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Metabolism of Reactive Nitrogen Species in Pea Plants Under Abiotic Stress Conditions

Francisco J. Corpas [1,*], Mounira Chaki [2], Ana Fernández-Ocaña [2], Raquel Valderrama [2], José M. Palma [1], Alfonso Carreras [2], Juan C. Begara-Morales [2], Morad Airaki [1], Luis A del Río [1] and Juan B. Barroso [2]

[1] *Departamento de Bioquímica, Biología Celular y Molecular de Plantas, Estación Experimental del Zaidín, Consejo Superior de Investigaciones Científicas (CSIC), E-18080 Granada, Spain*
[2] *Grupo de Señalización Molecular y Sistemas Antioxidantes en Plantas, Unidad Asociada al CSIC, Área de Bioquímica y Biología Molecular, Universidad de Jaén, E-23071 Jaén, Spain*

Nitric oxide (·NO) is a key signaling molecule in different physiological processes of animals and plants. However, little is known about the metabolism of endogenous ·NO and other reactive nitrogen species (RNS) in plants under abiotic stress conditions. Using pea plants exposed to six different abiotic stress conditions (high light intensity, low and high temperature, continuous light, continuous dark and mechanical wounding), several key components of the metabolism of RNS including the content of ·NO, *S*-nitrosothiols (RSNOs) and nitrite plus nitrate, the enzyme activities of L-arginine-dependent nitric oxide synthase (NOS) and *S*-nitrosoglutathione reductase (GSNOR), and the profile of protein tyrosine nitration (NO_2-Tyr) were analyzed in leaves. Low temperature was the stress that produced the highest increase of NOS and GSNOR activities, and this was accompanied by an increase in the content of total ·NO and *S*-nitrosothiols, and an intensification of the immunoreactivity with an antibody against NO_2-Tyr. Mechanical wounding, high temperature and light also had a clear activating effect on the different indicators of RNS metabolism in pea plants. However, the total content of nitrite and nitrate in leaves was not affected by any of these stresses. Considering that protein tyrosine nitration is a potential marker of nitrosative stress, the results obtained suggest that low and high temperature, continuous light and high light intensity are abiotic stress conditions that can induce nitrosative stress in pea plants.

Keywords: Low temperature — Nitric oxide — Nitric oxide synthase (NOS) — Nitrosative stress — Nitrotyrosine — *S*-nitrosothiols.

Abbreviations: BSA, bovine serum albumin; CLSM, confocal laser scanning microscopy; DAF-FM DA, 4-aminomethyl-2′,7′-difluorofluorescein diacetate; GSNO, *S*-nitrosoglutathione; GSNOR, *S*-nitrosoglutathione reductase; L-NAME, L-N^G-nitroarginine methyl ester; ·NO, nitric oxide; NOA, nitric oxide analyzer; NOS, nitric oxide synthase; NO_2-Tyr, 3-nitrotyrosine; NR, nitrate reductase; PBS, phosphate-buffered saline; PTIO, 2-phenyl-4,4,5,5-tetramethylimidazoline-1-oxyl; ROS, reactive oxygen species; RNS, reactive nitrogen species; RSNO, *S*-nitrosothiol.

Introduction

Nitric oxide (·NO) is a gaseous free radical with important physiological functions in higher plants (Corpas et al. 2001, Lamattina et al. 2003, Neill et al., 2003, del Río et al. 2004, Shapiro 2005, Corpas et al. 2007a, Corpas et al. 2007b, Besson-Bard et al. 2008, Neill et al. 2008). In the past decade, many plant biologists searched intensively for an NO-generating enzyme similar to the nitric oxide synthase (NOS) identified in mammalian systems (Wendehenne et al. 2001, del Río et al. 2004). However, at present, the enzymatic source of ·NO production in plant cells under normal or stress conditions is still a controversial issue (Corpas et al. 2004a, Corpas et al. 2006, Crawford et al. 2006, Zemojtel et al. 2006, Neill et al. 2008).

In recent years, the term reactive nitrogen species (RNS) was introduced in the biological literature to designate NO and other NO-related molecules, such as *S*-nitrosothiols (RSNOs), *S*-nitrosoglutathione (GSNO) and peroxynitrite ($ONOO^-$), among others, which have relevant roles in multiple physiological processes of animal and plant cells (Corpas et al. 2007b, Halliwell and Gutteridge 2007). However, in higher plants, the information available on the metabolism of RNS is very limited compared with animal systems. *S*-Nitrosoglutathione (GSNO) is an *S*-nitrosothiol which is formed by the reaction of ·NO with reduced glutathione (GSH) in the presence of oxygen and is thought to function as a mobile reservoir of ·NO bioactivity in animal and plant cells (Durner et al. 1999, Díaz et al. 2003, Ng and Kubes 2003, Wang et al. 2006). This molecule could have more relevance than would be expected considering that GSH is one of the major low molecular weight soluble antioxidants

*Corresponding author: E-mail, javier.corpas@eez.csic.es; Fax, +34-958-129600.

of plant cells and is involved in the ascorbate–glutathione cycle (Noctor and Foyer 1998) and detoxification of xenobiotics (Edwards et al. 2000), and also acts as an independent redox signaling molecule (Foyer and Noctor 2005). In recent years, the glutathione-dependent enzyme formaldehyde dehydrogenase (FALDH; EC 1.2.1.1) has been demonstrated to have GSNO reductase (GSNOR) activity in bacteria, yeast and mammals (Liu et al. 2001) and to be involved in the mechanism of protein *S*-nitrosylation in mammalian cells (Haqqani et al. 2003). The enzyme GSNOR catalyses the NADH-dependent reduction of GSNO to GSSG and NH_3 (Liu et al. 2001, Lamotte et al. 2005). In plants, GSNOR activity has been detected in *Arabidopsis* (Sakamoto et al. 2002, Achkor et al. 2003, Feechan et al. 2005, Espunya et al. 2006), tobacco (Díaz et al. 2003) and peas (Barroso et al. 2006), and the gene coding for this enzyme has been reported to be modulated in response to wounding, jasmonic acid and salicylic acid (Díaz et al. 2003).

The implication of ·NO in the mechanism of response against some abiotic stresses has been reported in different plant species (for a review, see Corpas et al. 2007a, Corpas et al. 2007b). However, there is little information on other NO-derived molecules; much of the data available have been obtained by indirect studies using exogenous ·NO donors, and some of the results are contradictory depending on the type of ·NO donors employed (Murgia et al. 2004, Floryszak-Wieczorek et al. 2006).

