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Production of Nitric Oxide under Ultraviolet-B Irradiation is Mediated by Hydrogen Peroxide through Activation of Nitric Oxide Synthase

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Excised leaves of kidney bean (*Phaseolus vulgaris*) were used to investigate the mechanism of NO generation under UV-B stress. We showed that two signaling molecules, NO and H_2O_2, were produced in the irradiated leaves. NO release was blocked by LNNA, an inhibitor of NOS. Application of CAT (EC 1.11.1.6) not only effectively eliminated H_2O_2 in the leaves, but also inhibited the activity of NOS and the emission of NO. In contrast, treatment with exogenous H_2O_2 increased both of those events. Therefore, we suggest that, under UV-B stress, NO production is mediated by H_2O_2 through greater NOS activity.

Keywords: catalase, hydrogen peroxide, nitric oxide, nitric oxide synthase, ultraviolet B

Industrial development is causing more polluted waste to be released into the air, leading to a breakdown of the ozone layer. This thinner layer has induced the enhancement of ultraviolet B (UV-B) radiation (208 to 320 nm) on Earth (Blumthaler and Ambach, 1990). Moreover, it has caused more reactive oxygen species [ROS, including O_2^-, hydrogen peroxide (H_2O_2), and ·OH] to accumulate, thus destroying the reductive status in plant cells, which extensively influences normal morphometrics and organellar structures. These phenomena can impact plant growth and development, their photomorphogenesis, photosynthesis, respiration, flowering, and pollination (Jordan, 1996). In the chloroplasts especially, UV-B can oxidize and degrade the Photosystem II protein complex (Friso et al. 1994), reduce Rubisco activity (Jansen et al., 1998), decompose chlorophyll and carotenoids (Vass et al., 1996), and inhibit the expression of photosynthetic genes (Strid et al., 1994), thereby leading to diminished fertility (Vega and Pizarro, 2000).

The accumulation of ROS not only elicits oxidative stress in those cells, but it also acts as a signaling molecule to activate plant resistance (Foyer et al., 1997). In the presence of UV-B irradiation, the production of salicylic acid, ethylene, and jasmonate then induces the expression of defense genes (Mackerness, 2000). For instance, in *Arabidopsis* seedlings, O_2^- and H_2O_2 can potentiate *PDF 1.2* and *PR-1* expression, respectively, to resist UV-B damage (Mackerness et al., 2001).

Nitric oxide (NO) is a bioactive molecule involved in many physiological processes in animals, such as vasorelaxation, platelet inhibition, neurotransmission, cytotoxicity, and immunoregulation (Anbar, 1995). NO also acts as an important signaling molecule in plants, inducing germination in lieu of red right (Beligni and Lamattina, 2001), affecting tissue growth and development (Leshem and Haramaty, 1996; Durner and Klessing, 1999), and enhancing cell senescence (Pedroso and Durzan, 2000; Pedroso et al., 2000). This molecule also may mediate plant responses to biotic and abiotic stresses; its role has been suggested in responses to drought, salt, and heat stresses, as well as disease resistance and apoptosis (Leshem et al., 1998; Durner and Klessing, 1999; Beligni and Lamattina, 2001; Garcia-Mata and Lamattina, 2001; Zhao et al., 2007). NO is synthesized by NO synthase (NOS) in animal cells. NOS is also active in plants (Ninnemann and Maier, 1996), but can be hindered by N^{ω}-nitro-l-Arg (LNNA) and N_G-monomethyl-l-Arg, two known inhibitors of mammalian NOS. This enzyme is extensively activated in plants under numerous stresses (Zhao et al., 2004, 2007; Foresi et al., 2007). NO is also closely related to plant resistance to UV-B stress, inducing programmed cell death and inhibiting mesocotyl elongation under irradiation (Rao and Davis, 2001; Zhang et al., 2003). A defense gene, *Chs*, is regulated by NO to resist UV-B (Mackerness et al., 2001). However, the precise mechanism for generating nitric oxide remains elusive.

