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RESEARCH PAPER

Mechanical wounding induces a nitrosative stress by down-regulation of GSNO reductase and an increase in *S*-nitrosothiols in sunflower (*Helianthus annuus*) seedlings

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Abstract

Nitric oxide (NO) and related molecules such as peroxynitrite, *S*-nitrosoglutathione (GSNO), and nitrotyrosine, among others, are involved in physiological processes as well in the mechanisms of response to stress conditions. In sunflower seedlings exposed to five different adverse environmental conditions (low temperature, mechanical wounding, high light intensity, continuous light, and continuous darkness), key components of the metabolism of reactive nitrogen species (RNS) and reactive oxygen species (ROS), including the enzyme activities L-arginine-dependent nitric oxide synthase (NOS), *S*-nitrosogluthathione reductase (GSNOR), nitrate reductase (NR), catalase, and superoxide dismutase, the content of lipid hydroperoxide, hydrogen peroxide, *S*-nitrosothiols (SNOs), the cellular level of NO, GSNO, and GSNOR, and protein tyrosine nitration [nitrotyrosine (NO_2-Tyr)] were analysed. Among the stress conditions studied, mechanical wounding was the only one that caused a down-regulation of NOS and GSNOR activities, which in turn provoked an accumulation of SNOs. The analyses of the cellular content of NO, GSNO, GSNOR, and NO_2-Tyr by confocal laser scanning microscopy confirmed these biochemical data. Therefore, it is proposed that mechanical wounding triggers the accumulation of SNOs, specifically GSNO, due to a down-regulation of GSNOR activity, while NO_2-Tyr increases. Consequently a process of nitrosative stress is induced in sunflower seedlings and SNOs constitute a new wound signal in plants.

Key words: Abiotic stress, mechanical wounding, nitric oxide, nitrotyrosine, peroxynitrite, protein tyrosine nitration, reactive nitrogen species, *S*-nitrosoglutathione, *S*-nitrosothiols.

Introduction

Higher plants are exposed to continuous adverse environmental conditions such as extreme temperature, drought, light intensity, ultraviolet (UV) radiation, ozone, mechanical injury, salinity, and heavy metals which can affect many physiological aspects including germination, growth, development, and reproduction (Potters *et al.*, 2007). Furthermore, many of these adverse environmental conditions inflict extensive yield losses in agriculture, and consequently economic losses (Boyer, 1982; Mittler, 2006). At the cell level, many of these environmental conditions can also induce oxidative stress in plants by the abnormal generation of reactive oxygen species (ROS) such as superoxide radicals ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2), and hydroxyl radicals ($^{\cdot}OH$) that can cause oxidative damage to biomolecules including lipids, proteins, and nucleic acids, the latter being a consequence of oxidative stress (Mittler, 2002; Apel and Hirt, 2004).

At present, the physiological relevance of nitric oxide (NO) in plants under optimal and stressful conditions is

well recognized (Lamattina *et al.*, 2003; Shapiro, 2005, Qiao and Fan, 2008). However, NO belongs to a family of NO-derived molecules designated reactive nitrogen species (RNS), for which the significance in plants is less well known. Among these molecules, interest in *S*-nitrosothiols (SNOs) has increased considerably in recent years (Feechan *et al.*, 2005; Gaston *et al.*, 2006; Corpas *et al.*, 2008; Lindermayr *et al.*, 2008). The main reason is that the half-life of NO *in vivo* is very short whereas SNOs are generally more stable in solution. SNOs can participate in the transport, storage, and delivery of NO, as well as in post-translational modifications involved in cell signalling and stress conditions (Lindermayr and Durner, 2009). In plant cells, ROS and RNS formation and turnover appear to be involved in the mechanism of plant disease resistance (Feechan *et al.*, 2005; Chaki *et al.*, 2009*a*) and abiotic stress (Valderrama *et al.*, 2007; Corpas *et al.*, 2008). Among the different SNOs, *S*-nitrosoglutathione (GSNO), which is formed by the *S*-nitrosylation reaction of NO with glutathione (GSH), could have significant physiological relevance in plants, since GSNO is thought to function as a mobile reservoir of NO bioactivity (Durner and Klessig, 1999; Díaz *et al.*, 2003). So far the presence of GSNO has been reported in several plant species under biotic and abiotic stress conditions (Barroso *et al.*, 2006; Valderrama *et al.*, 2007; Chaki *et al.*, 2009*a*). Researchers have demonstrated the existence of a glutathione-dependent enzyme formaldehyde dehydrogenase (FALDH; EC 1.2.1.1), which has GSNO reductase (GSNOR) activity. This enzyme catalyses the NADH-dependent reduction of GSNO to GSSG and NH_3 (Liu *et al.*, 2001; Lamotte *et al.*, 2005). In plants, the presence of GSNOR activity has been reported in different species (Sakamoto *et al.*, 2002; Achkor *et al.*, 2003; Díaz *et al.*, 2003). Moreover, the relevance of this enzyme in several physiological and stress situations has been shown, such as cadmium stress (Barroso *et al.*, 2006), cold stress (Corpas *et al.*, 2008), heat stress (Lee *et al.*, 2008), biotic stress (Rusterucci *et al.*, 2007; Chaki *et al.*, 2009*a*), or regulating cell death in plant cells (Chen *et al.*, 2009).

In the present work, using sunflower plants as a model and exposing them to five different adverse conditions (low temperature, mechanical wounding, high light intensity, continuous light, and continuous darkness), a biochemical, molecular, and cellular study of the key components involved in the homeostasis of RNS was undertaken. Taken together, the results demonstrate that mechanical wounding was the only condition assayed that drastically affects the homeostasis of RNS metabolism. Moreover, the data indicate that tyrosine nitration of proteins is augmented after mechanical wounding. Therefore, using protein tyrosine nitration as a marker, it is proposed that GSNOR and SNOs mediate the process of nitrosative stress.

