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High temperature triggers the metabolism of *S*-nitrosothiols in sunflower mediating a process of nitrosative stress which provokes the inhibition of ferredoxin–NADP reductase by tyrosine nitration

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ABSTRACT

High temperature (HT) is considered a major abiotic stress that negatively affects both vegetative and reproductive growth. Whereas the metabolism of reactive oxygen species (ROS) is well established under HT, less is known about the metabolism of reactive nitrogen species (RNS). In sunflower (*Helianthus annuus* L.) seedlings exposed to HT, NO content as well as *S*-nitrosoglutathione reductase (GSNOR) activity and expression were down-regulated with the simultaneous accumulation of total *S*-nitrosothiols (SNOs) including *S*-nitrosoglutathione (GSNO). However, the content of tyrosine nitration (NO_2-Tyr) studied by high-performance liquid chromatography with tandem mass spectrometry (LC–MS/MS) and by confocal laser scanning microscope was induced. Nitroproteome analysis under HT showed that this stress induced the protein expression of 13 tyrosine-nitrated proteins. Among the induced proteins, ferredoxin–NADP reductase (FNR) was selected to evaluate the effect of nitration on its activity after heat stress and *in vitro* conditions using 3-morpholinosydnonimine (SIN-1) (peroxynitrite donor) as the nitrating agent, the FNR activity being inhibited. Taken together, these results suggest that HT augments SNOs, which appear to mediate protein tyrosine nitration, inhibiting FNR, which is involved in the photosynthesis process.

Key-words: heat stress; nitric oxide; nitroproteome; nitrotyrosine; peroxynitrite; protein tyrosine nitration; reactive nitrogen species; *S*-nitrosoglutathione.

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Sequence data from this article have been deposited in the EMBL/GenBank data libraries under accession number AY941250 for sunflower GSNOR.

Abbreviations: APF, 3′-(*p*-aminophenyl) fluorescein; CLSM, confocal laser scanning microscopy; cPTIO, 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide; DAF-2 DA, 4,5-diaminofluorescein diacetate; DAF-FM DA, 4-aminomethyl-2′,7′-difluorofluorescein diacetate; DAR-4M AM, diaminorhodamine-4M acetoxymethyl ester; DHE, dihydroethidium; GSNO, *S*-nitrosoglutathione; GSNOR, *S*-nitrosoglutathione reductase; HT, high temperature; LC–MS/MS, high-performance liquid chromatography with tandem mass spectrometry; NO, nitric oxide; NO_2-Tyr, tyrosine nitration; $ONOO^-$, peroxynitrite; PMSF, phenylmethylsulphonyl fluoride; PVPP, polyvinylpolypyrrolidone; RNS, reactive nitrogen species; ROS, reactive oxygen species; SNOs, *S*-nitrosothiols; TMP, 2,2,6,6-tetramethyl-piperidine.

INTRODUCTION

Plants respond to biotic and abiotic stress by activating a wide variety of localized systemic defences. Among these defences, the free radical NO has been shown to be an intermediate signalling molecule (Delledonne *et al*. 1998; Hancock *et al*. 2002; Mur *et al*. 2006; Valderrama *et al*. 2007; Corpas *et al*. 2008, 2011; Chaki *et al*. 2009a; Airaki *et al*. 2011). Conversely, it has been hypothesized that SNOs, GSNO being the most abundant, could function as a NO carrier throughout the plant, thus conferring NO with the property of a long-distance signalling molecule (Durner & Klessig 1999). Furthermore, the participation of SNOs in the mechanism of response against pathogens has been demonstrated in plant cells (Feechan *et al*. 2005; Chaki *et al*. 2009a). However, there is little information on the participation of SNOs in the mechanism of plant response to abiotic stress.

In animal systems, GSNO is one of the major endogenous metabolites of NO that have been detected both in

intracellular and extracellular spaces, and, although GSNO is often used as a NO donor, several pieces of evidence support the idea that it is a biomolecule with physiological and clinical implications more than simply a source of NO (Hogg 2000). NO released from SNOs or GSNO may go through different non-enzymatic mechanisms (Singh *et al*. 1996a,b; Trujillo *et al*. 1998; Hogg 2000). In addition, GSNO can be metabolized *in vivo* by an evolutionarily conserved glutathione-dependent formaldehyde dehydrogenase designated as GSNOR, which seems to be critical for NO homeostasis and protection against nitrosative stress from bacteria to humans (Liu *et al*. 2001). In higher plants, this enzyme has also been described as a key enzyme in NO signalling, plant development and response to several types of environmental stress (Sakamoto, Ueda & Morikawa 2002; Díaz *et al*. 2003; Barroso *et al*. 2006; Lee *et al*. 2008; Chaki *et al*. 2009a; Chen *et al*. 2009; Leterrier *et al*. 2011).

HT is a major abiotic stress that negatively affects both vegetative and reproductive growth. In general, HT can generate heat stress, which in turn produces specific families of proteins known as heat shock proteins (HSPs) (Howarth & Ougham 1993; Wang *et al*. 2004; Larkindale & Vierling 2008). However, HT can also cause an overproduction of ROS, which could be involved in triggering defence responses against potentially damaging temperatures (Nobuhiro & Mittler 2006; Volkov *et al*. 2006; Kotak *et al*. 2007; Locato *et al*. 2008). Sunflower shows wide adaptability, although HTs (38–40 °C) can lower seed yield, as well as oil and protein content (Hewezi, Leger & Gentzbittel 2008). In the present study, we examined the metabolism of RNS, showing that HT provokes nitrosative stress, reflected by the rise of both NO_2-Tyr and tyrosine-nitrated proteins, which appears to be mediated by SNOs.

MATERIALS AND METHODS

Plant material and growth conditions

Sunflower (*Helianthus annuus* L. line X55) 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 HT. Thus, the seedlings were sequentially exposed to 30 °C for 1 h, 35 °C for 1 h and finally 38 °C for 4 h (DeRocher & Vierling 1995; Leterrier, del Río & Corpas 2007).

Crude extract of sunflower hypocotyls

Hypocotyls were ground using a mortar and pestle in liquid nitrogen. The resulting coarse powder was transferred to different extraction buffer, depending on the analysis. For NOS activity the coarse powder was transferred into 1/5 (w/v) 0.1 M Tris–HCl buffer, pH 8.0, containing 0.1 M NaCl, 7% (w/v) PVPP, 1 mM ethylenediaminetetraacetic acid (EDTA), 1 mM PMSF, 15 mM dithiothreitol (DTT) and protease inhibitor cocktail (2×; Sigma, St Louis, MO, USA). 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). Then, 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 Biosciences Piscataway, NJ, USA) to remove salts and low-molecular weight components. For protein precipitation, the coarse powder was transferred into 1/2 (w/v) extraction medium of 0.1 M Tris–HCl, buffer, pH 7.6, containing 5% (w/v) sucrose, 7% (w/v) PVPP, 0.05% (v/v) Triton X-100, 0.1 mM EDTA, 15 mM DTT, 1 mM PMSF and a commercial cocktail of protease inhibitors (AEBSF, 1,10-phenantroline, pepstatin A, leupeptin, bestatin and E-64) (Sigma). Then, the crude extracts were filtered through one layer of miracloth (Calbiochem, San Diego, CA, USA), centrifuged at 3000 *g* for 6 min (4 °C) and the supernatants were used for immunoblot analyses.

Determination of lipid hydroperoxides

The content of lipid hydroperoxides was determined 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.

Determination of chlorophyll and photosynthetic rate

Chlorophyll content, after extraction with 80% acetone, was determined according to Arnon (1949). Photosynthetic rate was examined with the Li-Cor 6400 infrared gas analyser (IRGA) (LI-COR Corporate, Lincoln, NE, USA).

Determination of total nitrite, nitrate and SNOs

Total nitrite and nitrate was assayed by a chemiluminescence method using a NO analyser (NOA 280i; Sievers Instruments, Boulder, CO, USA) (Corpas *et al*. 2008). Total SNOs was determined by a chemiluminescence method, as described by Jourd'heuil *et al*. (2005) with some modifications (Corpas *et al*. 2008).

Enzyme activity

L-Arginine-dependent NOS activity was estimated by an ozone chemiluminescence method using a NO analyser (Sievers NOA 280i) according to Corpas *et al*. (2008). The activity was expressed as nmol of NO min^{-1} g^{-1} FW. To validate this method of determining the NOS activity of hypocotyl samples, commercial rat neuronal NOS from Calbiochem (2.9 U) was assayed separately as a positive control.

Nitrate reductase (NR) activity was assayed spectrophotometrically as described by Lea *et al*. (2004). Hypocotyl

samples (1 g) were homogenized with 30 mg PVPP in 1 mL 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*). Then, 300 μL of assayed 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 stopped by adding 400 μL of 1:1 mixture of 1% (w/v) sulphanilamide in 1.5 M HCl and 0.2% (w/v) *n*-naphtylethylenediamine dihydrochloride. After colour development for 15 min, the nitrite formed was determined spectrophotometrically by measuring absorbance at 540 nm.

