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Research paper

Ultraviolet-B-induced flavonoid accumulation in *Betula pendula* leaves is dependent upon nitrate reductase-mediated nitric oxide signaling

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Nitric oxide (NO) is an important signaling molecule involved in many physiological processes in plants. Nitric oxide generation and flavonoid accumulation are two early reactions of plants to ultraviolet-B (UV-B) irradiation. However, the source of UV-B-triggered NO generation and the role of NO in UV-B-induced flavonoid accumulation are not fully understood. In order to evaluate the origin of UV-B-triggered NO generation, we examined the responses of nitrate reductase (NR) activity and the expression levels of *NIA1* and *NIA2* genes in leaves of *Betula pendula* Roth (silver birch) seedlings to UV-B irradiation. The data show that UV-B irradiation stimulates NR activity and induces up-regulation of *NIA1* but does not affect *NIA2* expression during UV-B-triggered NO generation. Pretreatment of the leaves with NR inhibitors tungstate (TUN) and glutamine (Gln) abolishes not only UV-B-triggered NR activities but also UV-B-induced NO generation. Furthermore, application of TUN and Gln suppresses UV-B-induced flavonoid production in the leaves and the suppression of NR inhibitors on UV-B-induced flavonoid production can be reversed by NO via its donor sodium nitroprusside. Together, the data indicate that *NIA1* in the leaves of silver birch seedlings is sensitive to UV-B and the UV-B-induced up-regulation of *NIA1* may lead to enhancement of NR activity. Furthermore, our results demonstrate that NR is involved in UV-B-triggered NO generation and NR-mediated NO generation is essential for UV-B-induced flavonoid accumulation in silver birch leaves.

Keywords: *Betula pendula* Roth (silver birch), flavonoids, nitric oxide, nitrate reductase, UV-B irradiation.

Introduction

The increased ultraviolet-B (UV-B) radiation reaching the earth's surface due to the decrease in the thickness of the ozone layer has attracted considerable attention in recent years. Enhanced UV-B radiation (280–320 nm) may have diverse effects on plants. Ultraviolet-B has the highest energy of any part of the daylight spectrum and has the potential to damage macromolecules, including DNA, and to impair cellular processes (Björn 1996, Frohnmeyer and Staiger 2003, Brown et al. 2005). Organisms have therefore evolved mechanisms to protect against UV-B and to repair UV damage (Rozema et al. 1997). One of the most important protective mechanisms in higher plants is the accumulation of UV-absorbing compounds in epidermal tissues (Jordan 1996, Rozema et al. 1997). On the other hand, UV-B is not simply an agent of damage, but a key environmental signal that regulates diverse processes in a range of organisms (Björn 1996, Jansen et al. 1998, Frohnmeyer and Staiger 2003, Ulm and Nagy 2005). Given the broad significance of regulatory responses to UV-B, it is important to understand the underlying mechanisms of UV-B perception and signal transduction.

Nitric oxide (NO) is emerging as an important signaling molecule with many biological functions in plants, such as stimulation of seed germination and root growth, induction of

plant defense responses, and defense gene activation (Neill et al. 2003, Besson-Bard et al. 2007, Wilson et al. 2008). Enhanced UV-B radiation triggers rapid NO generation in plants (He et al. 2005, Qu et al. 2006, Tossi et al. 2009). However, the source of UV-B-induced NO is still unclear. In mammals, NO production is mainly mediated by NO synthase (NOS), which catalyzes the conversion of L-Arg to L-citrulline and NO (Furchgott 1995). A NOS-like activity has been suggested by pharmacological studies in plants (Durner and Klessig 1999, Corpas et al. 2004, 2008, Jasid et al. 2006, Tian et al. 2007). However, the molecular identity of plant NOS is unknown. An Arabidopsis (*Arabidopsis thaliana*) *AtNOS1* gene that encoded a protein with sequence similarity to a protein that is involved in NO synthesis in snails was isolated by Guo et al. (2003). However, further studies have discounted the possibility that AtNOS1 per se is an Arg-dependent NOS enzyme (Crawford et al. 2006, Zemojtel et al. 2006). Accordingly, AtNOS1 was renamed AtNOA1 for NO Associated1 (Crawford et al. 2006). Recently, Flores-Pérez et al. (2008) demonstrated that the accumulation of plastid-targeted enzymes of the methylerythritol pathway conferring resistance to fosmidomycin in an isolated *noa1* allele named *rif1* (for *resistant to inhibition by fosmidomycin1*) is insensitive to NO donor, thus suggesting that the loss of NOA1/RIF1 function affects physiological processes unrelated to NO synthesis. Several studies have shown that NOA1/RIF1 is a plastid GTPase that is not directly related to NO production; rather, it may be required for proper protein synthesis in plastids (Flores-Pérez et al. 2008, Moreau et al. 2008).