Materials and methods

Plant material and growth conditions

Sunflower (*Helianthus annuus* L. line XA89) seeds were obtained from Koipesol Seeds SA (Seville, Spain). Seedlings were sown in wet vermiculite, and grown under optimum conditions for 9 d, with 16/8 h light/dark at 20 °C. Healthy and vigorous 9-day-old seedlings were selected and exposed to different adverse conditions that in other plant species cause oxidative stress (Leterrier *et al.*, 2005, 2007). In brief, for low temperature treatment, hypocotyls were incubated for 48 h at 8 °C. For mechanical wounding, hypocotyls were injured *in planta* by pinching them with a striped-tip forceps and, after 4 h, damaged hypocotyls were collected and analysed. For high light intensity treatment, seedlings were irradiated for 4 h at 1189 μE s^{-1} m^{-2}, using a GE 300 W-230V PAR 56/WFL lamp (General Electric, Madrid, Spain). To avoid the heating of seedlings, a Petri dish (19 cm diameter) containing cold water was placed 4 cm above the plants, and the water was replaced every 30 min. For continuous light treatment, seedlings were continuously illuminated for 48 h at 190 μE s^{-1} m^{-2}. For continuous darkness, seedlings were kept in darkness in a growth chamber for 48 h. In all cases, the control seedlings were kept in the growth chamber under optimal conditions, being processed at the same time as plants subjected to the different stress conditions.

Crude extract of sunflower hypocotyls

Hypocotyls were ground using a mortar and pestle in liquid nitrogen. The resulting coarse powder was added to different extraction buffers depending on the analysis. For L-arginine-dependent nitric oxide synthase (NOS) activity the coarse powder was placed in 1/5 (w/v) 0.1 M TRIS-HCl buffer, pH 8.0, containing 0.1 M NaCl, 7% (w/v) polyvinyl polypyrrolidone (PVPP), 1 mM EDTA, 1 mM phenylmethylsulphonyl fluoride (PMSF), 15 mM dithiothreitol (DTT), and protease inhibitor cocktail (2×; Sigma). For GSNOR activity the buffer was 0.1 M TRIS-HCl, buffer, pH 7.6, containing 5% sucrose, 7% (w/v) PVPP, 0.05% Triton X-100, 0.1 mM EDTA, 15 mM DTT, 1 mM PMSF, and protease inhibitor cocktail (2×; Sigma). The crude extracts were centrifuged at 3000 *g* for 6 min (4 °C) and the supernatants were passed through Sephadex G-25 gel filtration columns (NAP-10 from Amersham) to remove salts and low molecular weight components.

Measurement of lipid hydroperoxide and H_2O_2 content

The content of lipid hydroperoxides was measured using a PeroxiDetect kit (Sigma) according to the manufacturer's instructions. Lipid hydroperoxide levels were measured by absorbance at 560 nm calculated from a standard curve prepared using *tert*-butylhydroperoxide.

In hypocotyl crude extracts, the H_2O_2 content was determined by a spectrofluorometric assay, as described by Creissen *et al.* (1999).

Enzyme activity

NOS activity assay was carried out using ozone chemiluminescence methods with a nitric oxide analyser (NOA™ 280i, Sievers Instruments, Boulder, CO, USA) according to Valderrama *et al.* (2007). The activity was expressed as pmol of NO mg^{-1} protein min^{-1}.

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 hypocotyl extracts were incubated in an assay mixture containing 20 mM TRIS-HCl (pH 8.0), 0.2 mM NADH, and 0.5 mM EDTA, whereupon the reaction was started by adding GSNO (Calbiochem) to the mixture to a final concentration of 400 μM. The activity was expressed as nmol NADH consumed per min per mg protein (ϵ_{340}=6.22 mM^{-1} cm^{-1}).

Catalase activity (EC 1.11.1.6) was determined by measuring the disappearance of H_2O_2, as described by Aebi (1984).

Nitrate reductase (NR) activity was assayed spectrophotometrically as described by Lea *et al.* (2004). Hypocotyl samples (1 g) were homogenized in 30 mg of PVPP in 1 ml of extract buffer (50 mM HEPES-KOH, pH 7.5, 1 mM DTT, 1 mM EDTA, and

7 mM cysteine), and the homogenate was centrifuged at 4 °C for 10 min (10 000 *g*). A 300 µl aliquot of assay mixture (50 mM HEPES-KOH, pH 7.5, 100 µM NADH, 5 mM KNO_3, 6 mM $MgCl_2$) was added to 100 µl of hypocotyl extract. After incubation at 30 °C for 30 min, the reaction was terminated by adding 400 µl of a 1:1 mixture of 1% (w/v) sulphanilamide in 1.5 M HCl and 0.2% (w/v) *n*-naphthylethylenediamine dihydrochloride. After colour development for 15 min, nitrite formed was determined spectrophotometrically by measuring the absorbance at 540 nm.

Protein concentration was determined with the Bio-Rad Protein Assay, using bovine serum albumin (BSA) as a standard.

Electrophoretic methods and western blot analyses

Native PAGE was performed using 6% acrylamide gels in TRIS-boric-EDTA buffer, pH 8.0, as described by Laemmli (1970). Staining for GSNOR activity was performed as described by Corpas *et al.* (2008). Briefly, 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 (Calbiochem). After 10 min, the filter paper was removed and gels were exposed to UV light and analysed for the appearance of the GSNOR activity bands.

Superoxide dismutase (SOD) isozymes were separated by non-denaturing PAGE on 10% acrylamide gels and visualized by a photochemical NBT (nitroblue tetrazolium) reduction method (Beauchamp and Fridovich, 1971). To identify the type of SOD isozyme, gels were incubated separately at 25 °C for 30–45 min in 50 mM K-phosphate, pH 7.8, in the presence or absence of 5 mM KCN (Corpas *et al.*, 1998).

SDS–PAGE was carried out in 12% acrylamide slab gels. Samples were prepared in 62.5 mM TRIS-HCl, pH 6.8, containing 2% (w/v) SDS, 10% (v/v) glycerol, and 10 mM DTT, and were heated at 95 °C for 5 min. For immunoblot analyses, the polypeptides were transferred onto polyvinylidene difluoride membranes (Immobilon P, Millipore, Bedford, MA, USA) using a semi-dry transfer system (Bio-Rad Laboratories). For immunodetection, rabbit polyclonal antibodies against cytosolic CuZn-SOD from spinach (1:3000 dilution; Kanematsu and Asada 1989), pumpkin catalase (1:2000 dilution; Yamaguchi and Nishimura, 1984), and GSNOR (1:200 dilution; Chaki *et al.*, 2009*a*) were used, and immunoreactive bands were detected with a photographic film (Hyperfilm; Amersham Pharmacia Biotech) with an enhanced chemiluminescence kit (ECL-PLUS, Amersham Pharmacia Biotech).