GSNOR activity was assayed spectrophotometrically at 25 °C by monitoring the oxidation of NADH at 340 nm, as described by Barroso *et al*. (2006). The activity was expressed as nmol NADH consumed per min per mg protein ($\varepsilon_{340} = 6.22$ mM^{-1} cm^{-1}).

The cytochrome *c* reductase activity of ferredoxin–NADP reductase (FNR; EC 1.18.1.2) was measured spectrophotometrically at 550 nm following the reduction of cytochrome *c* (Decottignies *et al*. 2003), and the rates were calculated using a molar absorption coefficient of 15 300 M^{-1} cm^{-1}.

Electrophoretic methods and Western blot analyses

Polypeptides were separated by 12% sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE), and proteins were transferred to polyvinylidene fluoride (PVDF) membranes (Immobilon P; Millipore, Bedford, MA, USA) using a semi-dry transfer system (Bio-Rad, Hercules, CA, USA). For immunodetection, a rabbit polyclonal antibody against GSNOR (Chaki *et al*. 2009b) diluted 1:200 was used. For immunodetection of Hsp70 protein, a polyclonal antibody against cucumber PMP72 (Corpas & Trelease 1997) diluted 1:300 was used, and immunoreactive bands were detected with a photographic film (Hyperfilm; Amersham Pharmacia Biotech) with an enhanced chemiluminescence kit (ECL-PLUS; Amersham Pharmacia Biotech).

RNA isolation and real-time quantitative PCR

Total RNA was isolated from hypocotyls with the Trizol Reagent (Gibco BRL, Paisley, UK) as described in the manufacturer's manual. RNA was quantified spectrophotometrically. One microgram 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, 3.2 μg random primer p(dN)6, 1× RT buffer, 50 U RNase inhibitor, 20 U AMV RT (FIZZYMES). The reaction was carried out at 25 °C for 10 min, 42 °C followed by a 5 min step at 99 °C, and then cooling to 4 °C.

Real-time quantitative PCR analysis for sunflower GSNOR (accession no. AY941250) 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 unit 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 (5′-TCTTGGTCATGAGGCTGCTGGGATTGTTG-3′ and 5′-TGGCTCCCCTAATTTTGCCACACAGGTTG-3′) and SYBR Green I, 8 nM fluorescein, using an 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. RNA 18S was employed as an internal control using the following primer set 5′-TTTGATGGTACCT GCTACTCGGATAACC-3′ and 5′-CTCTCCGG AATCG AACCC TAA TTC TCC-3′.

Detection of NO, superoxide radical ($O_2^{\cdot-}$) and peroxynitrite ($ONOO^-$) by CLSM

NO was detected in hypocotyl transversal sections of approximately 2.5 mm long that were incubated for 1 h at 25 °C, in darkness, with different fluorescent probes: (1) 10 μM DAF-2 DA (Calbiochem) prepared in 10 mM Tris–HCl (pH 7.4) (excitation 495 nm; emission 515 nm); (2) 10 μM DAF-FM DA (Calbiochem) prepared in 10 mM Tris–HCl (pH 7.4) (excitation 495 nm and emission 515 nm). It is stable in a pH range of 5.5–12.0 (Zhang, Czymmek & Shapiro 2003); or (3) 5 μM DAR-4M AM (Calbiochem) prepared in 100 mM potassium phosphate buffer (PB), pH 7.4 (excitation 543 nm; emission 575 nm) (Tun *et al*. 2006). After incubation, samples were washed twice in the same buffer for 15 min each and processed as described elsewhere (Chaki *et al*. 2009a). The samples were examined with a confocal laser scanning microscope system (Leica TCS SL; Leica Microsystems, Wetzlar, Germany). Background staining, routinely negligible, was controlled with hypocotyl sections unstained and, as control, sections were pre-incubated with 200 μM cPTIO, a NO scavenger, at 25 °C for 30 min.

For superoxide radicals ($O_2^{\cdot-}$), the hypocotyl samples were incubated at 37 °C for 30 min with 10 μM DHE (Sigma-Aldrich-Fluka, St. Louis, MO, USA) (excitation 488 nm; emission 520 nm). Background staining was controlled with hypocotyl sections pre-incubated with the superoxide radical scavenger TMP (1 mM) for 1 h.

Peroxynitrite ($ONOO^-$) was detected with 10 μM 3′-(*p*-aminophenyl) fluorescein (APF, Invitrogen, Carlsbad, CA, USA) prepared in 10 mM Tris–HCl at pH 7.4 (Gomes, Fernandes & Lima 2006; Chaki *et al*. 2009a). Hypocotyl cross sections were incubated at 25 °C for 1 h in darkness.

Detection of SNOs by CLSM

It is well established that SNOs can release NO in a controlled manner in the living system either spontaneously or by the interaction with various biological reductants such as glutathione and ascorbate. Moreover, the reduced metal ion

(e.g. Cu^{+}) breaks down SNOs more rapidly than does the oxidized metal ion (e.g. Cu^{2+}), indicating that reducing agents such as glutathione and ascorbate can stimulate the breakdown of *S*-nitrosothiol by chemical reduction of contaminating transition metal ions (Singh *et al*. 1996a; Williams 1996; Smith & Dasgupta 2000). Therefore, SNOs were detected using the fluorescent reagent Alexa fluor 488 Hg-link phenylmercury under two conditions in the presence and absence of reductants.

Detection of SNOs in the absence of reductants

Hypocotyl cross sections were incubated at 25 °C for 2 h in 100 μM diethylenetetraminepentaacetic acid (DTPA) and 10 mM *N*-ethyl-maleimide (NEM) which blocks free sulfhydryl groups. Then, samples were washed three times in 10 mM Tris–HCl buffer, pH 7.4, for 15 min each. Afterwards, they were incubated with 10 μM Alexa fluor 488 Hg-link phenylmercury (Molecular Probes, Eugene, OR, USA, cat. no H30462) for 1 h at 25 °C, in darkness and washed three times in 10 mM Tris–HCl buffer, pH 7.4, for 15 min each. Finally, the samples were embedded in a mixture of 15% acrylamide–bis-acrylamide stock solution and processed as described below.

Detection of SNOs in the presence of reductants

Hypocotyl cross sections were pre-incubated in a reductant solution containing 1 mM ascorbate, 10 μM CuCl and 200 μM cPTIO in 10 mM phosphate-buffered saline (PBS) at room temperature for 1 h. Afterwards, the samples were washed three times for 15 min each (two washes with PBS and other one with 10 mM Tris–HCl buffer, pH 7.4). Then, they were incubated at 25 °C for 2 h in darkness with 100 μM DTPA and 10 mM NEM prepared in ethanol, and then washed three times in 10 mM Tris–HCl buffer, pH 7.4, for 15 min each. Next, the samples were incubated with 10 μM Alexa fluor 488 Hg-link phenylmercury for 1 h at 25 °C, in darkness and washed three times in 10 mM Tris–HCl buffer, pH 7.4, for 15 min each. Finally, the samples were embedded in a mixture of 15% acrylamide–bis-acrylamide stock solution and processed as described below.

In both situations, sections were analysed with a CLSM system using standard filters for Alexa fluor 488 green fluorescence (excitation 495 nm; emission 519 nm).

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

Sunflower hypocotyls ($n = 5$ for each experimental group) were cut into pieces of 4–5 mm and fixed in 4% (w/v) *p*-formaldehyde in 0.1 M PB, pH 7.4, for 3 h at room temperature. Then, they were cryoprotected by immersion in 30% (w/v) sucrose in PB overnight at 4 °C. Serial sections, 60 μm thick, were made by means of a cryostat (2800 Frigocut E; Reichert-Jung, Vienna, Austria). Immunohistochemistry was carried out by confocal analysis of immunofluorescence-stained sections, as described by Valderrama *et al*. (2006) using a GSNO rat antisera (Barroso *et al*. 2006) diluted 1:2500, purified IgG of a rabbit polyclonal antibody against GSNOR diluted 1:50 (Chaki *et al*. 2009a) and a rabbit polyclonal antibody against NO_2-Tyr (Chaki *et al*. 2009a) diluted 1:300. Controls for background staining, which was usually negligible, were performed replacing the primary antibody with an equivalent concentration either of the incubation buffer or of normal rabbit serum.

Determination of NO_2-Tyr and tyrosine (Tyr)

Tyr and NO_2-Tyr were identified and quantified in sunflower hypocotyls from control and subjected to HT seedlings using LC–MS/MS according to Chaki *et al*. (2009b).