In this work, using pea plants exposed to six different abiotic stress conditions, several components of the metabolism of RNS in plant cells, including the content of ·NO, RSNOs and nitrite plus nitrate, L-arginine-dependent nitric oxide synthase (NOS) activity, GSNOR activity and protein tyrosine nitration, were analyzed. The results obtained showed that in pea plants several abiotic stresses activated the metabolism of RNS, and low and high temperature, continuous light and high light intensity seemed to induce nitrosative stress in this plant species.

Results

In plants, ·NO can be generated by enzymatic and non-enzymatic sources (Corpas et al., 2004a, del Río et 2004, Wilson et al. 2007). The present work is focused on the NOS activity as one of the enzymatic sources of ·NO in plants under abiotic stress conditions. Thus, in pea leaf extracts, the effect of inhibitors of NOS and nitrate reductase (NR) on the enzymatic production of ·NO from L-arginine (designated as L-arginine-dependent NOS activity) is shown in Fig. 1. Prior to the enzymatic assays, and in order to avoid potential interference by endogenous metabolites, leaf extracts were passed through Sephadex G-25 columns (NP-10, Amersham) which eliminated low molecular weight compounds, including endogenous nitrites, nitrates and

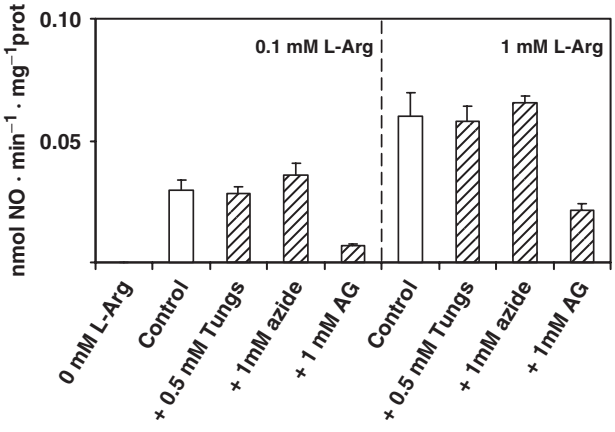

Fig. 1 Characterization of L-arginine-dependent nitric oxide synthase (NOS) activity in pea leaf extracts by ozone chemiluminiscence. Reaction mixtures containing pea leaf extracts, NADPH (1 mM) and the NOS cofactors (10 μM FAD, 10 μM FMN and 10 μM BH_4) were incubated in the absence and presence of L-arginine (1 or 0.1 mM). As controls, pea leaf extracts were pre-incubated for 30 min, separately, with 1 mM aminoguanidine (an animal NOS inhibitor), and with 0.5 mM Na-tungstate and 1 mM Na-azide (two inhibitors of nitrate reductase). Results are means of determinations from three different samples. AG, aminoguanidine.

L-arginine. Thus, the L-arginine-dependent NOS activity was only dependent on the L-arginine concentration and was 50% reduced at a 0.1 mM L-arginine concentration compared with the activity obtained with 1 mM L-arginine. In contrast, when samples were pre-incubated with 1 mM aminoguanidine (an NOS inhibitor), the activity was reduced 64% with 1 mM L-arginine and 75% with 0.1 mM L-arginine as substrates. In contrast, pre-incubation with either 1 mM Na-azide or 0.5 mM Na-tunsgtate, two inhibitors of NR, did not affect the L-arginine-dependent NOS activity.

The cellular localization of endogenous ·NO in cross-sections of pea leaves was studied by confocal laser scanning microscopy (CLSM) using 4-aminomethyl-2′,7′-difluorofluorescein diacetate (DAF-FM DA) as fluorescent probe (Fig. 2). The green color corresponding to ·NO was localized mainly in the vascular tissue and epidermal cells, and the red-orange color which corresponds to the autofluorescence, was distributed in the cells of palisade and spongy mesophyll tissues (Fig. 2A). When the leaf sections were pre-incubated with 200 μM 2-phenyl-4,4,5,5-tetramethylimidazoline-1-oxyl (PTIO, an ·NO scavenger) the green fluorescence was strongly reduced mainly in vascular tissues (Fig. 2B) compared with the control sections incubated only with DAF-FM DA (Fig. 2A). On the other hand, when leaf sections were pre-incubated with 5 mM L-N^G-nitroarginine methyl ester (L-NAME), an inhibitor of animal NOS, the green fluorescence was also strongly reduced (Fig. 2C).

The endogenous content and cellular distribution of ·NO were also studied in leaves of pea plants exposed to six

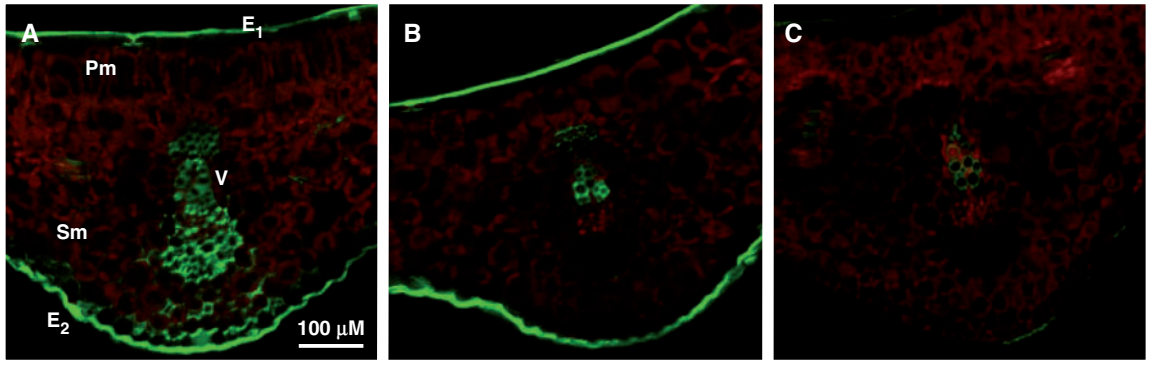

Fig. 2 Representative images illustrating the CLSM detection and visualization of endogenous ·NO in leaves of pea plants. DAF-FM DA (10 μM) was used as fluorescent probe. (A) Cross-section of pea leaf incubated with DAF-FM DA. (B) Cross-section of pea leaf pre-incubated for 30 min with 200 μM PTIO (an ·NO scavenger) and then incubated with DAF-FM DA. (C) Cross-section of pea leaf pre-incubated for 60 min with 5 mM L-NAME (a NOS inhibitor) and then incubated with DAF-FM DA. Each picture was prepared from at least 20 cross-sections analyzed by CLSM. Strong and bright green fluorescence corresponds to ·NO. The red-orange color corresponds to the chlorophyll autofluorescence. Adaxial epidermis (E_1), abaxial epidermis (E_2), main vein (V), palisade mesophyll (Pm) and spongy mesophyll (Sm). Scale bar = 100 μm.