Determination of SNOs

Total SNOs were estimated by a chemiluminescence method (Chaki *et al.*, 2009*a*). The detection of SNOs is based on the reductive decomposition of nitroso species by an iodine/triiodide mixture to release NO, which is subsequently measured by gas-phase chemiluminescence upon reaction with ozone. SNOs are sensitive to mercury-induced decomposition, in contrast to other nitroso species including nitrosamines (RNNOs) and nitrosyl haems. The samples were homogenized in the buffer described above containing 100 µM DTPA (diethylenetetraminepentaacetic acid) (1:5; w/v), and centrifuged at 3000 *g* for 10 min. The supernatants were then incubated with 10 mM NEM (*N*-ethylmaleimide) for 15 min at 4 °C. For each sample, two aliquots were prepared: (i) treated with 10 mM sulphanylamide for 15 min at 4 °C, to eliminate nitrite; and (ii) treated with 10 mM sulphanylamide and 7.3 mM $HgCl_2$ for 15 min at 4 °C to eliminate nitrite and SNOs, respectively. Then, these samples were analysed in an NOA 280i. The data from (i–ii) represented the total SNO concentration. The whole procedure was performed under a red safety light to protect SNOs from light-dependent decomposition.

Determination of total nitrite and nitrate

Total nitrite and nitrate from hypocotyl samples were assayed by a chemiluminescence method using an NOA 280i according to Corpas *et al.* (2008).

RNA isolation and real-time quantitative PCR

Total RNA was isolated from hypocotyls with the Trizol Reagent (Gibco-BRL) as described in the manufacturer's manual. RNA was quantified spectrophotometrically. A 1 µg aliquot of total RNA was used as a template for the reverse transcriptase reaction. It was added to a mixture containing 5 mM $MgCl_2$, 1 mM dNTPs, 3.2 µg of random primer p(dN)6, 1× reverse transcriptase buffer, 50 U of RNase inhibitor, and 20 U of AMV reverse transcriptase (FIZZYMES). The reaction was carried out at 25 °C for 10 min at 42 °C followed by a 5 min step at 99 °C, and then cooling to 4 °C.

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 of each dNTP, 2.4 mM $MgCl_2$, 0.5 µM of gene-specific primers for sunflower GSNOR (accession no. AY941250) also designated as glutathione-dependent formaldehyde dehydrogenase (5′-TCTT-GGTCATGAGGCTGCTGGGATTGTTG-3′ and 5′-TGGCT-CCCCTAATTTTGCCACACAGGTTG-3′), SYBR Green I, and 8 nM fluorescein, using an iCycler iQ system (Bio-Rad). Amplifications were performed under the following conditions: initial polymerase activation at 95 °C for 4 min; then 30 cycles of 30 s at 95 °C, 30 s at 60 °C and 1 min at 72 °C. 18S RNA was employed as an internal control using the primer set 5′-TTTGATGGTACCTGCTACTCGGATAACC-3′ and 5′-CTCTCCGGAATCGAACCCTAATTCTCC-3′.

Other assays

To estimate the statistical significance between means, the data were analysed by Student's *t*-test.

Histochemical analyses of plasma membrane integrity

Histochemical detection of loss of plasma membrane integrity in hypocotyls was performed by the method described by Yamamoto *et al.* (2001). For this analysis, the sunflower seedlings were incubated in 15 ml of Evans blue solution [0.02% (w/v) in water] for 10 min and then they were washed three times in distilled water for 10 min each. Blue colour indicates damage to the plasma membrane.

Detection of NO by confocal laser scanning microscopy (CLSM)

NO was detected in hypocotyl transversal sections of ~2.5 mm long that were incubated for 1 h at 25 °C in the dark with 10 µM 4,5-diaminoflorescein diacetate (DAF-2 DA; Calbiochem) prepared in 10 mM TRIS-HCl (pH 7.4), according to Chaki *et al.* (2009*a*).

Immunolocalization of GSNO, GSNOR, and nitrotyrosine (NO_2-Tyr) by CLSM

Sunflower hypocotyls (n=5 for each experimental group) were cut into 4–5 mm pieces and fixed in 4% (w/v) *p*-formaldehyde in 0.1 M phosphate buffer (PB), pH 7.4, 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). Immunohistochemistry was carried out by confocal analysis of immunofluorescence-stained sections using a GSNO rat antiserum (Calbiochem, cat. no. 487932) diluted 1:2500; purified IgG of a rabbit polyclonal antibody against GSNOR

diluted 1:50 (Chaki *et al.*, 2009*a*); and a rabbit polyclonal antibody against NO_2-Tyr (Chaki *et al.*, 2009*b*) diluted 1:300. Hypocotyl sections were examined with a laser confocal scanning microscope (Leica TCS SL). Controls for background staining, which was usually negligible, were performed by replacing the primary antibody with an equivalent concentration either of the incubation buffer or of normal rabbit serum.

Results

A wide range of adverse environmental conditions can increase the production of ROS, and the overproduction of ROS can provoke oxidative damage to some macromolecules such as lipids or proteins. Therefore, the presence of oxidized molecules can be used as a maker of the process of oxidative stress. To evaluate whether there is oxidative stress in hypocotyls of 9-day-old sunflower seedlings exposed to several adverse environmental conditions (low temperature, mechanical wounding, high light intensity, continuous light, and continuous darkness), the content of lipid hydroperoxide was analysed. Figure 1 shows the lipid hydroperoxide content in hypocotyls of sunflower seedlings exposed to the aforementioned adverse environmental conditions. Under these environmental conditions, the lipid hydroperoxide content was increased by 35% by wounding and by 65% due to high light intensity. Based on this marker of oxidative stress, in the present experimental system, only mechanical wounding and high light intensity appear to generate oxidative stress in sunflower.

