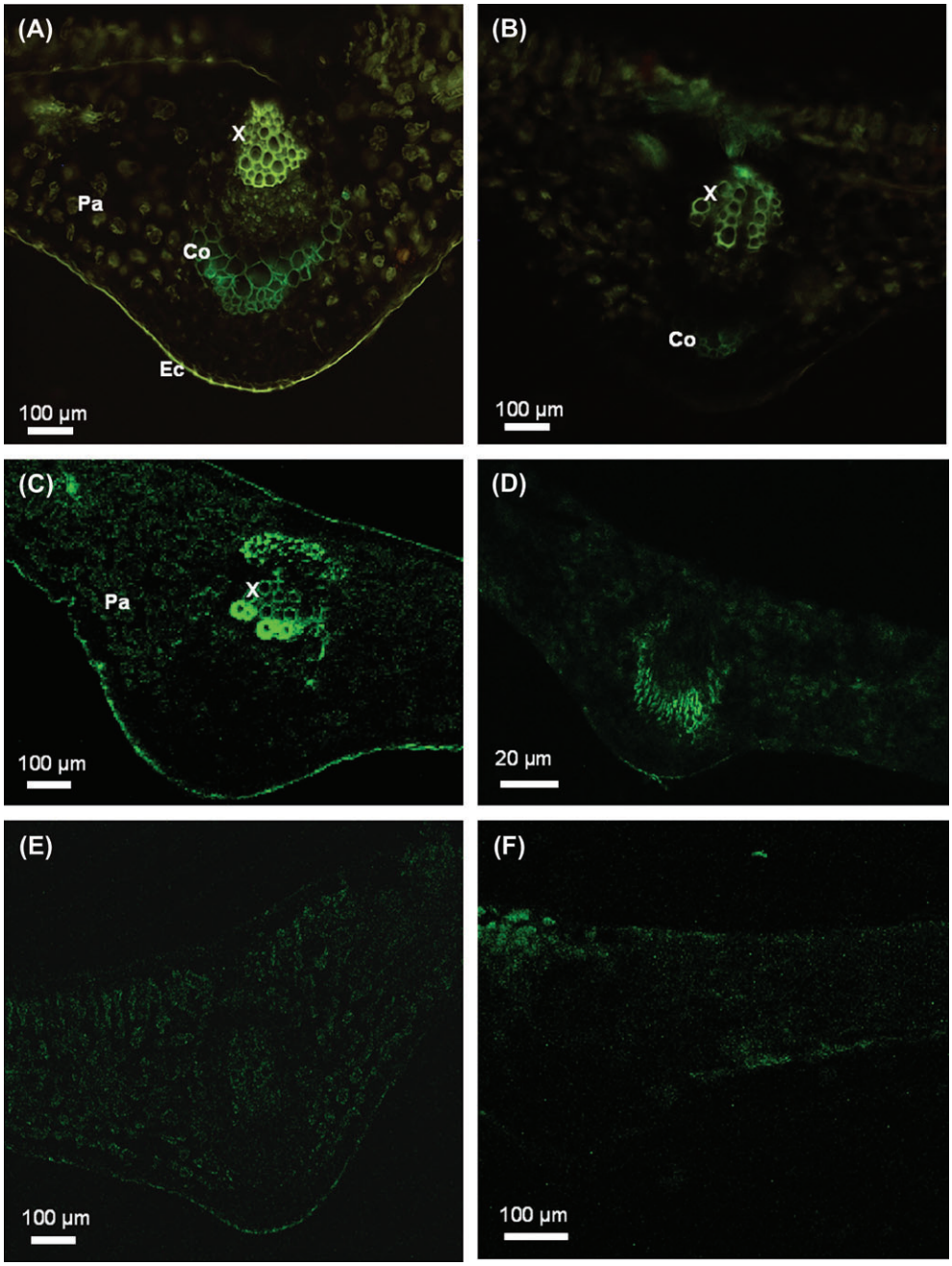

Fig. 1. Localization of GSNO and NO· in pea leaves. GSNO was localized by immunofluorescence histochemistry using anti-GSNO and Cy2-conjugated antibody. The intense green immunofluorescence attributable to anti-GSNO observed by fluorescence microscopy was mainly localized in the collenchyma cells of leaves from control plants (A). (B) Plants grown with 50 µM $CdCl_2$. Autofluorescence appears as a yellowish colour (A, B) and a change in its intensity is only appreciable in the epidermal cuticle of leaves from cadmium-treated plants. The level of endogenous NO· in pea leaves was detected by confocal laser scanning microscopy (CLSM) using the fluorescent probe 4,5-diaminofluorescein diaceate (DAF-2 DA). NO· was identified by its bright green fluorescence. (C) Control plants. (D) Plants treated with 50 µM $CdCl_2$. (E) Control of background staining where the anti-GSNO was omitted. (F) Control of background staining where DAF-2 DA was omitted. Xylem (X), phloem (P), collenchyma (Co), parenchyma (Pa), and epidermal cuticle (Ec) cells.

