

# Chloroplasts as a Nitric Oxide Cellular Source. Effect of Reactive Nitrogen Species on Chloroplastic Lipids and Proteins<sup>1</sup>

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Nitric oxide (NO) generation by soybean (*Glycine max* var. ADM 4800) chloroplasts was studied as an endogenous product assessed by the electron paramagnetic resonance spin-trapping technique. Nitrite and L-arginine (Arg) are substrates for enzymatic activities considered to be the possible sources of NO in plants. Soybean chloroplasts showed a NO production of  $3.2 \pm 0.2$  nmol min<sup>-1</sup> mg<sup>-1</sup> protein in the presence of 1 mM NaNO<sub>2</sub>. Inhibition of photosynthetic electron flow by 3-(3,4-dichlorophenyl)-1,1-dimethyl urea resulted in a lower rate ( $1.21 \pm 0.04$  nmol min<sup>-1</sup> mg<sup>-1</sup> protein) of NO generation. Chloroplasts incubated with 1 mM Arg showed NO production of  $0.76 \pm 0.04$  nmol min<sup>-1</sup> mg<sup>-1</sup> protein that was not affected either by omission of Ca<sup>2+</sup> or by supplementation with Ca<sup>2+</sup> and calmodulin to the incubation medium. This production was inhibited when chloroplasts were incubated in the presence of NO synthase inhibitors N<sub>ω</sub>-nitro-L-Arg methyl ester hydrochloride and N<sub>ω</sub>-nitro-L-Arg. In vitro exposure of chloroplasts to an NO donor (250 μM S-nitrosoglutathione) decreased lipid radical content in membranes by 29%; however, incubation in the presence of 25 μM peroxynitrite (ONOO<sup>-</sup>) led to an increase in lipid-derived radicals (34%). The effect of ONOO<sup>-</sup> on protein oxidation was determined by western blotting, showing an increase in carbonyl content either in stroma or thylakoid proteins as compared to controls. Moreover, ONOO<sup>-</sup> treatment significantly affected both O<sub>2</sub> evolution and chlorophyll fluorescence in thylakoids. Data reported here suggest that NO is an endogenous metabolite in soybean chloroplasts and that reactive nitrogen species could exert either antioxidant or prooxidant effects on chloroplast macromolecules.

Nitric oxide (NO) is both a gaseous free radical and a bioactive molecule that plays important roles in diverse processes in plants (for review, see Neill et al., 2002; Lamattina et al., 2003). NO is involved in plant growth regulation (Beligni and Lamattina, 2001), cell differentiation (Ferrer and Ros-Barcelo, 1999), stomatal closure (Garcia-Mata et al., 2003), phytoalexin accumulation (Noritake et al., 1996), and plant responses against a variety of abiotic stresses, such as wounding, salinity, drought, and hypoxia (Neill et al., 2003). NO was identified as an endogenous metabolite in biological systems, and it has been proposed to have either antioxidant or prooxidant effects (Caro and Puntarulo, 1998; Beligni and Lamattina, 1999; Boveris et al., 2000).

It is widely accepted that the protective role against oxidative stress is due to the fact that NO can prevent the Fenton reaction by scavenging iron and thus avoiding the formation of one of the most deleterious reactive oxygen species, the hydroxyl radical (·OH; Wink et al., 1995). The cytoprotective role for NO in plants has been clearly tested in DNA, lipids, proteins, and chlorophyll (for review, see Lamattina et al., 2003). However, the reaction of NO with superoxide anion generates peroxynitrite (ONOO<sup>-</sup>), a species with potent oxidizing power (Blough and Zafiriou, 1985).

It has been shown that NO synthesis in plants (for review, see Lamotte et al., 2005) depends on the activity of nitrate reductase located in the cytosol and on the activity of nitrite-NO reductase on the plasma membrane in roots (Dean and Harper, 1986; Yamasaki and Sakihama, 2000; Stöhr et al., 2001). In addition, in plant tissues, nonenzymatic reduction of inorganic oxidized nitrogen forms (NO<sub>2</sub><sup>-</sup>, NO<sub>2</sub>) takes place in acidic compartments, such as the apoplastic space where phenolic compounds may catalyze the reaction (Bethke et al., 2004). Moreover, the presence of carotenoids or other conjugated molecules can accelerate light-mediated NO production (Cooney et al., 1994). Mitochondria also support nitrite-dependent NO synthesis (Tischner et al., 2004; Modolo et al., 2005; Planchet et al., 2005). In this regard, Guo and Crawford (2005) showed that the Arabidopsis (*Arabidopsis thaliana*) protein NO synthase 1 (AtNOS1) is targeted to the

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mitochondria. This protein, which catalyzes the NADPH-dependent oxidation of Arg to L-citrulline and NO, does not show any homology to previously described mammalian NO synthase (NOS; Guo et al., 2003). In addition to AtNOS1, a distinct NOS-like protein has been reported in plant peroxisomes (Corpas et al., 2004), cytosol, and microsome fractions (Qu et al., 2006). Interestingly, in tobacco (*Nicotiana tabacum*) cells elicited with cryptogein, it was reported that chloroplasts were the first organelle where NO increased within the first 3 min after elicitation (Foissner et al., 2000). In addition, when leaf or cell suspensions of tobacco were loaded with 4,5-diaminofluorescein diacetate and subjected to an abiotic stressor, fluorescence appeared first in the plastids (Gould et al., 2003).

Chloroplasts are among the more active organelles involved in free energy transduction in plants (photo-phosphorylation). It has been reported that photosynthesis can be affected not only by generation of reactive oxygen species (for review, see Asada, 1999), but also by reaction with NO and other NO-related species. Takahashi and Yamasaki (2002) have shown that NO is capable of inhibiting chloroplast electron transport in a reversible manner. Previously, studies of air pollution had suggested that NO may reduce CO<sub>2</sub> assimilation (Hill and Bennett, 1970). Both photosynthesis and photorespiration have been found to be affected by NO in different plants (Yamasaki, 2000; Takahashi and Yamasaki, 2002). The aim of this work was to identify endogenous sources of NO generation and NO and ONOO<sup>-</sup>-dependent oxidative damage in isolated soybean (*Glycine max*) chloroplasts.

## RESULTS

### Characterization of the Isolated Fraction of Chloroplasts

Chloroplasts were isolated from soybean leaves by differential centrifugation and purified by Percoll centrifugation (Bartoli et al., 2004). The recovery of chlorophyll was employed as a biochemical marker for the chloroplast fraction (Table I). Purity of the isolated chloroplast fraction was assessed by determination of enzymatic activities considered to be specific markers. Isolated chloroplasts exhibited negligible contamination with cytosol, mitochondria, or peroxisomes (Table I). Intactness of isolated chloroplasts was determined

as ferricyanide-dependent O<sub>2</sub> evolution according to Edwards et al. (1979). Preparations employed for the experiments showed integrity of not less than 70% to 80%.