different abiotic stress conditions (Fig. 3). The ·NO content, detected by its characteristic green color, showed an increase in plants subjected to low temperature (Fig. 3D), where ·NO was present in almost all cell types including adaxial and abaxial epidermis, main vein, palisade mesophyll and spongy mesophyll. Mechanical wounding also produced a considerable increase of ·NO especially in vascular tissue (Fig. 3G). In contrast, high temperature (Fig. 3C), darkness (Fig. 3F) and continuous light (Fig. 3E) induced a decrease in the ·NO content of leaves compared with control plants (Fig. 3A). High light intensity (Fig. 3B) did not appear to produce significant changes in the ·NO content of leaves.

In plants, ·NO can also be generated from nitrite and nitrate by enzymatic or non-enzymatic sources (del Río et al. 2004, Wilson et al. 2007). To analyze the possible contribution of nitrites and nitrates to the generation of the ·NO detected in leaves, the total content of nitrites and nitrates in leaves of pea plants exposed to the six different abiotic stress conditions was determined (Fig. 4). No statistically significant changes were observed between the concentration of nitrites and nitrates in leaves from plants subjected to different abiotic stresses compared with control plants.

The L-arginine-dependent generation of ·NO (NOS activity) in leaves of pea plants exposed to six different abiotic stress conditions is shown in Fig. 5. L-Arginine-dependent NOS activity was measured by ozone chemiluminiscence and, when the different activities were compared with those of the control plants (53.7 ± 9.2 pmol ·NO mg^{-1} protein min^{-1}), it was found that the L-arginine-dependent NOS activity was significantly enhanced (6-fold) by low temperature, 3-fold by mechanical wounding and 2-fold by high light intensity. However, high temperature, continuous light and dark did not affect the NOS activity significantly.

In leaves of pea plants subjected to the same abiotic stress conditions mentioned above the GSNOR activity was determined by both spectrophotometry and native PAGE (Fig. 6). The activity was also significantly increased (25–67%) in all the abiotic conditions studied except in the stress by high light intensity which did not produce any significant effect on the GSNOR activity. The highest increase in GSNOR activity (67%) was observed in plants subjected to low temperature stress, a similar situation to that of the L-arginine-dependent NOS activity mentioned above. This pattern was confirmed when the activity was analyzed by native PAGE and specific gel staining. The results showed that the GSNOR activity was enhanced by all the abiotic stress conditions compared with the control plants (Fig. 6, upper panel).

In plants exposed to the six abiotic stress conditions, the total content of RSNOs was determined by a chemiluminiscence method (Fig. 7). In control plants, the content of RSNOs was about 300 pmol mg^{-1} protein. The content of RSNOs was increased 5-fold by low temperature and high light intensity stress, 4-fold by mechanical wounding, and 3-fold by high temperature and continuous dark. However, continuous light did not modify the level of RSNOs.

The protein tyrosine nitration of plants grown under different abiotic stress conditions was studied in leaf extracts by immunoblot analysis, using an antibody against 3-nitrotyrosine (NO_2-Tyr) (Fig. 8). In control plants, a pattern of six immunoreactive bands with molecular masses of 59, 42, 39, 37, 33 and 29 kDa was found. This pattern was similar in pea plants subjected to the six abiotic stress conditions, but with an intensification of the immunoreactive