Enzymatic production of NO from L-arginine-dependent NOS activity and content of SNOs

Taking account of the fact that the existence of putative NOS activity could be considered the most controversial, Fig. 2A shows the biochemical characterization of the enzymatic production of NO from L-arginine (NOS activity) in hypocotyl extracts, measured by ozone chemiluminescence. The NO production was strictly dependent on the L-arginine concentration, tetrahydrobiopterin (BH_4), calmodulin, and calcium, and the NOS activity was strongly reduced (82–97%) in the absence of NADPH, FAD, and FMN. In addition, when the samples were pre-incubated with the animal NOS inhibitors aminoguanidine (AG) or L-N^G-monomethyl arginine citrate (L-NMMA), the NOS activity was reduced by 97%. Thus, the sunflower NOS activity requires the same cofactors as the mammalian NOS isoforms (Alderton *et al.*, 2001; Corpas *et al.*, 2009*b*) and the NOS activity reported in other plant species such as pea (Corpas *et al.*, 2006) or olive (Valderrama *et al.*, 2007).

Based on these data, this activity was analysed. Figure 2B shows the L-arginine-dependent generation of NO (NOS activity) in hypocotyls of sunflower seedlings exposed to the above-mentioned adverse environmental conditions. When the different activities were compared with those of the control plants (634.0±5.0 pmol NO mg^{-1} protein min^{-1}), it

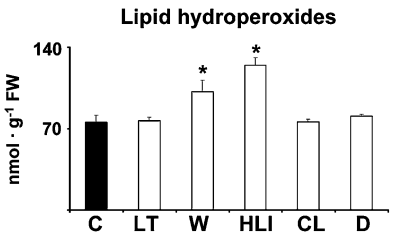

Fig. 1. Lipid hydroperoxide content in hypocotyls of sunflower seedlings exposed to several adverse environmental conditions: low temperature (LT), mechanical wounding (W), high light intensity (HLI), continuous light (CL), and continuous darkness (D). Lipid hydroperoxide contents were determined as described in the Materials and methods. The data are the mean ±SD of at least three independent experiments. *Differences from control values were significant at P <0.05.

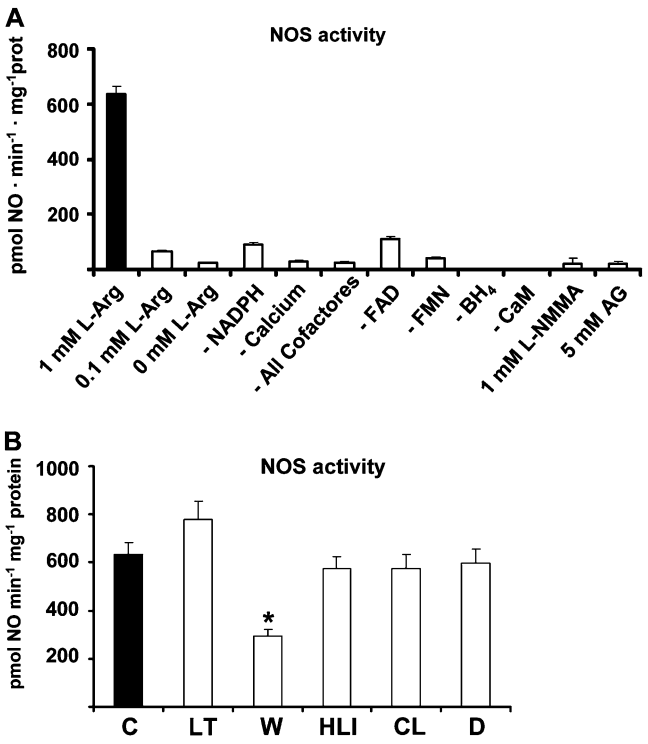

Fig. 2. (A) Biochemical characterization of L-arginine-dependent nitric oxide synthase (NOS) activity in hypocotyls of *Helianthus annuus* L. Reaction mixtures containing hypocotyl samples were incubated in the absence and presence of L-arginine (1 or 0.1 mM), NADPH (1 mM), EGTA (0.5 mM), cofactors (10 μM FAD, 10 μM FMN, and 10 μM BH_4), calmodulin (CaM), L-N^G-monomethyl arginine citrate (L-NMMA; a competitive and irreversible inhibitor of all three animal NOS isoforms), and 1 mM aminoguanidine (AG; an animal NOS activity inhibitor). The NOS activity was quantified from the NO produced, which was determined by the ozone chemiluminescence method. L-Arg, L-arginine. (B) L-Arginine-dependent NOS activity in hypocotyls of sunflower seedlings exposed to several adverse environmental conditions: low temperature (LT), mechanical wounding (W), high light intensity (HLI), continuous light (CL), and continuous darkness (D). The data are the mean ±SD of at least three independent experiments. *Differences from control values were significant at P <0.05.

was found that the NOS activity was reduced 54% by mechanical wounding and increased 22% by low temperature. The other conditions did not affect the NOS activity.

Figure 3 shows the total content of SNOs in hypocotyls of sunflower seedlings exposed to the same five adverse conditions described above. Among these adverse conditions, only mechanical wounding led to an increase (4.5-fold) in the total content of SNOs.

GSNOR

Considering that GSNO is an SNO, GSNOR activity and its protein and gene expression were studied (Fig. 4). In hypocotyls of sunflower seedlings, GSNOR activity was determined by both spectrophotometry and native PAGE (Fig. 4A, B). The activity was significantly reduced (50%) only in plants that underwent mechanical wounding. The other conditions had no significant effect on the GSNOR activity. Figure 4C shows the GSNOR protein expression evaluated by immunoblots, and again only mechanical wounding produced a significant reduction. Figure 4D shows the analysis by real-time quantitative reverse transcription–PCR of the GSNOR gene expression; a similar behaviour was observed showing that mechanical wounding, with a reduction of 58% in gene expression, was the only stress that affected its expression. Taken together, all the data indicate that mechanical wounding was the stress that provoked a significant reduction in activity, protein, and gene expression of GSNOR.