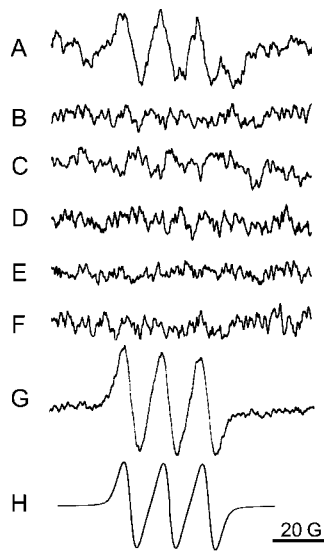
### Arg-Dependent NO Generation

NO generation by chloroplasts was assessed in the presence of Arg and NADPH, according to Galatro et al. (2004). Isolated chloroplasts were subjected to osmotic shock and immediately incubated up to 10 min in the presence of the spin trap (sodium-*N*-methyl-*D*-glucamine dithiocarbamate [MGD])<sub>2</sub>-Fe(II), and the required cofactors described for assaying the activity of plant NOS. A three-line spectrum provided unequivocal evidence for Arg-dependent NO generation (Fig. 1A). A well-known NO donor, *S*-nitrosoglutathione (GSNO), in the presence of the spin trap solution was recorded at identical setting parameters (Fig. 1G). The amount of spin adduct was assessed using an aqueous solution of 4-hydroxy-2,2,6,6-tetramethyl piperidine *N*-oxyl (TEMPOL) as standard. Electron paramagnetic resonance (EPR) spectra of samples and TEMPOL solutions were recorded at the same EPR spectrometer settings and first-derivative EPR spectra were double integrated to obtain the area intensity; then, the concentration of spin adduct in the sample was calculated using the ratio of these areas. Under these experimental conditions, an NO generation rate of  $7.6 \pm 0.4$  nmol mg<sup>-1</sup> protein during a 10-min incubation period was obtained for Arg-dependent synthesis by chloroplasts. Nondetectable NO generation was observed when the spin trap was incubated in the presence of cofactors but in the absence of chloroplasts (Fig. 1D), or in the absence of NADPH (Fig. 1C). The EPR signal was completely absent if the chloroplasts were previously boiled, suggesting that the Arg-dependent signal was due to an enzymatic activity (Fig. 1B). When chloroplasts were incubated with known NOS inhibitors, such as *N*<sub>ω</sub>-nitro-*L*-Arg methyl ester hydrochloride (*L*-NAME; 5 mM) or *N*<sub>ω</sub>-nitro-*L*-Arg; (*L*-NNA; 5 mM), in the presence of all the required cofactors for NOS activity, the rate of NO generation was completely inhibited (Fig. 1, E and F) as compared to controls. Controls performed without addition of Arg were assessed and nonsignificant differences were obtained in the rate of NO generation as compared to the complete

**Table I.** Characterization of the preparation of isolated chloroplasts from soybean leaves

Chlorophyll content was expressed as mg chlorophyll mg<sup>-1</sup> protein. Hydroxypyruvate reductase and phosphoenolpyruvate carboxylase activities were expressed as nmol consumed of NADH min<sup>-1</sup> mg<sup>-1</sup> protein; fumarase activity was expressed as nmol fumarate min<sup>-1</sup> mg<sup>-1</sup> protein. nd, Nondetectable.

Biochemical Marker	Location	Fraction		Ratio
		Homogenate	Chloroplasts	
Chlorophyll content	Chloroplasts	0.38 ± 0.02	1.84 ± 0.04	4.84
Hydroxypyruvate reductase	Peroxisomes	248 ± 14	24 ± 5	0.09
Phosphoenolpyruvate carboxylase	Cytosol	91 ± 5	nd	0
Fumarase	Mitochondria	72 ± 3	3 ± 1	0.04



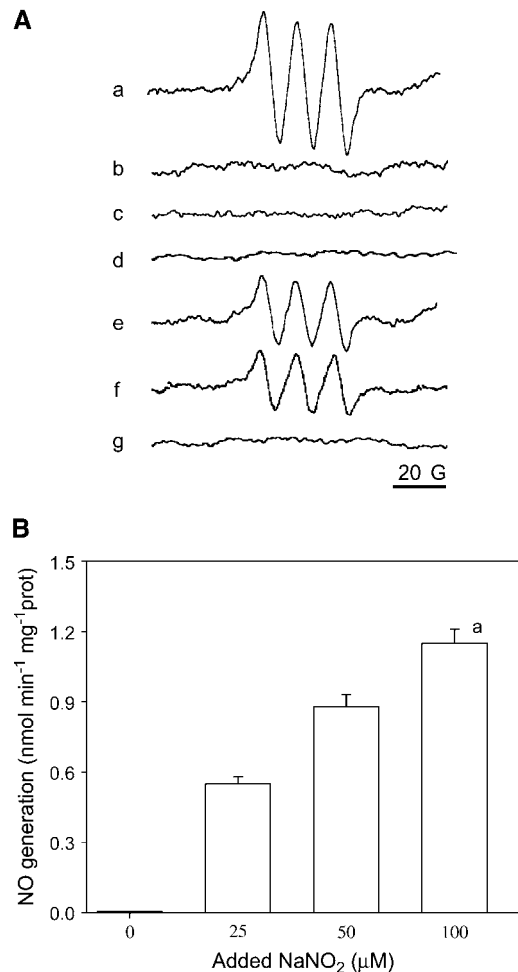
**Figure 1.** Arg-dependent NO generation. A to H, EPR spectra of the  $(\text{MGD})_2\text{-Fe(II)-NO}$  adduct from chloroplasts incubated for 10 min in the presence of MGD-Fe (10:1 mM), 1 mM Arg, 1 mM  $\text{CaCl}_2$ , 5 mM  $\text{MgCl}_2$ , and 0.1 mM NADPH (A); previously boiled chloroplasts were incubated under the conditions indicated above (B); chloroplasts were incubated under the conditions indicated above in the absence of NADPH (C); the reaction medium indicated above incubated in the absence of chloroplasts (D); chloroplasts incubated for 10 min simultaneously with 5 mM L-NAME or L-NNA and all the substrates indicated above (E and F); 50  $\mu\text{M}$  GSNO and 50  $\mu\text{M}$  DTT incubated 10 min in the presence of MGD-Fe (10:1 mM; G); and computer-simulated spectrum employing the following spectral parameters:  $g = 2.03$  and  $a_N = 12.5$  G (H). Measurements were performed at room temperature and four scans, except for GSNO (one scan).

system. This effect could be attributed to an important endogenous supply of Arg. In this regard, total amino acid content in chloroplasts was studied and Arg was shown to be the most abundant amino acid in chloroplast stroma with a concentration of  $56.7 \pm 0.8$  nmol  $\text{mg}^{-1}$  stromal protein. Moreover, the reported synthesis of NO was not affected either by omission or addition of  $\text{Ca}^{2+}$  or by supplementation with  $\text{Ca}^{2+}$  and 10  $\mu\text{g mL}^{-1}$  calmodulin to the incubation medium.

