UV-B–Induced PR-1 Accumulation Is Mediated by Active Oxygen Species

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Depletion of the stratospheric ozone layer may result in an increase in the levels of potentially harmful UV-B radiation reaching the surface of the earth. We have found that UV-B is a potent inducer of the plant pathogenesis-related protein PR-1 in tobacco leaves. UV-B fluences required for PR-1 accumulation are similar to those of other UV-B–induced responses. The UV-B–induced PR-1 accumulation was confined precisely to the irradiated area of the leaf but displayed no leaf tissue specificity. A study of some of the possible components of the signal transduction pathway between UV-B and PR-1 induction showed that photosynthetic processes are not essential, and photoreversible DNA damage is not involved. Antioxidants and cycloheximide were able to block the induction of PR-1 by UV-B, and treatment of leaves with a generator of reactive oxygen resulted in the accumulation of PR-1 protein. These results demonstrate an absolute requirement for active oxygen species and protein synthesis in this UV-B signal transduction pathway. In contrast, we also show that other elicitors, notably salicylic acid, are able to elicit PR-1 via nonreactive oxygen species–requiring pathways.

INTRODUCTION

Recent measurements of ozone levels have led to concern that the stratospheric ozone layer is being depleted as a result of contamination with man-made chlorofluorocarbons (Anderson et al., 1991; Manney et al., 1994). Concomitantly, the amounts of solar UV-B radiation (280 to 320 nm) reaching the surface of the earth is increasing (Kerr and McElroy, 1993). UV-B radiation has been shown to be harmful to living organisms, damaging DNA, proteins, and lipids (Sancar and Sancar, 1988; Kochevar, 1990). Plants, which use sunlight for photosynthesis and are unable to avoid exposure to enhanced levels of UV-B radiation, are particularly at risk. The effects of UV-B radiation on plants include the destruction of plasma membrane–associated ATPases (Imbrie and Murphy, 1984), damage to the D1 reaction center protein of photosystem II (Greenberg et al., 1989; Jansen et al., 1993), and general growth inhibition (Tevini et al., 1989). Thus, mechanisms by which plants may protect themselves from UV-B radiation are of particular interest.

One important defense mechanism appears to be the accumulation of UV-B–absorbing flavonoids and sinapate esters in leaf epidermal cells (Beggs et al., 1986; Li et al., 1993). Genes encoding key enzymes involved in the biosynthesis of flavonoids are induced by UV irradiation (Chappell and Hahlbrock, 1984; Dangl et al., 1987; Douglas et al., 1987). UV-inducible elements have been described in promoters from one of these genes, chalcone synthase, in several species of plants (Schulze-Lefert et al., 1989; Wingender et al., 1990; Fritze et al., 1991). In addition, the enzymes catalyzing phenylproponoid

and flavonoid biosynthesis in plants are induced by a range of other stimuli, including wounding and pathogen attack (for review, see Hahlbrock and Scheel, 1989). Little is known about the signal pathways involved in UV-B–mediated induction of defense genes.

Another class of defense-related proteins that have been found to be induced by a range of elicitors are the pathogenesis-related (PR) proteins. These proteins have been shown to be induced in response to a variety of stimuli, including fungal and bacterial infection and exogenously applied elicitors, such as salicylic acid (SA), ethylene, and xylanase (Asselin et al., 1985; Bol et al., 1990; Lotan and Fluhr, 1990; Eyal et al., 1992). In addition, irradiation with the shorter wavelength, UV-C (200 to 280 nm), has been previously shown to cause induction of all PR protein families (Brederode et al., 1991). However, this induction by UV-C was correlated with extensive leaf damage, which itself might induce a pathogenesis response (Brederode et al., 1991). Furthermore, UV-C is not considered to be a true model for UV-B–induced physiological responses in plants (Stapleton, 1992).