Histochemical observation of mechanical wounding in sunflower hypocotyls

Taking into consideration that mechanical wounding is the adverse condition that provokes an oxidative stress and significantly affects the metabolism of NO and NO-derived molecules this stress was the focus of further studies.

Figure 5A shows the appearance of sunflower seedlings after just 4 h of wounding *in planta* by pinching the hypocotyls with striped-tip forces. Figure 5B shows the histochemical analyses of plasma membrane integrity using staining with Evan Blue solution. It was found that, after 4 h of mechanical wounding, a strong blue colour is observed in the injured area of the hypocotyl which indicates the loss of cell membrane integrity.

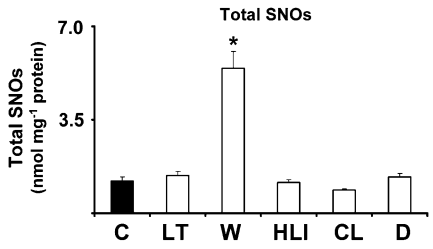

Fig. 3. *S*-nitrosothiol (SNO) content in sunflower seedlings exposed to several adverse environmental conditions: low temperature (LT), mechanical wounding (W), high light intensity (HLI), continuous light (CL), and continuous darkness (D). The data are the mean ±SD of at least three independent experiments. *Differences from control values were significant at P <0.05.

H_2O_2 content and antioxidant enzymatic activities under mechanical wounding

Due to the fact that mechanical wounding produced a rise in lipid hydroperoxide (Fig. 1), other components involved in the metabolism of ROS such as H_2O_2 content and antioxidant enzymes were also studied (Fig. 6). Figure 6A shows that the content of H_2O_2 was increased 2.8-fold after mechanical wounding. However, the analysis of catalase activity and its protein expression did not show any critical

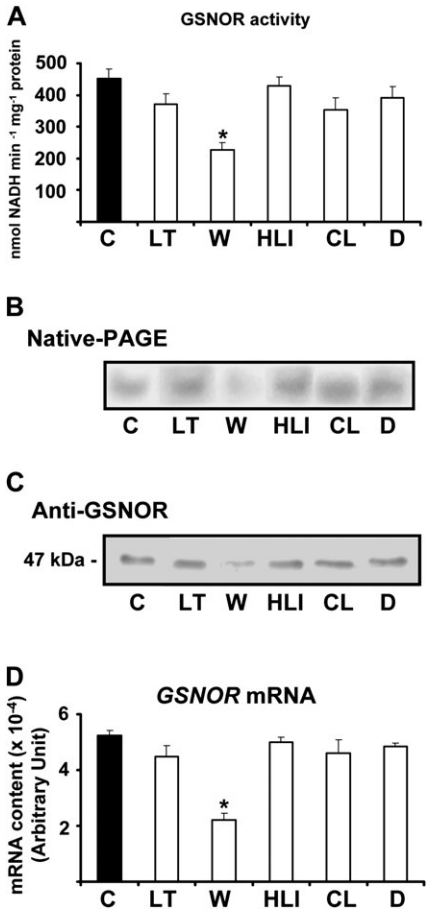

Fig. 4. Effect of adverse environmental conditions on *S*-nitrosoglutathione reductase (GSNOR) activity, protein and gene expression in hypocotyls of sunflower seedlings. (A) Spectrophotometric assay of GSNOR activity. (B) Native PAGE (100 μg of protein per lane) and staining for GSNOR activity. (C) Immunoblot of hypocotyl crude extracts tested with purified IgGs of a rabbit polyclonal antibody against GSNOR (dilution 1:200). Proteins (10 μg protein) were separated by 10% SDS–PAGE and transferred onto a PVDF membrane. (D) GSNOR gene expression analysis and real-time quantitative reverse transcription-PCR using 18S RNA as an internal control. Data are the mean ±SD of at least three independent RNA samples. C, control; LT low temperature, W, mechanical wounding; HLI, high light intensity; CL, continuous light; D, continuous darkness. *Differences from control values were significant at P <0.05.

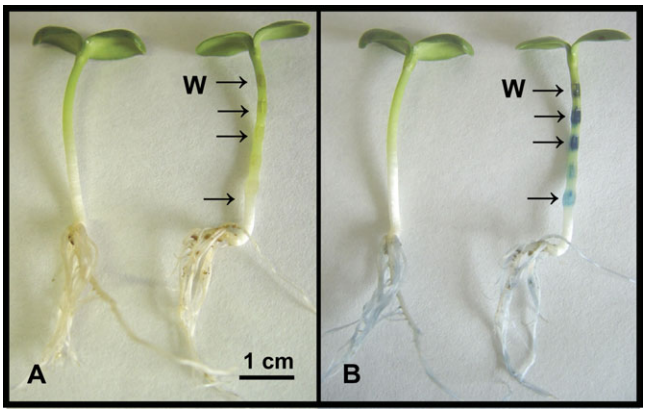

Fig. 5. (A) Representative images of the appearance of control sunflower seedlings and after 4 h of mechanical wounding (W). (B) Histochemical detection of plasma membrane integrity by staining with Evan blue solution in control sunflower seedlings and after 4 h of mechanical wounding. Arrows indicate the hypocotyl area injured *in planta* by pinching them with a striped-tip forceps.