### Nitrite-Dependent NO Generation

Intact chloroplasts incubated under light conditions in the presence of  $\text{NaNO}_2$  and  $(\text{MGD})_2\text{-Fe(II)}$  as a spin trap generated a significant amount of NO (Fig. 2A, a). Previous boiling of the chloroplasts (Fig. 2A, b), incubations performed in the presence of  $\text{NaNO}_3$  instead of  $\text{NaNO}_2$  (Fig. 2A, c), or omission of chloroplasts in the incubation medium (Fig. 2A, d) led to no detection of the  $(\text{MGD})_2\text{-Fe(II)-NO}$  complex. Inhibition of photosynthetic electron flow by 3-(3,4-dichlorophenyl)-1,1-dimethyl urea (DCMU) results in lower NO generation by illuminated chloroplasts as compared to controls over a 10-min period ( $12.1 \pm 0.4$  nmol  $\text{mg}^{-1}$  protein

and  $32 \pm 2$  nmol  $\text{mg}^{-1}$  protein, respectively; Fig. 2A, e). Stroma and thylakoid fractions were separated by osmotic shock and, to assess the purity of the fractions, chlorophyll content was measured, with nondetectable results in stroma and  $5.1 \pm 0.1$  mg chlorophyll  $\text{mg}^{-1}$  protein in thylakoids. When incubations were carried out in the presence of stroma, NO generation



**Figure 2.** Nitrite-dependent NO generation. A, EPR spectra of the  $(\text{MGD})_2\text{-Fe(II)-NO}$  adduct. a, Chloroplasts were incubated for 3 min in the presence of MGD-Fe (10:1 mM) and 1 mM  $\text{NaNO}_2$ . b, Boiled chloroplasts were incubated under the conditions indicated above. c, Chloroplasts were incubated for 3 min in the presence of the spin trap solution and 1 mM  $\text{NaNO}_3$ . d, Isolation buffer was incubated for 3 min in the presence of MGD-Fe (10:1 mM) and 1 mM  $\text{NaNO}_2$ . e, Chloroplasts were incubated for 3 min in the presence of MGD-Fe (10:1 mM), 1 mM  $\text{NaNO}_2$ , and 1  $\mu\text{M}$  DCMU. f, Thylakoids were incubated for 3 min in the presence of MGD-Fe (10:1 mM) and 1 mM  $\text{NaNO}_2$ . g, Stroma were incubated for 3 min in the presence of MGD-Fe (10:1 mM) and 1 mM  $\text{NaNO}_2$ . B, Nitrite-dependent NO generation in isolated chloroplasts in the presence of  $\text{NaNO}_2$  up to 100  $\mu\text{M}$ . Quantification was performed by double integration of the EPR signals. Data are expressed as means  $\pm$  SE of six independent experiments with two replicates in each experiment. Superscript a, Significantly different from values for isolated chloroplasts incubated in the presence of 25  $\mu\text{M}$   $\text{NaNO}_2$ ;  $P \leq 0.05$  (Statview for Windows Version 5.0; SAS Institute).

was nondetectable (Fig. 2A, g), whereas NO generation was detectable when thylakoids were employed (Fig. 2A, f). These results suggested that thylakoids were the main component involved in nitrite reduction. Because levels of nitrite of 10  $\mu\text{M}$  have been reported in spinach (*Spinacia oleracea*) leaves (Rockel et al., 2002), and assuming that nitrite concentration in chloroplasts should be in the same order of magnitude, the rate of generation of NO was measured as a function of  $\text{NaNO}_2$  concentration in the incubation medium in the micromolar range (Fig. 2B).

### Effect of NO and ONOO<sup>-</sup> on Lipid Peroxidation

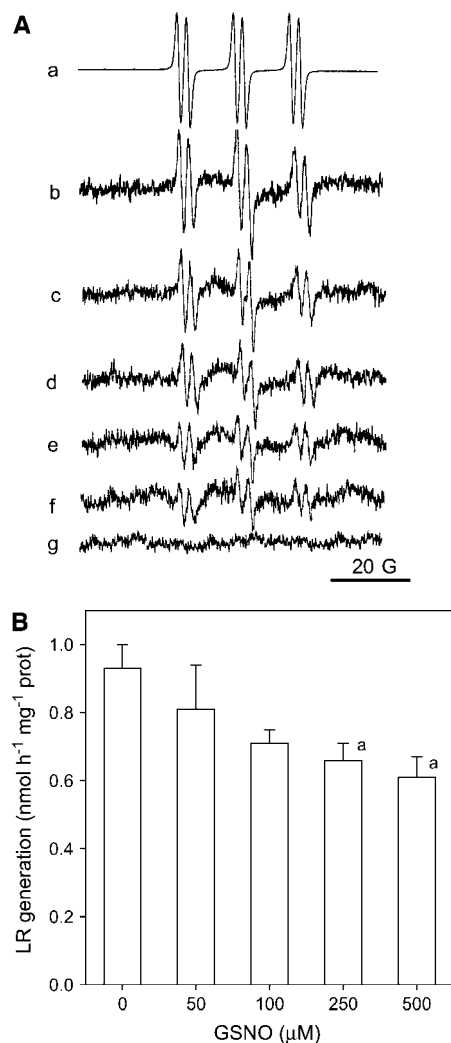
Lipid radical generation, as an index of free radical chain reaction extent in the membranes, was assessed employing EPR. Chloroplastic lipid radicals combined with the spin trap  $\alpha$ -(4-pyridyl 1-oxide)-*N*-*t*-butyl nitron (POBN) resulted in adducts that gave a characteristic EPR spectrum with hyperfine coupling constants of  $a_{\text{N}} = 15.8$  G and  $a_{\text{H}} = 2.6$  G (Buettner, 1987; Fig. 3A, b). Because POBN itself may be photochemically active, POBN alone was examined and no signal of the POBN spin adduct was observed (Fig. 3A, g). Isolated chloroplasts exposed to 50 to 500  $\mu\text{M}$  GSNO for 30 min resulted in a lower EPR signal as compared to control chloroplasts (Fig. 3A, c–f). Quantification of the EPR signals showed that exposure to 250  $\mu\text{M}$  GSNO (approximately 2  $\mu\text{M}$  NO steady-state concentration; Fig. 6) for 30 min led to a decrease of 29% in the rate of generation of chloroplastic lipid radicals (Fig. 3B).