There is significant overlap between the NO- and H_2O_2-signaling pathways in plants (Jih et al., 2003; Zeier et al., 2004; Zago et al., 2006), but some contradictory results have been reported about the relationship between NO and H_2O_2 production, with several studies showing that NO regulates ROS existence in plants (Clark et al., 2000; de Pinto et al., 2002) and others indicating that this generation of NO and H_2O_2 is interdependent (Neill et al., 2002; Bright et al., 2006; Zhao et al., 2007). Our previous research with leaves of kidney bean (*Phaseolus vulgaris*) demonstrated that NO could stimulate the activities of SOD, APX, and CAT to eliminate H_2O_2 (Shi et al., 2005). This led us to suggest that hydrogen peroxide possibly affects the generation of NO. The objective of our current study was to investigate this functioning under UV-B stress.

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Abbreviations: CAT, catalase; FW, fresh weight; H_2O_2, hydrogen peroxide; LNNA, N^{ω}-nitro-l-Arginine; NO, nitric oxide; NOS, nitric oxide synthase; ROS, reactive oxygen species; SNP, sodium nitroprusside; UV-B, ultraviolet-B.

MATERIALS AND METHODS

Plant Material

Seeds of kidney bean (*Phaseolus vulgaris*) from the GanSu Seed Company (GanSu Province, China) were surface-sterilized with 0.5% NaOCl solution for 20 min. After being washed for 10 min in tap water, they imbibed in distilled water for 12 h, then were placed overnight between layers of moistened cheesecloth at 25°C. These new germinants were planted in sterilized sand and grown for 10 d in a controlled-environment culture chamber, under conditions of 25°C, 60% relative humidity, and a 12-h photoperiod (photosynthetic flux of 120 μmol m^{-2} s^{-1}). Expanded leaves were excised for our treatments.

UV-B, LNNA, and Catalase (CAT) Treatments

Leaf samples were initially floated on distilled water, 200 μM LNNA, or 200 U mL^{-1} CAT (these two reagents prepared with sterilized water). After being pumped for 3 min, the dishes were transferred to a chamber with UV-B irradiation sources (TL-40W/12UV; Philips, Eindhoven, The Netherlands) for a time course of 0, 2, 6, 12, or 24 h of exposure. The lamps were filtered with 0.13-nm-thick cellulose diacetate (transmission down to 280 nm) for UV-B radiation to remove any ultraviolet C component emitted by the UV-B source. Spectral irradiance was determined with a spectroradiometer (Optronics Model 742; Optronics Laboratories, Orlando, FL, USA); light intensity at the sample surface was 15 μmol m^{-2} s^{-1}. During all treatment periods with UV-B, LNNA, or CAT, the solutions were refreshed every 12 h. When the experiments were concluded, the excised leaves were collected for either immediate use or storage at -80°C.

H_2O_2 Treatment

Excised leaves were incubated in cultured dishes containing H_2O_2 at a concentration of 0, 1, 2, 5, or 10 mM. Following their pumping for 3 min, the dishes were exposed to only visible light (120 μmol m^{-2} s^{-1}) for 20 min. Afterward, the leaves were collected for analyses of NO production and NOS activity.

Determination of H_2O_2 Content

H_2O_2 content was determined via the POD-coupled assay protocols described by Veljovic-Jovanovic et al. (2002). Excised leaves (approx. 1 g) were ground in liquid N_2 and the powder was extracted in 2 mL of 1 M $HClO_4$ with insoluble polyvinylpyrrolidone (5%, w/v). The homogenate was centrifuged at 12,000g for 10 min, and the supernatant was neutralized with 5 M K_2CO_3 to pH 5.6 in the presence of 100 μL of 0.3 M phosphate buffer (pH 5.6). This solution was centrifuged at 12,000g for 1 min, and sampled were incubated for 10 min with 1 unit of ascorbate oxidase prior to our assays. The reaction mixture was composed 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 units of peroxidase. The reaction was initiated by adding 200 μL of sample. Changes in absorbance at 590 nm were monitored at 25°C.