plants (Foissner *et al*., 2000; Pedroso *et al*., 2000; Gould *et al*., 2003; Lamattina *et al*., 2003; Neill *et al*., 2003; Corpas *et al*., 2004*b*; Gabaldón *et al*., 2005). The main goal of this work was to study the metabolic relationships of GSNO using as a model pea plants grown with cadmium which has previously been shown to induce oxidative stress in this plant species (Sandalio *et al*., 2001). In order to get a deeper knowledge into NO and its metabolism in plant cells, biochemical, molecular, and cellular approaches have been used and the content of nitric oxide (NO),

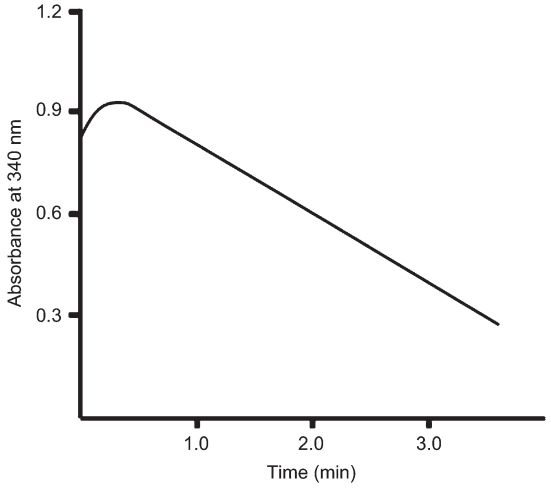

Fig. 2. GSNO-dependent oxidation of NADH by pea leaf extracts. The reaction was carried out by following the oxidation of NADH at 340 nm, as described by Sakamoto *et al*. (2002). Following the addition of GSNO to the reaction mixture, the pea leaf extracts (300 μg protein) rapidly oxidized NADH.