Two-dimensional (2D) gel electrophoresis and immunoblot analysis

Hypocotyls (approx. 168 g) of 1000 sunflower seedlings from control and subjected to HT were processed for crude extracts, as mentioned above. Protein precipitation was made with acetone according to Chaki *et al*. (2009b) and proteins were separated by 2D gel electrophoresis. Isoelectric focusing was carried out with precast IPG-gels pH 3–10. Each gel was loaded with 100 μg of proteins. Second-dimensional separation was performed by glycine–SDS–PAGE. Gels were SYPRO Ruby stained, scanned and analysed with Bio-Rad PD Quest software. Immunoblot of 2D gels was performed as described above, using an antibody against NO_2-Tyr (dilution 1:10 000).

In situ digestion of 2D spots and protein identification by MALDI TOF–TOF analysis

The spots identified in the gel from control samples and subjected to HT were automatically recovered using Investigator ProPic Protein Picking Workstation (Genomic Solutions Ltd, Huntingdon, UK) and digested with trypsin using an Investigator ProGest Protein Digestion Station (Genomic Solutions) as described previously by Chaki *et al*. (2009b). Peptides were purified using a ProMS station (Genomic Solutions) with a C18 column (ZipTip; Millipore) and eluting with α-cyano-4-hydroxycinnamic acid (3 mg mL^{-1}) in 70% acetonitrile/0.1% TFA in a MALDI plate (1 μL). After crystallization, the samples were analysed by MALDI TOF–TOF mass spectrometer in a range mass-to-charge ratio (*m*:*z*) of 800–4000 Da using a spectrometer 4700 Proteomics Analyzer (Applied Biosystems, Foster City, CA, USA) in automatic mode. Internal calibration of the mass spectrums was performed using the *m*:*z* of the peptides from porcine trypsin autolysis (mass $MH^{+} = 842.509$, mass $MH^{+} = 2211.104$), given a precision in the *m*:*z* ratio of 20 ppm. From each sample, the three spectra with the highest *m*:*z* ratios were selected. The protein was identified by combining the MS spectrum with the corresponding MS/MS using the MASCOT

program from the database of MatrixScience (http://www.matrixscience.com/). The following search parameters were applied, limiting the taxonomic category to green plants: a mass tolerance of 100 ppm and one incomplete cleavage were allowed; complete alkylation of cysteine by carbamidomethylation and partial oxidation of methionine resulted.

Treatment with 3-morpholinosydnonimine (SIN-1) (peroxynitrite donor) of hypocotyl samples and activity assay

The molecule SIN-1 spontaneously releases both NO and $O_2^{\cdot-}$ with the concomitant generation of $ONOO^-$. For this reason, a protein-nitrating compound is used (Daiber *et al*. 2004; Natal *et al*. 2008; Chaki *et al*. 2009b). Thus, hypocotyl samples were passed through Sephadex G-25 gel-filtration columns (NAP-10 from Amersham) equilibrated and eluted with 100 mM K-PB, pH 7.2, containing 2 mM potassium cyanide (inhibitor of CuZn-SOD) and 2 mM sodium azide (inhibitor of peroxidases). Aliquots of these samples were incubated at 37 °C for 2 h with increased concentrations (0–5 mM) of SIN-1 (Calbiochem) made up fresh before use. Then, the samples were passed again through NAP-10 column to avoid any interference of SIN-1 and assayed for FNR activity.

Other assays

The protein concentration was determined with the Bio-Rad protein assay, using BSA as a standard. To estimate the statistical significance between means, the data were analysed by Student's *t*-test.

RESULTS

The goal of this study was to understand the function of the NO and NO-derived molecules under environmental stress, specifically HT, using sunflower seedlings as model plants. It is well established that a wide range of environmental stress can augment the production of ROS in plants and thus provoke oxidative damage at the cell level, such as lipid hydroperoxidation, leading to membrane damage (Yoshimura *et al*. 2004). Figure 1a shows the lipid hydroperoxide content in hypocotyls of sunflower seedlings exposed to HT. As a result, HT raised the lipid hydroperoxide content 48%, indicating that our experimental conditions (38 °C for 4 h) cause oxidative stress in sunflower hypocotyls. Additionally, to test whether this HT induces the generation of HSPs, we evaluated it by immunoblot using an antibody which recognizes the heat shock 70 protein (Hsp70) family (Corpas & Trelease 1997). Supporting Information Fig. S1 shows the immunoblot analyses that detected an immunoreactive band of 72 kDa, which was induced in response to 38 °C for 4 h, indicating that this condition also induced heat stress.

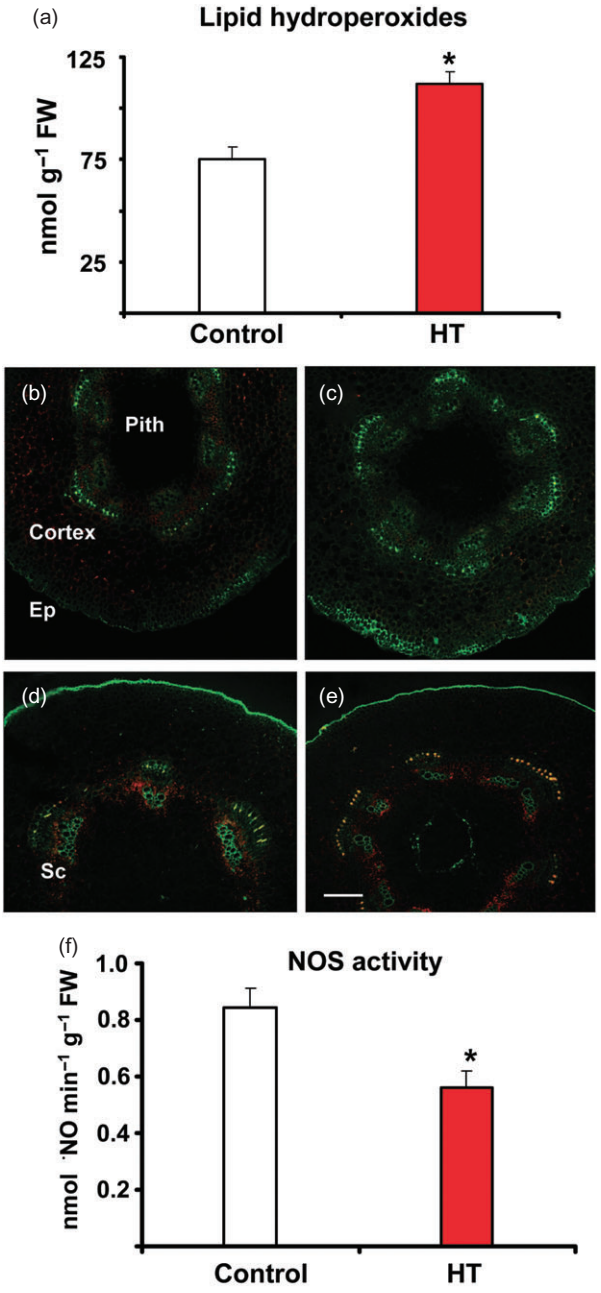

Figure 1. Effect of HT in lipid hydroperoxide, superoxide radical ($O_2^{\cdot-}$) and NO content, and NOS activity in sunflower seedlings. (a) Lipid hydroperoxide contents in sunflower hypocotyls. (b and c) Representative images illustrating the CLSM detection of $O_2^{\cdot-}$ in cross sections of hypocotyls from control and subjected to HT sunflower seedlings, respectively. The bright green fluorescence corresponds to the detection of $O_2^{\cdot-}$ with 10 μM DHE. (d and e) Representative images illustrating the CLSM detection of NO in cross sections of hypocotyls from control and subjected to HT sunflower seedlings, respectively. The bright green fluorescence corresponds to the detection of NO with 10 μM DAF-2 DA. (f) L-Arginine-dependent NOS activity in hypocotyl extracts from sunflower seedlings subjected to HT and control. Results are the mean of four different experiments ± SEM. *Differences from control values were significant at $P < 0.05$. Bar = 200 μm. Ep, epidermis; Sc, sclereids.

Cell localization of superoxide radical ($O_2^{\cdot-}$) and NO: enzymatic production of NO by L-arginine-dependent NOS activity

The localization of $O_2^{\cdot-}$ in cross sections of sunflower hypocotyls was assayed by CLSM using the fluorescent probe DHE. The green fluorescence corresponding to the presence of $O_2^{\cdot-}$ was localized mainly in epidermal cells and vascular tissue in control plants (Fig. 1b), and after HT treatment, the $O_2^{\cdot-}$ showed an intensification in vascular tissue as well as epidermal and cortex cells (Fig. 1c). As control, the hypocotyl sections were pre-incubated with the superoxide radical scavenger TMP, and the fluorescence was considerably reduced (Supporting Information Fig. S2a).

