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ORIGINAL RESEARCH

Protective Effect of Nitric Oxide against Oxidative Damage in *Arabidopsis* Leaves under Ultraviolet-B Irradiation

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Abstract Nitric oxide (NO) is a key molecule involved in many physiological processes. To characterize its roles in the tolerance of *Arabidopsis thaliana* to ultraviolet-B (UV-B), we investigated the effect of a reduced endogenous NO level on oxidative damage to wild-type and mutant (*Atnoa1*) plants. Under irradiation, hydrogen peroxide was accumulated more in mutant leaves than in the wild type. However, the amounts of UV-B-absorbing compounds (flavonoids and anthocyanin) and the activities of two antioxidant enzymes—catalase (CAT, EC 1.11.1.6) and ascorbate peroxidase (APX, EC 1.11.1.11)—were lower in leaves of the former. Supplementing with sodium nitroprusside, an NO donor, could alleviate the oxidative damage to mutant leaves by increasing flavonoid and anthocyanin contents and enzyme activities. In comparison, N^{ω} – nitro – *l* – arginine, an inhibitor of nitric oxide synthase, had the opposite effects on oxidation resistance in wild-type leaves. All these results suggest that nitric oxide acts as a signal for an active oxygen-scavenging system that protects plants from oxidative stress induced by UV-B irradiation.

Keywords Nitric oxide · Oxidative damage · Ultraviolet-B

Abbreviations

APX	ascorbate peroxidase
CAT	catalase
H_2O_2	hydrogen peroxide
LNNA	N^{ω} – nitro – *l* – arginine
MP	membrane permeability
NO	nitric oxide
NOS	nitric oxide synthase
SNP	sodium nitroprusside
UV-B	ultraviolet-B

More ultraviolet-B (UV-B) irradiation now reaches Earth through a thinner ozone layer, causing increased levels of reactive oxygen species (ROS, including O_2^-, H_2O_2, and ·OH) to accumulate in plants. This O_2^- can be catalyzed to H_2O_2, and if the latter form is not degraded immediately, it is inverted to the more toxic ·OH. All of these ROS severely affect the normal structure and functioning of plant organelles. Simultaneously, plants utilize antioxidant materials and enzymes to negate these influences but must maintain a stable cellular redox state to avoid damage (Mackerness et al. 2001). However, the precise mechanism for initiating these protective measures remains elusive.

Nitric oxide (NO), a bioactive molecule, can be swiftly diffused into neighboring cells. In the plant kingdom, this important signal can be used instead of red light to induce germination (Beligni and Lamattina 2001), and it can also affect growth and development (Durner and Klessing 1999) and enhance cell senescence (Pedroso and Durzan 2000;

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Pedroso et al. 2000). NO can also mediate plant responses to biotic and abiotic stresses, e.g., drought, salt, and heat; diseases; and apoptosis (Leshem et al. 1998; Durner and Klessing 1999; Beligni and Lamattina 2001; Mata and Lamattina 2001; Zhao et al. 2004; Wu et al. 2007). It is synthesized by NO synthase (NOS) in animals and plants. In the latter organism, NOS activity (Ninnemann and Maier 1996) can be inhibited by $N^{\omega} - \text{nitro} - l - \text{Arg}$ (LNNA) and N_G-monomethyl-*l*-Arg, both inhibitors of mammalian NOS enzymes. NO synthase is extensively activated by numerous stresses (Zhao et al. 2004, 2007; Foresi et al. 2007), including UV-B irradiation. Although it induces programmed cell death, inhibits mesocotyl elongation, and regulates the defense gene *Chs* under such exposure (Rao and Davis 2001; Mackerness et al. 2001; Zhang et al. 2003), it has remained unclear how NO protects plants against irradiation.