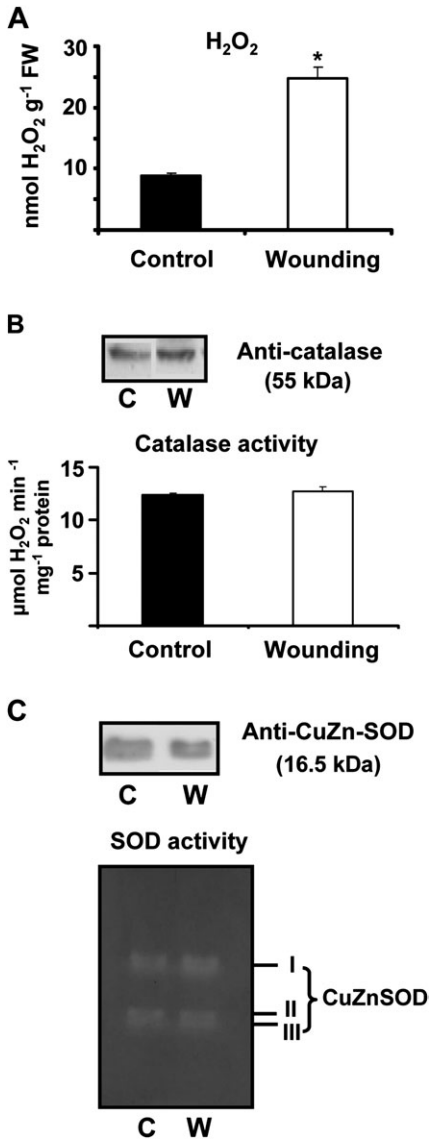

Fig. 6. Hydrogen peroxide content and antioxidant enzymatic activities in hypocotyls of sunflower seedlings exposed to mechanical wounding (W). (A) Hydrogen peroxide content in hypocotyls of sunflower seedlings exposed to mechanical wounding. (B) Immunoblot probed with polyclonal antibodies against pumpkin catalase (1/2000 dilution) and catalase activity. (C) Immunoblot blot analysis using antibodies against spinach CuZn-SOD (1/3000 dilution) and the activity of CuZn-SOD isozymes separated by native PAGE and stained by a photochemical method. For each sample, 20 µg of protein was loaded per lane. Data are the mean ±SD of at least three different experiments. *Differences from control values were significant at $P < 0.05$.

difference (Fig. 6B). The analysis of SOD activity by native PAGE showed the presence of three isozymes which were identified with specific inhibitors (CN^- and H_2O_2) as three CuZn-SODs (I, II, and III). Mechanical wounding did not produce significant change in the CuZn-SOD activity pattern and its protein expression (Fig. 6C).

NR activity and total nitrite and nitrate content under mechanical wounding

The increase of SNOs (GSNO) was not well correlated with the down-regulation of NOS activity, and taking into account that NO could also be generated from nitrite and nitrate from enzymatic or non-enzymatic sources, the potential contribution of these molecules to the generation of the NO which could contribute to the formation of SNOs was analysed. Table 1 shows the total content of nitrites and nitrates detected in control hypocotyls and after mechanical wounding. No statistically significant change was observed between the concentration of nitrites and nitrates in hypocotyls from seedlings subjected to this stress compared with control plants. Additionally, the NR activity, which is considered as another potential source of NO (Fig. 7), was also analysed; however, this activity also did not show any difference after mechanical wounding.

Cellular localization and level of NO, GSNO, GSNOR, and NO_2-Tyr under mechanical wounding

Figure 8 shows the cellular localization and level of NO (Fig. 8A, B), GSNO (Fig. 8C, D), GSNOR (Fig. 8E, F), and NO_2-Tyr (Fig. 8G, H) in cross-sections of hypocotyls of sunflower exposed to this stress. The location and level of NO were analysed by CLSM using the fluorescent probe DAF-2 DA where the green fluorescence corresponds to the location of NO. NO was found to be localized mainly in the epidermal cells and vascular tissue in control plants (Fig. 8A) and, after 4 h of mechanical wounding, the NO localization and content were very similar to those of the control but less intense (Fig. 8B). As a control of NO production, hypocotyl sections were pre-incubated with cPTIO (an NO scavenger) and the green fluorescence was greatly reduced (Supplementary Fig. 1A available at *JXB* online). The cellular location and level of GSNO, GSNOR, and NO_2-Tyr were analysed in sunflower hypocotyl sections

Table 1. Total nitrite and nitrate in hypocotyl extracts from sunflower plants subjected to mechanical wounding

A chemiluminiscence method was used and the total nitrite and nitrate of extracts was expressed as nmol mg^{-1} protein. Results are means ±SD of samples from at least three different experiments.

Condition	Total nitrate and nitrite
Control	248.6±15.4
Mechanical wounding	239.8±16.3

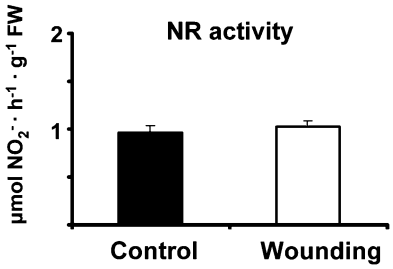

Fig. 7. Nitrate reductase (NR) activity in hypocotyls of sunflower seedlings exposed to mechanical wounding. The data are the mean ±SD of at least three independent experiments.

using specific antibodies (Chaki *et al.*, 2009*a*) and a green fluorescence was noted in the corresponding panels. In control plants, the GSNO was localized in vascular tissue and epidermis cells (Fig. 8C) and, after wounding it increased noticeably, mainly in cortex cells and vascular tissues (Fig. 8D). GSNOR in control hypocotyls was detected in cortex cells and mainly in vascular tissue (Fig. 8E) and, after mechanical wounding, the content of GSNOR protein was clearly reduced (Fig. 8F), this behaviour being identical to that observed in immunoblots (Fig. 4C). In the case of the content of NO_2-Tyr, the control hypocotyl shows a general distribution in cortex cells and vascular tissues (Fig. 8G). However, after wounding, clear fluorescence intensification appeared in all cell types (Fig. 8H). As a control for background staining, the primary antibodies against GSNO, GSNOR, and NO_2-Tyr were replaced by the respective pre-immune serum (Supplementary Fig. 1A–D). Under these conditions, no immunoflourescence background was observed.