In addition to NOS-mediated NO production, several other NO synthesis pathways have been reported in plant cells (Yamasaki 2000, Berthke et al. 2004, Tun et al. 2006, Rumer et al. 2009, Gupta and Kaiser 2010, Gupta et al. 2011*a*, 2011*b*), among which nitrate reductase (NR), usually associated with nitrogen assimilation, can mediate NO generation from nitrate in an NAD(P)H-dependent manner (Dean and Harper 1988, Yamasaki et al. 1999, Rockel et al. 2002). Nitrate reductase activity can be triggered by both biotic and abiotic stresses (Yamamoto et al. 2003, Salgado et al. 2007, Lozano-Juste and Leon 2010). The involvement of NR-mediated NO production in physiological processes in plants has been demonstrated using *Arabidopsis* mutants defective in NR activity (Desikan et al. 2002, Bright et al. 2006, Modolo et al. 2006). Recently, NR-mediated NO generation has been reported to play roles in cold acclimation and freezing tolerance (Cantrel et al. 2011, Gupta et al. 2011*a*, 2011*b*).

The rapid accumulation of UV-absorbing substances such as flavonoids is one of the early reactions of plants to UV-B (Li et al. 1993). Their physiological relevance as UV-B sunscreens of flavonoids was confirmed by the UV-B hypersensitive phenotype of mutants devoid of these compounds on the one hand and the increased resistance to UV radiation of mutants with enhanced flavonoid levels on the other hand (Li et al. 1993, Landry et al. 1995, Bieza and Lois 2001). Although the accumulation of flavonoid is one of the most effective protection responses of plants to UV-B, the underlying mechanisms of UV-B signal transduction leading to flavonoid accumulation are poorly understood (Frohnmeyer and Staiger 2003, Brown et al. 2005). Reprogramming of metabolism and accumulation of secondary metabolites are the common reactions of plants to biotic and abiotic stresses. It has been well characterized that stress-triggered secondary metabolite production is mediated by plant endogenous signaling, among which NO plays an important role (Hahlbrock et al. 2003, Xu and Dong 2005, Xu et al. 2005). For example, NO has been reported to be involved in taxol production in *Taxus yunnanensis* cells challenged with a fungal-derived cerebroside (Wang et al. 2007), hypericin production of *Hypericum perforatum* cells induced by heat shock (Xu et al. 2008) and artemisinin production in *Artemisia annua* hairy roots triggered by oligosaccharide (Zheng et al. 2008). Given that NO is involved in plant secondary metabolite biosynthesis, it is not unexpected that NO plays a role in UV-B-induced flavonoid accumulation. However, the number of detailed studies to date addressing the source of UV-B-triggered NO generation and the role of NO in UV-B-induced flavonoid accumulation is quite low, especially with tree species.

The objective of this work is to examine the role of NR in UV-B-induced NO generation and flavonoid accumulation in plants. We assayed the responses of NR activity and expression levels of *NIA1* and *NIA2*, two genes encoding NR, in the leaves of *Betula pendula* Roth (silver birch) seedlings to UV-B irradiation and examined the effects of NR inhibitors on UV-B-induced NO generation and flavonoid accumulation. The data indicate that UV-B irradiation induces up-regulation of *NIA1* and stimulates NR activity. Furthermore, we demonstrate that UV-B-induced NO generation is at least partially dependent on NR and that NR-mediated NO signaling is essential for UV-B-induced flavonoid accumulation in the leaves of silver birch seedlings.

Materials and methods

Seedling cultivation and treatments

Seeds of *B. pendula* Roth (silver birch) obtained from the Chinese Academy of Forestry (Dongxiaofu 1, Haidian, Bejiang, China) were sown on trays containing per-fertilized nursery pert B6, sand and vermiculite (7:2:1). The seeds were germinated in the greenhouse at 22 °C and 50% relative air humidity from 06:00 to 20:00 and at 18 °C and 40% relative humidity from 20:00 to 06:00. During this initial stage, plants received only sunlight passing through the 4 mm glass of the roof of the greenhouse. One month after germination, the

seedlings were transferred to growth chambers (ZSX650GS, Wuhan Ruihua Scientific Co., China) characterized by a 12 h photoperiod, day/night temperatures of 22/18 °C, ~50% humidity and 300 μmol m^{-2} s^{-1} photon flux density (PAR 400–700 nm). The seedlings were fertilized with nutrients twice a week (100 ml), according to Ingestad (1962).

One week after the transfer, plants were irradiated for 2 h with 5.3 kJ m^{-2} of biologically effective UV-B radiation (UV-BBE) supplied by fluorescent lamps (40 W/12; Beijing Lighting Research Institute, Beijing, China). The lamps were placed directly above the seedlings and filtered with either 0.13 mm thick cellulose diacetate (transmission down to 290 nm) for UV-B irradiance or 0.13 mm polyester plastic films (absorbing all radiation <320 nm) which acted as the control. Cellulose diacetate filters were presolarized. The desired irradiation was obtained by changing the distance between the lamps and the seedlings. The spectral irradiance from the lamps was determined with an Optronics Model 720 (Beijing Normal University Optronics Factory, Beijing, China) spectroradiometer. The spectral irradiance was weighted using a generalized plant response action spectrum (Caldwell 1971) and normalized at 300 nm to obtain the desired level of biologically effective UV-B radiation. In order to evaluate the possible role of NO in UV-B-induced flavonoid accumulation pharmaceutically with NO scavengers and inhibitors, we exposed plants to UV-B just once.

