Plant, Cell and Environment (1998) **21,** 1163–1171

The nopaline synthase (*nos*) promoter is inducible by UV-B radiation through a pathway dependent on reactive oxygen species

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ABSTRACT

The molecular mechanism of plant response to UV-B radiation was studied using the nopaline synthase (*nos*) promoter, which has been shown to be inducible by methyl jasmonate (MJ) and reactive oxygen species (ROS). In the leaves of transgenic tobacco (*Nicotiana tabacum* L.) plants that carried a fusion between the *nos* promoter and the chloramphenicol acetyltransferase (*cat*) gene, 2 h of UV-B treatment resulted in a transient increase in the level of *cat* mRNA, a maximum being reached at 6 h after the UV-B treatment. It was also found that MJ and UV-B enhance *nos* promoter expression via separate pathways. Diethyldithiocarbamic acid, a potent inhibitor of jasmonate production, had little effect on UV-B stimulation of the *nos* promoter. In contrast, antioxidants, such as dimethylthiourea, reduced glutathione, cysteine, N-acetylcysteine and DTT, blocked UV-B induction of the *nos* promoter, but did not affect MJ induction of the *nos* promoter. These results suggest that UV-B induction of the *nos* promoter is mediated via a pathway that requires reactive oxygen species and is distinct from the jasmonate or MJ mediating pathway.

Key-words: methyl jasmonate; *nos* promoter; UV-B radiation; reactive oxygen species; transgenic tobacco leaves.

INTRODUCTION

The destruction of the ozone layer has accelerated during the past two generations, mainly as a result of the release of synthetic chlorofluorocarbons (Kerr & McElroy 1993; Lubin & Jensen 1995). The ozone layer is vital to life on earth because it absorbs a large amount of ultraviolet-B (UV-B) light. As it becomes depleted, the amount of UV-B radiation reaching the earth is increasing. UV-B radiation has therefore received much attention in recent years.

The molecular mechanism of plant response to UV-B radiation is complex. The absorption of UV-B radiation by nucleic acids damages DNA because it leads to production of cyclobutane-type pyrimidine dimers and pyrimidine(6,4)pyrimidone dimers (Chen, Mitchell & Britt 1994; Landry *et al*. 1997; Pang & Hays 1993). In addition, UV-B irradiation, depending on the dosage, influences gene expression through cellular signal transduction pathways. Studies on the chalcone synthase (CHS) gene have shown that UV-B induction of this gene is mediated via signal transduction pathways associated with calcium/calmoduline and reversible protein phosphorylation (Christie & Jenkins 1996; Frohnmeyer, Bowler & Schäfer 1997). Moreover, deletion analysis of the CHS promoter showed that the UV-responding DNA motif is an element that is present in many plant promoters and that it can be activated by other biotic and abiotic stresses (Block *et al*. 1990; Hartmann *et al*. 1998; Kircher *et al*. 1998; Schulze-Lefert *et al*. 1989; Staiger, Kaulen & Schell 1989; van der Meer *et al*. 1990).

Response of plant genes to biotic and abiotic stresses is often mediated by several rapid transduction processes that are associated with the formation of reactive oxygen species (ROS) and induction of lipid peroxidation (Mehdy 1994; Levine *et al*. 1994; Vick & Zimmerman 1987). When plants are attacked by viral or microbial pathogens, the recognition of pathogens or elicitors leads to a rapid formation of ROS. The generation of ROS plays a part in the activation of defence genes (Mehdy 1994; Jabs *et al*. 1997). In addition to ROS, the induction of lipid hydrolysis and peroxidation increases certain compounds that are thought to have stress signal functions. A striking release of jasmonate and its methyl ester derivative, methyl jasmonate (MJ), which are octadecanoic acid derivatives of linolenic acid, has been observed after lipid hydrolysis and peroxidation of plant cells (Creelman & Mullet 1995; Vick & Zimmerman 1987). Rapid lipid hydrolysis has been observed after wounding of plant tissues, and the metabolites hydrolysed from unsaturated fatty acids such as linoleic and linolenic acids are converted to jasmonate and MJ, acting as potent inducers of several plant defensive genes (Creelman, Tierney & Mullet 1992; Farmer & Ryan 1992).

The nature of the molecular mechanism by which UV-B light starts the signal transduction pathway responsible for the activation of defence genes is under debate. Exposure to UV-B radiation also induces ROS production and lipid

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peroxidation, which affect many signal transduction pathways and transcription factors (Stapleton 1992). On one hand, Green & Fluhr (1995) reported that UV-B induction of a pathogenesis-related gene, PR-1a of tobacco, was mediated via an ROS-requiring pathway. On the other hand, a group of pathogenesis-related proteins (PR-1a, PR-3a, and PR-3) of tomato and *Arabidopsis*, which were not inducible by the jasmonate, did not respond to UV irradiation (Conconi *et al*. 1996; Epple, Apel & Bohlmann 1995). Conconi *et al*. (1996) reported that proteinase inhibitors I and II responded to UV-B radiation via the octadecanoid pathway. This implies that UV-B induced expression of plant defense genes is mediated by the same signalling pathway that participates in the jasmonate-mediated response.