The AtNOS1 gene encodes a protein similar to that involved in NO synthesis in the snail *Helix pomatia*. *Atnos1*, a homozygous mutant line with T-DNA insertion in the first exon of *NOS1*, has lower in vivo NOS activity and NO content than in the wild type (Guo et al. 2003). Critical questions have been raised about the nature of AtNOS1 as a protein associated with NO biosynthesis, such that it might require renaming as AtNOA1 for NO-associated 1 (see Crawford et al. 2006; Guo 2006).

The *Atnoa1* mutant is a convenient plant material for studies of NO functioning. Here, we used it as a loss-of-function mutant to examine the relationship between NO and oxidative stress under UV-B and to investigate the role of nitric oxide for inducing adaptive responses.

Materials and Methods

Plant Materials and UV-B Irradiation

Plants of *Arabidopsis thaliana* L. Heynh. ecotype Columbia and the *Atnoa1* mutant were grown in a greenhouse at 22°C under a 14-h photoperiod (120 μmol m^{-2} s^{-1}). For treatments, 100 μM sodium nitroprusside (SNP) or 100 μM potassium ferricyanide (as the control of SNP because of their similar chemical attributes) was sprayed on the leaf surfaces of 4-week-old *Atnoa1* plants at 6-h intervals. Wild-type plants of the same age were treated with 100 μM LNNA, while only water was applied to the controls. After 12 h, all plants were exposed to UV-B irradiation from fluorescent F40 UV-B lamps (Phillips, Holland) over a time course of 0, 6, 12, 24, or 36 h. The lamps were filtered with 0.13-mm-thick cellulose diacetate (transmission reduced to 280 nm) to remove any ultraviolet-C component emitted by the UV-B source. Spectral irradiance was measured with an Optronics Model 742 spectroradiometer (Optronics Laboratories, Orlando, FL, USA), and light intensity at the sample surface was 15 μmol m^{-2} s^{-1}. After treatments, the expanded leaves were excised for immediate analysis.

Determination of H_2O_2 Content

H_2O_2 contents were assayed with POD-coupled protocols described by Veljovic-Jovanovic et al. (2002). Briefly, 1 g of excised leaves was ground in liquid N_2, and the powder was extracted in 2 ml of 1 M $HClO_4$ in the presence of insoluble polyvinylpyrrolidone (5%, *w*/*v*). The homogenate was centrifuged at 12,000×*g* for 10 min, and the supernatant was neutralized with 5 M K_2CO_3 to pH 5.6 in the presence of 0.1 ml of 0.3 M phosphate buffer (pH 5.6). The solution was centrifuged at 12,000×*g* for 1 min, and the sample was incubated for 10 min with 1 U of ascorbate oxidase prior to assay. The reaction mixture consisted of 0.1 M phosphate buffer (pH 6.5), 3.3 mM 3-(dimethylamino) benzoic acid, 0.07 mM 3-methyl-2-benzothiazoline hydrazone, and 0.3 U of peroxidase. This reaction was initiated by the addition of 0.2 ml of sample. Changes in absorbance at 590 nm were monitored at 25°C.

Determination of Flavonoid and Anthocyanin Contents

Flavonoids and anthocyanin were measured as described by Mirecki and Teramura (1984) with some modifications. Briefly, 5 g of excised leaves was homogenized with a mortar and pestle at 4°C in 5 ml of extraction buffer (99:1 methanol–HCl, *v*:*v*). The homogenate was centrifuged at 2,000×*g* for 10 min at 4°C, and the supernatant was used for assays. Flavonoid and anthocyanin contents were presented according to their absorbances at 300 and 530 nm, respectively.

Determination of Antioxidant Enzyme Activity

To measure the activity of antioxidant enzymes, 0.5 g of leaves was homogenized with a mortar and pestle at 4°C in 5 ml of 50 mM phosphate buffer (pH 7.8) that contained 1 mM EDTA and 2% PVP. The homogenate was centrifuged at 15,000×*g* for 30 min at 4°C, and the supernatant was used for assaying activities. Protein content was determined according to the method of Bradford (1976), using bovine serum albumin as our standard.

