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Nitric Oxide Alleviates Oxidative Damage in the Green Alga *Chlorella pyrenoidosa* Caused by UV-B Radiation

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ABSTRACT. The effect of ultraviolet-B radiation (UV-B; 280–320 nm) on induction of nitric oxide was estimated in the suspensions of green alga *Chlorella pyrenoidosa* with or without the NO scavenger *N*-acetyl-L-cysteine, and reductants such as 1,4-dithiothreitol, glutathione (reduced form), and ascorbic acid. Exogenously added sodium nitroprusside (NO donor), glutathione, 1,4-dithiothreitol, and ascorbic acid were able to prevent chlorophyll loss mediated by UV-B. Addition of NO to algal suspensions irradiated by UV-B increased the activity of catalase and superoxide dismutase but lowered the activity of phenylalanine ammonia-lyase. UV-B thus appears to be a strong inducer of NO production, exogenously added NO and reductants protecting the green alga against UV-B-induced oxidative damage.

Many environmental factors, such as trioxygen (ozone), air pollution, irradiation or heavy metal stress, cause plants to produce reactive oxygen species and lead to oxidative stress. Reactive oxygen species act as transduction signals in plant defense response, but also effect cellular damage. Reactive oxygen species such as the superoxide radical-anion ($O_2^{\bullet -}$), hydroxyl radical ($HO^{\bullet}$) and H_2O_2, are generated during normal biochemical processes that include mithochondrial or chloroplast transfer of electrons (Schreck and Bacuerle 1991). Reactive oxygen species are reduced and turned into O_2 by antioxidant enzymes such as superoxide dismutase (EC 1.15.1.1) and catalase (EC 1.11.1.6). Plants usually increase the activity of antioxidant enzymes when threatened by oxidative damage.

Many studies showed that UV-B radiation causes oxidative stress in plants (Danilov and Ekelund 2000). The reactive oxygen species are not only responsible for UV-B radiation induced damage but can act as second messengers. Many measurements have shown that in a variety of aquatic systems algal cells can be damaged by current UV-B radiation levels, which have deleterious effects on growth, pigmentation, carbon fixation and motility (Jeanine *et al.* 1999). There is strong evidence that exposure to UV-B results in the generation of reactive oxygen species within the chloroplast, and in very rapid perturbation of thylakoid membranes (Chow *et al.* 1992).

Nitric oxide as a simple molecule has been proved to be involved in many physiological processes (Sigler *et al.* 1999; Koutný 2000). Its physiological action in many events involves a number of redox and additive interactions with target molecules (Stamler 1994; Majumdar *et al.* 2000; Mittal *et al.* 2001, 2002; Trebichavský *et al.* 2001). Klepper (1991) found emissions of NO from several plants after herbicide treatment or under anaerobic conditions in darkness. Nitric oxide can induce hypersensitive resistance response in soybean cells inoculated with avirulent *P. syringae* pv. *glycinea*, NO generation corrrelating with H_2O_2 accumulation (Delledonne *et al.* 1998). Mallick *et al.* (2000*a*,*b*) found that the green alga *Scenedesmus obliquus* produces NO when exposed to herbicides, Cu^{II} or Fe^{III}. Nitric oxide and reactive oxygen species were shown as early signaling components of responses to UV-B in *Arabidopsis* (Mackerness *et al.* 2001).

Here we report on NO production by the green alga *Chlorella pyrenoidosa* in response to UV-B radiation and on the effect of agents such as sodium nitroprusside, *N*-acetyl-L-cysteine, 1,4-dithiothreitol, glutathione and ascorbic acid.

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MATERIALS AND METHODS

Cell culture. Chlorella pyrenoidosa (*Fresh Alga Cultures* at the *Institute of Hydrobiology, Chinese Academy of Sciences*, Wuhan, Peoples Republic of China) was cultured in axenic BBM cultures (Bischoff and Bold 1963), under about 60 μmol m^{-2} s^{-1} white light/dark cycle (12/12 h) at 25 ± 1 °C. Before treatment, the suspensions were filtrated by vacuum on 1.2-μm Millipore filters (*Sigma*), washed four times with deionized water and incubated again in fresh culture medium (in mg/L: $NaNO_3$ 250, KH_2PO_4 175, NaCl 125, K_2HPO_4 75, $CaCl_2$ 25, $MgSO_4$ 7.5; pH 7.0). The fresh culture suspension was divided into parts (A_{540} 0.401 ± 0.001) containing about 1.83×10^7 cells per mL.

UV-B irradiation. The cell suspensions were irradiated from above in open glass vessels (diameter 150 mm, depth 20 mm), containing 25 mL algal suspension over a 1.2-μm Millipore filter. Below the filter was 25 mL identical medium but without algae. The UV-B radiation was provided by a UV-B lamp (*BaoJi Lamp Factory*, China). UV-B light passed through a narrow-band filter (about 10-nm band, transmission maximum being at 297 nm). Irradiation time was 4, 8 and 12 h, intensity 0.2 J m^{-2} s^{-1}.The spectral irradiance from the lamps was determined with an Optronics Model 742 (*Optrons Labs*, USA) spectroradiometer. The suspension was continuously stirred.