We have previously demonstrated that the promoter of the nopaline synthase (*nos*) gene, which comes from an *Agrobacterium* tumour-inducing (Ti) plasmid, responds to various inducing agents including wounding, MJ, auxins, salicylic acid, and H_2O_2 (An, Costa & Ha 1990; Kim, Kim & An 1993; Dai & An 1995). The *nos* promoter had been used for construction of plant selective markers as it was believed to be constitutively active in plant organs (Lichtenstein & Fuller 1987). However, it was later found that *nos* promoter activity is weak in transgenic tobacco plants unless the plants are treated with inducing agents or stress (An, Costa & Ha 1990; Kim, Kim & An 1993; Dai & An 1995). In the present study, we have demonstrated that the *nos* promoter is inducible by UV-B and that the induction is associated with the ROS-mediated pathway, which is distinct from the jasmonate-mediated signalling pathway.

MATERIALS AND METHODS

Plant material and growth conditions

Transgenic tobacco (*Nicotiana tabacum* L.) plants carrying the *nos* promoter–CAT (chloramphenicol acetyltransferase) fusion were used in the present study (An *et al*. 1988). Kanamycin-resistant transgenic plants were selected on agar plates and grown in greenhouse conditions. All of the experiments were performed with the third generation of transgenic plants.

Plant treatments

Fully expanded leaves taken from 8-week-old plants were cut into 1 cm^2 segments. As a control, leaf segments were incubated on a Murashige & Skoog (MS) medium (Murashige & Skoog 1962) in Petri dishes under white light (80–100 $\mu mol\ m^{-2}\ s^{-1}$). White light free of UV light was obtained placing a UV shielding plate (Plexiglas, Röhm-Pharma, Germany) below cool-white fluorescent bulbs (Osram, Germany). UV-B radiation was obtained from two 8 W ULTRA-LUM 300 nm UV-B lamps (ULTRA-LUM UV B-28 lamp, ULTRA-LUM, USA). The fluence rate was measured using an ultraviolet intensity monitor (CDR-1, ULTRA-LUM, USA), and adjusted by varying the distance between the leaf segments and the UV-B light source. All experiments were performed using a range of UV-B fluence rates between 0·12 and 0·24 $\mu mol\ m^{-2}\ s^{-1}$. For treatment with UV-B radiation, the leaf segments were floated on an MS medium, exposed to UV-B radiation for 2 h, and further incubated under white light without activation of the UV-B lamp. The leaf segments were harvested at various time points during the incubation period, frozen in liquid nitrogen, and stored at – 80 °C until processing. MJ was dissolved in ethanol at a concentration of 25 $mmol\ m^{-3}$ and added to an MS medium at a final concentration of 50 $\mu mol\ m^{-3}$. Dimethylthiourea (DMTU) dissolved in ethanol at 100 $mmol\ m^{-3}$ was diluted to final concentrations of 0·1 $mmol\ m^{-3}$ and 1 $mmol\ m^{-3}$ in an MS medium. Cycloheximide (CHX) was dissolved in an MS medium at a final concentration of 300 $\mu mol\ m^{-3}$. Cysteine (CYS), N-acetyl-L-cysteine (NAC), a reduced form of glutathione (GSH), and dithiothreitol (DTT) were dissolved in an MS medium at 5 $mmol\ m^{-3}$ or 30 $mmol\ m^{-3}$, after which the solutions were adjusted to pH 5·7 with NaOH. Diethyldithiocarbamic acid (DIECA) was dissolved in an MS medium at final concentrations between 0·2 and 2·5 $mmol\ m^{-3}$. All of the solutions were prepared fresh immediately before use.

RNA preparation and gel blot analysis

Total RNA was extracted using the Tri-Reagent (Molecular Research Center, Cincinnati, OH, USA) according to the manufacture's instructions. Twenty μg of RNA were separated in an agarose-formaldehyde gel and blotted onto a Hybond-N^+ nylon membrane (Amersham) by capillary blotting (Sambrook, Fritsch & Maniatis 1989). Radioactive probes were synthesized by randomly priming the *cat*-coding region using the Rediprime system (Amersham). Hybridization was performed at 60 °C according to Church & Gilbert (1984). The blots were washed twice in 2 × SSPE at 60 °C for 10 min, followed by a final wash in 0·2 × SSPE at 60 °C for 5 min. The intensity of the hybridization was quantified with the Fuji bio-imaging analyser BAS 1000.