In mammalian cells, UV irradation has been reported to activate a number of defense genes whose regulatory regions contain a specific DNA sequence motif recognized by the transcription factor AP-1 (Devary et al., 1991). UV irradiation was shown to induce the expression of the c-*jun* gene, which encodes a major component of the AP-1 complex (Devary et al., 1991). The pathway that involves UV irradiation–induced c-*jun* expression requires the activation of Src tyrosine kinases followed by the reactive oxygen species (ROS)–dependent activation of Ha-Ras and Raf-1 (Devary et al., 1992).

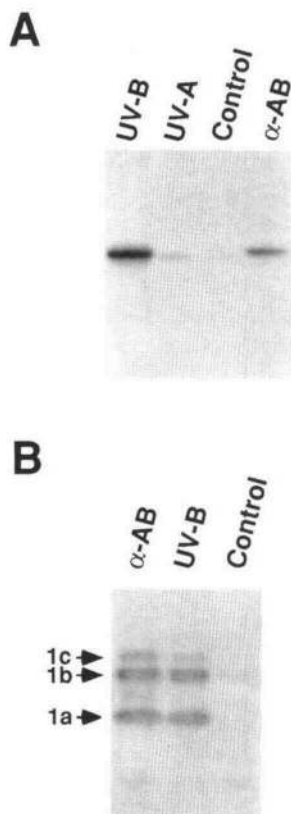[1] To whom correspondence should be addressed.

A

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Figure 1. Induction of PR-1 Accumulation by UV-B.

Protein was extracted from detached leaves that had been treated with 5 mM α-aminobutyric acid (α-AB) or 0.2 μmol m^{-2} sec^{-1} UV-B or UV-A. Each was treated for 22 hr or left untreated (Control). Aliquots (30 μg) from each sample were fractionated using PAGE and immunoblotted with the anti–PR-1 antibody.

(A) Samples were separated using denaturing SDS-PAGE.

(B) Samples were separated using native PAGE. Arrows indicate the different isoforms of PR-1.

Here we definitively show that UV-B induces the accumulation of acidic-type PR-1 protein and start to elucidate some of the steps in the signal transduction pathway between UV-B irradiation and gene expression. We show that ROS and protein synthesis are required in this pathway and compare it with signal transduction pathways induced by other elicitors of defense genes.

RESULTS

Acidic PR-1 Protein Accumulates after UV-B Irradiation

Irradiation of tobacco (cv Samsun NN) leaves with UV-B (280 to 320 nm) caused the accumulation of high levels of acidic-type PR-1 protein (Figure 1A). UV-B appeared to be a potent inducer, consistently inducing equal or higher levels of acidic-type PR-1 protein synthesis than α-aminobutyric acid, which itself has been reported to be an effective elicitor of PR-1 protein induction (Lotan and Fluhr, 1990). Irradiation with UV-A (320 to 360 nm) did not cause induction of PR-1 protein, suggesting that this response is specific to the shorter wavelengths of UV. Treatment with UV-C has been shown to cause extensive leaf damage and was not examined here (Brederode et al., 1991).

Acidic-type PR-1 is encoded by a multigene family. At least three distinct isoforms of PR-1 can be resolved by native PAGE. Figure 1B shows that all three acidic-type PR-1 proteins accumulated after UV-B and α-aminobutyric acid treatment. UV-B also caused the induction, albeit very weakly, of other PR proteins (data not shown).

Analysis of the kinetics of PR-1 mRNA induction by continuous UV-B irradiation showed that the accumulation of PR-1 transcripts could be seen after 10 hr, with high levels of transcript after 24 hr (Figure 2). This is comparable to the time course for PR-1 transcript accumulation seen after other elicitor treatments in tobacco (Ohshima et al., 1990; Brederode et al., 1991; Eyal et al., 1992).