Catalase activity (CAT, EC 1.11.1.6) was assayed as described by Durner and Klessing (1996) and was defined as the decrease in absorbance at 240 nm for 1 min following the decomposition of H_2O_2. Ascorbate peroxidase activity (APX, EC 1.11.1.11) was measured according to the method of Nakano and Asada (1981) by monitoring the rate of ascorbate oxidation at 290 nm.

Determination of Lipid Peroxidation

Lipid peroxidation was evaluated in terms of malonaldehyde (MDA) content, following the method of Buege and Aust (1978) with some modifications. Briefly, 0.5 g of leaves was homogenized with a mortar and pestle in 10% trichloroacetic acid, and the homogenate was centrifuged at 4,000×*g* for 30 min. A 2 ml aliquot of the supernatant was then mixed with 2 ml of 10% trichloroacetic acid containing 0.5% thiobarbituric acid. This mixture was heated at 100°C for 30 min. The absorbance of the supernatant was measured at 532 nm, with a reading at 600 nm subtracted from it to account for nonspecific turbidity. The amount of MDA was calculated using an extinction coefficient of 155 mM^{-1} cm^{-1}.

Determination of Membrane Permeability

Membrane permeability (MP) was determined as described by Sairam and Srivastava (2002). With some modifications, 0.5 g of leaves was incubated in Petri dishes for 2 h with 10 ml of deionized water at 25°C. Afterward, conductivity in this bathing solution was determined (C1). The samples were then heated at 80°C for 2 h, and conductivity was read again (C2). Electrolyte leakage was expressed as a percentage of the total conductivity after heating at 80°C ($MP = C1/C2 \times 100$).

Results

Effect of NO on H_2O_2 Production under UV-B Irradiation

Because UV-B exposure causes the generation of ROS, we first determined H_2O_2 contents in leaves of *Arabidopsis*. In the absence of irradiation, that content was 51% higher in the mutant than in the wild type (Fig. 1). Under UV-B treatment, H_2O_2 contents increased gradually with time. At 36 h, contents were greatest in both the mutant (99%) and the wild type (95%) compared with the control.

To elucidate the relationship between nitric oxide and this accumulation of H_2O_2, we applied SNP as a NO donor or LNNA as a NOS inhibitor. SNP reduced H_2O_2 contents in the mutant leaves, with only 81% remaining after 36 h of irradiation. Treatment with LNNA led to the reverse in the wild-type leaves, stimulating more H_2O_2 production, i.e., to 133% of the control after 36 h. To further determine if NO is the compound from SNP that reduces H_2O_2 content, we conducted control experiments with potassium ferricyanide, which has a structure similar to SNP but lacks the ability to produce NO. This treatment had no obvious affect on H_2O_2 (Fig. 1). All of these results indicated that NO inhibits H_2O_2 accumulation under UV-B.

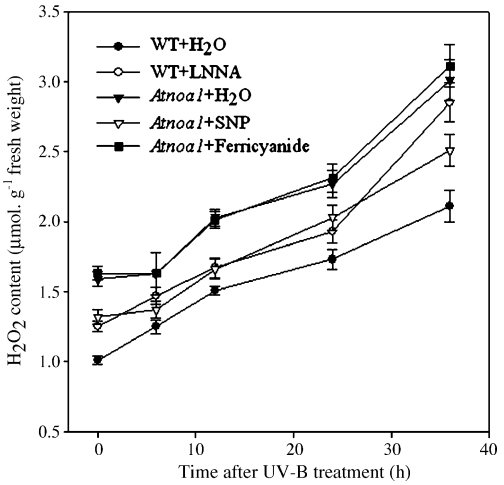

Fig. 1 Effect of NO on H_2O_2 contents in *Arabidopsis* leaves under UV-B irradiation. Means and SE were calculated from three independent experiments

Effect of NO on Flavonoid and Anthocyanin Contents under UV-B Irradiation

Flavonoids and anthocyanin absorb UV-B irradiation and weaken its damaging effect on leaf tissue (Day and Vogelmann 1995). Because the enhancement of H_2O_2 via UV-B was suppressed by nitric oxide here, we decided to assess NO stimulation of antioxidant reactions.