Because NO could be generated in chloroplasts and may react with the photoproduced  $\text{O}_2^-$  to generate ONOO<sup>-</sup>, the effect of exogenous ONOO<sup>-</sup> on lipid peroxidation was assessed. Chloroplasts exposed to 5, 10, 25, and 50  $\mu\text{M}$  ONOO<sup>-</sup> showed an increase in the typical lipid radical POBN signal, with respect to control chloroplasts (Fig. 4A, b–e). Lipid radical content increased significantly by 34% and 39% in chloroplast membranes exposed to 25 and 50  $\mu\text{M}$  ONOO<sup>-</sup>, respectively, as compared to controls (Fig. 4B).

### Effect of NO and ONOO<sup>-</sup> on Protein Oxidation

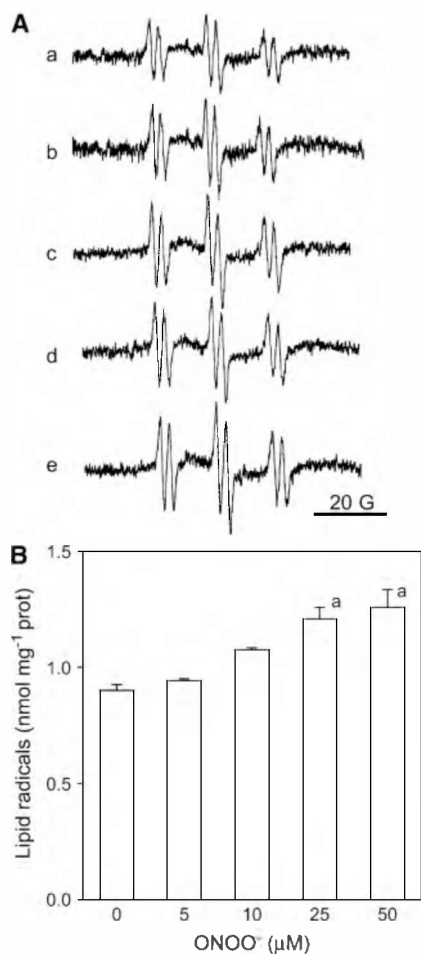
Oxidative modifications on chloroplastic proteins were studied by western-blot assays employing anti-dinitrophenyl (DNP) primary antibodies, which allow identification of previously derivatized protein carbonyls. Intact chloroplasts exposed to 500  $\mu\text{M}$  GSNO and 50  $\mu\text{M}$  DL-dithiothreitol (DTT) for 30 min showed a 10% decrease in carbonyl protein content as compared to control chloroplasts incubated over the same time period in the presence of DTT (Fig. 5A).

For treatments with ONOO<sup>-</sup>, chloroplasts were osmotically broken and separated into stroma and thylakoid fractions. Both fractions showed a significant increase of carbonyl group content (Fig. 5A). After exposure to 20 and 50  $\mu\text{M}$  ONOO<sup>-</sup>, stroma proteins



**Figure 3.** Effect of the NO donor on lipid radical generation in chloroplast membranes. A, Computer-simulated spectrum using hyperfine splitting characteristic of POBN lipid radical adducts,  $a_{\text{N}} = 15.8$  G and  $a_{\text{H}} = 2.6$  G (a). Chloroplasts were incubated at 30°C for 30 min in the presence of POBN as spin trap, 50  $\mu\text{M}$  DTT, and 0  $\mu\text{M}$  GSNO (b), 50  $\mu\text{M}$  GSNO (c), 100  $\mu\text{M}$  GSNO (d), 250  $\mu\text{M}$  GSNO (e), 500  $\mu\text{M}$  GSNO (f), and spectrum of POBN alone (g). All EPR measurements were made at room temperature. B, Rate of lipid radical generation by chloroplast membranes incubated in the presence of 50  $\mu\text{M}$  DTT and GSNO up to a concentration of 500  $\mu\text{M}$ . Quantification was performed by double integration of the EPR signals. Data are expressed as means  $\pm$  SE of six independent experiments with two replicates in each experiment. Superscript a, Significantly different from values for isolated chloroplasts incubated for 30 min in the absence of an NO donor;  $P \leq 0.05$  (Statview for Windows Version 5.0; SAS Institute).

showed an increase of 16% and 39% in total carbonyl content as compared to control samples, respectively. Thylakoid proteins exposed for 15 min to 20 and 50  $\mu\text{M}$  ONOO<sup>-</sup> showed an increase by 23% and 46% in total carbonyl content as compared to controls, respectively (including an identical concentration of NaOH as in treated samples; Fig. 5B).

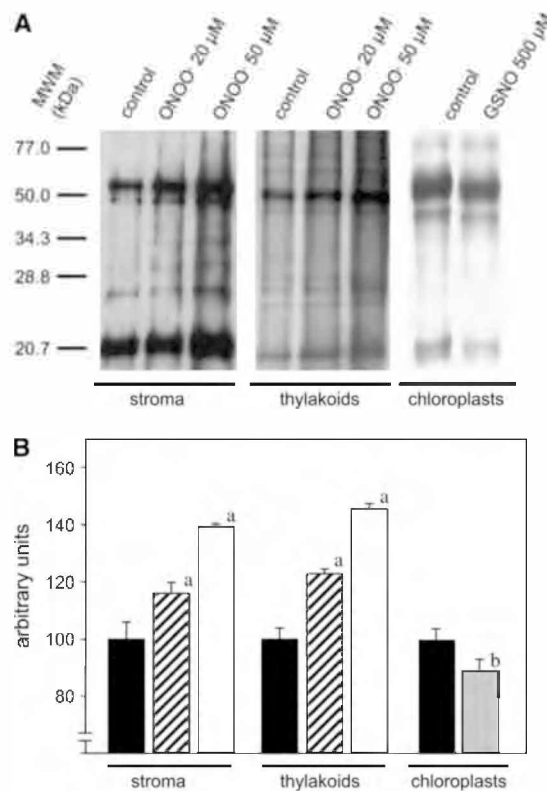


**Figure 4.** Effect of ONOO<sup>-</sup> on lipid radical content in chloroplast membranes. A, Chloroplasts were incubated at 30°C for 5 min in the presence of POBN as a spin trap and identical volumes of 10 mM NaOH with 0 μM ONOO<sup>-</sup> (a), 5 μM ONOO<sup>-</sup> (b), 10 μM ONOO<sup>-</sup> (c), 25 μM ONOO<sup>-</sup> (d), and 50 μM ONOO<sup>-</sup> (e). B, Quantification of lipid radical content in chloroplast membranes incubated for 5 min in the presence of ONOO<sup>-</sup>. Quantification was performed by double integration of EPR signals. Data are expressed as means ± SE of six independent experiments, with two replicates in each experiment. Superscript a, Significantly different from values for isolated chloroplasts incubated for 5 min in the absence of ONOO<sup>-</sup> (10 mM NaOH); *P* ≤ 0.05 (Statview for Windows Version 5.0; SAS Institute).