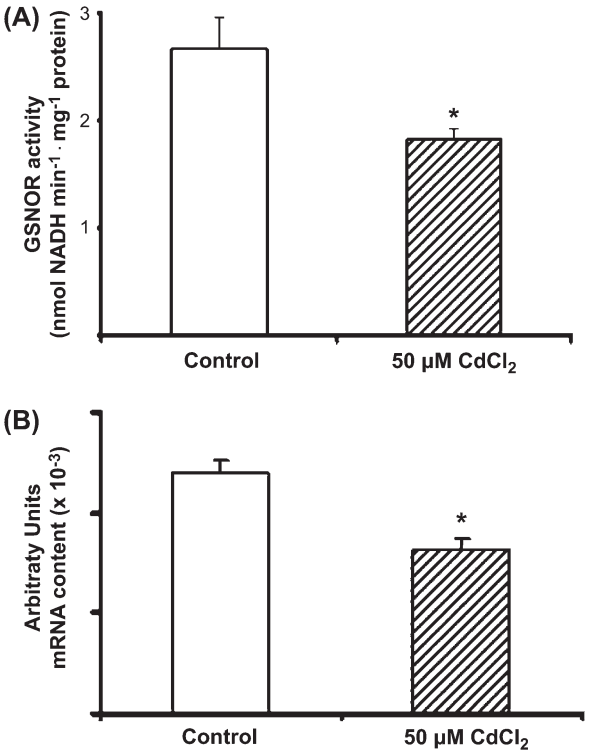

Fig. 3. *S*-nitrosoglutathione reductase (GSNOR) activity and expression in leaves from control and Cd-treated pea plants. (A) GSNOR activity in leaf homogenates from control plants and from plants grown with 50 μM $CdCl_2$. Data are mean ±SEM of at least four different replicates. * Differences from control value were significant at $P < 0.05$. (B) Real-time PCR transcript analysis (arbitrary units) of GSNOR in control and Cd-treated pea plants. Data are mean ±SEM of, at least, four independent preparations of RNA from pea leaves. Differences were significant at $P < 0.05$.

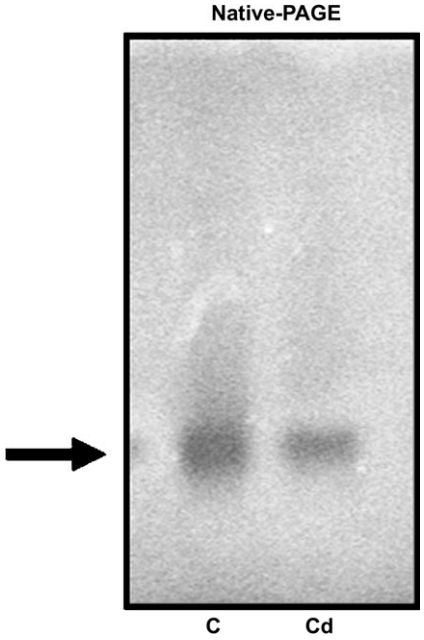

Fig. 4. Native polyacrylamide gel electrophoresis of pea leaf extracts and staining for GSNOR activity. Leaf extracts (200 μg protein) were subjected to electrophoresis on 6% acrylamide gels. C, control plants. Cd, plants treated with 50 μM $CdCl_2$.

glutathione (GSH), *S*-nitrosoglutathione (GSNO), and the activity and mRNA expression of GSNOR were analysed.

Pea plants have proved to be a good experimental model under normal and stress conditions. Pioneer studies on nitric oxide were carried out with this leguminous plant (Leshem and Haramaty, 1996), even when the enzymatic source(s) of nitric oxide had still to be identified (Corpas *et al*., 2004*a*). In previous studies in pea plants, it was demonstrated that Cd induces oxidative stress symptoms characterized by an increase in the generation of ROS, alterations in the antioxidative systems, lipid peroxidation, and oxidative modification of proteins (Sandalio *et al*., 2001; Romero-Puertas *et al*., 2002, 2004). These results, together with the ultrastructural data, pointed to an induction of leaf senescence by cadmium (McCarthy *et al*., 2001; Sandalio *et al*., 2001).

Results reported in this work showed that under the Cd-induced oxidative stress conditions, the content of soluble GSH in pea leaves was reduced more than 50% and the cellular analysis of NO by CLSM also revealed a significant reduction of this molecule in the vascular tissues. The ratio of reduced to oxidized glutathione has been proposed as a redox sensor in different signal transduction processes (Foyer and Noctor, 2003). Results recently obtained in pea plants treated with Cd have shown that the ratio GSH/GSSG in leaves was significantly decreased by metal treatment (MC Romero-Puertas *et al*., unpublished results). Therefore, the results reported in this work on the Cd-induced decrease of GSH content of leaves are consistent with the reduction in the GSNO level determined in the

same tissue by immunofluorescence histochemistry. On the other hand, during the natural senescence of pea leaves a decrease of the $NO^{\cdot}$ level was reported (Corpas *et al*., 2004*b*) which could contribute to the senescence symptoms induced by Cd in leaves of pea plants (McCarthy *et al*., 2001; Sandalio *et al*., 2001).