For chemical treatments, plants were sprayed with NO scavengers and inhibitors (0.5 mM 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (cPTIO), 0.1 mM tungstate (TUN), 5.0 mM glutamine (Gln), 0.5 mM N^G-nitro-L-Arg methyl ester (L-NAME) and 0.5 mM *S,S′*-1,3-phenylene-bis(1,2-ethanediyl)-bis-isothiourea (PBITU)) for a 1 h infiltration period before UV-B irradiation. After UV-B exposure, the youngest unfolded leaf was harvested from the plants at the time indicated in the figures for NR activity, NO, gene expression and flavonoid analysis, respectively. All chemicals were purchased from Sigma Corporation (St Louis, MO, USA) unless otherwise noted.

Quantification of NO by hemoglobin assay

NO accumulation was assayed and calculated by following the conversion of oxyhemoglobin (HbO_2) to methemoglobin (MetHb) spectrophotometrically at 401 and 421 nm, using an extinction coefficient of 77 mm^{-1} cm^{-1} (A_{401} HbO_2, A_{421} MetHb) (Murphy and Noack 1994, Pasqualini et al. 2009). Oxyhemoglobin was prepared as detailed previously (Clarke and Higgins 2000). Fresh leaves (150 mg FW) were snap frozen and homogenized in a mortar with 100 mm K-phosphate buffer (pH 7.0) and 0.6% (w:v) insoluble polyvinylpolypyrrolidone. The extract was clarified by adding powdered activated carbon and centrifuged at 11,000*g* for 10 min at 4 °C. The supernatant was filtered through a polytetrafluoroethylene Millipore membrane (0.45 μm) and immediately assayed for NO. Five minutes before oxyhemoglobin addition, samples were pretreated with catalase (100 U) and superoxide dismutase (100 U) to remove ROS. To evaluate the percentage of recovery of NO during extraction, 1 mm sodium nitroprusside (SNP), which releases 5 μm NO at room temperature, was added to the cells and the measured recovery after Millipore filtration ranged from 70 to 76%.

Determination of NR activity

NR activity was assayed following the method of Scheible et al. (1997) with some modifications. About 1 g of leaves was ground with liquid N_2 and then resuspended in extraction buffer containing 100 mM HEPES-KOH (pH 7.5), 1 mM EDTA, 10% (v/v) glycerol, 5 mM dithiothreitol, 0.1% Triton X-100, 0.5 mM phenylmethylsulfonyl fluoride, 20 μM flavin adenine dinucleotide 1 μM leupeptin, 5 μM Na_2MoO_4 and 1% polyvinylpyrrolidone. After centrifuging at 10,000*g* for 20 min at 4 °C, the supernatant was used for NR determination. The NR activity was measured by mixing 1 volume of extract with 5 volumes of prewarmed (25 °C) assay buffer (100 mM HEPES-KOH, pH 7.5, 5 mM KNO_3 and 0.25 mM NADH). The reaction was started by the addition of assay buffer, incubated at 25 °C for 30 min, and then stopped by adding 0.1 M zinc acetate. After 15 min, the tubes were centrifuged at 13,000*g* for 5 min. The nitrite produced was measured colorimetrically at 520 nm by adding 1 ml of 1% (w/v) sulfenilamide in 3 M HCl plus 1 ml of 0.02% (v/v) *N*-(1-naphthyl)-ethylenediamine in distilled water. The protein content of the extract was determined by the method of Bradford (1976).

Gene expression analysis by quantitative real-time polymerase chain reaction

Leaves of the seedlings with different treatments were collected in liquid nitrogen as described above. The gene expression pattern was assayed using real-time quantitative reverse transcription (RT)-polymerase chain reaction (PCR). Total RNA was extracted from the leaves with Trizol reagent (Invitrogen) and treated with RNase-free DNase I (Promega) at 37 °C for 30 min to eliminate contamination with genomic DNA, extracted with phenol/CHCl (1:1) and precipitated overnight at −80 °C with NaAc 1:10 with 2.5 volumes of absolute ethanol. The total RNA was reverse transcribed into first-strand cDNA with SuperScript II reverse transcriptase (Invitrogen), and the cDNAs obtained were used as templates for PCR amplification with specific primers. Primers for the genes in silver birch were designed with primer 3: www primer tool (University of Massachusetts Medical School, Worcester, MA, USA) and checked by PCR with the cDNA as template before they were used in real-time PCR. Reverse transcription PCR was carried out in a TL988C instrument (Tianlong Science and Technology) and monitored with SYBR-green I dye (Roche). Triplicates of

10 µl PCR reactions of each sample were done in a 384-well plate. Relative expression levels were calculated according to the $2^{-\Delta\Delta C_T}$ method (Livak and Schmittgen 2001) using actin as a reference gene.

Gene-specific primers used for real-time quantitative RT-PCR were 5′-AGTACGGTAAATTCTGGTGCTGGTG-3′ and 5′-CCCTATC TCTCCTCTATGAGGCTTG-3′ for *AtNIA1*, 5′-GACGCCGAACTCGC CGACGAAG-3′ and 5′-TGTCTCTCCACCATCTACCGTGACCTC-3′ for *AtNIA2*, and 5′-CCACATGCTATTCTGCGTTTGGACC-3′ and 5′-CATCCCTTACGATTTCACGCTCTGC-3′ for *Actin11*. The same amplification reaction was conducted with an *Actin11* gene and used as template RNA loading control.