The contents of both compounds were increased in a time-dependent manner under UV-B irradiation, peaking at 36 h of exposure. Accumulations were greater in the wild type, being 175% and 197% over the control for flavonoids and anthocyanin, respectively, versus 159% and 168%, respectively, in the mutant leaves (Figs. 2 and 3).

For *Atnoa1*, SNP intensified the production of flavonoids and anthocyanin by 17% and 18%, respectively, over

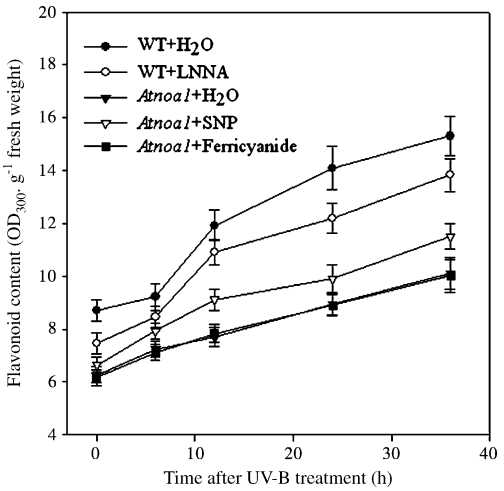

Fig. 2 Effect of NO on flavonoid contents in *Arabidopsis* leaves under UV-B irradiation. Means and SE were calculated from three independent experiments

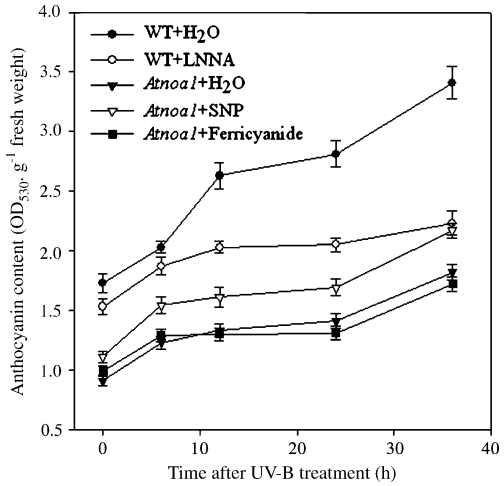

Fig. 3 Effect of NO on anthocyanin contents in *Arabidopsis* leaves under UV-B irradiation. Means and SE were calculated from three independent experiments

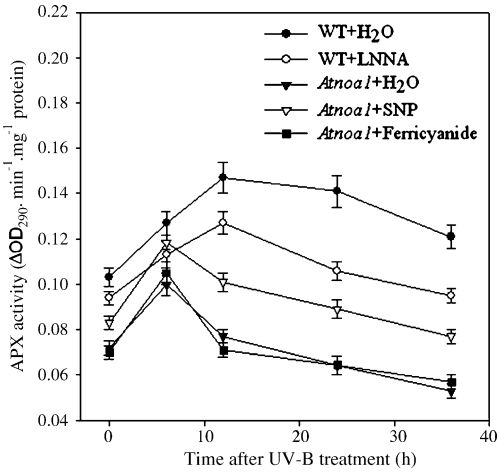

Fig. 5 Effect of NO on APX activity in *Arabidopsis* leaves under UV-B irradiation. Means and SE were calculated from three independent experiments

the control after 36 h, while potassium ferricyanide had no obvious effect. In contrast, their production in the wild type was inhibited by LNNA, with reductions of 13% (flavonoid) and 33% (anthocyanin). These results demonstrate that NO can enhance flavonoid and anthocyanin accumulations under UV-B.

Effect of NO on CAT and APX Activities under UV-B Irradiation

CAT and APX are two key antioxidant enzymes that eliminate excess H_2O_2 in plants. In the presence of irradiation, their activities changed in a similar pattern, with both increasing initially then decreasing over time (Figs. 4 and 5). Maximum values for CAT and APX in the wild type were 148% and 145% of the control, respectively, after 12 h. However, values peaked at 6 h in the mutant, measuring 128% and 150%, respectively, of the control. Activities were always greater in the wild type than in *Atnoa1*.