**Effect of NO and ONOO<sup>-</sup> on Photosynthesis**

The effect of NO on electron transport activities of thylakoid membranes was assessed by ferricyanide-dependent O<sub>2</sub> evolution and chlorophyll fluorescence measurements. Intact chloroplasts treated with NO showed a decline of 18% in the ferricyanide-dependent O<sub>2</sub> evolution rate after 30 min of incubation in the presence of 500 μM GSNO as compared to control chloroplasts (0.54 ± 0.05 nmol O<sub>2</sub> min<sup>-1</sup> mg<sup>-1</sup> chlorophyll), whereas PSII quantum yield ( $\phi_{PSII}$ ) was not affected in intact chloroplasts exposed up to 3 μM NO (50–500 μM GSNO; Table II). Even though ONOO<sup>-</sup> is able to cross the erythrocyte membrane (Denicola et al.,

1998), no data are available regarding ONOO<sup>-</sup> diffusion across the chloroplastic double membrane. Thus, broken chloroplasts were employed to assess the effect of ONOO<sup>-</sup> on chloroplast functionality. Ferricyanide-dependent O<sub>2</sub> evolution was inhibited by 15% when a concentration of 100 μM ONOO<sup>-</sup> was employed during 10 min of incubation as compared to control chloroplasts (0.32 ± 0.02 nmol O<sub>2</sub> min<sup>-1</sup> mg<sup>-1</sup> chlorophyll). PSII quantum yield ( $\phi_{PSII}$ ) was reduced in ONOO<sup>-</sup>-treated samples. When chloroplasts were exposed for 10 min to 50 μM ONOO<sup>-</sup>, the ratio  $F_v'/F_m'$  significantly decreased by 28% as compared to control samples (Table II).



**Figure 5.** Extent of protein oxidation in stroma, thylakoids, and intact chloroplasts exposed to reactive nitrogen species. A, Protein carbonyl content was revealed by derivatization with dinitrophenylhydrazine. Samples (1 μg protein) were run in 10% (w/v) SDS-PAGE, transferred to nitrocellulose membrane, and protein oxidation was determined by western blotting using anti-DNP antibodies. B, Quantification of relative carbonyl content of stroma and thylakoid proteins exposed to 0 μM (black bars), 20 μM (hatched bars), and 50 μM (white bars) ONOO<sup>-</sup> for 15 min, and total chloroplastic proteins exposed to 0 μM (black bar) and 500 μM (gray bar) GSNO for 30 min. Exposed films were quantified with a densitometer and the carbonyl content of proteins was expressed as a percentage of the corresponding values in control samples. Data are expressed as means ± SE of six independent experiments with two replicates in each experiment. Superscript a, Significantly different from values for samples incubated for 15 min in the absence of ONOO<sup>-</sup> (10 mM NaOH); *P* ≤ 0.05 (Statview for Windows Version 5.0; SAS Institute). Superscript b, Significantly different from values for samples incubated for 30 min in the absence of GSNO (50 μM DTT); *P* ≤ 0.1 (Statview for Windows Version 5.0; SAS Institute).

**Table II.** Effect of reactive nitrogen species on photosynthesis

PSII quantum yield ( $\phi_{PSII}$ ) was determined on light-adapted chloroplasts exposed for 30 min to the NO donor or for 10 min to ONOO<sup>-</sup>. Data are expressed as means  $\pm$  SE of six independent experiments with two replicates in each experiment.

Treatment	$F_v'/F_m'$
Control chloroplasts (50 $\mu$ M DTT)	0.20 $\pm$ 0.02
Chloroplasts + 50 $\mu$ M DTT + 500 $\mu$ M GSNO	0.21 $\pm$ 0.02
Control chloroplasts (NaOH)	0.28 $\pm$ 0.03
Chloroplasts + 25 $\mu$ M ONOO <sup>-</sup>	0.23 $\pm$ 0.01
Chloroplasts + 50 $\mu$ M ONOO <sup>-</sup>	0.20 $\pm$ 0.02 <sup>a</sup>

<sup>a</sup>Significantly different from values for isolated chloroplasts incubated for 10 min in the absence of ONOO<sup>-</sup> (medium containing the same amount of NaOH) at  $P \leq 0.05$  (Statview for Windows Version 5.0; SAS Institute).

## DISCUSSION

The results presented here employing EPR spectroscopy support the hypothesis that chloroplasts participate in NO synthesis in plants, as was suggested by previous nonquantitative studies employing fluorescence microscopy (Foissner et al., 2000; Gould et al., 2003) and immunogold electron microscopy (Barroso et al., 1999). Because NO production has been previously detected in several compartments within the plant cell (Lamotte et al., 2005), a highly purified preparation was required to assess a role for chloroplasts in the subcellular generation of NO. Thus, chloroplasts isolated from soybean leaves were assayed for biochemical markers of cytosol, mitochondria, and peroxisomes to confirm the effectiveness of the isolation procedure.

The total rate of generation of NO could be written as indicated in Equation 1.