On the other hand, the localization of NO in cross sections of sunflower hypocotyls was also analysed by CLSM using the fluorescent probe DAF-2 DA, where the green fluorescence corresponds to the location of NO and red/yellow to the autofluorescence. The green fluorescence corresponding to the presence of NO was localized mainly in the epidermal cells and vascular tissue in control plants (Fig. 1d), and after HT treatment the NO distribution was very similar to that of control, but the fluorescent intensity was slightly lower in vascular tissue (Fig. 1e). For an evaluation of the reliability of this fluorescent probe in the detection of NO, the hypocotyl sections were pre-incubated with 200 μM cPTIO (a NO scavenger) and the green fluorescence was strongly reduced (Supporting Information Fig. S2b). These results were corroborated using alternative fluorescent probes to detect NO such as DAF-FM DA, which has been shown to be stable in a pH range of 5.5–12.0 (Zhang *et al*. 2003; Corpas *et al*. 2006), and DAR-4M AM (Corpas *et al*. 2009), which has different excitation and emission wavelengths to the DAF-2 DA or DAF-FM DA. With these two probes, the results showed that the fluorescent intensity of NO was also slightly lower after HT treatments (Supporting Information Fig. S3a–d).

Although the NOS gene has not yet been identified in higher plants, in a previous study, the presence of L-arginine-dependent NOS activity in sunflower hypocotyls was detected and characterized at the biochemical level (Chaki *et al*. 2009a, 2011). Thus, the L-arginine-dependent NOS activity was measured by ozone chemiluminescence, and, when the NOS activity in control plants (0.846 nmol NO min^{-1} g^{-1} FW) was compared with HT-treated plants, it was found that NOS activity was significantly reduced in the latter (32%; Fig. 1f).

In plants, NO can also be generated from nitrite and nitrate by enzymatic or non-enzymatic sources (del Río, Corpas & Barroso 2004; Gupta *et al*. 2011). To analyse the possible contribution of these molecules to the generation of the NO detected in hypocotyls, we determined the total content of nitrites and nitrates (Table 1). No statistically significant changes were detected between the concentration of nitrites and nitrates in hypocotyls from seedlings subjected to HT stress compared with control plants. NR is considered another potential source of NO in plant cells (Gupta *et al*. 2011), but the activity did not show any difference after HT stress (Table 1).

Table 1. Nitrate reductase (NR) activity, total nitrite and nitrate in hypocotyl extracts from sunflower plants subjected to HT (38 °C)

Condition	NR activity (μmol NO_2^- h^{-1} g^{-1} FW)	Total nitrate and nitrite (nmol g^{-1} FW)
Control	0.97 ± 0.07	386.3 ± 25.6
HT	0.91 ± 0.08	417.8 ± 35.9

Results are means ± SEM of samples from at least three different experiments.
FW, fresh weight.

Content and cellular analysis of SNOs and GSNO

SNOs may function as a major intracellular NO reservoir or vehicle for NO. Figure 2a shows the analysis of the SNOs content according to the chemiluminescence method, where the total content of SNOs increased 2.8-fold in hypocotyls of sunflower seedlings exposed to HT. Additionally, the cell locations of SNOs in cross sections of sunflower hypocotyls were also analysed by CLSM using the fluorescence probe Alexa Fluor 488 Hg-link (Chaki *et al*. 2009a). In control sunflower seedlings, the SNOs were detected mainly in vascular tissue (Fig. 2b); however, in sunflower seedlings exposed to HT, the SNOs showed a highly significant increase in fluorescence intensity in all cell types, including vascular tissues and cortex cells (Fig. 2c). As a control to corroborate the specificity of this fluorescence probe, hypocotyl sections from control plants were exposed to HT and pre-incubated in the presence of a solution containing 1 mM ascorbate, 10 μM ClCu (molecules that can break down SNOs) and 200 μM cPTIO (a NO scavenger) (Fig. 2d,e). In both cases, the location was similar to control, but the fluorescence intensity of SNOs was considerably reduced mainly in the seedling samples exposed to HT (Fig. 2e), indicating the specificity of this fluorescence probe.

Considering that GSNO could be the most abundant SNOs in plants, we also studied the cell location of GSNO using CLSM with a specific antibody. Figure 2f,g shows the cell location and abundance of GSNO in the cross section of hypocotyls of sunflower exposed to HT where green fluorescence was noted in the corresponding panels. In control plants, the GSNO was located in vascular tissue and epidermal cells (Fig. 2f), and after HT it noticeably rose, mainly in cortex cells (Fig. 2g). This behaviour was similar to that found in total SNOs (Fig. 2b,c).

GSNOR activity, protein and gene expression, and cell location

As part of the analysis of GSNO metabolism, GSNOR activity and expression were studied. GSNOR activity was

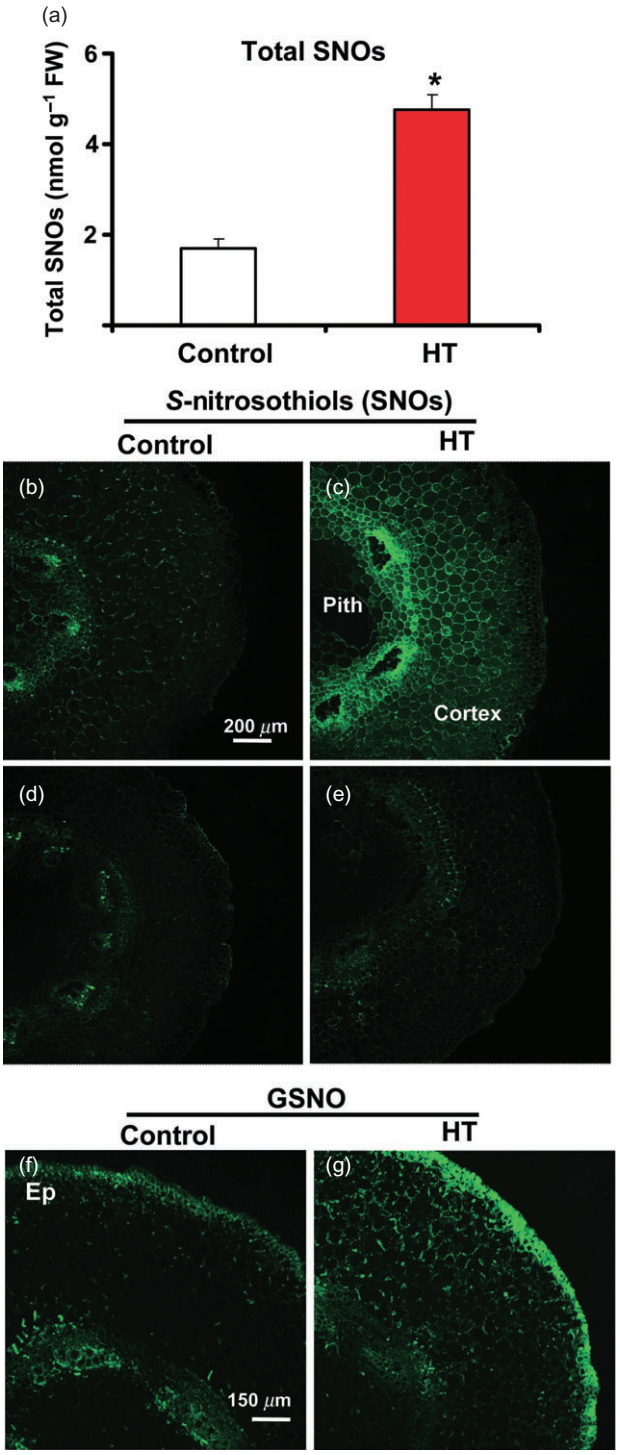

Figure 2. Effect of HT in SNOs content and location in sunflower seedlings. (a) SNOs content in hypocotyl extracts from sunflower seedlings subjected to HT and control. Results are the mean of four different experiments ± SEM. *Differences from control values were significant at $P < 0.05$. (b–e) Representative images illustrating the CLSM detection of SNOs in cross sections of hypocotyls of sunflower seedlings from control and subjected to HT in the absence of reductants (b and c) and in the presence of reductants (d and e). (f and g) Representative images illustrating the CLSM detection of GSNO using a specific antibody against GSNO diluted 1:2500.

reduced by 25% in hypocotyls of sunflower seedlings exposed to HT (Fig. 3a), and similar behaviour was found with the protein content, which was reduced 37% according to the immunoblot assay (Fig. 3b). In addition, the cell location of GSNOR was also studied by CLSM using the same antibody. Figure 3c,d shows the cell location and abundance of GSNOR in the cross section of hypocotyls of sunflower exposed to HT where a green fluorescence was noted in the corresponding panels. The location of GSNOR in control hypocotyls was detected in cortex cells and mainly in vascular tissue (Fig. 3c), and after HT the content of GSNOR protein was clearly reduced (Fig. 3d), this behaviour being identical to that observed in immunoblots (Fig. 3b).