Determination of NOS Activity

NOS activity was assayed according to the method of Murphy and Noack (1994), with some modifications. Excised leaf tissues (approx. 2 g) were homogenized in 5 mL of homogenization buffer (50 mM triethanolamine hydrochloride at pH 7.5) that also contained 0.5 mM EDTA, 1 μM leupeptin, 1 μM pepstatin, 7 mM glutathione, and 0.2 mM phenylethylsulfonyl fluoride. After centrifugation at 9000 g for 30 min, the supernatant was collected and re-centrifuged at 10,000 g for 45 min at 4°C. This supernatant was used for NOS determinations. Activity was analyzed by a hemoglobin assay (Murphy and Noack, 1994), and protein concentration was obtained as described by Bradford (1976).

Determination of NO Content

NO content was determined with some modifications to the method of Murphy and Noack (1994). Briefly, 15 disks (1-cm diam.) of excised leaves were incubated for 5 min with 100 U of catalase and 100 U of superoxide dismutase to remove endogenous ROS before the addition of 10 mL of oxyhemoglobin (5 mM). After 2 min of incubation, nitric oxide was measured spectrophotometrically by calculating the conversion of oxyhemoglobin to methemoglobin per the formula: C (mM) = (OD577-OD591)/11.2.

Western-Blot Analysis

SDS-PAGE was performed as described by Laemmli (1970), solubilizing and then separating 50 μg of total protein on a 7.5% (w/v) acrylamide gel. After electrophoresis, the proteins were electro-transferred to a nitrocellulose membrane, which was blocked for 60 min with 5% (w/v) nonfat milk in 0.05% (w/v) Tween 20, 10 mM Tris (pH 8.0), and 150 mM NaCl. A polyclonal antibody raised against NOS was added and incubated with the membrane overnight, then an alkaline phosphatase-coupled secondary antibody was added before incubating for 1.5 h. Color was developed with a solution containing nitroblue tetrazolium and 5-bromo-4-chloro-3-indolylphosphate.

RESULTS

NO Production and NOS Activity under UV-B Irradiation

Flux of nitric oxide was stimulated by UV-B exposure (Figure 1A). The rate of NO release from excised kidney bean leaves increased gradually with lengthening time of radiation, reaching its maximum value, about 250% of the control, after 24 h of treatment.

Effects of LNNA on NO Production under UV-B Irradiation

Generation of NO in response to UV-B was obviously hindered by LNNA. After 12 h and 24 h of such treatment, rates of release were inhibited by 55% and 50%, respectively (Figure 1B).