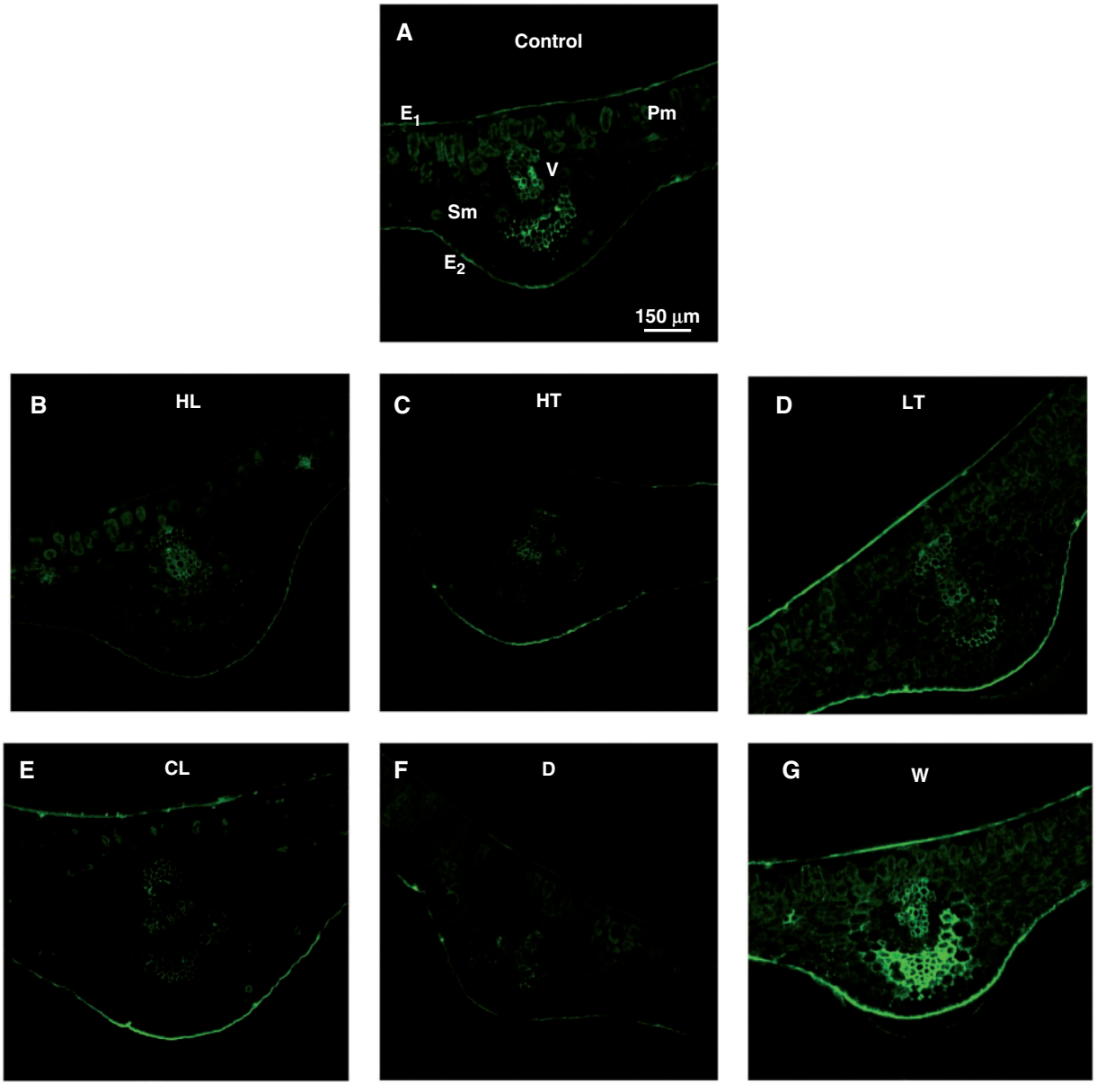

Fig. 3 Representative images illustrating the CLSM detection of endogenous ·NO in leaves of pea plants exposed to different abiotic stress conditions. Cross-sections of pea leaves (100 μm thick) were incubated with 10 μM DAF-FM DA as fluorescent probe. (A) Control pea plants. (B) Plants subjected to high light intensity (HL). (C) Plants exposed to high temperature (HT). (D) Plants exposed to low temperature (LT). (E) Plants subjected to continuous light (CL). (F) Plants subjected to continuous dark (D). (G) Plants damaged by mechanical wounding (W). Adaxial epidermis (E_1), abaxial epidermis (E_2), main vein (V), palisade mesophyll (Pm) and spongy mesophyll (Sm). Scale bar = 150 μm.

bands of 59, 42 and 29 kDa by low temperature, high temperature, high light and continuous light. A new immunoreactive band of 73 kDa, which was not present in control plants, was detected in plants subjected to high and low temperature, and high light stress. A densitometric quantification of the relative optical density of the immunoreactive bands detected with the antibody against NO_2-Tyr is shown in Table 1. Assigning a value of 1 to each immunoreactive band in the control plants, the number-fold increase of each specific band under stress conditions was calculated. The results showed that low and high temperature, continuous light and high light intensity were the stresses than induced a higher intensification of each specific band compared with control conditions (Table 1).

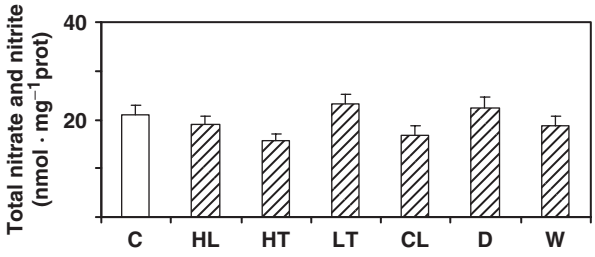

Fig. 4 Total nitrite and nitrate in leaf extracts from pea plants subjected to different abiotic stress conditions. A chemiluminiscence method was used and the total nitrite and nitrate of extracts was expressed as nmol mg^{-1} protein. C, control; HL, high light intensity; HT, high temperature; LT, low temperature; CL, continuous light; D, continuous dark; W, wounding. Results are means ± SEM of samples from at least three different experiments.

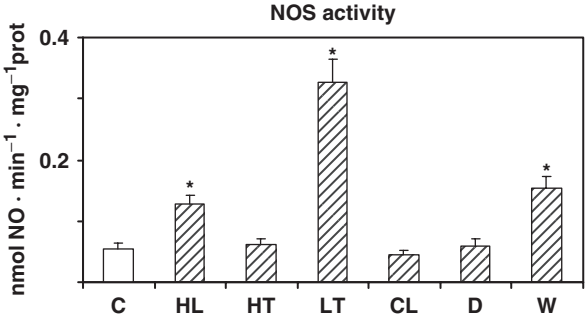

Fig. 5 L-Arginine-dependent NOS activity in leaf extracts from pea plants subjected to different abiotic stress conditions. C, control; HL, high light intensity; HT, high temperature; LT, low temperature; CL, continuous light; D, continuous dark; W, wounding. Results are means ± SEM of samples from at least three different experiments. In all cases the NOS activity was measured in the presence of 1 mM L-arginine. Asterisks mean that the increases in NOS activity were statistically significant ($P > 0.005$; $n \geq 3$) compared with control plants (C).

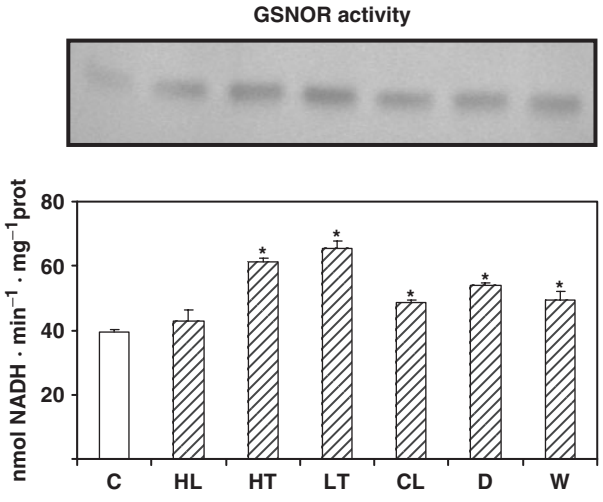

Fig. 6 Assay of *S*-nitrosoglutathione reductase (GSNOR) activity in leaf extracts from pea plants exposed to different abiotic stress conditions. Upper panel: native PAGE (100 µg of protein per lane) and staining for GSNOR activity. Lower panel: spectrophotometric assay of GSNOR activity. C, control; HL, high light intensity; HT, high temperature; LT, low temperature; CL, continuous light; D, continuous dark; W, wounding. Results are means ± SEM of samples from at least three different experiments. Asterisks mean that the increases of GSNOR activity were statistically significant ($P > 0.005$; $n \geq 3$) compared with control plants (C).