The analysis of the gene expression of *GSNOR* by real-time quantitative RT-PCR in hypocotyls of sunflower seedlings exposed to HT showed a reduction of 58% (Fig. 3e).

Content of Tyr and NO_2-Tyr

Table 2 shows the concentration of NO_2-Tyr and Tyr in hypocotyl extracts from sunflower control plants and those subjected to HT (38 °C) determined by LC–MS/MS. Under HT, the content of NO_2-Tyr increased 2.5-fold; however, the content of Tyr did not change. It is important to point out that the content of NO_2-Tyr was in the range of picomol, whereas the content of Tyr was in the micromol range.

Cell location and content of Tyr-nitrated proteins and peroxynitrite ($ONOO^-$)

Figure 4a–d shows the cell location of Tyr nitration of proteins using an antibody against NO_2-Tyr, which recognizes nitrated proteins (Chaki *et al.* 2009b). In the control samples, green fluorescence was visible mainly in vascular tissues and in some cortex cells (Fig. 4a). However, in sunflower seedlings exposed to HT, the green fluorescence was present in almost all cell types with a clear intensification in vascular tissues, cortex and epidermal cells (Fig. 4b). For an evaluation of whether SNOs could mediate the process of the Tyr nitration, hypocotyl samples from control sunflower seedlings and those exposed to HT were pre-incubated in a solution containing 1 mM ascorbate, 10 μM CuCl (molecules that can break down SNOs) and 200 μM cPTIO (a NO scavenger) (Fig. 4c,d, respectively). In both cases, the location and content of NO_2-Tyr were very similar to control (Fig. 4a).

Peroxynitrite ($ONOO^-$) has been shown to mediate protein–Tyr nitration (Radi 2004). This molecule was also detected in hypocotyl sections using the fluorescence probe APF to study the potential correlation of Tyr nitration and peroxynitrite (Fig. 4e–h). In control samples, peroxynitrite was detected mainly in vascular tissue and epidermal cells (Fig. 4e). However, under HT the green fluorescence was also present in almost all cell types with a significant intensification in vascular tissues (Fig. 4f). Thus, the cell localization of Tyr nitration and peroxynitrite was very similar under HT, indicating a potential correlation between these

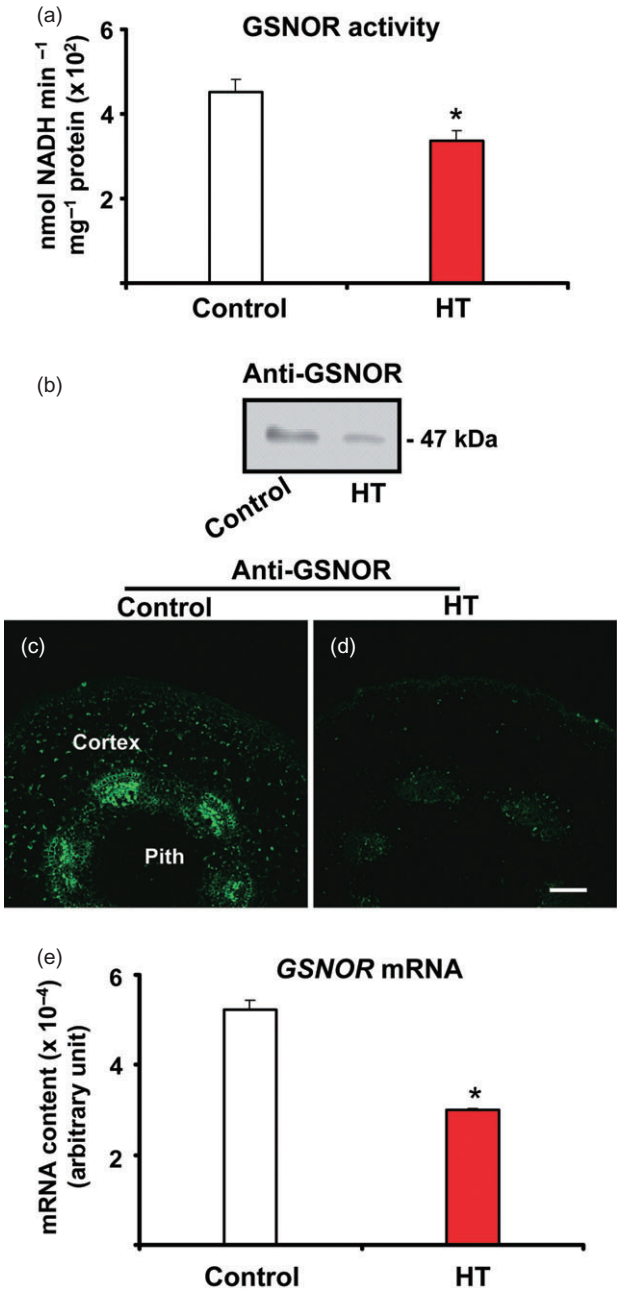

Figure 3. Effect of HT in GSNOR activity (a), protein expression (b), cell location (c and d) and gene expression (e) in hypocotyl samples from sunflower seedlings subjected to HT and control. For immunoblots, 20 μg protein of hypocotyl samples was applied in each lane and resolved by SDS–PAGE. Proteins were electroblotted onto PVDF membranes and then incubated with polyclonal antibodies against GSNOR (1:200). For CLSM analysis, the GSNOR IgG was diluted 1:50 as described in Materials and methods. Gene expression was studied by real-time quantitative RT-PCR using 18S RNA as an internal control. Data are the mean ± SEM of at least three independent RNA samples. *Differences from control values were significant at $P < 0.05$.

Table 2. Concentration of nitrotyrosine (NO_2-Tyr) and tyrosine (Tyr) in sunflower hypocotyls from sunflower plants exposed to HT (38 °C) and control

Condition	NO_2-Tyr (pmol mg^{-1} prot)	Tyr (μmol mg^{-1} prot)	NO_2-Tyr/Tyr (μmol mol^{-1})
Control	0.19 ± 0.02	0.56 ± 0.06	33.9 ± 3.2
HT	0.48 ± 0.03*	0.51 ± 0.03	94.1 ± 6.32*

The analyses were achieved by LC–MS/MS. Data are the mean ± SEM of at least three independent experiments. *Differences from control values were significant at $P < 0.01$.

two parameters. In the determination of whether SNOs could be involved in peroxynitrate generation, the hypocotyl sections (control and HT treated) were also pre-incubated in a solution containing ascorbate, CuCl and cPTIO (Fig. 4g,h). The location was almost identical to that of control (Fig. 4e), but the peroxynitrite content was lower, suggesting that SNOs could mediate the formation of peroxynitrite in this plant tissue.

Proteomic identification of Tyr-nitrated proteins in sunflower seedlings exposed to HT

In a previous study, a proteomic protocol was set up to enable the identification of protein targets of Tyr nitration in hypocotyls of healthy sunflower seedlings (Chaki *et al.* 2009b). Using this protocol, we analysed the proteins that undergo Tyr nitration under HT. Supporting Information Fig. S4 shows the protein staining of 2D gel of hypocotyl samples from sunflower seedlings subjected to HT and control (Fig. 4a), and its corresponding immunoblots probed with an antibody against NO_2-Tyr (Fig. 4b). Thus, a total of 22 immunoreactive spots were detected under HT conditions. Figure 4c shows zoom boxes that illustrate in more detail which immunoreactive spots were most intense under HT. The analysis with Bio-Rad PD Quest software revealed that the spots 10–22 were clearly more intense under HT, with only spot number 15 being a new one in comparison to a previous identification under healthy conditions (Chaki *et al.* 2009b). All 22 of these spots were analysed by MALDI-TOF mass spectrometry after trypic digestion using the MASCOT search engine to analyse MS data in order to identify proteins from primary sequence databases (Perkins *et al.* 1999). The immunoreactive proteins identified are listed in Table 3, the protein score confidence interval (CI) in all cases being higher than 99%, indicating that the proteins were not identified by random matches of peptide mass data. Thus, all the spots under HT were identical to control conditions with the exception of a new spot identified with the number 15, which was a carbonic anhydrase (CA). It is well established that stress by HT induces many proteins; in our case, many of the induced proteins, detected by protein staining, were within the range of 45.0–66.2 kDa (Fig. 4a). However, it was noted that there was no direct