Chlorophyll fluorescence and pigment analysis

Chlorophyll fluorescence was measured at room temperature with a portable modulated fluorometer (Hansatech, UK). The leaf segments were dark-adapted for 15 min, and readings were taken for 5 s as described previously (Oh *et al*. 1996). The ratio Fv/Fm was used to show the potential quantum yield of photochemical reactions of photosystem II (Raggi 1995). Chlorophyll was extracted from leaf segments (0·1 g fresh wt) with 80% (v/v) acetone, and the chlorophyll concentration measured by spectrophotometric quantification (Arnon 1949). For determination of the non-covalently bound haem, leaf segments (0·1 g fresh wt) were ground under liquid nitrogen and the resulting powder

extracted with 250 μm^3 of cold acid–acetone [80% (v/v) acetone containing 5% (v/v) concentrated HCl]. This mixture was centrifuged for 5 min at 13 500 *g* and the pellets re-extracted with two 250 μm^3 portions of acid–acetone solution. The three extracts were then combined. Purification and quantification of haem were performed using a Waters Sep-Pak C-18 cartridge (200 mg, Milford, MA) and reverse-phase HPLC as previously described (Yu & Weinstein 1997).

Other procedures

The CAT assay was performed with 0·5–5 μg of soluble protein and [^{14}C]chloramphenicol at 37 °C for 30 min, and reaction products were separated by thin-layer chromatography (An 1987). Soluble protein was quantified by the Bradford method using bovine serum albumin as a standard (Bradford 1976). Every reported experiment was repeated at least three times with a separated preparation of samples.

RESULTS

UV-B dosage effect

We have studied the effect of UV-B dosage on the *nos* promoter-driven CAT expression in transgenic leaf (Fig. 1). Leaves of transgenic plants that carried the *nos* promoter-*cat* reporter fusion were cut into 1 cm^2 segments, pooled, and then incubated on an MS medium. It was previously reported that leaf segment assays provided homogeneity and accessibility of chemicals, and that the procedure caused mild wounding (An, Costa & Ha 1990). The duration and dosage of UV-B irradiation were chosen to provide conditions optimal for inducing the *nos* promoter-driven CAT activities. As a control, transgenic plants carrying a fusion between the cauliflower mosaic virus (CaMV) 35S promoter and the *cat* coding region were used.

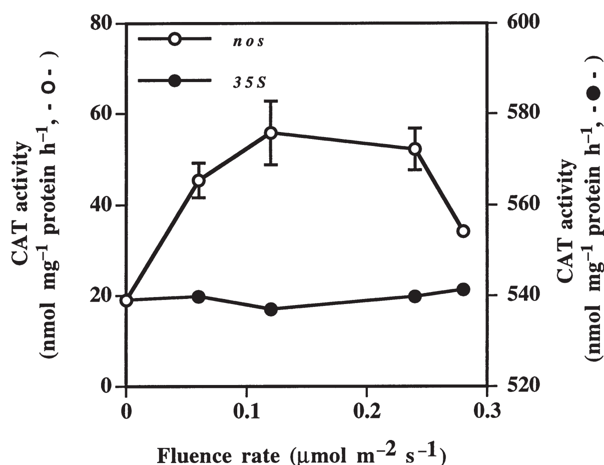

Figure 1. Activities of CAT (chloramphenicol acetyltransferase) driven by the *nos* promoter and the CaMV 35S promoter in response to varied fluence rates of UV-B. Leaf segments from transgenic tobacco plants carrying *nos–cat* and 35S–*cat* fusions were treated with varied fluence rates of UV-B for 2 h and subsequently incubated under white light for 10 h. Values are means ± SE, $n = 3$.

Samples were exposed to various fluence rates of UV-B radiation for 2 h, and CAT activity was measured 10 h later. No significant difference in the CAT level driven from the 35S promoter was found for UV-B fluence rates of 0–0·28 μmol m^{-2} s^{-1}. However, the *nos* promoter-driven CAT activities were dependent on UV-B dosage. The optimum response was observed when leaf segments were exposed to fluence rates of 0·12–0·24 μmol m^{-2} s^{-1} UV-B. At rates above 0·28 μmol m^{-2} s^{-1}, the CAT expression driven from both promoters declined sharply (data not shown). Therefore, UV-B fluence rates of 0·12–0·24 μmol m^{-2} s^{-1} were used in the present study.