Sequence data from this article can be found in the GenBank/EMBL data libraries under accession numbers NM_106425 (*AtNIA1*), NM_103364 (*AtNIA2*) and NM_112046 (*Actin11*).

Determination of flavonoids

Flavonoids were extracted and hydrolyzed from the samples according to the method reported by Hertog et al. (1992) with minor modifications. Briefly, 0.5 g dried samples were weighed into a 100 ml Erlenmeyer flask and dispersed in 40 ml of 62.5% aqueous methanol containing 2 g/l 2,(3)-*tert*-butyl-4-hydroxyanisole. The mixture was then ultrasonicated for 5 min. To this extract 10 ml of 6 M HCl was added. The sample was bubbled with nitrogen for ~40–60 s, after which the flask was sealed tightly. Hydrolysis was carried out in a shaking water-bath at 90 °C for 2 h. After hydrolysis the sample was allowed to cool; then it was filtered, made up to 100 ml with methanol and ultrasonicated for 5 min. Before flavonoid quantification the sample was filtered through a 0.2 µm membrane filter. Flavonoids were quantified by the high-performance liquid chromatographic method as reported previously (Mattila et al. 2000). It has been reported that quercetin is one of the main flavonoids in silver birch (Kotilainen et al. 2009, Morales et al. 2010). The flavonoids in leaves of the silver birch were quantified as quercetin at 340 nm (Yao et al. 2004).

Statistics

All experiments were repeated three times with seedlings of the same age. Data from experiments were analyzed by the *t*-test for simple comparisons between each treatment and its control and by Tukey's test for multiple comparisons between means. The assumptions of analysis of variance were considered to be statistically significant at $P < 0.05$.

Results

Involvement of NO in UV-B-induced flavonoid accumulation

It has been characterized that accumulation of flavonoids is a common reaction of plants to UV-B (Li et al. 1993). Figure 1 shows the time course of flavonoid accumulation of silver birch leaves after UV-B irradiation. As shown in the figure, flavonoid content in the leaves of silver birch irradiated with UV-B is significantly increased as compared with those of the control without UV-B irradiation, which is in agreement with the previous reports that UV-B radiation stimulates flavonoid biosynthesis in plants (Kotilainen et al. 2009, Morales et al. 2010). Since the UV-B-induced flavonoid accumulation lasts for a few days in silver birch leaves, the data of UV-B-induced flavonoids are presented on a daily timescale (Figure 1).

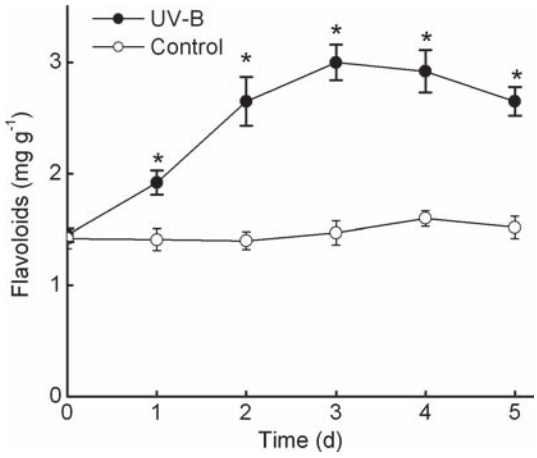

Figure 1. Flavonoid content in silver birch leaves. Seedlings irradiated with UV-B as described in Materials and methods were harvested at the time indicated in the figure for determining flavonoids. Seedlings that received no UV-B irradiation served as control. Data are means ± SE of three replicates. Asterisks show significant differences ($P < 0.05$) for the samples between UV-B treatment and control taken at the same time point.

Results also show that NO generation of silver birch leaves is significantly increased immediately after UV-B irradiation (Figure 2), which suggests that UV-B radiation may trigger rapid NO burst in the leaves. Since the UV-B-triggered NO burst occurs within a few hours, the data of UV-B-triggered NO generation are presented on an hourly timeline (Figure 2).

The above results indicate that flavonoid accumulation and NO generation are two responses of silver birch leaves to UV-B radiation. Moreover, UV-B triggers NO burst before flavonoid accumulation occurs, which suggests that NO signaling might be upstream of UV-B-induced flavonoid accumulation. To further evaluate the role of NO signaling in UV-B-induced flavonoid accumulation, we examined the effects of cPTIO, a specific NO scavenger that has been widely used in plants to abolish stress-induced NO generation (Besson-Bard et al. 2007, Wilson et al. 2008), on UV-B-induced flavonoid accumulation. As shown in Figure 3, pretreatment of the leaves with cPTIO significantly inhibits UV-B-induced flavonoid accumulation, while treatment of the control leaves with cPTIO has no effects on flavonoid content of the control leaves. The results demonstrate that NO is essential for UV-B-induced flavonoid accumulation.