Effect of CAT on H_2O_2 Production under UV-B Irradiation

Although H_2O_2 production was stimulated by UV-B expo-

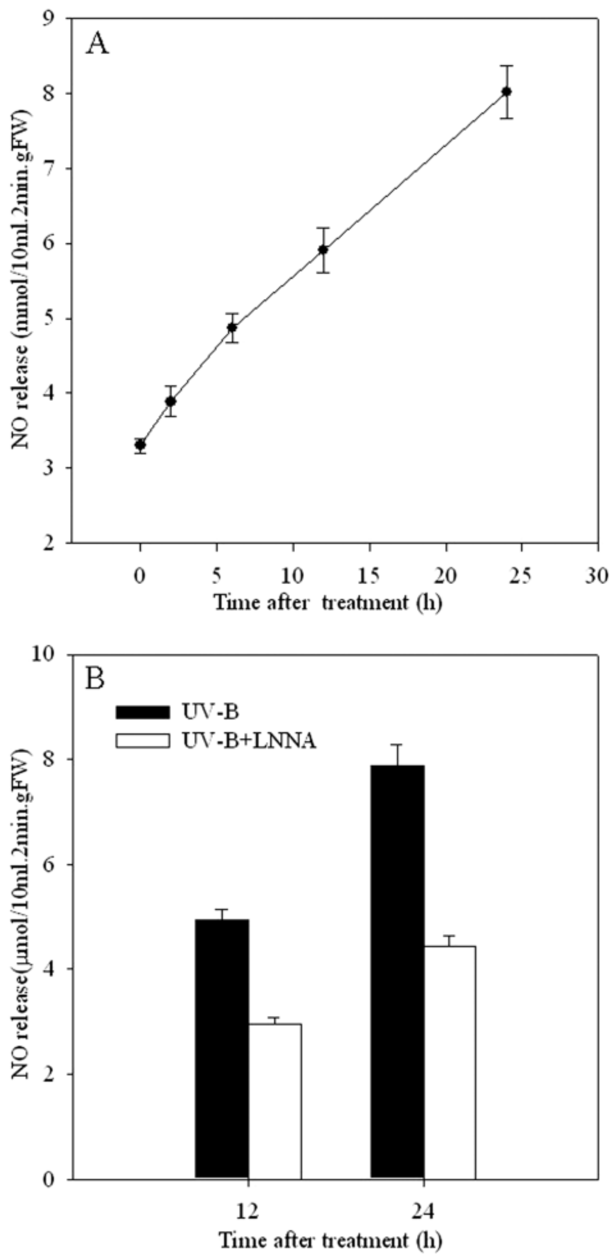

Figure 1. NO production in excised leaves from kidney bean, under UV-B irradiation alone (**A**) and together with LNNA treatment (**B**). FW: Fresh weight. Mean values and SE were calculated from 3 independent experiments.

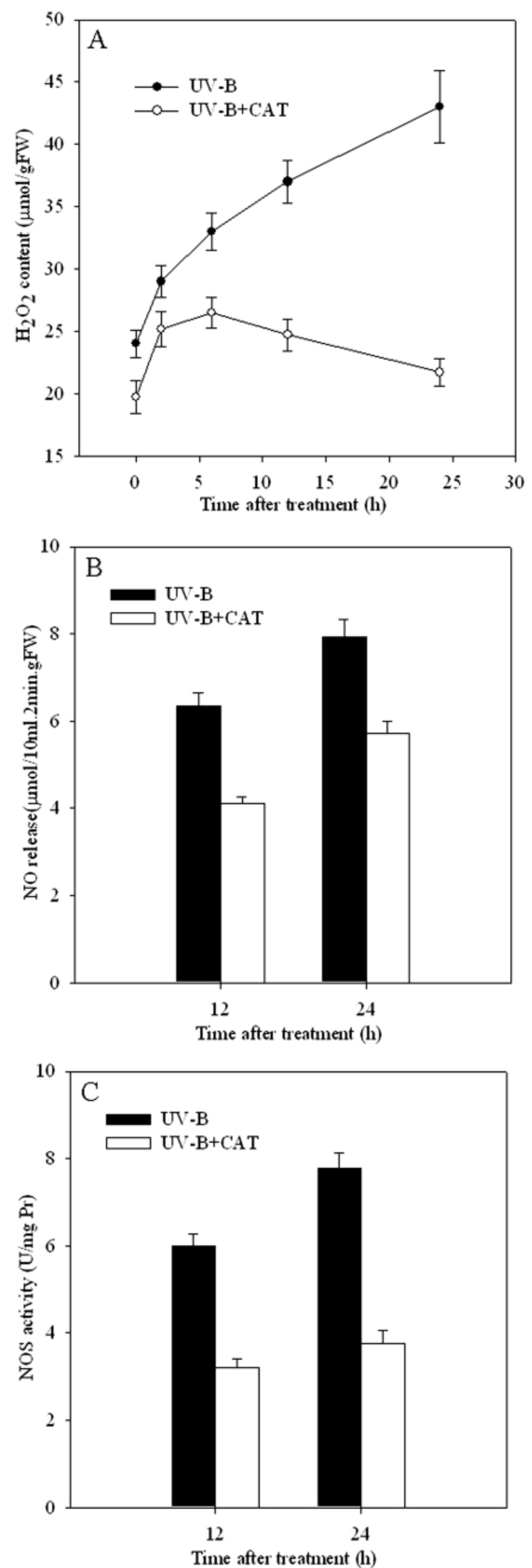

Figure 2. Effects of CAT on H_2O_2 production (**A**), NO production (**B**), and NOS activity (**C**) in excised leaves from kidney bean under UV-B irradiation. Mean values and SE were calculated from 3 independent experiments.

sure, this trend was inhibited in the presence of CAT (Figure 2A). Peroxide content increased gradually, in a time-dependent manner, reaching a peak, 198% of the control, after 24 h of irradiation. Treatment with 200 U mL^{-1} CAT dramatically inhibited this trend. After only 6 h of UV-B application, the H_2O_2 content was at its maximum value before declining gradually to a level close to normal at 24 h post-treatment.