UV-B induction time course

To study the kinetics of *nos* promoter induction by UV-B treatment, segments of a fully grown leaf were treated with UV-B radiation for 2 h and further incubated under white light. As a control, leaf segments were incubated under white light during the entire period. These segments demonstrated a progressive increase in *nos* promoter (Fig. 2a). In contrast, UV-B radiation increased CAT activity, the effect starting 4 h after the treatment; after 11 h, the increase was ≈ 4-fold.

RNA blot analysis was performed to study the induction kinetics more accurately. The *cat* mRNA was detectable 2 h after the UV-B treatment (Fig. 2c), whereas it was visible after 8 h in the control leaf segments (Fig. 2b). A maximum level of the transcript was observed 6 h after UV-B treatment and the level decreased thereafter. Therefore it is evident that the UV-B light pulses produce a signal that is sustained for several hours and is available to stimulate *nos* promoter expression. As mRNA accumulation reached a maximum 6 h after the UV-B treatment, this time point was chosen for the experiments described below.

UV-B effect on photosynthetic efficiency

As the leaf segments sustained no visible damage during UV-B treatment, we assessed damage to photosystem II, which is a sensitive target of UV-B and chromophores such as chlorophyll and haem that are associated with photosynthesis (Table 1). UV-B treatment reduced photosystem II efficiency (F_v/F_m) by 13%, but had no effect on chlorophyll content. This result is consistent with the previous observation that chlorophyll reduction did not occur unless plants were exposed to extremely high fluence rates of UV-B (Brandle *et al.* 1977). In contrast, a marked reduction in haem was seen under UV-B light, ≈ 40% of total haem being lost after the UV-B treatment. As haem is a prosthetic component of the electron transport chain in chloroplasts (Beal & Weinstein 1990), the UV-B-induced degradation of haem may be correlated with the reduction in photosystem II efficiency.

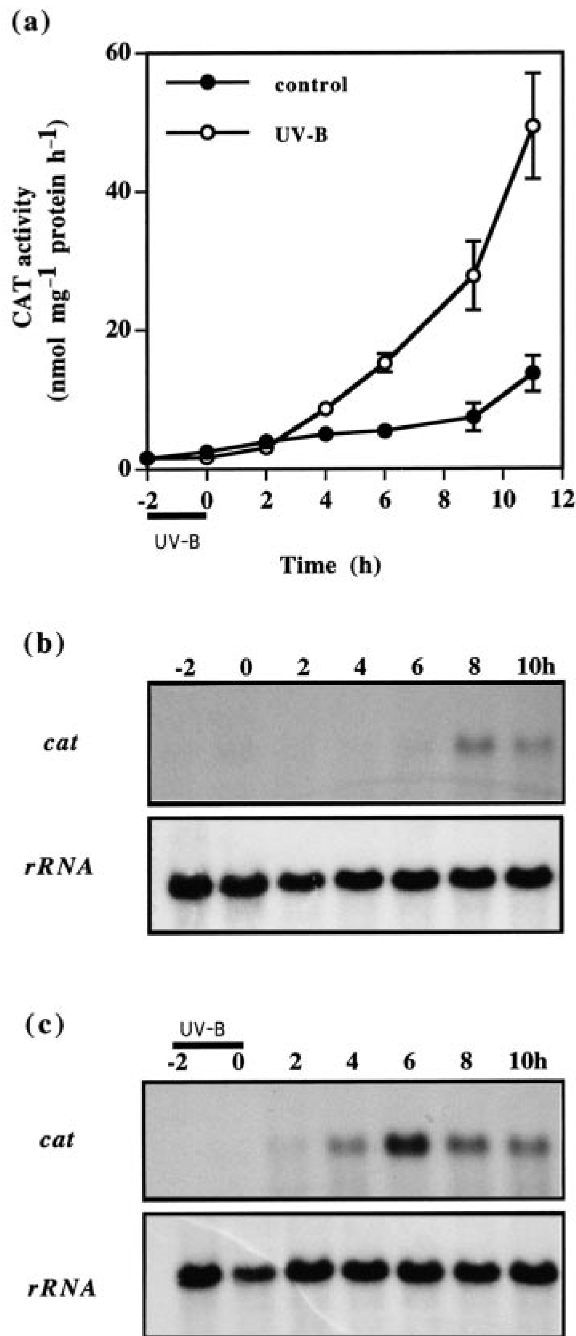

Figure 2. Time course of UV-B induction of the *nos* promoter. Leaf segments from transgenic tobacco plants were treated with UV-B for 2 h and then incubated under white light. Details for UV-B treatment are described in Materials and Methods. (a) CAT activity in control and UV-B treated segments. CAT activity was measured using 5 μg of total soluble protein. Values are means ± SE, n = 3. (b) *cat* mRNA expression in the segments. (c) *cat* mRNA expression in UV-B treated segments. In the experiements illustrated by this and subsequent figures, 20 μg of total RNA was hybridized with a ^{32}P-labelled *cat* probe. Equal loading was confirmed by re-hybridizing with a labelled ribosomal RNA probe.