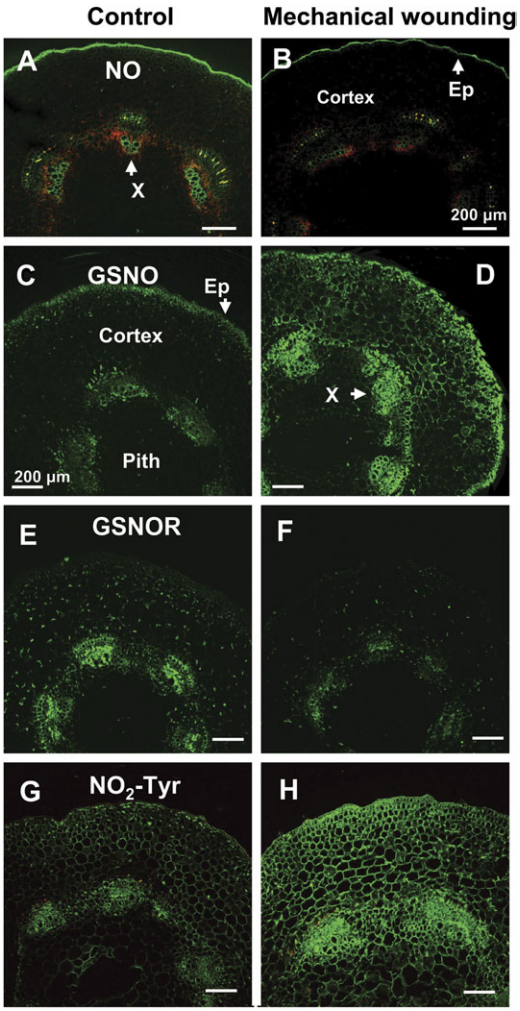

Fig. 8. Representative images illustrating the CLSM detection of nitric oxide (NO), nitrosoglutathione (GSNO), *S*-nitrosoglutathione reductase (GSNOR), and protein tyrosine nitration (NO_2-Tyr) in cross-sections of sunflower hypocotyls exposed to mechanical wounding. (A and B) The bright green fluorescence corresponds to the detection of NO using the fluorescence probe DAF-2 DA; (C and D) GSNO using a specific antibody against GSNO diluted 1:2,500; (E and F) GSNOR using IgG against sunflower GSNOR diluted 1:50; and NO_2-Tyr (G and H) using a specific antibody with a dilution of 1:300 as described in the Materials and methods. Ep, epidermis; X, xylem. Bar=200 µm.

Discussion

So far, most of the studies in plants have been focused on the analysis of NO while less attention has been paid to other NO-derived molecules, designated as RNS. In previous studies using as model plants sunflower seedlings exposed to a biotic stress by the fungus *Plasmopara halstedii* (Chaki *et al.*, 2009*a*) and pea plants exposed to different abiotic stress conditions (Corpas *et al.*, 2008), it was demonstrated that an increase in the tyrosine nitration of proteins provokes nitrosative stress. The goal of the present work was to find out whether there is a common mechanism of response, independent of the plant species or the analysed organs, that provokes nitrosative stress after both biotic and abiotic stress. Therefore, using specifically hypocotyls of sunflower seedlings exposed to five adverse environmental conditions NO homeostasis was analysed.

Mechanical wounding provokes oxidative stress and affects NO homeostasis

Plant injury (whether by mechanical wounding, herbivorous insects, etc.), provokes long-distance signals that also trigger responses in unwounded tissues. In this process, a cascade of signals could show some variations in different plant species, but it includes, for example, systemin, ipomoelin, oligouronides, abscisic acid, ethylene, and jasmonic acid

(León et al., 2001; Jih et al., 2003; Stratmann, 2003). Moreover, the injury can also lead to an overproduction of ROS, which can result in oxidative stress (Orozco-Cárdenas and Ryan, 1999; Guan and Scandalios, 2000; Chang et al., 2004; Reyes et al., 2007; Miller et al., 2009).

Among the adverse conditions assayed in sunflower, only high light intensity and mechanical wounding appeared to cause oxidative damage based on the high content of lipid hydroperoxides, which are typical markers of oxidative stress (Requena et al., 1996). Additionally, the hypocotyls after wounding showed a higher content of H_2O_2 that was not accompanied by an increase in the catalase and SOD activities, which could suggest that the antioxidative capacity was not sufficient to control the overproduction of ROS, allowing the oxidative damage to occur.

However, the generation of NO from the L-arginine-dependent NOS activity and its cellular content evaluated by CLSM were down-regulated, indicating that there is no direct correlation between oxidative stress and NO generation in the present experimental system. These results are in contrast to those reported in other plant species. For example, in pea leaves damaged by mechanical wounding, the L-arginine-dependent NOS activity and NO content showed an opposite behaviour because both parameters increased (Corpas et al., 2008). In *Arabidopsis*, in the marine macroalga *Dasycladus vermicularis*, or in *Pelargonium* leaves, similar results have been reported, given that, after wounding, NO generation restricted to the site of injury was found (Garces et al., 2001; Huang et al., 2004; Ross et al., 2006; Arasimowicz et al., 2009), indicating a direct correlation between injury and a high production of NO. In sweet potato, mechanical wounding causes the generation of methyl jasmonate, which activates an NADPH oxidase to generate H_2O_2. At the same time, wounding induces a NOS-like protein to generate NO; as a consequence, both molecules are overproduced (Jih et al., 2003). In another case, the application of exogenous NO to wounded tomato leaves inhibits the synthesis of proteinase inhibitor I protein, which is an important element of the plant's defence. In addition, this inhibition was reversed by the application of an NO scavenger (Orozco-Cárdenas and Ryan, 2002). On the other hand, the application of exogenous NO has been reported to protect sunflower leaves against UV-B irradiation (Tossi et al., 2009). Consequently, the low level of NO observed after mechanical wounding as a consequence of the NOS inhibition indicates that an appropriate level of NO is required to act as a protectant. In plants there are alternative sources of NO. Thus, in barley aleurone the apoplast can generate NO from nitrite (NO_2^-) under acidic conditions by a non-enzymatic mechanism (Bethke et al., 2004). Alternatively, NO could be generated as a side product during NO_3^- assimilation through the NADH-dependent reduction of NO_2^- by NR. Moreover, under anoxia, plant mitochondria can produce NO from nitrite and NADH, as can those of animals (Gupta et al., 2005; Planchet et al., 2005). In this sense, it is considered that the NO production capacity of NR at saturating NADH and NO_2^- concentrations is 1% of its nitrate reduction capacity, and *in vivo* the NO production depends on the total NR activity, the enzyme activation state, and the intracellular accumulation of NO_2^- and NO_3^- (Rockel et al., 2002). However, NR activity did not show any significant change in sunflower hypocotyls after mechanical wounding. Additionally, the total content of nitrite and nitrate also showed the absence of significant changes after mechanical wounding. Therefore, even when the NR activity, NO_2^-, and NO_3^- can contribute to the pool of NO, these molecules did not seem to be the major source under the present experimental conditions. However, other potential sources that could be involved, such as polyamines, cannot be discarded because there are two lines of evidence which could suggest their participationl; it has been described that polyamines induce biosynthesis of NO (Tun et al., 2006, 2008) and that the polyamine content increased significantly after wounding (Kim et al., 2008).