Effect of CAT on NOS Activity and NO Release under UV-B Irradiation

CAT treatment reduced NOS activities and the rates of NO release in excised leaves under UV-B stress. For example, 200 U mL^{-1} CAT depressed those rates to 64% and 74%

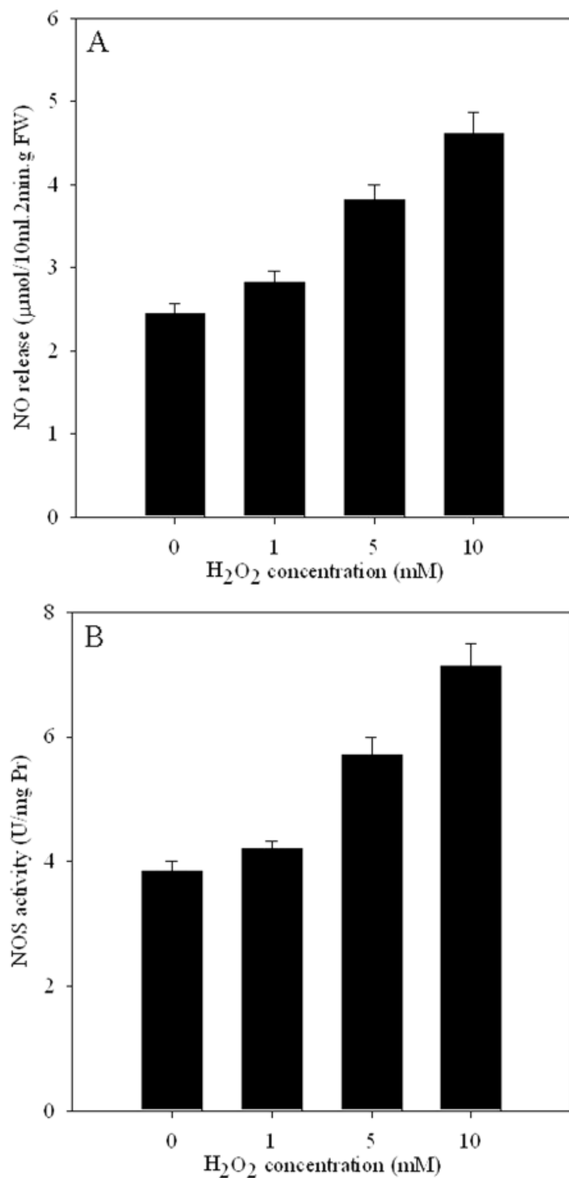

Figure 3. Effects of H_2O_2 on NO production (**A**) and NOS activity (**B**) in excised leaves from kidney bean. Mean values and SE were calculated from 3 independent experiments.