Relationship between MJ and UV-B induction of the *nos* promoter

It was previously reported that the *nos* promoter was inducible by MJ (Kim, Kim & An 1993). In the present study, we studied the relationship between MJ and UV-B radiation in affecting *nos* promoter activity. In the leaf segments that were treated with MJ and UV-B radiation, the level of the *nos* promoter-driven *cat* mRNA was approximately equivalent to the sum of their effects (Fig. 3a,b). This indicates that the effects of MJ and UV-B radiation are additive, and therefore implies that the effects of MJ and UV-B are mediated by independent pathways.

As it was reported that UV-B induced lipid peroxidation and, as a result, jasmonate and MJ (Stapleton 1992), it was necessary to determine whether the UV-B effect on the *nos* promoter was mediated by increasing the jasmonate level. DIECA is a potent inhibitor of jasmonate production in the octadecanoid pathway (Farmer *et al*. 1994). We therefore studied effects of DIECA on UV-B induction of the *nos* promoter (Fig. 3c). DIECA did not inhibit the UV-B effect on the *nos* promoter, indicating that UV-B induction of the *nos* promoter is mediated by a pathway unrelated to jasmonate.

Effects of antioxidants

We have previously shown that the *nos* promoter is H_2O_2 inducible (Dai & An 1995). As UV-B radiation stimulates ROS production, we have explored whether UV-B induction of the *nos* promoter is mediated by active oxygen radicals. DMTU, a synthetic antioxidant that removes ROS inside cells (Curtis *et al*. 1988), effectively inhibited UV-B induction of the *cat* mRNA at concentrations above 0·1 mmol m^{-3} (Fig. 4a). We also looked at the effects of physiological reducing agents (Fig. 4b). We found that DTT, GSH, CYS, and NAC inhibited UV-B induction of the *nos* promoter-driven *cat* mRNA. These results indicate that UV-B induction of the *nos* promoter activity is mediated by ROS. We also studied the effects of the antioxidants on MJ induction of *nos* promoter activity. MJ inducibility was not affected by GSH and DMTU after 2 h or 6 h treatment (Fig. 5). Neither did CYS, NAC or DTT reduce the inducibility of *nos* promoter activity by MJ (data not shown).

These antioxidants are not likely to cause non-specific degradation of mRNAs, because the presence of antioxidants alone or in combination with MJ application had almost no effect on *cat* mRNA levels (Fig. 5). As a reference, we monitored the expression of the 60S ribosomal (r)-protein L25, which is developmentally and environmentally regulated (Gao *et al*. 1994). We observed that antioxidant treatment caused a slight increase in accumulation of the r-protein L25 transcript (data not shown).

Effect of cycloheximide

To test whether the responses of the *nos* promoter require synthesis of a cytoplasmic protein, we examined the effect

Treatment	PS II efficiency (F_v/F_m)	Chlorophyll $a+b$ (mg mg^{-1} protein)	Haem (μg mg^{-1} protein)
Control	0·8 ± 0·01	1·1 ± 0 4	4·2 ± 0·4
UV-B	0·7 ± 0·01	1·1 ± 0 2	2·5 ± 0·2

Table 1. UV-B effect on the efficiency of the photosystem (PS) II open centres (F_v/F_m) and on total chlorophyll and haem content. Leaf segments from transgenic tobacco plants were exposed to UV-B for 2 h and then incubated under white light for 6 h. Values are means ± SE; n = 3–5

of CHX on *nos* promoter induction in response to UV-B and MJ (Fig. 6a,b). The presence of CHX did not inhibit *nos* promoter activity, but it resulted in superinduction of the promoter. CHX caused an increase of the *cat* mRNA level in the presence and absence of the stimuli. These results indicate that synthesis of a new protein is not required for the induction of the *nos* promoter by UV-B and MJ.

DISCUSSION

The results of this study provide evidence that the *nos* promoter is inducible by UV-B radiation. Transgenic plants carrying the *nos* promoter-*cat* reporter gene exhibited an enhanced levle of *cat* mRNA after 2 h of UV-B pulse. It was reported earlier that CHS promoter-driven GUS activity was stimulated significantly after 30 min of UV-B treatment (Frohnmeyer, Bowler & Schäfer 1997). These observations suggest that signals produced from a UV-B pulse persist for several hours and stimulate certain promoters in plants. Some UV-induced responses are reversible by irradiation with white light. This reversible response may be caused by DNA damage through production of cyclobutane-type pyrimidine dimers and pyrimidine(6,4)pyrimidone dimers (Beggs, Stolzer-Jehle & Wellmann 1985; Chen, Mitchell & Britt 1994; Landry *et al*. 1997). UV-B irradiation, however, influences the cellular signal transduction pathways responsible for gene activation. Thus, induction of the *nos* promoter by UV-B radiation can be regulated by a more specific response which is mediated via a specific signalling pathway.