The GSNOR activity has been described to be associated with the enzyme glutathione-dependent formaldehyde dehydrogenase (Sakamoto *et al*., 2002). Very recently it has been reported that mutations of *AtGSNOR1*, an *Arabidopsis thaliana* GSNOR, modulate the extent of cellular *S*-nitrosothiol formation and turnover, which seems to regulate multiple modes of plant disease resistance (Feechan *et al*., 2005). In this work to study the GSNO modulation by Cd in pea leaves, the GSNOR activity (total and isozymic) and mRNA expression were analysed. Under cadmium stress, a down-regulation in both activity and transcripts was found in pea leaves (Fig. 3), and these results were well correlated with the observed decrease of the NO and GSH contents. This suggests that Cd stress down-regulates both activity and expression of GSNOR, probably because, under these conditions, the formation of GSNO, the enzyme's substrate, is also depressed as checked by immunofluorescence histochemistry. The plant GSNOR, as proposed in microbes and mammals (Liu *et al*., 2001), might have a dual role in turning off GSNO-derived NO signalling and in the cellular protection against nitrosative stress by controlling excess *S*-nitrosylation (Liu *et al*., 2001; Sakamoto *et al*., 2002; Lamotte *et al*., 2005). Under the conditions used in this work, Cd is known to induce oxidative stress in pea plants (McCarthy *et al*., 2001; Sandalio *et al*., 2001; Romero-Puertas *et al*., 2002, 2004), and the results obtained indicate that it is very unlikely that Cd can cause nitrosative stress in pea plants. This suggests that oxidative and nitrosative stress do not necessarily go hand in hand.

In *Arabidopsis* the expression of the GS-FDH/GSNOR gene is down-regulated by mechanical wounding and activated by salicylic acid (Díaz *et al*., 2003). However, in preliminary results obtained in our laboratory with pea plants under different environmental stresses, an increase in the GSNOR activity was found by low and high temperature, continuous light, darkness, and mechanical wounding (FJ Corpas *et al*., unpublished results). These results contrast with those reported in this work for Cd and suggests under that abiotic stress conditions, the GSNOR activity is differentially regulated depending on the stress type.

An important question to elucidate is the physiological function of GSNO in the collenchyma cells and its down-regulation by cadmium stress. In pea leaves, it has previously been observed that $NO^{\cdot}$ was localized mainly in the xylem of vascular tissue but not in collenchyma cells (Corpas *et al*., 2004*b*), and similar results were reported in the xylem of *Zinnia elegans* (Gabaldón *et al*., 2005). The collenchyma is a typical supporting tissue of the primary plant body and growing plant organs. Often either phloem or xylem of the vascular bundles is associated with collenchyma cells. The localization of H_2O_2 and peroxidase in collenchyma tissues of *Zinnia elegans* stems has been reported (Ferrer and Ros-Barceló, 1999) which could indicate that they are involved in the cross-linking of pectins and hemicelluloses that predominate in the walls of these cells. In addition, a glutathione peroxidase-like protein was localized in the collenchyma of *Solanum lycopersicum*, and this protein was induced by mechanical stimulation, suggesting that it could regulate apoplastic ROS accumulation (Herbette *et al*., 2004). Considering that $NO^{\cdot}$ can inhibit the H_2O_2-metabolizing enzymes catalase and ascorbate peroxidase (Clark *et al*., 2000), the presence in the collenchyma of the $NO^{\cdot}$ donor GSNO could allow the persistence of hydrogen peroxide, a metabolite involved in the lignification process in vascular tissues (Ros-Barceló, 1997, 2005; Gabaldón *et al*., 2005). Thus the $NO^{\cdot}$ level regulated by GSNOR might contribute to the gradual lignification of the adjacent cells of collenchyma during the cell proliferation and elongation processes.

In summary, in this work the presence of GSNO and its tissue localization was demonstrated in pea plants. The presence of *S*-nitrosoglutathione reductase activity was verified in leaves from pea plants and this activity and GSNO were found to be decreased under Cd stress conditions. If it has been demonstrated in *Arabidopsis* that a decrease in GSNOR activity causes an accumulation of SNO (Feechan *et al*., 2005), results reported in this work suggest an alternative possibility: that the GSNOR activity could be induced by its substrate GSNO. Further research is necessary to demonstrate this hypothesis, and to localize and characterize the GSNO metabolic pathways in plant cells. This would open new avenues in the research of the physiological function of $NO^{\cdot}$ in higher plants.

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