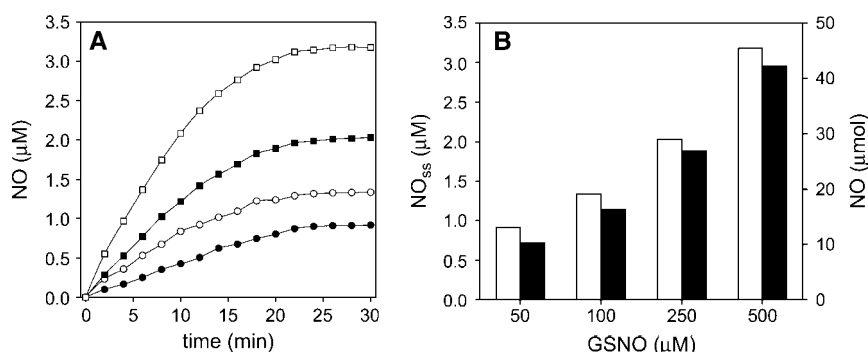
$$\frac{d[\text{NO}]}{dt} = \left(\frac{d[\text{NO}]}{dt}\right)_{\text{NOS like}} + \left(\frac{d[\text{NO}]}{dt}\right)_{\text{NO}_2^-} + \left(\frac{d[\text{NO}]}{dt}\right)_{\text{other sources}} \quad (1)$$

In this work, two independent pathways for NO generation were identified in soybean chloroplasts, one pathway dependent on the activity of a NOS-like enzyme employing Arg/NADPH and another dependent on nitrite. Other alternative sources could be relevant under certain physiological or pathological conditions. Thus, further experiments would be required to assess the relative contribution of these sources, such as NO release from endogenous GSNO (Barroso et al., 2006).

The rate of the reaction of NO with O<sub>2</sub><sup>-</sup> to generate ONOO<sup>-</sup> seems to be the main pathway related to the disappearance of NO, even though other cellular compounds, such as glutathione, which is present in high concentrations (around 25 mM) in the chloroplasts (Asada, 1999), could participate in NO metabolism through quenching reactions. Even though a complex kinetic approach would require more detailed knowledge of the endogenous reactions taking place *in vivo* in the chloroplasts, it could be assumed from the value of the kinetic constant for the NO reaction with O<sub>2</sub><sup>-</sup> ( $k = 6.9 \cdot 10^9 \text{ M}^{-1} \text{ s}^{-1}$ ; Huie and Padmaja, 1993) that ONOO<sup>-</sup> generation is the main pathway leading to NO disappearance. Thus, the rate of disappearance of NO could be estimated according to Equation 2.

$$-\frac{d[\text{NO}]}{dt} = \left(\frac{d[\text{ONOO}^-]}{dt}\right) + \left(-\frac{d[\text{NO}]}{dt}\right)_{\text{quenching reactions}} \quad (2)$$

The data presented here under unrestricted availability of substrates indicate a generation rate of NO by the activity of a NOS-like enzyme of 0.76 nmol min<sup>-1</sup> mg<sup>-1</sup> protein (1 mM Arg, 100  $\mu$ M NADPH). Interestingly, the lack of dependence of NO generation by Ca<sup>2+</sup> seems a distinctive characteristic from other Arg-dependent NO activities described in plants (Guo et al., 2003); however, it should be considered that intact chloroplasts could contain the required Ca<sup>2+</sup> for enzymatic activity. Regarding nitrite, it may accumulate



**Figure 6.** Electrochemical detection of NO. A, Measurement of the amount of NO released from GSNO in 100 mM phosphate buffer, pH 7.4, in the presence of 50  $\mu$ M DTT at room temperature: 50  $\mu$ M GSNO ( $\bullet$ ), 100  $\mu$ M GSNO ( $\circ$ ), 250  $\mu$ M GSNO ( $\blacksquare$ ), and 500  $\mu$ M GSNO ( $\square$ ). A standard curve adding different volumes of nitrite solution to potassium iodide dissolved in sulfuric acid was used to calibrate the electrode, according to the manufacturer's instructions. B, Kinetic parameters of NO release: NO steady-state concentration was evaluated between 15 to 30 min (white bars); total amount of NO generated in 10-mL phosphate buffer during the whole incubation period was obtained as the integrated area of NO concentration as a function of time (black bars).

in compartments with a pH more alkaline than the cytosol, such as illuminated chloroplasts (Morot-Gaudry-Talarmin et al., 2002). Consistently, micromolar concentrations of nitrite have been described in extracts from spinach leaves (Rockel et al., 2002). Under our experimental conditions, the chloroplastic NO generation rate in the presence of  $100 \mu\text{M NO}_2^-$  was  $1.15 \text{ nmol min}^{-1} \text{ mg}^{-1} \text{ protein}$ . On the other hand, chloroplastic ONOO<sup>-</sup> generation would need optimal electron transport because its generation depends on O<sub>2</sub><sup>-</sup> and NO steady-state concentrations. Considering that under in vivo conditions chloroplasts may contain the required substrates for NO synthesis, the assays presented here strongly suggest that feasible NO production could take place inside the chloroplasts, as well as ONOO<sup>-</sup>. In this scenario, which implicates the presence of both NO and ONOO<sup>-</sup> in the surroundings of the chloroplasts, the study of the effect of these species on chloroplastic lipids and proteins is of interest. Previous data from Shi et al. (2005) showed that treatment with an NO donor prevented ion leakage increase and chlorophyll loss and alleviated the UVB-mediated increase in carbonyl groups in thylakoid membrane proteins. The basal content of carbonyl groups observed in control plants could be the result of radical generation as by-products of normal physiological processes, which produce small, but detectable, levels of oxidative modified proteins in the tissue. In this study, incubation of chloroplasts in the presence of the NO donor GSNO (about  $3 \mu\text{M NO}$ ) caused a slight, but significant, decrease (10%) in the content of carbonyl groups in proteins as compared to control chloroplasts. Thus, NO could exert a protective role against protein oxidation as it was previously reported in relation to lipid oxidation (Radi, 1998). In this sense, lipid radical content in chloroplasts was significantly decreased (29%) by GSNO exposure (about  $2 \mu\text{M NO}$ ) as compared to control chloroplasts.

In our experimental conditions, NO treatment did not produce any inhibitory effect on maximal photochemical efficiency of PSII, as was observed by Takahashi and Yamasaki (2002) in spinach thylakoids exposed to NO donors. However, a detrimental effect on the O<sub>2</sub> evolution rate in intact chloroplasts treated with NO was detected, suggesting that other reactions could be operative in the presence of excess NO.