In the mutant leaves, SNP strongly induced CAT and APX activities, peaking at 21% and 19% higher than the control, respectively (Figs. 4 and 5). Potassium ferricyanide had no obvious influence, whereas treatment with LNNA inhibited their activities by as much as 78% and 79% of the control, respectively. All of these results suggest that NO has a positive effect on antioxidant enzymes.

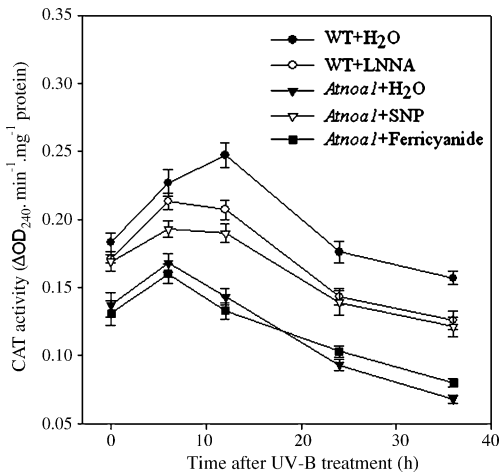

Fig. 4 Effect of NO on CAT activity in *Arabidopsis* leaves under UV-B irradiation. Means and SE were calculated from three independent experiments

Effect of NO on MDA Content and MP under UV-B Irradiation

MDA values are used as an index of membrane peroxidation, which indicates the degree of injury to membranes due to free radicals. Here, UV-B exposure dramatically potentiated MDA and MP values as a function of time (Figs. 6 and 7). After 36 h of irradiation, MDA and MP were greatest in the mutant, at 713% and 821% over the control, respectively, compared with wild-type at values that were 280% and 639% higher than the control, respectively. These data were always greater in the mutant leaves than in the wild type.

In *Atnoa1*, MDA and MP values were suppressed by SNP, with only 73% and 64%, respectively, remaining after 36 h of irradiation. Potassium ferricyanide had no obvious effect. LNNA treatment on wild-type leaves led to MDA and MP values that were enhanced 111% and 28% higher, respectively, over the control, after 36 h. These results demonstrate that NO severely inhibits the stimulation of MDA and MP under UV-B.

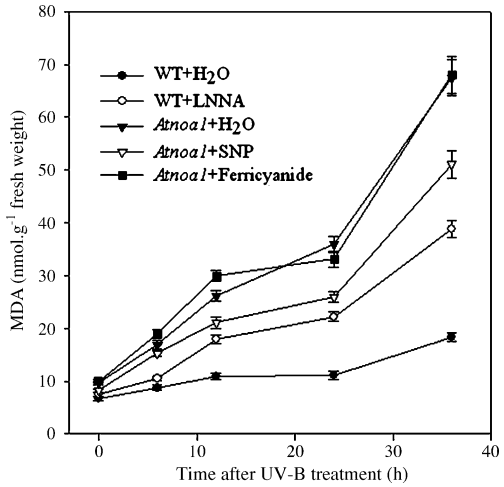

Fig. 6 Effect of NO on MDA in *Arabidopsis* leaves under UV-B irradiation. Means and SE were calculated from three independent experiments

Discussion

UV-B irradiation promotes ROS formation and exerts oxidative stress in plants. As a countermeasure, a series of protective reactions are triggered. Nitric oxide serves as a second messenger in multiple resistance responses and can also be stimulated by UV-B. Therefore, it is reasonable to infer NO resistance to active oxygen induced by irradiation. Here, we tested the *Atnoa1* mutant, which has a lower endogenous NO level, and compared it with wild-type *Arabidopsis*.