Measurement of nitric oxide. The culture solution beneath the filter was incubated with 200 U catalase and 200 U superoxide dismutase (*Sigma*) for 5 min to remove reactive oxygen species before addition to oxyhemoglobin (10 μmol/L). After a 5-min incubation, NO was quantified by spectrophotometric measurement (conversion of oxyhemoglobin to methemoglobin; Murphy and Noack 1994; Delledonne *et al.* 1998).

Determination of activity of superoxide dismutase, catalase, and phenyalanine ammonia-lyase. After the treatment, the cells were collected by centrifugation (3000 ***g***, 15 min) and homogenized in 5 mL homogenization buffer (2 % polyvinylpyrrolidone (*W*/*V*), pH 7.8, 4 °C); the homogenizate was centrifuged (10 000 ***g***, 20 min, 2 °C). The supernatant was used as the reaction fraction. Superoxide dismutase activity was assayed according to Zhou *et al.* (1986) with pyrogallol (1,2,3-benzenetriol) at 30 °C. The reaction medium consisted of 50 mmol/L Tris–HCl (2.8 mL, pH 8.2) and 0.2 mL crude extract. Superoxide dismutase activity was expressed as the increase in A_{325} per min in the crude extract. Catalase activity was assayed according to Aebi (1984) by measuring the drop in hydrogen peroxide at 240 nm at 25 °C in a reaction medium consisting of 50 mmol/L potassium phosphate buffer (pH 7.0), 14 mmol/L hydrogen peroxide (2.8 mL) and 0.2 mL crude extract. Phenylalanine ammonia-lyase (EC 4.3.1.5) activity was estimated according to Zimmermann and Hahlbrock (1975) by measuring ammonia at 290 nm in the crude extract at 30 °C. The reaction medium consisted of 50 mmol/L Tris–H_2SO_4 (2.8 mL, pH 8.8), 20 μL phenylalanine solution and 0.2 mL crude extract.

Data analysis was performed by use of MS Excel 97 (*Microsoft*). Every experiment was done in triplicate.

RESULTS AND DISCUSSION

The *Chlorella* cells produced NO under UV-B radiation. Table I shows that when irradiated by UV-B light, the alga produced NO. *N*-Acetyl-L-cysteine (the scavenger of NO) obviously reduced the NO production. Glutathione, 1,4-dithiothreitol and ascorbic acid also blocked NO formation. There are many sources of NO production in plants. Mallick *et al.* (2000*a*,*b*) showed that enhanced activity of nitrate reductase under optimal light intensity may cause increased nitrite production and give rise to a high rate of NO formation. Corn nitrate reductase produced NO when nitrite and NADPH were provided as substrates (Yamasaki and Sakihama 2000). Some data suggest that NO is an intrinsic radical molecule which also occurs in photosystem II (Guittet *et al.* 1999). Mackerness *et al.* (2001) showed that in *Arabidopsis* sp. UV-B exposure leads to NO production through increased nitric-oxide synthase activity.

Exogenous NO prevented chlorophyll decay in *C. pyrenoidosa* under UV-B irradiation (Table I). It was reported that the reaction of NO with reactive oxygen species could prevent the injury to the chloroplast membrane and loss of chlorophyll (Beligni and Lamattina 1999).

The activity of antioxidant enzymes (superoxide dismutase, catalase) was increased while the activity of phenylalanine ammonia-lyase was reduced by exogenous NO, 1,4-dithiothreitol, *N*-acetyl-L-cysteine, ascorbic acid and glutathione in response to UV-B radiation. Phenylalanine ammonia-lyase is an enzyme induced by irradiation and by elicitors.

Table I. Effect of UV-B radiation (0.2 J m^{-2} s^{-1}) on the nitric oxide production (10^{-2} μmol/L) in algal suspension, chlorophyll content (μg/L algal suspension[a]) after 4, 8 and 12 h, and enzyme activity (all per g protein per min) of catalase (CAT, ΔA_{240}), superoxide dismutase (SOD, ΔA_{325}), and phenylalanine ammonia-lyase (PAL, ΔA_{290}) in algal cells (mean values of three different experiments ± SD)