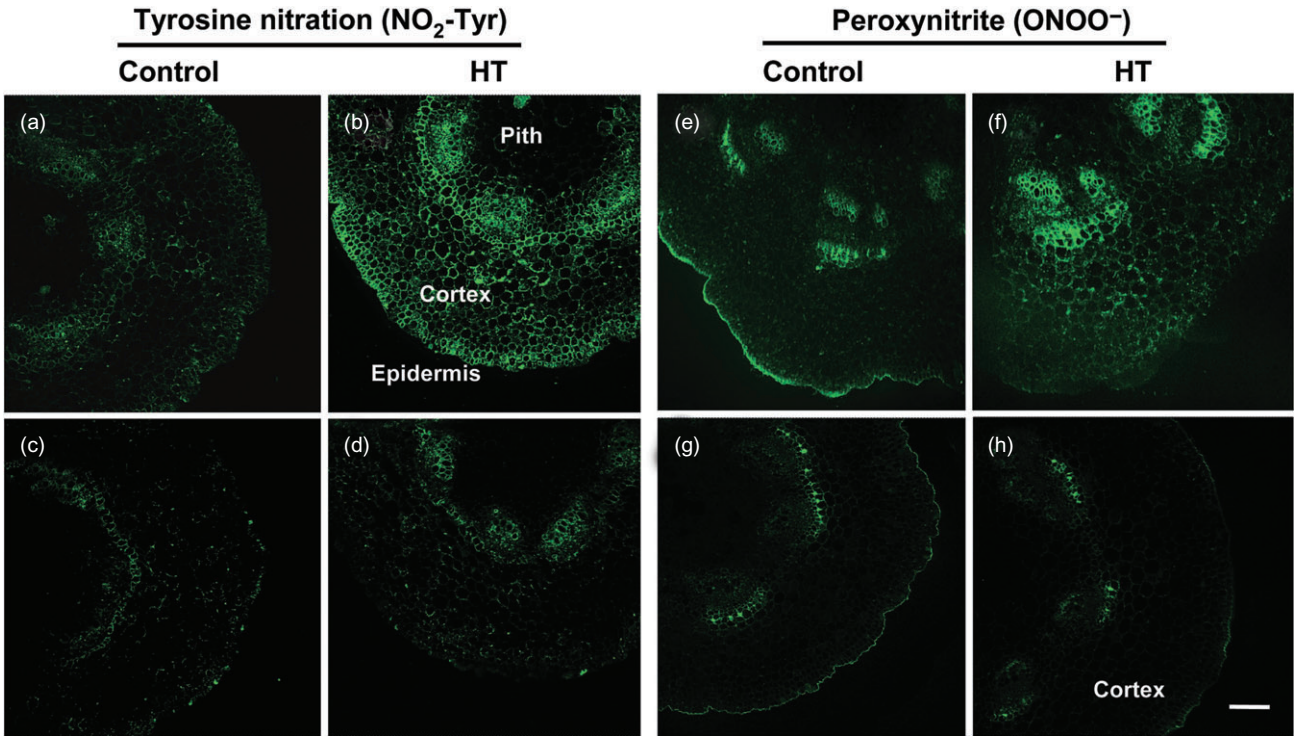

Figure 4. Representative images illustrating the CLSM detection of protein 3-nitrotyrosine (a–d) and peroxynitrite (e–h) in cross sections of hypocotyls from sunflower seedlings subjected to HT and control. NO_2-Tyr was detected using a specific antibody with a dilution of 1:300 and $ONOO^-$ was carried out using APF as described in Materials and methods. To evaluate the potential involvement of SNOs in the generation of protein nitration and peroxynitrite, the hypocotyl samples from sunflower seedlings exposed to HT (d and h) and control (c and g) were pre-incubated in a solution containing 1 mM ascorbate, 10 μM CuCl (molecules which can discompose SNOs) and 200 μM cPTIO (a NO scavenger). Bar = 200 μm.

correlation between the induced proteins detected by protein staining (Supporting Information Fig. S4a) and the nitrated protein identified by immunoblots after HT stress (Supporting Information Fig. S4b).

Effect of HT on photosynthesis

To gain fuller knowledge of Tyr nitration under HT stress, we analysed the effect of HT in some photosynthetic parameters in cotyledons of sunflower seedlings. Thus, the analysis of photosynthetic rate and the chlorophyll content was diminished by 26 and 58%, respectively (Fig. 5a,b), indicating that photosynthesis is affected in this heat stress condition. Then, among the identified nitrated proteins (Table 3), we selected FNR, which is involved in photosynthesis. Thus, Fig. 5c shows that HT caused 31% inhibition on FNR activity. Afterwards, by *in vitro* assay, we evaluated the effect of different concentrations of SIN-1 (0, 0.5, 1.0 and 5 mM), a peroxynitrite-generating system which has been shown to mediate Tyr nitration (Daiber *et al*. 2004; Radi 2004; Larrainzar *et al*. 2008; Chaki *et al*. 2009b) in this activity. Figure 5d depicts the effect of peroxynitrite on FNR activity in sunflower hypocotyl samples showing greater inhibition with the increase of SIN-1 concentration, inhibition reaching 91% with 5 mM SIN-1.

DISCUSSION

It is well established that plants, in response to environmental stress, can modulate the antioxidative system to avoid the potential damage of an overproduction of ROS (Nobuhiro & Mittler 2006). However, an impairment of the antioxidant defence system can provoke oxidative damage to biomolecules and prompt a process of oxidative stress. However, less is known about the involvement of NO-derived molecules designated as RNS in response to environmental stress and their direct or indirect interaction with specific proteins that can undergo post-translational modifications that affect their function. The goal of this work was to establish whether the abiotic stress caused by HT triggers nitrosative stress and how it occurs.

HT causes oxidative stress and impairs NO metabolism in hypocotyls of sunflower seedlings

HT has been shown to cause oxidative damage manifested in lipid peroxidation that, among other effects, impairs mitochondrial and chloroplastic functions (Larkindale & Knight 2002; Vacca *et al*. 2004; Takahashi & Murata 2008). In sunflower, the significant rise of lipid hydroperoxide moves us to conclude that our experimental conditions (38 °C for

Table 3. Identified 3-nitrotyrosine proteins from sunflower hypocotyls under HT

Spot no.	Identified protein	Acc.no.	Protein score CI %/pep. count	MW/pI	Functional grouping
1	Alpha-mannosidase	Q9FFX7	100/6	116918.1/6.0	Mannose metabolic process, carbohydrate metabolism
2	Transketolase precursor	S58083	100/12	75577.1/5.79	Carbohydrate metabolism
3	ATPase-like protein	H85431	99.89/6	69731.6/6.99	Energy metabolism, ATP binding, oxidative phosphorylation
4	Molecular chaperone HSP68	T07024	99.99/11	73317.1/6.37	Protein folding
5	CAD ATPase (AAA1), 35570–33019	B96835	99.918/12	57485.9/6.95	Energy metabolism, ATP binding, oxidative phosphorylation
6	Ferredoxin–nitrite reductase	*S16603*	*99.59*/12	*67093.2/6.51*	Photosynthetic carbon assimilation
7	Glutathione reductase	Q6F4I4	100/14	61175.5/8.75	Antioxidant metabolism
8	Putative serine/threonine–protein kinase	Q84YQ2	99.716/11	53561.8/9.74	Post-translational modifications
9	Alternative oxidase	Q9SC31	99.303/8	40246.2/7.75	Energy metabolism
10*	Putative cytochrome P450	Q9FW84	99.808/11	49131.6/9.64	Biosynthetic reactions of fatty acid conjugates, plant hormones and defensive compounds
11*	Putative dehydratase/deaminase	Q8W314_ ORYSA	99.966/13	65954.7/5.77	Isoleucine biosynthetic process
12*	Ferredoxin–NADP reductase precursor	S04030	99.723/22	40453.7/8.56	Photosynthesis
13*	Hypothetical protein	Q75L89	99.72/12	66365.6/5.31	Unknown protein
14*	Transposon protein, putative, CACTA, En/Spm subclass	Q2QN81	99.609/16	49380.1/6.07	DNA metabolism
15*	Carbonic anhydrase (CA)/carbonate dehydratase (EC 4.2.1.1) precursor, chloroplast- *Arabidopsis thaliana*	S28412	100/14	36520.5/5.46	Photosynthetic carbon assimilation
16*	Acetyl-CoA carboxylase	Q9FEH8	99.407/17	201697.5/6.05	Fat metabolism in higher plants
17*	Putative DNA damage–repair/toleration protein DRT102	Q2PEP7	99.799/14	33287.6/5.22	DNA metabolism
18*	*S*-adenosyl-homocysteine hydrolase	Q9SDP1	100/15	34216.2/4.99	Amino acid biosynthesis
19*	14-3-3-like protein	T12951	99.994/5	29043.5/4.65	Regulating cell signalling
20*	20S proteasome subunit C8	G84667	100/9	27644.9/5.93	Proteasome pathway
21*	Proteasome endopeptidase complex (EC 3.4.25.1) chain PAA2	T51967	100/6	27446.9/5.75	Proteasome pathway
22*	Calmodulin-like protein	Q67TZ4	99.864/8	21417.7/8.68	Calcium-binding protein

*Proteins induced by heat stress.
Concentrated sunflower hypocotyl extracts were subjected to 2D electrophoresis and immunoblot probed with an antibody against 3-nitrotyrosine. The identified spots were analysed by MALDI-TOF mass spectrometry after trypsine digestion. The MASCOT search engine was used to parse MS data in order to identify proteins from primary sequence databases. The value of protein score confidence interval (CI) closest to 100% indicates that the protein is most likely correctly matched. Pep. Count., number of identified peptides.
MW, molecular weight; pI, isoelectric point.