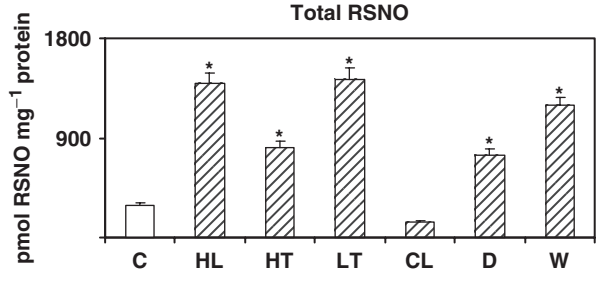

Fig. 7 Analysis of total *S*-nitrosothiols (RSNOs) by ozone chemiluminiscence in leaf extracts from pea plants subjected to different abiotic stress conditions. C, control; HL, high light intensity; HT, high temperature; LT, low temperature; CL, continuous light; D, continuous dark; W, wounding. Results are means ± SEM of samples from at least three different experiments. Asterisks mean that the increases of RSNOs were statistically significant ($P > 0.005$; $n \geq 3$) compared with control plants (C).

Discussion

NO is a key molecule in plant physiology where it has been demonstrated to be involved in many metabolic and developmental processes (Lamattina et al. 2003, Neill et al. 2003, Corpas et al., 2004b, del Río et al. 2004, Shapiro, 2005, Corpas et al. 2006, Wilson et al. 2007, Corpas et al., 2008b). However, little is known about the metabolism of endogenous ·NO and other RNS in plant cells under abiotic stress conditions. Therefore, the main goal of this work was to analyze the possible effect on the metabolism of RNS of certain environmental stress conditions which previously have been shown to alter the activity and gene expression of proteins involved in the cellular defense against ROS, such as antioxidative enzymes (Leterrier et al. 2005, Leterrier et al. 2007). For this purpose, pea plants were also used as a model because in previous studies the enzymatic source of ·NO was biochemically characterized as an L-arginine-dependent NOS (Barroso et al. 1999, Corpas et al., 2004b, Corpas et al. 2006). Using an ozone chemiluminiscence method, the L-arginine-dependent NOS activity of pea plants was not affected by either azide or tungstate, two well-known inhibitors of NR (Deng et al. 1989, Corpas et al. 2004b, Foresi et al. 2007), indicating that the ·NO production was not dependent on NR. On the other hand, the animal NOS inhibitor

aminoguanidine reduced the NOS activity of pea leaves between 64 and 75%, indicating that the ·NO generated was due to an L-arginine-dependent NOS activity (Fig. 1).

Additionally, ·NO accumulation was analyzed by CLSM using DAF-FM DA as fluorescent probe (Zhang et al. 2003), and ·NO was localized mainly in vascular tissue and epidermal cells, as has been previously described in leaves of several plant species (Corpas et al. 2004b, Corpas et al. 2006, Valderrama et al. 2007). When the sections of pea leaves were pre-incubated with PTIO (an ·NO scavenger) the level of ·NO detected was considerably reduced, and when tissue sections were pre-incubated with L-NAME (an NOS inhibitor) the presence of ·NO was almost undetectable. This indicates that, under the experimental conditions used in this work, an NOS activity is responsible for the ·NO produced in pea leaves. However, the possibility that part of the ·NO normally generated in pea plants could arise from other sources different from the NOS activity cannot be discarded. Thus, the ·NO production capacity of NR, at saturating NADH and NO_2^- concentrations, is considered to be 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). For this reason, in this work the total content of nitrite and nitrate in leaves of pea plants exposed to different abiotic stress conditions was analyzed. The results showed the absence of significant changes in the content of NO_2^- and NO_3^- as a result of the abiotic stress conditions applied.

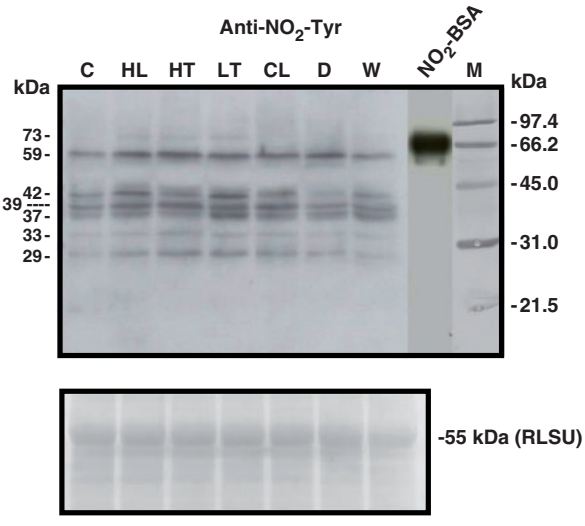

Fig. 8 Upper panel: representative immunoblot showing the protein tyrosine nitration in leaves from pea plants subjected to different abiotic stress conditions. C, control; HL, high light intensity; HT, high temperature; LT, low temperature; CL, continuous light; D, continuous dark; W, wounding. Leaf samples (75 μg of protein per lane) were subjected to SDS–PAGE and Western analysis using an antibody against 3-nitrotyrosine (NO_2-Tyr) (dilution 1 : 8,000). Commercial nitrated BSA (NO_2-BSA) (2 μg of protein) was used as a positive control. M, molecular mass markers. The numbers on the left side of the immunoblot indicate the relative molecular masses of the detected immunoreactive bands, and the relative molecular masses of the protein markers are shown on the right side. Lower panel: visualization of Rubisco large subunit (RLSU) as an equal loading control. Leaf samples (75 μg of protein) were subjected to SDS–PAGE in 10% acrylamide gels, and proteins were stained with Coomassie Brilliant Blue G-250.

With this basic information, the accumulation and distribution of ·NO in leaves of pea plants under the six abiotic stress conditions were studied by CLSM. It was observed that mechanical wounding and low temperature were the stresses that induced a generalized increase of ·NO content (Fig. 3D, G). Mechanical wounding showed a higher intensification of ·NO in the vascular tissue, whereas under low temperature stress the presence of ·NO was increased in all cell types without intensification in any specific tissue.