Although there was no visible damage, the UV-B treatment reduced the electron transport capacity of photosystem II. In addition, a parallel reduction in haem was observed. In the presence of ROS, haem can mediate oxidative degradation of proteins and membrane lipids (Aft & Mueller 1984; Kim & Sevanian 1991). During these processes, haem is also modified and destroyed (Yu & Weinstein 1997). No reduction in chlorophyll content was observed following the UV-B treatment. Our observation is consistent with the earlier study that chlorophyll concentration was not significantly reduced by UV-B irradiation, even though there was a notable decline of photosynthesis (Brandle *et al*. 1977).

The *nos* promoter is MJ inducible (Kim, Kim & An 1993); therefore it can be postulated that the octadecanoid pathway may be involved in the UV-B induction of the *nos* promoter. It was postulated that a group of plant defensive genes, proteinase inhibitors I and II, responded to UV-B by producing jasmonate from lipid peroxidation through the

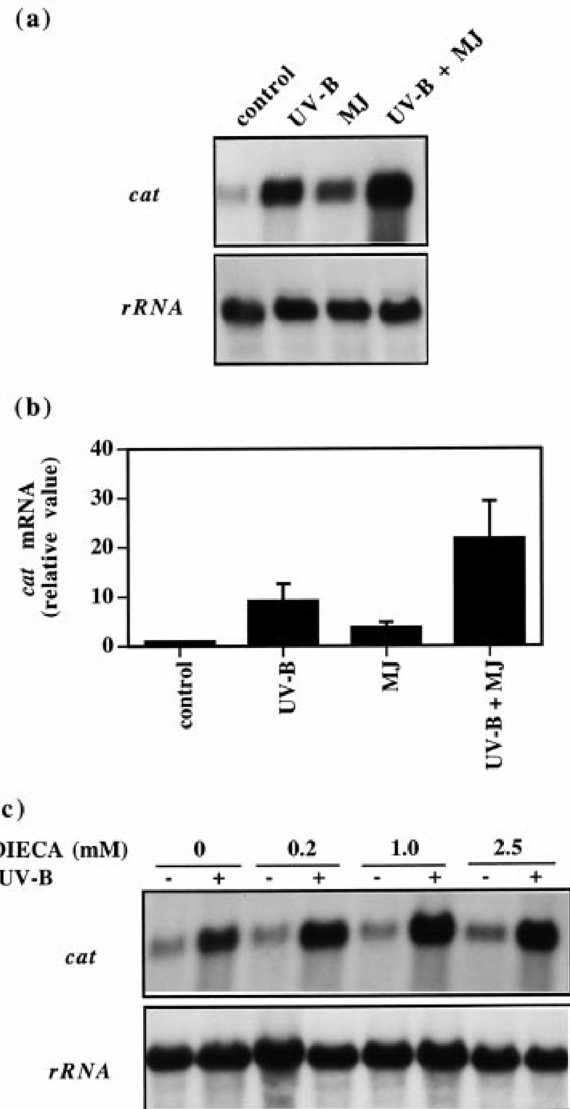

Figure 3. Effects of methyl jasmonate (MJ) and diethyldithiocarbamic acid (DIECA) on UV-B induction of the *nos* promoter. Leaf segments from transgenic tobacco plants were treated with 50 μmol m^{-3} MJ or various concentrations of DIECA and then immediately exposed to UV-B for 2 h, and to white light for a further 6 h. (a) RNA blot analysis for the effect of MJ on UV-B induction; (b) quantitation of RNA blot analysis shown in (a). Relative RNA levels on the basis of the ribosomal RNA signals are plotted. Values are means ± SE, n = 3. (c) RNA blot analysis for the effect of DIECA on the UV-B induction.