ONOO<sup>-</sup>, the product of the reaction between O<sub>2</sub><sup>-</sup> and NO and its conjugated acid, peroxyntrous acid (ONOOH; pK<sub>a</sub> = 6.8), is a potent oxidant known to be formed in vivo. At pH 7.4, 80% of ONOO<sup>-</sup> is present in the anionic form. However, the biological half-life of ONOO<sup>-</sup> is low (<1 s; Denicola et al., 1998). The chemistry of ONOO<sup>-</sup> is complex and strongly dependent on pH. Denicola et al. (1998) provided evidence that ONOO<sup>-</sup> could react at a distance from its site of production (i.e. 1–2 cell diameters), even in the presence of excess target molecules. ONOO<sup>-</sup> is especially known to cause nitration of phenolic rings, including Tyr residues in proteins (Alamillo and Garcia-Olmedo, 2001), and to increase protein carbonyl content

(Väänänen et al., 2005). The results presented here showed a correlation between ONOO<sup>-</sup> exposure and the content of oxidatively modified proteins both in stroma and thylakoids from soybean chloroplasts. However, direct evidence for ONOO<sup>-</sup> formation in vivo in chloroplasts is still under study. Although oxidation of proteins and other molecules might contribute directly to lowering overall plant vigor, it is becoming increasingly evident that oxidation of target or signal molecules is an intrinsic part of how plants perceive and respond to environmental and developmental triggers (Foyer and Noctor, 2005). Our results showed that chloroplasts exposed to ONOO<sup>-</sup> ( $25 \mu\text{M}$ ) increased lipid radical content by 34% and reduced both the O<sub>2</sub> evolution rate and the  $\phi_{\text{PSII}}$ , indicating damage to the electron transport activities of thylakoid membranes.

## CONCLUSION

Taken as a whole, these data suggest that at least two pathways for NO production are operative in chloroplasts, one dependent on NOS-like enzyme activity and another on nitrite, as suggested by in vitro exposure assays. In situ generation of NO could play a protective role in preventing the oxidation of chloroplastic lipids and proteins; however, the reaction of NO with O<sub>2</sub><sup>-</sup> leading to ONOO<sup>-</sup> production may result in a potential source of damage, even under physiological conditions. Careful adjustments will take place to control chloroplastic integrity upon changes in cellular oxidative conditions. Under circumstances of high NO steady-state concentration (i.e. high nitrite content in chloroplasts), generation of reactive nitrogen species (i.e. ONOO<sup>-</sup>) may lead to impairment of the photosynthetic machinery. On the other hand, because chloroplasts are the main site of carbon and nitrogen metabolism, as well as reactive oxygen species production, NO and related species can potentially affect and regulate a wide range of downstream signals through their effects on chloroplasts (Lum et al., 2005). Further studies are required to analyze the effect of environmental stress conditions on NO and ONOO<sup>-</sup> steady-state concentration and triggering signaling mechanisms.

## MATERIALS AND METHODS

### Plant Material

Soybean (*Glycine max* var. ADM 4800) seeds were grown for 12 d at 24°C to 26°C under 16-h-light/8-h-dark cycles. Plants were grown in a greenhouse chamber receiving  $300 \mu\text{mol m}^{-2} \text{ s}^{-1}$  of photosynthetically active radiation (400–700 nm) supplied by a bank of Philips 40-W daylight-fluorescent light.

### Electrochemical Detection of NO

The NO donor GSNO was synthesized by the reaction of 10 mM NaNO<sub>2</sub> in 100 mM HCl with equal volumes of 10 mM reduced glutathione in 100 mM phosphate buffer, pH 7.4. The mixing solution containing 5 mM GSNO was

kept on ice and used immediately. For electrochemical detection of NO, different volumes of GSNO stock solution were added over 10 mL of 100 mM phosphate buffer, pH 7.4, in the presence of 50  $\mu\text{M}$  DTT. NO release from GSNO was detected at room temperature and recorded as a function of time employing a selective electrode in conjunction with a DUO 18 data acquisition system (ISO-NO Mark II WPI; Fig. 6A). The NO steady-state concentration was evaluated after 15 min of NO release and the total amount of NO generated during the whole incubation period was determined by integration of NO concentration as a function of time in GSNO solutions up to 0.5 mM (Fig. 6B).

### Synthesis of ONOO<sup>-</sup>

ONOO<sup>-</sup> synthesis was performed according to a modified procedure described by Koppenol et al. (1996). Equal volumes of ice-cold 2 M hydrogen peroxide prepared in 1.85 M HNO<sub>3</sub> and 2 M NaNO<sub>2</sub> were combined and instantly added to the same volume of cold 4 M NaOH. Residual hydrogen peroxide was removed by passage over MnO<sub>2</sub> and the stock solution was immediately fractionated and frozen under liquid nitrogen. ONOO<sup>-</sup> concentration (approximately 250 mM) was determined spectrophotometrically at 302 nm ( $\epsilon = 1,670 \text{ M}^{-1} \text{ cm}^{-1}$ ).

### Chloroplast Isolation

Chloroplasts were isolated from leaves homogenized in 50 mM HEPES, pH 7.6, containing 330 mM sorbitol, 2 mM EDTA, 1 mM MgCl<sub>2</sub>, 5 mM ascorbic acid, 0.05% (w/v) bovine serum albumin, and protease inhibitors (40  $\mu\text{g mL}^{-1}$  phenylmethylsulfonyl fluoride, 0.5  $\mu\text{g mL}^{-1}$  leupeptin, 0.5  $\mu\text{g mL}^{-1}$  aprotinin), and filtered through a 20- $\mu\text{m}$  pore mesh at 4°C. The homogenate was loaded on a Percoll cushion (50 mM HEPES, pH 8.0, 330 mM sorbitol, 35% [v/v] Percoll) and centrifuged at 2,500g for 10 min. The pellet containing intact chloroplasts was washed in 50 mM HEPES, pH 8.0, 330 mM sorbitol, and centrifuged at 1,500g for 5 min (Bartoli et al., 2004). The fraction was characterized by measuring chlorophyll content (Lichtenthaler, 1987) and ferricyanide-dependent O<sub>2</sub> evolution (Edwards et al., 1979). Biochemical assays were carried out both on leaf homogenates and in isolated chloroplasts to determine the purity of the obtained fraction. Samples were assayed for hydroxypyruvate reductase activity for assessing peroxisomal contamination (Schwitzguébel and Siegenthaler, 1984). Phosphoenolpyruvate carboxylase activity, a cytosolic marker, was measured by coupling the reaction to NADH oxidation mediated by malate dehydrogenase (Quy et al., 1991), and fumarase was employed as a marker of mitochondria (Bartoli et al., 2004).

### In Vitro Treatments of Isolated Chloroplasts

Exposure to GSNO was performed by incubation of chloroplasts at 30°C for 30 min in buffer containing 50  $\mu\text{M}$  DTT and GSNO (0.05–0.5 mM as indicated). Exposure to ONOO<sup>-</sup> of the isolated chloroplasts was performed by incubation up to 15 min as indicated. ONOO<sup>-</sup> working solution (prepared from the stock solution in 10 mM NaOH) was added as a bolus, followed immediately by vortex shaking during 5 s. To avoid modifications due to increases in pH, ONOO<sup>-</sup> was added in volumes that were never higher than 1% of the reaction medium; control samples were performed by the addition of alkaline solution (10 mM NaOH) in the absence of ONOO<sup>-</sup>. After treatment, chloroplasts were washed twice with HEPES buffer, as was previously described. When required, stroma and thylakoid fractions were separated by osmotic shock and centrifugation at 3,000g for 5 min and the purity of the fraction was assessed by electrophoresis (data not shown). Protein content was performed according to Bradford (1976) and total chlorophyll was determined spectrophotometrically after 1-h extraction with 80% (v/v) acetone (Lichtenthaler, 1987).