4 h) provoke oxidative stress which agrees well with heat stress, considering the increase of protein expression of the Hsp of 72 kDa.

Previous data have shown a rise in NO production under HT. In alfalfa sprouts, heat stress (37 °C for 2 h) doubled the rate of NO emission (Leshem, Wills & Ku 1998). In tobacco, HT generates a rapid and significant rise in NO in adaxial epidermal cells after 7 min heat treatment at 40 °C and in suspension cells after 5 min heat treatment at 45 °C, evaluated by the fluorescence from DAF-2 DA (Gould *et al*. 2003). Conversely, pea plants exposed at 38 °C for 4 h decreased the NO content of leaves, but it did not significantly affect the NOS activity (Corpas *et al*. 2008). However, in sunflower under the same experimental conditions (38 °C for 4 h), cellular NO content was slightly lower while NOS activity was significantly reduced. Additionally, NR activity, total nitrite and nitrate, which can be alternative sources of NO under certain circumstances, did not change, although it cannot be ruled out that some post-translational modulation involved in the production of NO by NR (Kaiser *et al*. 2002) was affected specifically by HT.

SNOs are involved in generating $ONOO^-$ and protein nitration under HT, causing nitrosative stress

The half-life of NO is short, however, and its functionality can be extended and relatively modulated when NO reacts with low-molecular weight and protein-bound thiols to form SNOs. SNOs, including GSNO, can release NO in a controlled manner in the cells, either spontaneously or by interaction with various biological reductants such as

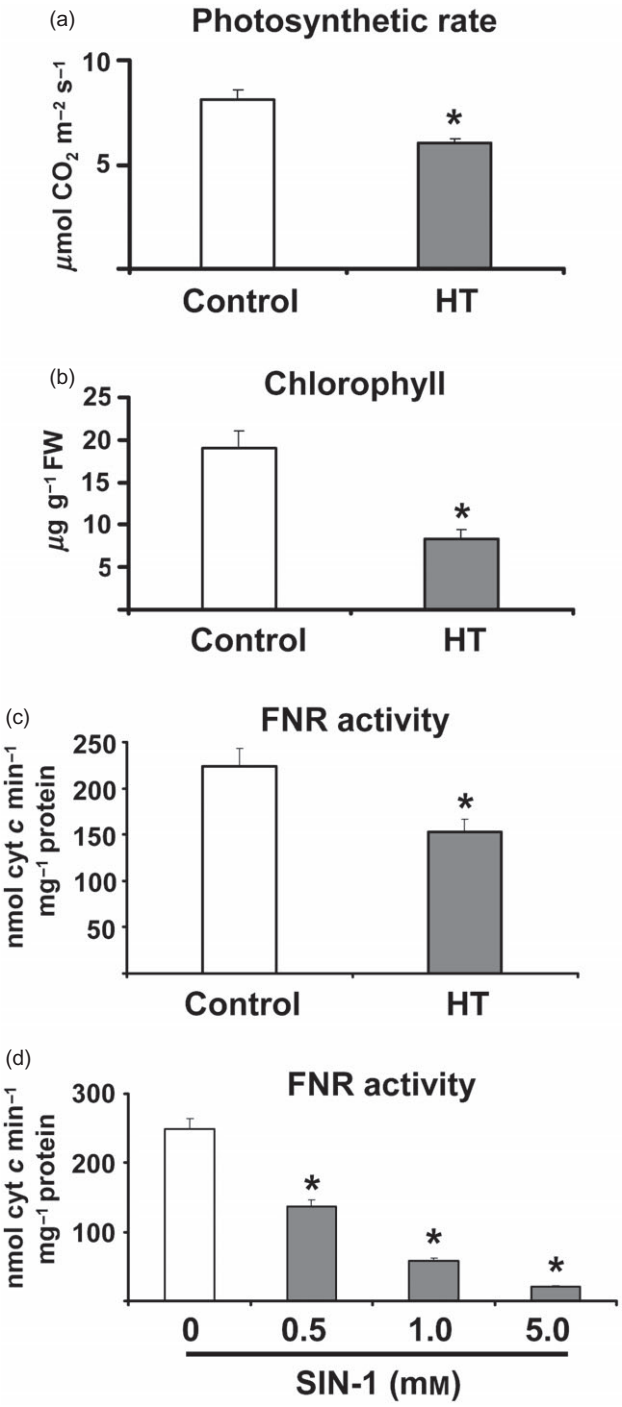

Figure 5. Effect of HT on photosynthesis and ferredoxin–NADP reductase (FNR) activity. (a) Photosynthetic rate. (b) Chlorophyll content in cotyledons of sunflower plants subjected to HT (38 °C). Data are the mean ± SEM of at least three different experiments. The light intensity condition was 177 μE m^{-2} s^{-1}. *Differences from control values were significant at $P < 0.01$. (c) FNR activity in sunflower hypocotyls from plants exposed to HT (38 °C) and control. *Differences from control values were significant at $P < 0.05$. (d) Effect of SIN-1 ($ONOO^-$ donor) on FNR activity.

glutathione and ascorbate. Furthermore, reduced metal ions such as copper (Cu^+) break down SNOs more rapidly than do oxidized metal ions (e.g. Cu^{2+}) (Dicks *et al*. 1996), indicating that reducing agents such as glutathione and ascorbate can stimulate the breakdown of SNOs by chemical reduction of contaminating transition metal ions (Singh *et al*. 1996b; Williams 1996; Smith & Dasgupta 2000). In sunflower, HT induced a significant increase in the generation of total SNOs either determined by ozone chemiluminescence or using the fluorescent reagent Alexa fluor 488 Hg-link phenylmercury as well as GSNO. The rise of SNOs has been detected in other plant species under different stress conditions. In olive, salinity stress significantly boosts total SNOs (Valderrama *et al*. 2007) and a similar situation has been found in pea plants under low temperatures, high light intensity and mechanical injury (Corpas *et al*. 2008).

Considering that SNOs, including GSNO, may function as an intracellular NO reservoir and as a vehicle for NO throughout the cells (Singh *et al*. 1996a,b; Wang *et al*. 2006; Arasimowicz & Floryszak-Wierczore 2007), it must be considered that the rise of SNOs observed in hypocotyls after HT stress could also come from other sunflower organs such as the cotyledons. Taking into consideration that total SNOs include high molecular weight (protein-bound thiols) and low molecular weight (principally GSNO), the histological analysis showed a differential distribution in the different cell types. Thus, total SNOs linked mainly to proteins (detected by a fluorescence probe) were present in almost all cell types with a special intensification in vascular tissue after HT stress (Fig. 2c). However, GSNO (detected by a specific antibody) was present in vascular tissue and epidermal cells, but after HT a higher expression was found in epidermal cells (Fig. 2g). In this sense, a similar behaviour has been reported in sunflower hypocotyls after infection by the pathogen *Plasmopara halstedii* (Chaki *et al*. 2009a) or mechanical injury (Chaki *et al*. 2011). Thus, it can be hypothesized that the highest abundance of GSNO in epidermal cells could act as a protection mechanism in these type of cells because they are in direct contact with the external environment. On the other hand, the enzyme GSNOR which breaks down GSNO (considered the most abundant low-molecular weight SNOs in plant cells) was down-regulated at a different level (i.e. gene and protein expression as well as its specific activity). Accordingly, the data suggest that the accumulation of GSNO, and subsequently of SNOs, must be caused by the inhibition of GSNOR. A similar behaviour has been noted in sunflower hypocotyls under mechanical injury (Chaki *et al*. 2011). Moreover, these data are consistent with the results reported in different organisms by other authors where, using genetic approaches, it is shown that in GSNOR knock-out mutants in mice, *Arabidopsis* and yeast, there is a rise in SNOs (Liu *et al*. 2001, 2004; Feechan *et al*. 2005; Rustérucci *et al*. 2007; Lee *et al*. 2008). In *Arabidopsis*, several mutants have been identified to have impairment in the *GSNOR1* gene, showing the involvement of this gene in physiological and stress processes. For example, the mutant HOT5 (sensitive to hot temperatures) showed that

GSNOR modulates the intracellular level of SNOs, which enables thermotolerance as well as regulation of plant growth and development (Lee *et al*. 2008). Similarly, the mutant paraquat resistant 2 (PAR2), which has a higher NO level, shows an anti-cell death phenotype (Chen *et al*. 2009).