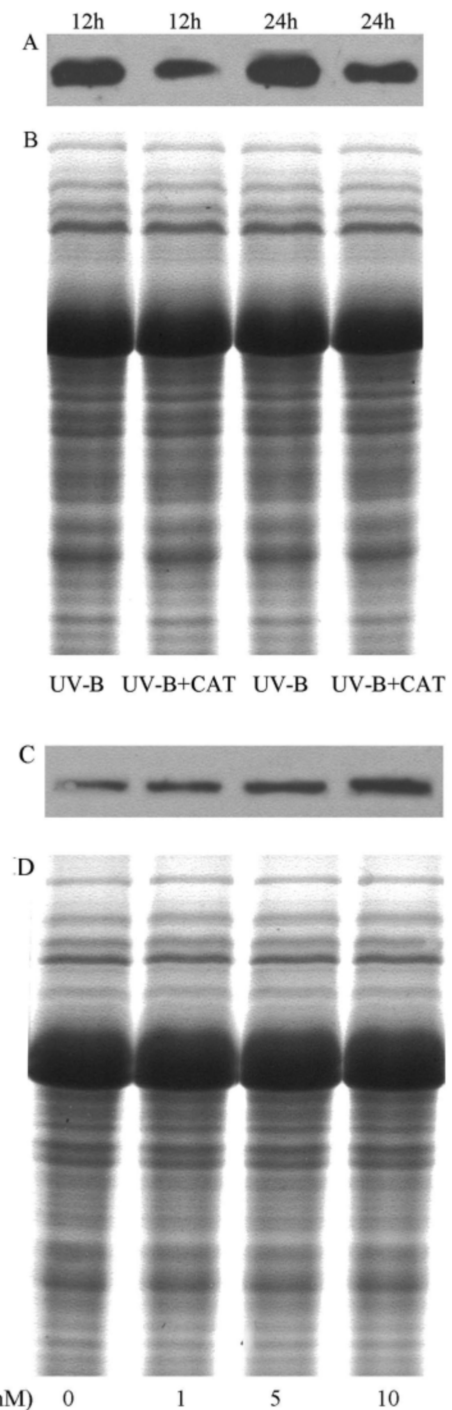

Figure 4. Western-blot analyses for effects of CAT (**A**) and H_2O_2 (**C**) on NOS expression under UV-B irradiation. Coomassie Bright Blue-stained gels (**B**, **D**) show equal loading of proteins. Immunoblotting results indicate similar trends for protein expression in 3 independent experiments.

of normal after 12 h and 24 h, respectively (Figure 2B), while NOS activities were reduced to 61% and 55% after 12 h and 24 h, respectively (Figure 2C).

Effect of H_2O_2 on NOS Activity and NO Production

Compared with our CAT results, exogenous application of H_2O_2 influenced NOS activities and NO production in an opposite manner, with both being gradually enhanced as peroxide concentrations increased. The highest levels were achieved upon treatment at 10 mM H_2O_2, where values for NOS activity and NO release were 182% and 188% of the control, respectively (Figure 3A, B).

Western-Blot Analysis of NOS Expression

Western-blot analysis showed that irradiation caused a decline in the steady-state protein of NOS after both 12 h and 24 h of CAT treatment. In contrast, the application of H_2O_2 increased NOS expression as the concentration rose (Figure 4A, C).

Our two Coomassie Bright Blue-stained acrylamide gels indicated that equal amounts of proteins were loaded for these western-blot analyses (Figure 4B, D).

DISCUSSION

Here, we provide evidence for the involvement of H_2O_2 in the production of nitric oxide by irradiated plants. Hydrogen peroxide, generated under UV-B stress, served as a second messenger for the induction of NOS expression, i.e., activating NO, another signaling molecule.

Nitric oxide functions in multiple plant-resistance reactions against environmental stresses (Leshem and Haramaty, 1996; Garcia-Mata and Lamattina, 2001; Mackerness et al., 2001; Wu et al., 2007; Zhao et al., 2007). Shi et al. (2005) have reported that the application of an NO donor could aid in resistance to UV-B and the avoidance of oxidative damage to kidney bean leaves. Because it is reasonable to think that NO responds to UV-B, we first measured its production in stressed leaves and found that its release was obviously higher during irradiation exposure. NO probably arises from a NOS-like enzyme under exogenous stimulations (Zhang et al., 2003; Zhao et al., 2004, 2007). Our data also demonstrated that the release of nitric oxide could be arrested by LNNA, a special NOS inhibitor. Although the generation of NO is thought to depend upon nitrate reductase (Garcia-Mata and Lamattina, 2003), our results indicated that the increased NO flux was primarily due to NOS activity in leaves excised from irradiated kidney bean plants.

Excessive ROS not only damages plants, but also induces their resistance reaction against harmful factors (Foyer et al., 1997). H_2O_2, a well-known oxidant, can be used as an index of oxidative damage, acting as a signaling molecule related to such resistance. Consequently, we also detected H_2O_2 production under UV-B stress, with contents becoming gradually enhanced as this stress period lengthened. Furthermore, catalase, a H_2O_2 scavenger, dramatically inhibited this enhancement.