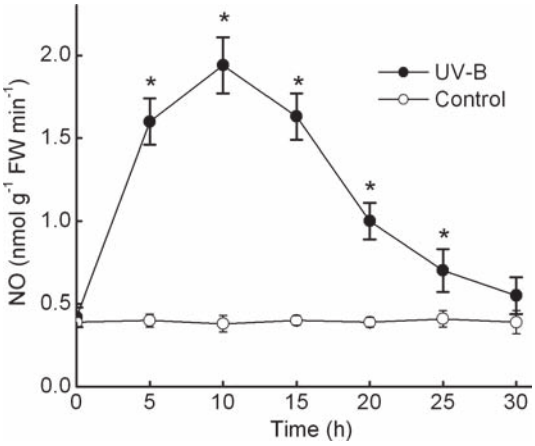

Figure 2. Ultraviolet-B-triggered NO generation of silver birch leaves. Seedlings irradiated with UV-B as described in Materials and methods were harvested at the time indicated in the figure for NO analysis. Seedlings that received no UV-B radiation served as control. Data are means ± SE of three replicates. Asterisks show significant differences ($P < 0.05$) for the samples between UV-B treatment and control taken at the same time point.

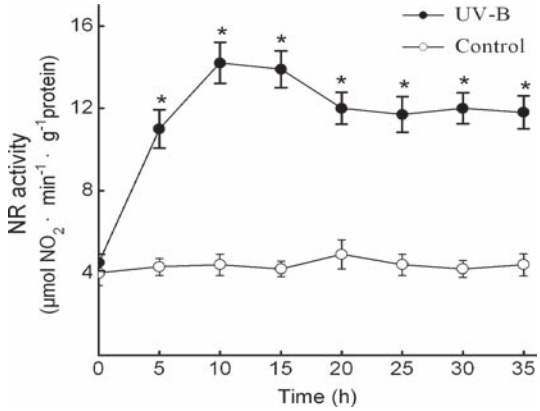

Figure 4. Ultraviolet-B-induced NR activity of silver birch leaves. Seedlings that were irradiated with UV-B as described in Materials and methods were harvested at the time indicated in the figure for NR analysis. Seedlings that received no UV-B radiation served as control. Data are means ± SE of three replicates. Asterisks show significant differences ($P < 0.05$) for the samples between UV-B treatment and control taken at the same time point.

Responses of NR and NIA1 and NIA2 genes to UV-B

In order to evaluate the possible role of NR in UV-B-triggered NO generation, we assayed the responses of NR activity in silver birch leaves to UV-B radiation. As shown in Figure 4, NR activity in leaves irradiated with UV-B displays a time-dependent increase, reaching the highest level at ~10 h after UV-B irradiation, while NR activity of control leaves without UV-B radiation remains unchanged during the same period, which indicates that the increases in NR activity in the UV-B-radiated leaves are not due to development-dependent changes in NR activity. Thus, the results clearly indicate that the UV-B radiation triggers NR activity in the leaves.

In addition to NR activity, we also examined the responses of *NIA1* and *NIA2*, two genes encoding NR in plants (Wilkinson and Crawford 1993), of silver birch leaves to UV-B radiation by quantitative real-time PCR. As shown in Figure 5, the expression of *NIA1* gene in the leaves irradiated with UV-B is significantly increased, being ~4.3-fold of the control 10 h after UV-B irradiation, while expression of *NIA1* of the control leaves was relatively constant during the same period. In contrast, the expression of *NIA2* gene of the leaves irradiated with UV-B remains unchanged from 0 to 20 h after UV-B irradiation as compared with that of control (Figure 5b), showing that *NIA2* expression in the leaves is not affected by UV-B during the period of UV-B-triggered NO generation (0–20 h after UV-B radiation), although the expression levels of *NIA2* increased 25 h after UV-B radiation.

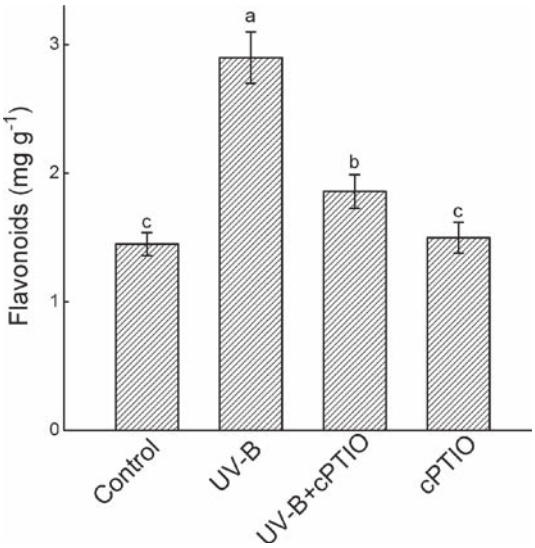

Figure 3. Inhibition of NO-specific scavenger (cPTIO) on UV-B-induced flavonoid accumulation. Seedlings that were pretreated with 0.5 mM cPTIO were irradiated with UV-B as described in Materials and methods and harvested 3 days after UV-B treatment for flavonoid analysis. Seedlings that received no UV-B radiation served as control. Data are means ± SE of three replicates. Different letters show significant differences between means ($P < 0.05$).

Dependence of UV-B-triggered NO generation on NR

The above results show that UV-B radiation triggers NR activity in the leaves. In order to further examine whether NR is involved in UV-B-triggered NO generation, we assayed the effects of NR inhibitors TUN and Gln on UV-B-induced NO generation. The results show that pretreatment of the leaves with TUN and Gln inhibits not only UV-B-triggered NR activity but also UV-B-induced NO generation (Figures 6 and 7), which suggests that NR is involved in UV-B-induced NO generation in the leaves. In addition, we examined the effects of NOS inhibitors L-NAME and PBITU on UV-B-induced NO generation. As shown in Figure 7, pretreatment of the leaves with NOS inhibitors has only slight effects on UV-B-induced NO generation.