H_2O_2, a well-known oxidant, can be an indicator of oxidative injury. Here, such damage was intensified with longer UV-B exposure, especially in the mutant leaves, showing that *Atnoa1* plants are more sensitive to irradiation and that NO might be involved in H_2O_2 production (Fig. 1). To elucidate the relationship between NO and H_2O_2 accumulation, we used foliar applications of SNP (NO donor) and LNNA (NOS inhibitor). The former compound diminished H_2O_2 contents, whereas the latter one enhanced production, thereby indicating that nitric oxide protects *Arabidopsis* leaves from oxidative stress (Fig. 1).

Plants utilize antioxidant materials and enzymes, including flavonoids and anthocyanin, to arrest the generation of reactive oxygen and decrease the effect of exogenous stresses (Vega and Pizarro 2000). In our *Atnoa1* mutant leaves, only small quantities of those two UV-B absorbers were mobilized in response to irradiation compared with the much higher amounts that were accumulated in wild-type leaves. This implies that the latter form blocked more irradiation. Production of those compounds was enhanced by SNP but inhibited by LNNA (Figs. 2 and 3), demonstrating that the NO donor stimulated both flavonoids and anthocyanin.

To avoid oxidative damage, antioxidant enzymes act against free radicals (McCord and Fridovich 1969). In both wild-type and *Atnoa1* leaves, CAT and APX were induced by UV-B irradiation, albeit to a lesser extent in the former type. This suggests that those mutant leaves were easily damaged by interior active oxygen. SNP intensified this enhancement, whereas LNNA suppressed it (Figs. 4 and 5), indicating that NO could potentiate the ability to eliminate free radicals.

UV-B absorbing compounds and antioxidant enzymes play decisive roles in protecting plants against UV-B stress. NO has a positive effect and could be considered an inducer of antioxidant reactions.

Membrane peroxidation and ion leakage are used to describe the degree of oxidative damage. UV-B irradiation caused higher levels of MDA and MP in our mutant leaves, which were more seriously damaged by oxidative stress than were the wild type. SNP decreased values for both MDA and MP, while LNNA increased them (Figs. 6 and 7). These results demonstrate that NO precludes oxidative damage.

Although we used SNP as NO donor here, it can also release CN, which has been shown to elicit some of the effects observed from NO (Boullerne et al. 1999). Therefore, to show that NO, and not CN, had mediated these responses, we applied potassium ferricyanide as a control and found that it did not influence the antioxidant reaction under UV-B irradiation.

In summary, nitric acid can induce the antioxidant system to prevent the accumulation of active oxygen and protect plants from oxidative damage under UV-B. It is highly possible that this response can be mediated through increased expression of genes that encode active oxygen-

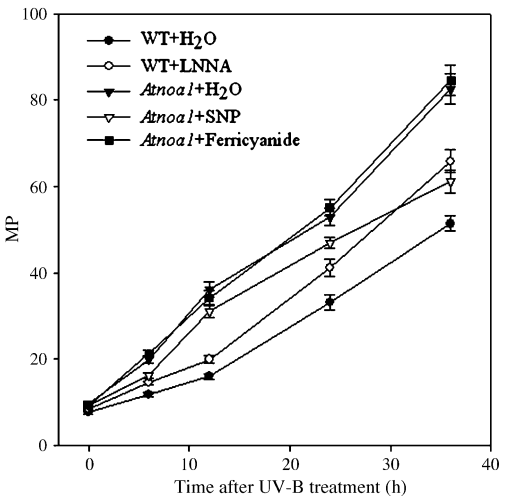

Fig. 7 Effect of NO on MP in *Arabidopsis* leaves under UV-B irradiation. Means and SE were calculated from three independent experiments

scavenging substances. We previously reported that H_2O_2 activates NOS, thus increasing the level of NO (Zhang and Zhao 2008). Those results along with our current data suggest a feedback loop between NO and H_2O_2. In the *Atnoa1* mutant, NO generation is partially blocked (Guo et al. 2003). There, antioxidant reactions cannot be activated in response to irradiation, such that free radicals are then accumulated and plant tissues damaged.

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