Accumulation of PR-1 Protein Is Fluence Dependent

Leaves were irradiated with UV-B light of varying fluence rates for 22 hr, and a fluence rate response curve was plotted for the accumulation of acidic-type PR-1 protein. Figure 3A shows that the level of protein accumulating depended upon the amount of UV-B given. The minimum fluence rate required for protein accumulation was between 0.08 and 0.2 μmol m^{-2} sec^{-1} UV-B irradiation (equivalent to total fluences of 6 and 16 mmol m^{-2}), and the maximum response was observed when leaves were irradiated with 1 μmol m^{-2} sec^{-1} UV-B (equivalent to a total fluence of 80 mmol m^{-2}). At a fluence rate of 10 μmol m^{-2} sec^{-1} UV-B, PR-1 accumulation decreased. This may have been a result of damage to the leaf. Figure 3B shows that leaves irradiated with 0.2 μmol m^{-2}

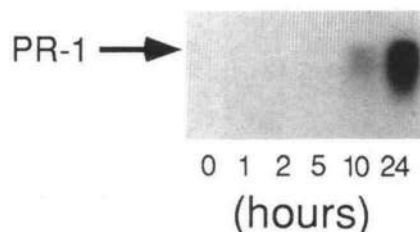

PR-1 ➡

0 1 2 5 10 24

(hours)

Figure 2. Time Course of PR-1 Transcript Accumulation Induced by UV-B.

Total RNA was extracted from detached leaves that had been irradiated with 0.2 μmol m^{-2} sec^{-1} UV-B for 0 to 24 hr. Aliquots (20 μg) from each sample were fractionated in agarose–formaldehyde denaturing gels and hybridized with a probe to PR-1M. The arrow indicates the PR-1 transcripts.
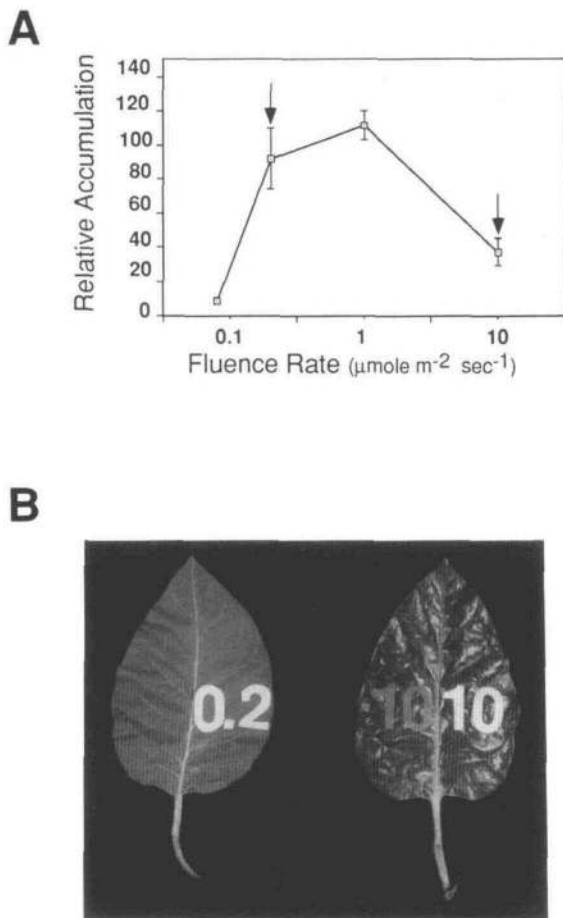
A

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Figure 3. Fluence Rate-Dependent PR-1 Induction and Leaf Bronzing Caused by UV-B.