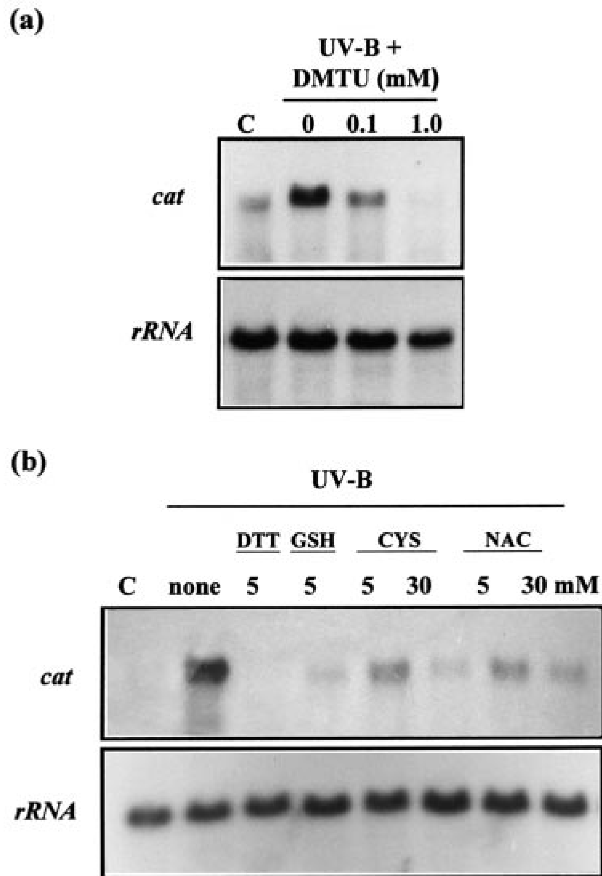

Figure 4. Effects of antioxidants on UV-B induction of the *nos* promoter. Leaf segments from transgenic tobacco plants were treated with (a) dimethylthiourea (DMTU) and (b) thiol antioxidants, and then immediately exposed to UV-B for 2 h, and to white light for a further 6 h. C, control.

octadecanoid pathway (Conconi *et al*. 1996). In support of this hypothesis, in a tomato plant that carried a mutation in the octadecanoid pathway, proteinase inhibitor genes did not respond to UV light. In the present study, we have examined the role of this pathway on UV-B induction of the *nos* promoter by using DIECA. DIECA is an active reducing agent that inhibits the octadecanoid pathway. It does this by efficiently converting 13-hydroperoxylinoleic acid, the product of lipoxygenase acting on linolenic acid, to 13-hydroxylinolenic acid, which is not a signalling intermediate; the pathwaty is thus shut down and becomes a dead end (Farmer *et al*. 1994). Farmer *et al*. (1994) reported that DIECA severely inhibited the accumulation of proteinase inhibitor proteins in wounded tomato plants. In contrast to these results, we observed no inhibition of *nos* promoter activity by DIECA. Therefore, it appears that a diverse array of signal transduction pathways operate during UV-B damage in plant cells. In the present study, we found that UV-B and MJ signals were additive, indicating that MJ and UV-B responses are mediated by separate pathways.

It has been established that UV-B induces ROS production, which affects many signal transduction pathways and transcription factors. Green & Fluhr (1995) have reported that, in tobacco plants, the UV-B induction of a pathogenesis-related gene, PR-1a, is mediated via the ROS-requiring pathway. However, the mechanism for the ROS-mediated UV-B signal transduction is not well understood. In this study, we have observed UV-B induced haem degradation. Heme can undergo enzymatic and non-enzymatic coupled oxidation, which results in ring opening. In both cases, haem degradation requires the presence of ROS (Aft & Mueller 1984; Kim & Sevanian 1991). To test whether

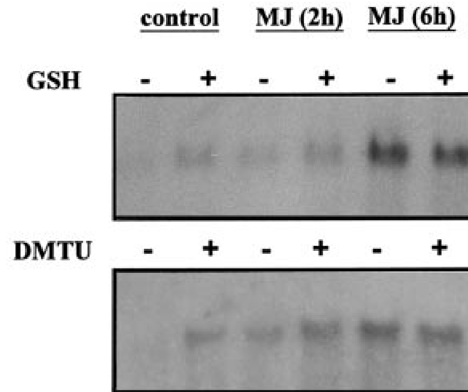

Figure 5. Effects of antioxidants on methyl jasmonate (MJ) induction of the *nos* promoter. Leaf segments from transgenic tobacco plants were treated with 50 μmol m^{-3} MJ for 2 h or 6 h in the presence and absence of antioxidants. Concentrations of reduced glutathione (GSH) and dimethylthiourea (DMTU) were 5 mmol m^{-3} and 1 mmol m^{-3}, respectively.