### EPR Detection of NO

Isolated chloroplasts (0.8 mg protein mL<sup>-1</sup>) were supplemented with equal volumes of 100 mM phosphate buffer containing the spin trap (10 mM MGD, 1 mM FeSO<sub>4</sub>; Komarov and Lai, 1995) and the appropriate cofactors as described in each experiment. For Arg-dependent NO generation, chloroplasts were incubated up to 10 min in the presence or absence of 1 mM Arg, 0.1 mM NADPH, 1 mM Ca<sup>2+</sup>, 10  $\mu\text{g mL}^{-1}$  calmodulin, and 5 mM L-NAME and L-NNA as Arg analogs. For nitrite-dependent NO generation, chloroplasts were

incubated up to 10 min in the presence of 25 to 100  $\mu\text{M}$  NaNO<sub>2</sub>; when indicated, 1  $\mu\text{M}$  DCMU or 1 mM NaNO<sub>3</sub> were employed.

Samples were transferred to bottom-sealed Pasteur pipettes before EPR analysis. Spectra were recorded at room temperature (18°C) in a Bruker ECS 106 EPR spectrometer, operating at 9.5 GHz. Instrument settings include a 200-G field scan, 83.886-s sweep time, 327.68-ms time constant, 5.983-G modulation amplitude, 50-kHz modulation frequency, and 20-mW microwave power. Quantification of the spin adduct was performed using an aqueous solution of TEMPOL. TEMPOL is a stable free radical used as a standard to obtain the concentration of other free radical adducts. TEMPOL solutions were standardized spectrophotometrically at 429 nm using  $\epsilon = 13.4 \text{ M}^{-1} \text{ cm}^{-1}$ ; the concentration of the NO-Fe<sup>2+</sup>-MGD adduct was obtained by double integration of the three lines and cross-checked with the TEMPOL spectra. The amount of TEMPOL spins placed in the EPR cavity ranged from 0.5 to 5 nmol (standard curve) because 50  $\mu\text{L}$  of a 10 to 100  $\mu\text{M}$  solution of TEMPOL were added to the cavity.

### Chloroplastic Content of Arg

Stroma from purified chloroplasts were extracted with 9 volumes of cold methanol, centrifuged at 10,000g for 15 min, and evaporated to half-volume (Lores-Amaiz et al., 2004). Thin-layer chromatography was performed to verify the purity of the sample before amino acid determination. Free amino acids were quantified by derivatization with phenylisothiocyanate and separation of the phenylthiocarbonyl amino acids was carried out by HPLC, using an amino acid analyzer (model 420; Applied Biosystems; LANAIS-PRO, CONICET-University of Buenos Aires).

### Detection of the Content of Lipid Radicals by EPR Spin Trapping

Isolated chloroplasts were suspended in 100 mM phosphate buffer, pH 7.4, containing 50 mM POBN in the presence of ONOO<sup>-</sup> or the NO donor and incubated at 30°C for 5 or 30 min, respectively. EPR spectra were obtained at room temperature using a Bruker spectrometer, ECS 106, operating at 9.81 GHz with a 50-kHz modulation frequency. EPR instrument settings for the spin-trapping experiments were as follows: microwave power, 20 mW; modulation amplitude, 1.232 G; time constant, 81.92 ms; receiver gain,  $2 \times 10^3$  (Jurkiewicz and Buettner, 1994). The content of the spin adduct was quantified as described above.

### Detection of Chloroplastic Oxidized Proteins

Carbonyl groups in proteins were derivatized as described by Levine et al. (1994). Thylakoids, stroma, or chloroplasts were mixed with an equal volume of SDS (12% [w/v]) and then with 2 volumes of 20 mM dinitrophenylhydrazine dissolved in 10% (v/v) trifluoroacetic acid. This mixture was incubated for 25 min at room temperature and the reaction was stopped by adding 1.5 sample volumes of 2 M Tris-HCl per 30% (v/v) glycerol. Proteins (1  $\mu\text{g}/\text{well}$ ) were loaded in 10% (w/v) acrylamide concentration minigels and run at room temperature under conditions of constant electrophoretic voltage (120 V) for 2 h. For western blotting, proteins were electrotransferred to nitrocellulose membranes at 120 V for 1 h. Blots were blocked with 5% (w/v) nonfat dry milk dissolved in phosphate-buffered saline plus Tween (PBST; 10 mM potassium phosphate buffer, pH 7.4, 150 mM NaCl, 0.1% [v/v] Tween 20), incubated overnight with primary antibody dissolved in blocking buffer (1:2,500), and washed several times with PBST. For carbonyl detection, the primary antibody was rabbit anti-DNP. Blots were then incubated for 2 h with the secondary antibody (goat anti-rabbit IgG conjugated to horseradish peroxidase) prepared 1:15,000 in PBST with 1% (w/v) nonfat dry milk, washed several times with PBST, and developed with a chemiluminescence detection kit (Bio-Rad). Band intensity was determined with Scion Image for Windows.

### Chlorophyll Fluorescence Measurements

Chlorophyll fluorescence measurements were performed placing chloroplasts in a solution containing 50 mM HEPES, pH 7.6, 330 mM sorbitol, 1 mM MnCl<sub>2</sub>, 0.5 mM K<sub>2</sub>HPO<sub>4</sub>, 2 mM EDTA, and 80  $\mu\text{M}$  K<sub>3</sub>Fe(CN)<sub>6</sub> added to stimulate photosynthetic electron transport. After switching on the lights (1,000  $\mu\text{mol photon m}^{-2} \text{ s}^{-1}$ ), the quantum yield of PSII ( $\phi_{\text{PSII}}$ ) was measured from the ratio of variable ( $F_v'$ ) to maximum ( $F_m'$ ) fluorescence in light-adapted chloroplasts using a fluorescence-modulated system (FMS2; Hansatech Instruments).



## Statistical Analyses

Data in the text, figures, and tables are expressed as means  $\pm$  SE of six independent experiments, with two replicates in each experiment. Effect of treatments on measured parameters was tested for significance using a single-factor ANOVA. Significantly different means were separated using the Fisher PLSD test (Statview for Windows Version 5.0; SAS Institute).

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