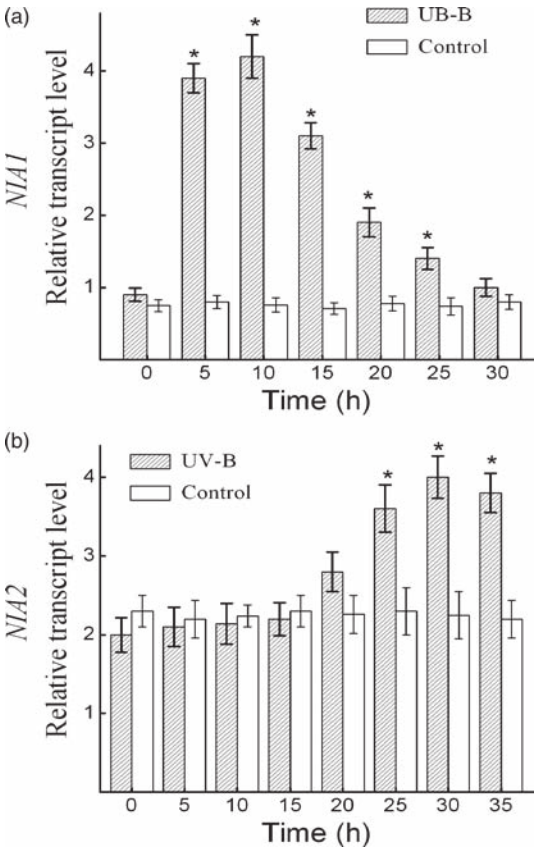

Figure 5. Effects of UV-B on expression levels of *NIA1* (a) and *NIA2* (b) genes of silver birch leaves. Seedlings irradiated with UV-B as described in Materials and methods were harvested at the time indicated in the figure for the determination of gene expression levels with real-time quantitative RT-PCR. Seedlings that received no UV-B radiation served as control. Data are means ± SE of three replicates. Asterisks show significant differences ($P < 0.05$) for the samples between UV-B treatment and control taken at the same time point.

Involvement of NR-mediated NO signaling in UV-B-induced flavonoid accumulation

To evaluate the possible role of NR in UV-B-induced flavonoid accumulation, we examined the effects of NR inhibitors TUN and Gln on flavonoid content of leaves irradiated with UV-B. The results show that the flavonoid content of leaves treated with UV-B + NR inhibitors (TUN or Gln) is significantly reduced compared with that of leaves irradiated with UV-B alone (Figure 8), which indicates that pretreatment with NR inhibitors may suppress UV-B-induced flavonoid accumulation. Furthermore, the data show that flavonoid content of leaves treated with UV-B + TUN or Gln + SNP (NO donor) is increased to the levels of the leaves treated with UV-B alone, although treatment of leaves with SNP alone has no effect on flavonoid content (Figure 8), which suggests that the suppression of NR inhibitors on UV-B-induced flavonoid accumulation (UV-B + TUN or Gln) can be reversed by application of NO via its donor SNP (UV-B + TUN or Gln + SNP). Moreover, the results show that the reversion of SNP on NR inhibitor-suppressed flavonoid accumulation in the UV-B-irradiated leaves (UV-B + TUN or Gln + SNP) can be abolished by NO-specific scavenger cPTIO (UV-B + TUN or Gln + SNP + cPTIO, Figure 8).

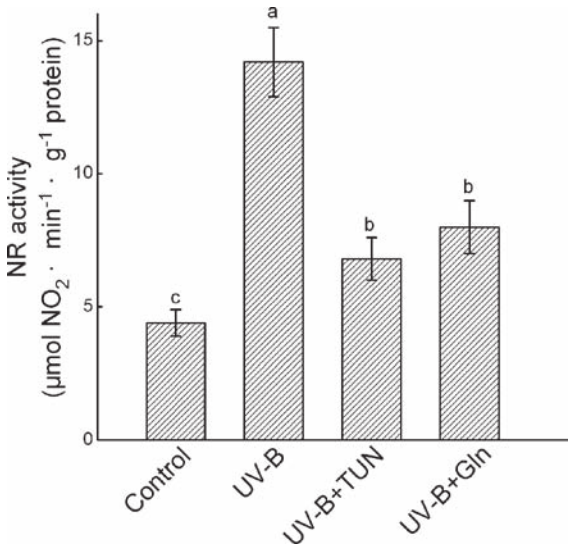

Figure 6. Effects of TUN and Gln on UV-B-induced NR activity. Seedlings that were pretreated with 0.5 mM TUN and 10 mM Gln were irradiated with UV-B as described in Materials and methods and harvested 10 h after UV-B treatment for NR analysis. Seedlings that received no UV-B radiation served as control. Data are means ± SE of three replicates. Different letters show significant differences between means ($P < 0.05$).