Two signaling molecules -- NO and H_2O_2 -- were produced in the presence of UV-B. Therefore, we utilized exogenous applications of CAT to examine the effect of peroxide on NO generation. Such treatment inhibited both NO production and NOS activity. Western-blot analysis provided strong evidence for this catalase-depressed NOS expression. NO is mainly synthesized by NOS; here, CAT eliminated H_2O_2 in excised leaves under UV-B. Therefore, it is possible that CAT restrained NOS expression so as to cause this NO release to be diminished due to the reduction in H_2O_2 content during irradiation.

To verify this, we also examined the effect of H_2O_2 on NO production and found a situation that was the reverse of that with CAT. Here, both NO production and NOS activity were excited by H_2O_2, the degree of response being correlated with its concentration. Western-blot analysis again confirmed that the amount of NOS protein was enhanced by hydrogen peroxide, providing further positive evidence for this deduction.

We believe that all of these results are the first to demonstrate that, under UV-B irradiation, H_2O_2 functions as a second messenger for inducing NOS expression. This may account for the enhanced release of nitric oxide. Based on our current and previous data (Shi et al., 2005), we propose that this is a feedback-regulation mechanism for NO production in response to UV-B exposure.

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LITERATURE CITED

Anbar M (1995) Nitric oxide: A synchronizing chemical messenger. Experientia **51**: 481-490

Beligni MV, Lamattina L (2001) Nitric oxide: A non-traditional regulator of plant growth. Trends Plant Sci **11**: 508-509

Blumthaler M, Ambach W (1990) Indications of increasing solar ultraviolet-B radiation flux in alpine regions. Science **248**: 206-208

Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Ann Biochem **72**: 248-254

Bright J, Desikan R, Hancock JT, Weir IS, Neill SJ (2006) ABA-induced NO generation and stomatal closure in *Arabidopsis* are dependent on H_2O_2 synthesis. Plant J **45**: 113-122

Clark D, Durner J, Navarre DA, Klessig DF (2000) Nitric oxide inhibition of tobacco catalase and ascorbate peroxidase. Mol Plant-Microbe Interact **13**: 1380-1384

de Pinto MC, Tomassi F, de Gara L (2002) Changes in the antioxidant systems as part of the signaling pathway responsible for the programmed cell death activated by nitric oxide and reactive oxygen species in tobacco bright-yellow 2 cells. Plant Physiol **130**: 698-708

Durner J, Klessig DF (1999) Nitric oxide as a signal in plants. Curr Opin Plant Biol **2**: 369-374

Foresi NP, Laxalt AM, Tonón CV, Casalongué CA, Lamattina L (2007) Extracellular ATP induces nitric oxide production in tomato cell suspensions. Plant Physiol **145**: 589-592

Foyer CH, Lopez Delgado H, Dat JF, Scott IM (1997) Hydrogen peroxide and glutathione-associated mechanisms of acclamatory stress tolerance and signaling. Physiol Plant **100**: 241-254

Friso G, Barbato R, Giacometti GM, Barber J (1994) Degradation of the D2 protein due to UV-B irradiation of the reaction center of photosystem II. FEBS Lett **339**: 217-221

Garcia-Mata C, Lamattina L (2001) Nitric oxide induces stomatal closure and enhances the adaptive plant responses against drought stress. Plant Physiol **126**: 1196-1204

Garcia-Mata C, Lamattina L (2003) Abscisic acid, nitric oxide and stomatal closure – Is nitrate reductase one of the missing links? Trends Plant Sci **8**: 20-26

Jansen MAK, Gaba V, Greenberg BM (1998) Higher plants and UV-B radiation: Balance damage, repair and acclimation. Trends Plant Sci **3**: 131-135

Jih PJ, Chen YC, Jeng ST (2003) Involvement of hydrogen peroxide and nitric oxide in expression of the ipomoelin gene from sweet potato. Plant Physiol **132**: 381-389

Jordan BR (1996) The effects of ultraviolet-B radiation on plants: A molecular perspective. Adv Bot Res **22**: 97-111

Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature **227**: 680-685

Leshem YY, Haramaty E (1996) The characterization and contrasting effects of the nitric oxide free radical in vegetative stress and senescence of *Pisum sativum* Linn. foliage. J Plant Physiol **148**: 258-263