Therefore, the data regarding NOS activity and ·NO content are clearly correlated, at least in plants subjected

Table 1 Relative quantification of Western blot of leaf extracts from pea plants probed with an antibody against 3-nitrotyrosine (NO_2-Tyr)

Polypeptide (kDa)	Abiotic stress condition						
	Control	HL	HT	LT	CL	D	W
73	0 (1)	8 (8.0)	10 (10)	9 (9)	0 (8)	0 (1)	0 (1)
59	39 (1)	68 (1.7)	67 (1.7)	63 (1.6)	55 (1.4)	73 (1.8)	36 (1)
42	34 (1)	62 (1.8)	52 (1.5)	67 (2.0)	49 (1.4)	24 (1)	33 (1)
39	38 (1)	60 (1.6)	69 (1.8)	67 (1.7)	65 (1.7)	44 (1.1)	45 (1.2)
37	28 (1)	43 (1.5)	46 (1.6)	64 (2.3)	49 (1.8)	43 (1.1)	48 (1.7)
33	5 (1)	12 (2.4)	27 (5.4)	18 (3.6)	16 (3.2)	9 (1.8)	7 (1.4)
29	10 (1)	23 (2.3)	38 (3.8)	33 (3.3)	29 (2.9)	18 (1.8)	9 (1)

Pea plants were subjected to different abiotic stress conditions including: HL, high light intensity; HT, high temperature; LT, low temperature; CL, continuous light; D, continuous dark; and W, wounding. These results are representative of different Western blots assayed. The intensity of bands was quantified using Adobe Photoshop software (version 6.0). Numbers in parentheses indicate the number-fold increase of each specific immunoreactive band compared with its control, to which a value of 1 was assigned.

to low temperature and mechanical wounding, where the NOS activity could be the main source of ·NO generation. The induction of NOS activity has been shown in other plant species by ozone and UV B radiation (Mackerness et al. 2001, Rao and Davis, 2001, An et al. 2005). On the other hand, some studies have described that ·NO production in certain plants is increased by a wide range of stress conditions including wilting, hypoxia, ozone, gravitational force, shear, wounding, salinity, and osmotic and heat stress, although the specific source of ·NO has not been determined (Leshem and Haramaty 1996; Pedroso and Durzan 2000, Dordas et al., 2003, Gould et al. 2003, Xing et al. 2004, Gong and Yuan 2005, Velikova et al. 2005). In contrast, there are reports showing that cadmium stress reduces the ·NO level in leaves and roots of pea plants (Barroso et al. 2006, Rodríguez-Serrano et al. 2006). Moreover, during natural senescence of pea leaves the arginine-dependent NOS activity and ·NO level are substantially reduced (Corpas et al. 2004b).

In sunflower plants, it has been shown that the ·NO emission by NR is higher under light conditions than in the dark (Rockel et al. 2002). This contrasts with the results reported in this work on L-arginine-dependent NOS activity (Fig. 5) which was not affected by either continuous dark or continuous light. These results support the idea that in different plant physiological processes the NOS and NR activity, apart from other non-enzymatic sources of ·NO, could function under distinct situations, perhaps depending on the plant species and/or the availability of the NO-generating system in a particular tissue. In any case, it would be interesting to know which factor(s) makes the final decision on the specific ·NO source to be used in a determined plant physiological process.

A characteristic of ·NO metabolism is its interaction with thiol groups present in protein or peptide cysteine residues to form RSNOs (Gaston 1999, Carver et al., 2005). At present, there is some evidence suggesting that *S*-nitrosylation of proteins may have an important function in plant biology (Lindermayr et al. 2006, Wang et al. 2006, Belenghi et al. 2007, Romero-Puertas et al. 2007, Romero-Puertas et al. 2008). RSNOs can participate in the storage and transport of ·NO in cells and, therefore, are good candidates to mediate *S*-nitrosylation (Wang et al. 2006). The RSNO content and turnover have been shown to control different types of plant disease resistance (Feechan 2005, Chaki 2007). In olive plants it was described that total RSNOs were increased 2-fold by salt stress (Valderrama et al. 2007). In our experimental conditions, the content of total RSNOs was induced 3- to 5-fold in all the stresses studied except for continuous light (Fig. 7). All these results suggest that RSNOs could be involved in the response mechanism of plants to abiotic stress.

Taking together the results of NOS activity, ·NO accumulation and RSNO content, it seems that low temperature and mechanical wounding are the only abiotic stresses that induce a clear increase of those parameters. However, the remaining stresses do not have a clear correlation, indicating that other factors must be involved in the response. In this respect, it must be born in mind that the RNS produced in response to abiotic stress could also lead to the stimulation of other NO-dependent signaling pathways, such as cGMP, protein kinase activation or gene expression (Pagnussat et al. 2004, Serpa et al. 2007). In this way, abiotic stress could also result in the activation of signals that migh help protect the plant from further stress.

In recent years the presence of GSNOR activity in plants has been demonstrated (Sakamoto et al. 2002, Díaz et al. 2003, Feechan et al. 2005, Barroso et al. 2006, Espunya et al. 2006) but there is still little information on this enzyme and much less is known about GSNO and its metabolism (Barroso et al. 2006). In our experimental conditions, GSNOR was induced by all the stresses studied except for high light intensity (Fig. 6). Recently, it was reported that GSNOR activity is necessary for the acclimation of plants to high temperature and for normal development and fertility under optimal growth conditions (Lee et al. 2008). However, in pea plants exposed to cadmium, both the activity and gene expression of GSNOR were down-regulated (Barroso et al. 2006). In *Arabidopsis*, the GSNOR gene has been shown to be regulated by wounding and salicylic acid (Diaz et al. 2003), and this gene is differentially expressed depending of the cell type and organ (Corpas et al. 2007d).

Tyrosine nitration is a covalent modification of proteins resulting from the addition of a nitro (-NO_2) group to one of the two equivalent ortho carbons of the aromatic ring of tyrosine residues (Ischiropolos 1998, Ischiropolos 2003, Gow et al. 2004, Radi 2004). In animal cells, this protein modification has been routinely used as marker of pathological diseases and oxidative stress (Ischiropoulos 2003) and it is also considered as a marker of nitrosative stress. In animal systems, it has been shown that tyrosine nitration can occur through different mechanisms, such as the peroxynitrite- or heme peroxidase-dependent routes (Radi 2004, Bartesaghi et al. 2007). However, in plant cells, much less information is available on protein tyrosine nitration (Perazzolli et al. 2004, Corpas et al. 2007c, Hebelstrup et al. 2008). Independently of the tyrosine nitration mechanisms operating in this work, the protein expression of the immunoreactive bands in the range 29–59 kDa was clearly intensified in pea plants exposed to low temperature, high temperature, high light intensity and continuous light (Fig. 8 and Table 1). In suspension cultures of *Taxus cuspidate*, an increase of 31% in the free NO_2-Tyr content during shear stress has been reported

(Gong and Yuan 2005). In olive plants under salt stress conditions, a significant increase of the L-arginine-dependent NOS activity and number of proteins that underwent tyrosine nitration in the molecular mass range 44–60 kDa has also been also described (Valderrama et al. 2007). In nitrite reductase antisense tobacco leaves, the induction of several tyrosine-nitrated polypeptides with molecular masses between 10 and 50 kDa was described (Morot-Gaudry-Talarmain et al. 2002). Moreover, in tobacco BY-2 suspension cells treated with a fungal elicitin, the induction of tyrosine nitration in proteins with molecular masses in the range 20–50 kDa was demonstrated (Saito et al. 2006). During the progression of the hypersensitive response in *Arabidopsis*, a rise in NO_2-Tyr immunoreactivity was found (Romero-Puertas et al. 2007) Conversely, in tobacco transgenic plants with a genetically increased cytokinin level, the content of tyrosine-nitrated proteins decreased (Wilhelmova et al. 2006). All these data indicate that an increase in the number of proteins or an intensification of specific proteins that experienced tyrosine nitration could be considered as an indicator of nitrosative stress, such as has been demonstrated in animal cells (Ischiropoulos 2003).