Discussion

Plants respond to UV-B radiation by activating various defense responses, among which NO generation and flavonoid accumulation are two early reactions. NO burst has been reported to be a common reaction of plants to multiple biotic and abiotic

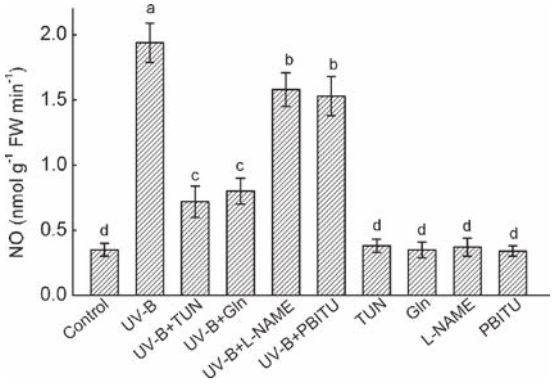

Figure 7. Effects of inhibitors on UV-B-triggered NO generation. Seedlings that were pretreated with 0.1 mM TUN, 5 mM Gln, 0.5 mM L-NAME and 0.5 mM PBITU were irradiated with UV-B as described in Materials and methods and harvested 10 h after UV-B treatment for NO analysis. Seedlings that received no UV-B radiation served as control. Data are means ± SE of three replicates. Different letters show significant differences between means ($P < 0.05$).

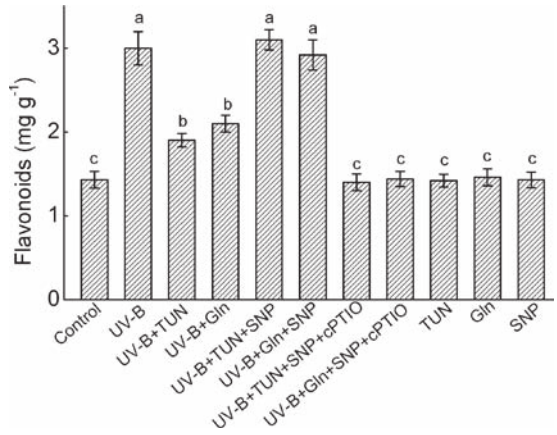

Figure 8. Effects of inhibitors and SNP on UV-B-induced flavonoid accumulation. Seedlings that were pretreated with 0.1 mM TUN, 5 mM Gln, 0.4 mM cPTIO and 10 μM SNP (NO donor) as indicated in the figure were irradiated with UV-B as described in Materials and methods and harvested 3 days after UV-B treatment for flavonoid analysis. Seedlings that received no UV-B radiation served as control. Data are means ± SE of three replicates. Different letters show significant differences between means ($P < 0.05$).

stresses (Neill et al. 2003, Besson-Bard et al. 2007, Wilson et al. 2008). However, the origin of stress-triggered NO generation is largely unknown. Several NO biosynthesis pathways have been reported to operate in plants, among which NR can mediate NO production from nitrite (Dean and Harper 1988, Yamasaki et al. 1999). Nitrate reductase activity can be triggered by multiple stresses and is considered to be an important source of NO in plants. The involvement of NR-mediated NO production in physiological processes in plants has been demonstrated using *Arabidopsis* mutants defective in NR activity (Bright et al. 2006, Modolo et al. 2006, Zhao et al. 2009). However, there has been no detailed study to evaluate the role of NR in UV-B-induced NO generation in plants. To investigate whether NR is involved in UV-B-induced NO generation of plants, we analyzed NR activity of silver birch leaves irradiated with UV-B and examined the effects of NR inhibitors on UV-B-induced NO generation. The results show that the NR activity of silver birch leaves irradiated with UV-B is significantly increased as compared with that of the control, indicating that NR activity is inducible by UV-B in the leaves. Furthermore, treatment of the leaves with NR inhibitor TUN not only suppresses UV-B-induced NR activity but also abolishes UV-B-triggered NO generation, which suggests that NR might be essential for UV-B-triggered NO generation in the leaves. Since the TUN treatment could also have inactivated other important molybdenum-containing enzymes and signaling components, a second strategy has been used to further evaluate the involvement of NR as a biosynthetic source of NO in UV-B-treated silver birch leaves, i.e., pretreatment of the leaves with Gln, a feedback NR inhibitor. As with TUN treatment, exogenous application of Gln suppresses both UV-B-induced NR activity and UV-B-triggered NO generation in the leaves. Thus, our data suggest that NR is involved in UV-B-triggered NO generation.

NR was encoded by two genes *NIA1* and *NIA2* in *Arabidopsis* (Wilkinson and Crawford 1993). Using mutants such as the double mutant *nia1*/*nia2*, Desikan et al. (2002) showed that NR was involved in abscisic acid (ABA)-induced NO generation in guard cells. Further experiments with the single mutants *nia1* and *nia2* indicated that *nia2* mutant was little affected in its ability to produce NO and the most influential NR enzyme in guard cell NO generation was that encoded by *NIA1* (Bright et al. 2006). The results of this work show that the expression level of *NIA1* in silver birch leaves irradiated with UV-B is significantly increased as compared with that of the control, while UV-B treatment has little effect on expression of *NIA2* during the period of UV-B-triggered NO generation. Moreover, UV-B-induced *NIA1* expression coincides with UV-B-triggered NO generation and NR activity. Thus, our results suggest that the UV-B-triggered NR activity during UV-B-triggered NO generation might be due to the up-regulation of *NIA1*. Together with the observation that expression of *NIA1*, but not *NIA2*, is essential to hormonal and developmental cues (Yu et al. 1998) and that *NIA1* is responsible for cold acclimation-induced NO generation in *Arabidopsis* (Zhao et al. 2009), it is suggested that the NR isoform NIA1 has a definite role in the production of NO in response to stimuli.