Considering that HT provokes a low NO content but a rise of SNOs in sunflower, we could hypothesize that these NO-derived molecules in an oxidative environment can directly or indirectly mediate the generation of $ONOO^-$, which participates in the rise of protein Tyr nitration. To evaluate this hypothesis, the sunflower samples exposed to HT were pre-incubated in a solution containing ascorbate, CuCl and cPTIO (which eliminates SNOs and scavenges the released NO, respectively), and a significant reduction was noted in the peroxynitrite content (Fig. 4h), which was well correlated with the reduction in the content of protein Tyr nitration (Fig. 4d), supporting the contention that SNOs could mediate the peroxynitrite production and consequently the generation of Tyr nitration. Thus, the present data agree with the results of Trujillo *et al*. (1998), who demonstrated that SNO breakdown was accompanied by peroxynitrite formation. These authors proposed that the mechanism involves the $O_2^{\cdot-}$-dependent reduction of SNOs to yield NO, which in turn reacts with a second $O_2^{\cdot-}$ molecule at very fast rates ($k = 6.7 \times 10^9$ M^{-1} s^{-1}) to yield peroxynitrite ($ONOO^-$).

Post-translational modifications are key elements to regulate the function of proteins. In particular, NO_2-Tyr modifications have become of particular interest in monitoring the onset and progression of disease because a rise of protein nitration has been reported in over 100 human pathologies and their animal or cell models (Ischiropoulos 2003; Bigelow & Qian 2008). At present, Tyr nitration has been shown to be a selective process that affects the function of the target protein in several ways: inhibition of functions being a much more common consequence of protein Tyr nitration (MacMillan-Crow *et al*. 1996; Bachschmid *et al*. 2003), gain of function (Ji *et al*. 2006) or no effect on function (Radi 2004). However, in other cases, it has been reported that nitration of a Tyr residue may either prevent further phosphorylation or stimulate phosphorylation (Rayala *et al*. 2007; Shi *et al*. 2007). In the case of plants, several reports have shown the rise of protein nitration under environmental stress conditions such as shear stress (Gong & Yuan 2005), salinity (Valderrama *et al*. 2007; Corpas *et al*. 2009) and pathogens (Saito *et al*. 2006; Romero-Puertas *et al*. 2007; Chaki *et al*. 2009a; Cecconi *et al*. 2009); however, no specific proteins have been identified so far. In some cases, the effect of nitration is reportedly mediated by peroxynitrite, which could have an inhibitory effect, for example inhibiting electron transport on the acceptor side of photosystem II (González-Pérez *et al*. 2008) or inhibiting *S*-adenosyl-L-homocysteine hydrolase activity (Chaki *et al*. 2009b).

Recently, the nitroproteome of sunflower hypocotyls was established under optimal growth conditions (Chaki *et al*. 2009b), where a total of 21 nitrated proteins were identified, this number of proteins being consistent with previous proteomic identification analyses in animal systems (Aulak *et al*. 2001; Tedeschi *et al*. 2005; Tyther *et al*. 2007). More recently, the nitroproteome of *Arabidopsis thaliana* seedlings has also been reported (Lozano-Juste, Colom-Moreno & León 2011). Under HT, only 13 of these proteins were induced (Table 3), and the feasibility and specificity of the antibody against NO_2-Tyr appear to be reliable because there is no direct correlation between the induced proteins detected by protein staining and the nitrated protein identified by immunoblot analysis after HT stress. Moreover, these 13 proteins are involved in different physiological processes, including photosynthesis, the proteasome pathway, signalling, and carbohydrate and fat metabolism, with CA being the only new candidates. Therefore, the rise in Tyr nitration observed under HT seems to confirm that it can be a reliable footprint of nitrosative stress in plants. However, in the present work, where there is a reduction in the NO production, a new step is added to the nitration process observed under HT because inhibition of GSNOR appears to mediate the accumulation of SNOs, the increase of peroxynitrite and consequently the rise of protein Tyr nitration.

Tyr nitration inhibits FNR activity in sunflower under HT

To gain greater insight into the physiological significance of Tyr nitration, among the identified proteins during the HT, we selected FNR because it is involved in photosynthesis. Likewise, this process is among the most thermosensitive aspects of plant function, because both light (electron transport) and dark (Calvin cycle) reactions of photosynthesis have thermolabile components (Allakhverdiev *et al*. 2008), and the decrease of the chlorophyll content and photosynthesis rate in sunflower after HT stress has implications in this direction. Ferredoxin–NADP oxidoreductase (FNR) mediates the final step of photosynthetic electron flow by transferring electrons from ferredoxin to NADP with the concomitant generation of reducing power (NADPH). This is used for carbon fixation, nitrogen metabolism, and lipid and chlorophyll biosynthesis, as well as for stromal redox regulation. Thus, the inhibition of FNR must affect the redox state of chloroplasts. Therefore, the nitrosative stress characterized by a rise of protein nitration under HT could, as a consequence, inhibit photosynthesis. In fact, in many plant species, photosynthesis is depressed by HT because of an inhibition of protein synthesis and inactivation of some chloroplast enzymes, as has been observed under our experimental conditions with the FNR. Accordingly, rice and wheat plants showed a reduction in the photosynthetic rate with the highest temperature at 32 °C (Al-Khatib & Paulsen 1999). In sunflower, HT provokes different changes in chloroplast structure perhaps because of the dehydration (Dekov, Tsonev & Yordanov 2000). On the other hand, HT induces changes in chloroplasts, affecting the reorganization of thylakoids with an increased excitation of photosystem I, accelerating cyclic electron transport while impeding the supply of NADPH and limiting carbon assimilation (Pastenesz & Horton 1996a,b). Moreover, ribulose 1·5-bisphosphate carboxylase/oxygenase (Rubisco) activity

declines with increasing temperature (Cen & Sage 2005). Therefore, the present results provide additional insight into the thermosensitivity of photosynthesis, and they reveal that HT yields nitrosative stress characterized by intensified nitration, which in sunflower leads to the inhibition of FNR (Fig. 5c,d).

In summary, it is proposed that HT causes both oxidative and nitrosative stress as a consequence of the rise in lipid oxidation and protein Tyr nitration, respectively. In the case of nitrosative stress, the proposed cascade of events is as follows: reduction of NO production, inhibition of GSNOR activity, accumulation of SNOs, formation of peroxynitrite and rise of protein nitration. Among the identified specific targets, nitration caused an inhibition of FNR activity, protein involved in photosynthesis process. Thus, these data provide new insights into the mechanism of photosynthesis inhibition under HT stress, which is mediated by a process of Tyr nitration where the GSNOR and SNOs are new key components.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Figure S1. Representative immunoblot showing the detection of Hsp72 protein in hypocotyl samples from control sunflower seedlings and subjected to HT. An antibody against cucumber PMP72 (diluted 1:300) which recognizes the Hsp70 protein family was used (Corpas & Trelease 1997). For each sample, 30 μg proteins was loaded.

Figure S2. Representative images illustrating the CLSM of the respective controls used for the detection of superoxide radical and NO in transversal sections of sunflower hypocotyls. (a) Hypocotyl section was pre-incubated for 60 min at

25 °C with TM P (a superoxide radical scavenger) and then with 10 μM DHE. (b) Hypocotyl section was pre-incubated for 30 min at 25 °C with 200 μM cPTIO (an NO scavenger) and then with 10 μM DAF-2 DA. Bar = 200 μm.

Figure S3. Representative images illustrating the CLSM detection of NO (green colour) with different fluorescent probes in cross sections of hypocotyls from control (a and c) and subjected to HT (b and d) sunflower seedlings. (a and b) Detection of NO with 10 μM DAF-FM DA (excitation 495 nm and emission 515 nm) as fluorescent probe. (c and d) Detection of NO with DAR-4M AM (excitation 543 nm; emission 575 nm) as fluorescent probe. Bar = 200 μm.

Figure S4. Detection of nitrated proteins in hypocotyl extracts from sunflower seedlings subjected to HT and control, by two-dimensional (2D) electrophoresis and immunoblot. (a) Representative 2D electrophoresis (pH 3–10 for the first dimension) of the hypocotyl extracts stained with Spypro Ruby. Molecular mass standards are indicated on the left in kDa. (b) The corresponding Western blot of hypocotyl crude extracts probed with a polyclonal antibody against nitrotyrosine (NO_2-Tyr) (dilution 1:10 000). Approximately 150 μg of proteins was loaded per gel. The arrows indicate all the immunoreactive spots, and the numbers refer to the proteins listed in Table 3. (c) Zoom boxes illustrate details of immunoreactive spots.