Mechanical wounding increases the content of SNOs and induces a down-regulation of GSNOR activity

SNOs are the result of the interaction between NO and thiol groups, and exhibit NO-mimetic activity. For that reason, SNOs may prolong and spatially extend the *in vivo* actions of locally produced NO (Hogg, 2002). In plant cells, there is little information on the metabolism of SNOs and still less is known about its modulation under physiological and abiotic stress conditions (Feechan et al., 2005; Corpas et al., 2008). Under the assayed experimental conditions, the SNO content exhibited a behaviour opposite to that of the NO content and NOS activity, given that whereas the total content of SNOs increased, the other two parameters were down-regulated after mechanical wounding. These results again are in contrast to those reported in other plant species under other stress conditions. For example, in olive trees under salinity stress, a significant increase in NO generation and NOS activity was accompanied by a rise in total SNOs (Valderrama et al., 2007). Similar behaviour was found in pea plants under low temperatures, high light intensity, and mechanical wounding where both NOS activity and SNOs increased (Corpas et al., 2008). In view of the fact that GSNO could be the most abundant SNO in plants, the analysis by CLSM of GSNO confirms that after mechanical wounding the hypocotyl cells also exhibited a high content of this SNO in all cells.

On the other hand, the enzyme GSNOR can decompose GSNO, and its analysis in hypocotyls showed that it was down-regulated at a different level, i.e. gene and protein expression as well as its specific activity. Consequently, the accumulation of GSNO and SNOs must be due to an inhibition of GSNOR. This is a reasonable explication based on data observed in GSNOR knockout transgenic mice where a significant accumulation of SNOs compared with wild type mice was detected (Liu et al., 2004). Related to GSNOR expression, a similar situation has been reported in tobacco leaves where after 2 h of mechanical damage a decrease was observed in both GSNOR mRNA and protein levels (Díaz et al., 2003). Unfortunately, GSNOR

activity and SNOs were not assayed so tobacco and sunflower cannot be compared. In pea leaves after mechanical wounding, an increased GSNOR activity and content of SNOs has been reported, accompanied by an increase in NOS activity (Corpas *et al.*, 2008). However, in pea plants grown with 50 μM cadmium, which produces an oxidative stress, GSNOR activity and its transcript expression were found to be reduced by 31% (Barroso *et al.*, 2006). Recently, in the *Arabidopsis thaliana* mutant HOT5 (sensitive to hot temperatures), which encodes a GSNOR, it has been shown that this enzyme is required for thermotolerance (Lee *et al.*, 2008). Nevertheless, in wild-type *Arabidopsis* exposed to heat stress, GSNOR protein expression was similar in both control and heat-stressed wild-type leaves. Therefore, these authors hypothesized that elevated levels of GSNO increase heat sensitivity due to the perturbation of pathways sensitive to ROS/RNS.

Mechanical wounding induces protein tyrosine nitration

Recently, tyrosine-nitrated proteins have been viewed as an established marker for the extent of RNS production and therefore cellular stress during physiological and pathological conditions in animal cells (Koeck *et al.*, 2005). Protein tyrosine nitration consists of the addition of a nitro group to one of the two equivalent ortho-carbons of the aromatic ring of tyrosine residues, and this post-translational modification *in vivo* involves either peroxynitrite ($ONOO^-$), nitrite/H_2O_2/haemperoxidase, or transition metals (Radi, 2004). Furthermore, when there is a physiological nitration in the plant cells, it has been proposed that an increase in protein tyrosine nitration could be considered a footprint of nitrosative stress (Corpas *et al.*, 2007). For example, in olive and *Arabidopsis* exposed to salinity stress, there is an oxidative and nitrosative stress which was characterized by a significant rise in the content of ROS, NO, and protein tyrosine nitration (Valderrama *et al.*, 2007; Corpas *et al.*, 2009*a*). In the present study, sunflower hypocotyls after mechanical wounding showed an oxidative stress, an increase in the content of SNOs, and a rise in protein tyrosine nitration; interestingly, the NO content and NOS activity were reduced. In this situation of oxidative stress, the SNOs could mediate the process of tyrosine nitration by a mechanism that implies the decomposition of SNOs, accompanied by $ONOO^-$ formation, from the $O_2^{\cdot-}$-dependent reduction of SNOs to yield NO, which in turn reacts rapidly with a second $O_2^{\cdot-}$ molecule to yield peroxynitrite (Trujillo *et al.*, 1998).

In summary, the results show that mechanical wounding causes both oxidative and nitrosative stress. In the latter case, the inhibition of GSNOR, at different levels including activity, and transcriptional and post-transcriptional levels, appears to provoke an accumulation of SNOs which in an oxidative situation could mediate peroxynitrite formation (Trujillo *et al.*, 1998), mediating the increased protein tyrosine nitration which is a footprint of nitrosative stress (Corpas *et al.*, 2007). Moreover, it is shown that in sunflower hypocotyls, this process is independent of the NO generated by the L-arginine-dependent NOS activity, NR, or the content of nitrite/nitrate. Therefore, it cannot be assumed to be a general concept that more NO in a specific plant tissue or species under a given adverse condition can provoke a process of nitrosative/oxidative stress. Likewise, it should be taken into account that GSNOR and, consequently, SNOs are key new elements in the wound signalling pathway.

Supplementary data

Supplementary data are available at *JXB* online.

Figure S1 Representative images illustrating confocal laser scanning microscopy of the respective controls used for the detection of NO, GSNO, GSNOR, and NO_2-Tyr in transversal sections of sunflower hypocotyls

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