In summary, the results reported in this work show that in pea plants the total ·NO and RSNO content, the L-arginine-dependent NOS and GSNOR activities, as well as protein tyrosine nitration are differentially modulated depending on the type of abiotic stress. Low and high temperature and high light intensity and continuous light were the abiotic stresses that produced the greatest effect on the metabolism of RNS, with a clear intensification of protein tyrosine nitration. On the basis of the tyrosine nitration of proteins as a marker of this type of stress, these results suggest that these environmental conditions induce nitrosative stress in pea plants. Research is under way in our laboratory directed at the characterization of proteins undergoing tyrosine nitration and the identification of the gene(s) coding for the protein responsible for the L-arginine-dependent NOS activity in pea plants.

Materials and Methods

Plant material and growth conditions

Pea (*Pisum sativum* L., cv. Lincoln) seeds were obtained from Royal Sluis (Enkhuizen, The Netherlands). Seeds were surface sterilized with 3% (v/v) commercial bleaching solution for 3 min, and then were washed with distilled water, and germinated in vermiculite for 3–4 d under the following growth chamber conditions: 24°C/18°C (day/night), 80% relative humidity, a 16 h photoperiod and a light intensity of 190 μE $m^{-2} s^{-1}$. Healthy and vigorous 3-week-old seedlings were selected and exposed to different stress conditions: (i) High light intensity. Plants were irradiated for 4 h at 1,189 μE $s^{-1} m^{-2}$, using a GE 300 W-230 V PAR 56/WFL lamp (General Electric, Madrid, Spain). To avoid heating of plants, a Petri dish (19 cm diameter) containing cold water was placed 4 cm above plants, and water was replaced every 30 min (Leterrier et al., 2005, Leterrier et al. 2007); (ii) High temperature. Plants were sequentially exposed to 30°C for 1 h, 35°C for 1 h and finally 38°C for 4 h (DeRocher and Vierling 1995). (iii) Low temperature. Plants were incubated for 48 h at 8°C. (iv) Continuous light. Plants were continuously illuminated for 48 h at 190 μE $s^{-1} m^{-2}$. (v) Darkness. Plants were kept in darkness in a growth chamber for 48 h. (vi) Mechanical wounding. Leaves were injured *in planta* by clicking them with a striped-tip forceps, and after 4 h damaged leaves were collected and analyzed (Leterrier et al. 2005, Leterrier et al. 2007). In all cases, the control plants were kept in the growth chamber under optimal conditions, being processed at the same time as plants subjected to the different stress conditions.

Crude extracts of plant tissues

Pea leaves were frozen in liquid N_2 and ground in a mortar with a pestle. The powder was suspended in a homogenizing medium containing 100 mM Tris–HCl, pH 8.0, 100 mM NaCl, 1 mM EDTA, 0.2 mM CHAPS, 1 mM phenylmethylsulfonyl fluoride (PMSF), 10 μM BH_4 and 10 μg ml^{-1} calmodulin (1 : 2; w/v). Homogenates were centrifuged at 17,000×*g* for 30 min, and the supernatants were passed through Sephadex G-25 desalting columns (NP-10 from Amersham, Biosciences Piscataway, NJ, USA) which removed low molecular weight substances (M_r <1.000). The different flow-throughs were immediately used for the assays.

·NO detection by CLSM

Pea leaf segments of approximately 25 mm^2 were incubated for 1 h at 25°C, in darkness, with 10 μM DAF-FM DA (Calbiochem, San Diego, CA, USA) prepared in 10 mM Tris–HCl (pH 7.4), a probe which is specific for ·NO (Zhang et al. 2003, Corpas et al. 2006). Then, leaf segments were washed twice in the same buffer for 15 min each and were embedded in a mixture of 15% acrylamide–bisacrylamide stock solution as described elsewhere (Corpas et al. 2006). Leaf sections (100 μm thick) were cut with a vibratome (Leica, Microsystems, Wetzlar, Germany) under 10 mM phosphate-buffered saline (PBS). Sections were then soaked in glycerol/PBS (containing azide) (1 : 1, v/v) and mounted in the same medium for examination with a CLSM system (Leica TCS SL; Leica Microsystems, Wetzlar, Germany), using standard filters and collection modalities for DAF-2 green fluorescence (excitation at 495 nm; emission at 515 nm) and chlorophyll autofluorescence (Chl *a* and *b*, excitation at 429 and 450 nm, respectively; emission at 650 and 670 nm, respectively) as red/orange. As controls, leaf sections were pre-incubated with the following compounds: (i) 5 mM L-NAME, a NOS competitive inhibitor, at 25°C for 1 h; and (ii) 200 μM PTIO, an ·NO scavenger, at 25°C for 30 min.

Enzyme activity assays

NOS activity was estimated by ozone chemiluminescence using an NO analyzer (NOA 280i, Sievers Instruments, Boulder, CO, USA) (Corpas et al. 2008a). This was performed in duplicate for each sample in a reaction medium containing 40 mM HEPES buffer, pH 7.2, 0.2 mM CHAPS, 10 μg ml^{-1} calmodulin, 1.25 mM $CaCl_2$, 1 mM β-NADPH, 10 μM FAD, 10 μM FMN, 10 μM BH_4 and 1 mM L-arginine. The reaction mixture was incubated at 37°C for 30 min. The ·NO produced in the enzymatic reaction is quickly oxidized to its stable end-products nitrite and nitrate. At 0 time and at 30 min, 200 μl of the reaction medium were deproteinated by the addition of 100 μl of 0.8 N NaOH and 100 μl of 16% $ZnSO_4$, and the mixture was shaken vigorously for 30 s and centrifuged for