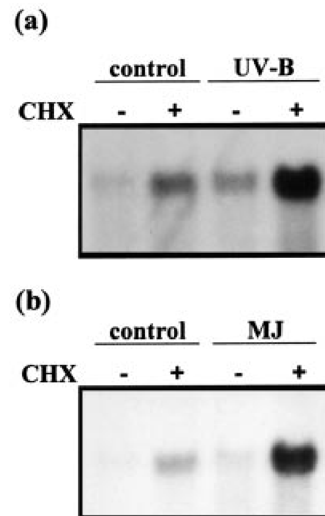

Figure 6. Effects of cycloheximide (CHX) on (a) UV-B and (b) methyl jasmonate (MJ) induction of the *nos* promoter. Leaf segments from transgenic tobacco plants were treated with 300 μmol m^{-3} CHX and then immediately exposed to UV-B for 2 h, and to white light for a further 6 h. In the case of MJ, leaf segments were treated with 300 μmol m^{-3} CHX and then immediately afterwards with 50 μmol m^{-3} MJ, before incubation for 8 h under white light.

ROS play a part in controlling UV-B induction of the *nos* promoter, a synthetic scavenger of oxygen radicals and several thiol antioxidants were used. We found that UV-B induction of the *nos* promoter activity was effectively blocked by the synthetic scavenger of oxygen radicals, DMTU, and by physiological thiol reducing agents such as GSH, precursors of GSH synthesis (CYS and NAC), and DTT. GSH plays a protective role in plants by acting as a reducing agent that removes electrophilic radicals and oxidants (Rennenberg 1982; Alscher 1989). Elevation of the GSH level in fungal elicitor-treated cells demonstrated a protective role by preventing accumulation of ROS (Edwards, Blount & Dixon 1991). This accompanied blocking of the transduction pathway of elicitor signals and the synthesis of phytoalexins (Apostol, Heinstein & Low 1989). In contrast, reducing the intracellular GSH level led to an increase in the level of ROS and phytoalexins (Guo *et al*. 1993). Therefore exogenous application of GSH and other thiol reducing agents can prevent ROS accumulation, thus interrupting the transduction process of the UV-B signal to the *nos* promoter. These antioxidants are not likely to enhance the turnover rate of mRNA, because the presence of antioxidants alone or in combination with MJ application had little effect on *cat* mRNA levels. It can be concluded from these findings that the *nos* promoter responds to UV-B through a ROS-requiring pathway that appears to be independent of the jasmonate-mediated or MJ-mediated induction pathway.

A functional analysis of the *nos* promoter upstream region identified at least three independently acting *cis*-elements that contribute to the *nos* promoter response to inducing agents; these included auxins, salicylic acid, mechanical wounding, MJ and H_2O_2 (An, Costa & Ha 1990; Kim, Kim & An 1993; Dai & An 1995). A 20-nucleotide regulatory element located between –131 and –112 is essential for all inducing agents. Deletion of the 12-nucleotide sequence between –112 and –101 or the CAAT box region significantly reduced responses from wounding, MJ, and H_2O_2. However, the auxin and salicylic acid responses were not significantly affected by the deletions. In the present study, we found that UV-B radiation and MJ induce *nos* promoter expression via separate pathways and that the UV-B signalling pathway used ROS as an intermediate signal. Although we have not identified UV-B-responding *cis*-elements of the *nos* promoter, we can speculate that the distinct UV-B and MJ signal transduction pathways may be regulated by transcription factors that interact with a common set of *cis*-elements in the *nos* promoter.

UV-B induced expression of tobacco PR-1a and *Arabidopsis* CHS was shown to require *de novo* protein synthesis because it was inhibited by CHX (Green & Fluhr 1995; Christie & Jenkins 1996). The feature of PR-1a gene induction by UV-B radiation is typical of late genes. Moreover, the response of CHS gene expression to UV light suggests the involvement of transcription factors that are synthesized early after the start of irradiation (Frohnmeyer *et al*. 1998; Kircher *et al*. 1998). In the case of the *nos* promoter, however, CHX treatment caused a substantial elevation of the transcript level and cotreatment with UV-B radiation or MJ resulted in a synergistic superinduction. The superinduction by CHX is probably mediated by an activation of pre-existing-transcription factors, and it has been observed from many primary response genes, including the mammalian *c-fos* gene (Herschman 1991), auxin-inducible genes (Abel & Theologis 1996), an immediate-early salicylic acid-inducible tobacco gene (Horvath & Chua 1996), CPRF family genes (Kircher *et al*. 1998), and the 35S transcript of CaMV (Qin *et al*. 1994). However, in our experimental conditions, the addition of CHX immediately before the treatments with UV-B radiation and MJ might not be sufficient to block early genes involved in UV-B and MJ responses of the *nos* promoter. Further studies will be required to verify whether the *nos* promoter response occurs via pre-existing transcription factors in the cell.

ACKNOWLEDGMENTS

This work was supported in part by a grant from the Ministry of Education of Korea. We thank Chahm An for critical reading of the manuscript.

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Received 14 May 1998; received in revised form 3 August 1998; accepted for publication 3 August 1998