In addition to NR-mediated NO generation, several other NO synthesis pathways have been reported in plants, among which NOS, the main source of NO in animals, is considered to be the possible source of NO in plants (Yamasaki 2000, Berthke et al. 2004). A NOS-like activity has been suggested by pharmacological studies in plants (Durner and Klessig 1999, Corpas et al. 2004, Tian et al. 2007) and proposed to be involved in cytokinin-triggered NO burst in *Arabidopsis*, parsley and tobacco (Tun 2001, Carimi et al. 2006). It has been reported that UV-B-induced NO generation in maize and *Ginkgo biloba* cells can be partially suppressed by NOS inhibitors (Hao et al. 2009, Tossi et al. 2009), suggesting that UV-B-induced NO generation might be dependent on NOS-like activity. In order to evaluate the possible role of NOS in UV-B-induced NO generation in silver birch leaves, we examined the effects of NOS inhibitors L-NAME and PBITU on UV-B-triggered NO generation of the leaves. The data show that treatment of the leaves with NOS inhibitors only has a slight effect on UV-B-triggered NO generation. In a previous study, we reported that the fungal elicitor induced NOS-like activity and NO generation in *H. perforatum* cells (Xu et al. 2005). However, the NOS-like activity and NO generation in the cells did not match kinetically, and the fungal elicitor-induced NOS-like activity was much lower than NO production (Xu et al. 2005), showing that the fungal elicitor-induced NO of the cells is not mainly dependent on NOS-like activity. Our results strongly suggest that NOS-like activity is not responsible for NO generation in UV-B-irradiated silver birch leaves.

Plants respond to UV-B radiation by triggering various defense responses, among which the defense-oriented reprogramming of metabolism is one of the common reactions. Among the major changes in cellular metabolism is the rapid accumulation of phenolic compounds in UV-B-irradiated plants (Li et al. 1993, Morales et al. 2010). Flavonoids, with absorption bands in the range 240–545 nm (Cerovic et al. 2002), act as sunscreens of plant leaves, protecting the inner cells from harmful radiation (Jordan 2002, Bassman 2004). Although flavonoid accumulation has been well characterized to be one of the effective defense reactions of plants to UV-B radiation, the molecular basis of UV-B-induced flavonoid accumulation is still not clearly known. It has been documented that the stress-induced secondary metabolite production is mediated by endogenous signaling, in which NO has been reported to play an important role (Zhao et al. 2005, Xu 2007). The results of our work show that irradiation of silver birch leaves with UV-B induces both flavonoid accumulation and NO generation. Moreover, our data demonstrate that NR is responsible for UV-B-triggered NO generation. Given that NO is involved in secondary metabolite production, it is therefore deduced that NR should play a role in UV-B-induced flavonoid accumulation. The data of the present work show that pretreatment of silver birch leaves with NR inhibitors not only suppresses UV-B-triggered NR activity but also abolishes UV-B-induced flavonoid accumulation, which provides definite evidence to support the deduction. Furthermore, our results show that the suppression of NR inhibitors on UV-B-induced flavonoid accumulation can be reversed by NO via its donor SNP and that the reversion of SNP on NR inhibitor-suppressed flavonoid accumulation can be abolished by the NO-specific scavenger cPTIO. Together, the results demonstrate that NR-mediated NO generation is involved in UV-B-induced flavonoid accumulation.

The dose-dependent effects of NO on secondary metabolite production of plants have been reported. In a previous study, we found that treatment of high-dose NO via its donor SNP stimulated matrine production of *Sophora flavescens* cells, while low concentrations of SNP had no effects on the production of secondary metabolites (Xu and Dong 2008). The results of the present work show that application of low concentrations of NO via its donor SNP has no effect on flavonoid accumulation in silver birch leaves but can reverse the NR inhibitor-suppressed flavonoid accumulation in UV-B-irradiated silver birch leaves (Figure 8). The data imply that some unknown components that are triggered in UV-B-irradiated leaves might be needed to act cooperatively with NO to mediate flavonoid accumulation. It is apparent that much more work is still needed to understand the molecular mechanism of UV-B-induced flavonoid accumulation.

It has been well characterized that the response of plants to UV-B may be affected by multiple factors. In order to check whether or not the UV-B signaling mechanism is appropriate for the other conditions, we repeated the experiments under high PAR, i.e., 800 $\mu mol\ m^{-2}\ s^{-1}$ photon flux density. The results obtained still supported the conclusion of this work (data not shown), although the data varied as compared with those under low PAR reported in the present work. However, we cannot yet conclude that the mechanism really reflects the natural world, considering that the response of plants to UV-B depends on the nature of the UV-B treatment, the extent of adaptation and acclimation to UV-B, and interaction with other environmental factors. Therefore, more experiments are needed to fully understand the UV-B signaling mechanism of plants under natural conditions.

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