Leshem YY, Wills RBH, Ku VV (1998) Evidence for the function of the free radical gas-nitric oxide (NO) as an endogenous maturation and senescence regulating factor in higher plants. Plant Physiol Biochem 36: 825-833

Mackerness S (2000) Plant responses to ultraviolet-B (UV-B: 280-320 nm) stress: What are the key regulators? Plant Growth Regul 37: 27-39

Mackerness SAH, John CF, Jordan B, Thomas B (2001) Early signaling components in ultraviolet-B responses: Distinct roles for different reactive oxygen species and nitric oxide. FEBS Lett 489: 237-242

Murphy ME, Noack E (1994) Nitric oxide assay using hemoglobin method. Methods Enzymol 233: 240-250

Neill S, Desikan R, Clarke A, Hurst RD, Hancock JT (2002) Hydrogen peroxide and *nitric oxide* as signaling molecules in plants. J Exp Bot 372: 1237-1247

Ninnemann H, Maier J (1996) Indications for occurrence of nitric oxide synthases in fungi and plants and involvement in photoconidiation of *Neurospora crassa*. Photochem Photobiol 64: 393-398

Pedroso MC, Durzan DJ (2000) Effects of different gravity environments on DNA fragmentation and cell death in Kalanchoe leaves. Ann Bot 86: 983-994

Pedroso MC, Magalhaes JR, Durzan DJ (2000) A nitric oxide burst precedes apoptosis in angiosperm and gymnosperm and foliar tissues. J Exp Bot 51: 1027-1036

Rao MV, Davis KR (2001) The physiology of ozone induced cell death. Planta 213: 682-690

Shi SY, Wang G, Wang YD, Zhang LG, Zhang LX (2005) Protective effect of nitric oxide against oxidative stress under ultraviolet-V radiation. Nitric Oxide 13: 1-9

Strid A, Chow WS, Anderson JM (1994) UV-B damage and protection at the molecular level in plants. Photosynth Res 39: 475-489

Vass I, Sass L, Spetea C, Bakou A, Ghanotakis DF, Petrouleas V (1996) UV-B induced inhibition of photosystem II electron transport studied by EPR and chlorophyll fluorescence: Impairment of donor and acceptor side components. Biochem 35: 8964-8973

Vega MP, Pizarro RA (2000) Oxidative stress and defence mechanisms of the freshwater cladoceran *Daphnia longispina* exposed to UV radiation. J Photochem Photobiol 54: 121-125

Veljovic-Jovanovic S, Noctor G, Foyer CH (2002) Are leaf hydrogen peroxide concentrations commonly overestimated? The potential influence of artefactual interference by tissue phenolics and ascorbate. Plant Physiol Biochem 40: 501-507

Wu CH, Tewari RK, Hahn EJ, Pa KY (2007) Nitric oxide elicitation induces the accumulation of secondary metabolites and antioxidant defense in adventitious roots of *Echinacea purpurea*. J Plant Biol 50: 636-643

Zago E, Morsa S, Dat JF, Alard P, Ferrarini A, Inzé D, Delledonne M, Breusegem FV (2006) Nitric oxide- and hydrogen peroxide-responsive gene regulation during cell death induction in tobacco. Plant Physiol 141: 404-411

Zeier U, Delledonne M, Mishina T, Severi E, Sonoda M, Lamb C (2004) Genetic elucidation of nitric oxide signaling in incompatible plant-pathogen interactions. Plant Physiol 136: 2875-2886

Zhang MX, An LZ, Feng HY, Chen T, Chen K, Liu YH, Tang HG, Chang JF, Wang XL (2003) The cascade mechanism of nitric oxide as second messenger of ultraviolet-B in inhibiting mesocotyl elongations. Photochem Photobiol 77: 219-225

Zhao L, Zhang F, Guo J, Yang Y, Li B, Zhang L (2004) Nitric oxide functions as a signal in salt resistance in the calluses from two ecotypes of reed. Plant Physiol 134: 849-857

Zhao MG, Tian QY, Zhang WH (2007) Nitric oxide synthase-dependent nitric oxide production is associated with salt tolerance in *Arabidopsis*. Plant Physiol 144: 206-217