10 min at 15,000×*g*. Aliquots (40 µl) of supernatants were injected into the purge chamber of the NOA containing 5 ml of 50 mM vanadium trichloride (VCl_3) in 1 M HCl at 90°C under an atmosphere of nitrogen. In these conditions, the NO-derived nitrite/nitrate of samples is reduced back to ·NO which was mixed with ozone, generated in the NOA, and the light emitted from the chemiluminiscence reaction between ·NO and ozone was detected by a photomultipler tube. The amount of ·NO generated was computed by referring the magnitude of these signals to that of those generated by nitrate standards. The production of ·NO in samples in the 30 min of reaction was calculated by subtracting the blank value (zero time), which represented the non-enzymatic ·NO production, and the activity was expressed as nmol of ·NO mg^{-1} protein min^{-1}. To validate this method in order to determine the NOS activity of plant samples, commercial neuronal NOS from Calbiochem, San Diego, CA, USA (2.9 U) was assayed separately as a positive control in each determination. As negative controls, samples were pre-heated at 95°C for 10 min. The NOS activity was also assayed using an L-arginine concentration of 0.1 mM and in the presence of 1 mM aminoguanidine which is an irreversible inhibitor of both constitutive and inducible NOS activity in animal cells (Laszlo et al. 1995). Additionally, the NOS activity was also assayed in the presence of 0.5 mM Na-tunsgtate and 1 mM Na-azide, two inhibitors of NR activity (Deng et al. 1989, Corpas et al. 2004b, Foresi et al. 2007).

GSNOR activity was assayed spectrophotometrically at 25°C by monitoring the oxidation of NADH at 340 nm (Sakamoto et al. 2002, Barroso et al. 2006). Leaf extracts were incubated in an assay mixture containing 20 mM Tris–HCl (pH 8.0), 0.2 mM NADH and 0.5 mM EDTA, and the reaction was started by adding GSNO (Calbiochem, San Diego, CA, USA) to the mixture at a final concentration of 400 µM. The activity was expressed as nmol NADH consumed per min per mg of protein ($\varepsilon_{340} = 6.22\,mM^{-1}\,cm^{-1}$).

For the separation of GSNOR isozymes, 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 using a modification of the method reported by Seymour and Lazarus (1989). Gels were soaked in 0.1 M sodium phosphate, pH 7.4, containing 2 mM NADH, for 15 min, in an ice-bath. Excess buffer was drained and gels were covered with filter paper strips soaked in freshly prepared 3 mM GSNO (Calbiochem, San Diego, CA, USA). After 10 min, the filter paper was removed and gels were exposed to UV light and analyzed for the appearance of the GSNOR activity bands (Fernández et al. 2003).

Determination of total nitrite and nitrate and RSNOs

Total nitrite and nitrate were determinated by a chemiluminescence method using an NOA (NOA 280i, Sievers Instruments, Boulder, CO, USA). Leaf samples were frozen in liquid N_2 and ground in a mortar with a pestle. The powder was suspended in a medium (1 : 3; w/v) containing 100 mM Tris–HCl, pH 7.6, 1.5 mM dithiothreitol (DTT), 5% saccharose (w/v) and 0.005% Triton X-100 (v/v). Homogenates were centrifuged at 17,000 × *g* for 30 min and 300 µl of supernatants (without being passed through Sephadex G-25 desalting columns) were deproteinated by the addition of 150 µl of 0.8 N NaOH and 150 µl of 16% $ZnSO_4$, and the mixture was shaken vigorously for 30 s and centrifuged for 10 min at 15,000×*g*. Aliquots (40 µl) of supernatants were injected into the purge chamber of the NOA containing 5 ml of 50 mM VCl_3 in 1 M HCl at 95°C. In these conditions, the total nitrites and nitrates of samples are reduced to ·NO which is transported to the analyzer by a stream of N_2 passed through the reaction cell and mixed with ozone, generated in the NOA. Finally, the light emitted from the chemiluminiscence reaction between ·NO and ozone was detected by a photomultipler tube, and the amount of ·NO generated (proportional to the total content of nitrite plus nitrate) was computed by referring the magnitude of these signals to those generated by a nitrite standard curve.

Total RSNOs were estimated by a chemiluminescence method, as described by Jourd'Heuil et al. (2005) with some modifications (Valderrama et al. 2007, Corpas et al. 2008a). The detection of RSNOs 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. RSNOs are sensitive to mercury-induced decomposition, in contrast to other nitroso species including nitrosamines (RNNOs) and nitrosyl hemes. The samples were homogenized in the previous buffer containing 100 µM DTPA (diethylenetetraminepentaacetic acid) (1 : 5; w/v), and centrifuged at 3,000×*g* for 10 min. Then, the supernatants were 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 sulfanilamide for 15 min at 4°C, to eliminate nitrite; and (ii) treated with 10 mM sulfanilamide and 7.3 mM $HgCl_2$ for 15 min at 4°C to eliminate nitrite and RSNOs, respectively. Then, these samples were analyzed in an NOA (NOA 280i, Sievers Instruments, Boulder, CO, USA). The data obtained from (i – ii) represented the total concentration of RSNOs. The whole procedure was performed under a red safety light to protect RSNOs from light-dependent decomposition.

SDS–PAGE and immunoblot analysis

SDS–PAGE was carried out according to the method of Laemmli (1970) in 10% acrylamide slab gels. Gels were stained with 0.1% (w/v) Coomassie Brilliant Blue G-250 prepared in 20% (v/v) ethanol, 1.6% (v/v) phosphoric acid and 8% (w/v) ammonium sulfate. For Western blot analysis, proteins were electroblotted to PVDF membranes with a semi-dry Trans-Blot cell (Bio-Rad, Hercules, CA, USA). After transfer, membranes were used for cross-reactivity assays with a rabbit polyclonal antibody against NO_2-Tyr (Uttenthal et al. 1998) diluted 1 : 8,000. For immunodetection, an affinity-purified goat anti-(rabbit IgG)–horseradish peroxidase conjugate (Bio-Rad, Hercules, CA, USA) and an enhanced chemiluminescence kit (ECLPLUS, Amersham Biosciences Piscatway, NJ, USA) were used. As a positive control, commercial nitrated bovine serum albumin (BSA; Sigma, St. Louis, MO, USA) was used.

The chemiluminescence was detected with a photographic film (Hyperfilm; Amersham Pharmacia Biotech). Films of immunoblots were scanned with a flat-bed scanner, and digital images were imported and quantified using Photoshop software (Adobe Photoshop ver 6.0). Then, the intensities of bands were compared according to their grayscale (http://www.lukemiller.org/journal/2007/08/quantifying-western-blots-without.html).

Other assays

Protein concentration was determined with the Bio-Rad Protein Assay, using BSA as standard. To estimate the statistical significance between means, the data were analyzed by Student's *t*-test.

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