Treatment	mmol/L	Nitric oxide production	Chlorophyll content						CAT	SOD	PAL
			4		8		12				
			Chl *a*	Chl *b*	Chl *a*	Chl *b*	Chl *a*	Chl *b*			
None[b]	–	0.70 ± 0.56	650 ± 30	1160 ± 50	920 ± 50	1560 ± 40	1170 ± 64	2100 ± 120	22.1 ± 1.0	14.8 ± 1.3	21.3 ± 10.3
+ UV-B		24.6 ± 2.31	400 ± 20	720 ± 40	370 ± 20	670 ± 40	320 ± 10	580 ± 30	59.3 ± 0.6	43.0 ± 0.7	442 ± 60.1
Sodium nitroprusside	0.5	30.3 ± 3.39	720 ± 40	1300 ± 70	930 ± 50	1680 ± 90	1230 ± 60	2220 ± 110	29.6 ± 1.2	24.1 ± 2.2	22.1 ± 35.0
+ UV-B		38.5 ± 2.79	610 ± 30	1100 ± 60	670 ± 40	1210 ± 70	710 ± 40	1280 ± 70	96.3 ± 1.5	78.0 ± 1.1	252 ± 50.1
1,4-Dithiothreitol	1	0.76 ± 0.28	630 ± 30	1140 ± 60	830 ± 50	1500 ± 60	1100 ± 60	1980 ± 200	40.8 ± 1.5	22.0 ± 2.1	20.3 ± 5.6
+ UV-B		16.8 ± 1.31	590 ± 20	1060 ± 60	620 ± 40	1120 ± 60	680 ± 40	1230 ± 40	81.5 ± 1.4	63.0 ± 1.5	210 ± 40.3
N-Acetyl-L-cysteine	1	0.53 ± 0.32	570 ± 30	1030 ± 60	860 ± 40	1550 ± 80	1050 ± 70	1890 ± 130	119 ± 1.6	70.5 ± 1.5	26.7 ± 15.4
+ UV-B		14.0 ± 1.44	500 ± 30	900 ± 240	460 ± 20	830 ± 40	430 ± 20	770 ± 40	29.6 ± 0.6	11.1 ± 1.0	321 ± 46.2
Ascorbic acid	1	0.62 ± 0.36	680 ± 40	1220 ± 70	850 ± 40	1520 ± 80	1100 ± 60	1980 ± 120	55.6 ± 1.1	44.5 ± 1.0	24.1 ± 4.3
+ UV-B		18.2 ± 2.16	580 ± 30	1050 ± 50	600 ± 40	1080 ± 80	630 ± 30	1100 ± 60	22.7 ± 0.9	14.8 ± 1.3	312 ± 51.0
Glutathione	1	0.47 ± 0.12	600 ± 40	1080 ± 40	860 ± 50	1550 ± 80	1000 ± 60	1800 ± 100	25.9 ± 2.1	22.3 ± 2.0	23.3 ± 5.8
+ UV-B		18.4 ± 1.30	630 ± 40	1140 ± 70	660 ± 50	1180 ± 90	680 ± 40	1230 ± 80	37.1 ± 1.2	31.0 ± 0.8	287 ± 60.4

[a]Initial values: Chl *a* 560, Chl *b* 1010. [b]Control.

Reactive oxygen species such as superoxide radical-anion ($O_2^{\bullet -}$), hydroxyl radical ($HO^{\bullet}$) and H_2O_2 are generated during normal biochemical processes that include mitochondrial or chloroplast transfer of electrons and other reactions. The reactive oxygen species are not only responsible for UV-B radiation-induced damage but can act as second messengers. NADPH oxidase produces superoxide radical-anion, which can be rapidly converted to H_2O_2 and O_2 by superoxide dismutase and catalase. Nitric oxide was shown to play a key function in plant disease resistance, plant defense response, and in programmed cell death (Delledonne *et al.*; Durner *et al.* 1998).

Tobacco mosaic virus (TMV) increased nitric-oxide synthase activity in tobacco plants (Durner *et al.* 1998). It was proved that NO modulated the activity of tobacco aconitase (Navarre *et al.* 1999). Nitric oxide, which serves as a key redox active signal molecule, has been implicated in the activation of plant defense. Some events mediated by reactive oxygen species during the infection of potato leaves by *Phytophthora* sp. have been examined, and NO was shown to decrease markedly ion leakage from the tissue (Beligni and Lamattina 1999).

The interaction of NO with reactive oxygen species suggests that it might have a physiological function in the hypersensitive disease-resistance response. Reactive oxygen species appear to play key roles in both the early and late stages of the resistance response (Pennell and Lamb 1997; Alvarez *et al.* 1998). In plants, NO was demonstrated to act synergistically with reactive oxygen species to increase host cell death in soybean suspension cells infected with *Pseudomonas syringae*. Nitric-oxide synthase inhibitors compromise the hypersensitive response in tobacco (Delledonne *et al.* 1998). Nitric oxide was able to reduce the oxidative injury produced by drought on wheat seedlings (Beligni and Lamattina 2001).

The changes of catalase, superoxide dismutase and phenylalanine ammonia-lyase activity in UV-B-irradiated *Chlorella* cells upon addition of NO points to the antioxidant properties of NO and its role as anti-stress molecule in plants. Here we showed that exogenously added NO was able to prevent UV-B radiation induced chlorophyll loss, enhance the activity of superoxide dismutase and catalase, and decrease the activity of phenylalanine ammonia-lyase, like the antioxidants 1,4-dithiothreitol, ascorbic acid, and glutathione.

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