(A) Protein was extracted from detached leaves that had been irradiated with varying fluences of UV-B for 22 hr. Aliquots (30 μg) from each sample were separated using denaturing SDS-PAGE and blotted with the anti–PR-1 antibody. The amount of acidic-type PR-1 protein in each lane was estimated using a gel scanner and calculated as a percentage of maximum accumulation using an α-aminobutyric acid–induced sample as a standard. Each point is the average of at least three experiments, with three separate leaves being treated in each experiment. The standard error of the mean is shown with error bars. Arrows indicate the rates of UV-B irradiation used to treat leaves in **(B)**.
(B) Detached leaves irradiated with 0.2 or 10 μmol m^{-2} sec^{-1} UV-B for 22 hr (total fluences of 16 and 800 mmol m^{-2}, respectively). Before irradiation, a small area of each leaf was covered by light-opaque foil (shaped as the numbers 0.2 or 10) to shade the leaf from UV-B. Before photographing, the shaded area was uncovered. The foil used for shading is shown on the right of each leaf; the shaded portion is on the left.

sec^{-1} UV-B for 22 hr, which induced high levels of PR-1 accumulation, showed no visible damage. However, in leaves irradiated with 10 μmol m^{-2} sec^{-1} UV-B for 22 hr, lower levels of acidic-type PR-1 accumulated (Figure 3A), and exposed

areas of the leaf showed the classic bronzing and glazing symptoms of UV-B–damaged leaves (Figure 3B; Teramura, 1983). By contrast, shaded areas of the leaf showed no visible damage (Figure 3B). Most subsequent experiments were performed using 0.2 μmol m^{-2} sec^{-1} UV-B for 22 hr, which is well below the range of fluences that caused detectable damage.

Localization of PR-1 Protein in UV-B–Treated Leaves

One characteristic of the induction of PR proteins by tobacco mosaic virus is that it is systemic; that is, treatment of one area of a plant induces a response in the rest of the plant (Vogeli-Lange et al., 1988). In contrast, after treatment with UV-B, PR-1 protein accumulation occurred only in areas of the leaves exposed to irradiation (Figure 4). No accumulation was found in shaded parts of the same leaves, which were protected from irradiation (as shown in Figure 3B), even within 5 mm of the exposed portion of the leaf.

UV-B–absorbing flavonoids and anthocyanins have been shown to accumulate in the epidermal cells of leaves, where they can act as a barrier to UV-B penetration of the underlying tissue (Beggs et al., 1986). To examine the localization of PR-1 protein in UV-B–irradiated tobacco leaves, leaf sections were treated with antibodies raised against PR-1 or the small subunit of ribulose bisphosphate carboxylase (RbcS) as a control. Figure 5C shows that accumulation of RbcS was, as expected, localized to the chloroplast-containing cells of the mesophyll and to the guard cells of the stomata; no RbcS protein was found in the rest of the epidermis. In contrast, PR-1 protein
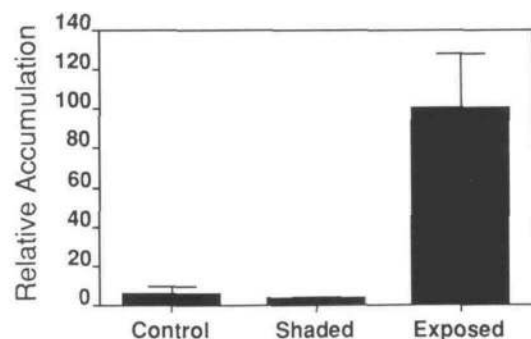

Figure 4. Localization of PR-1 Accumulation in Shaded and Exposed Areas of Leaves.

Leaves were irradiated with 1 μmol m^{-2} sec^{-1} UV-B for 22 hr or left untreated (Control). Before irradiation, a portion of each leaf was shaded using silver foil. Samples were taken from adjacent shaded and exposed sections of the leaves. Protein was extracted, and 30-μg aliquots from each protein sample were separated using denaturing SDS-PAGE and immunoblotted with the anti–PR-1 antibody. The amount of PR-1 protein was estimated using a gel scanner and calculated as a percentage of the accumulation in exposed leaves. Each point is the average of at least three experiments, with three leaves treated in each experiment. Standard error of the mean is shown with error bars.
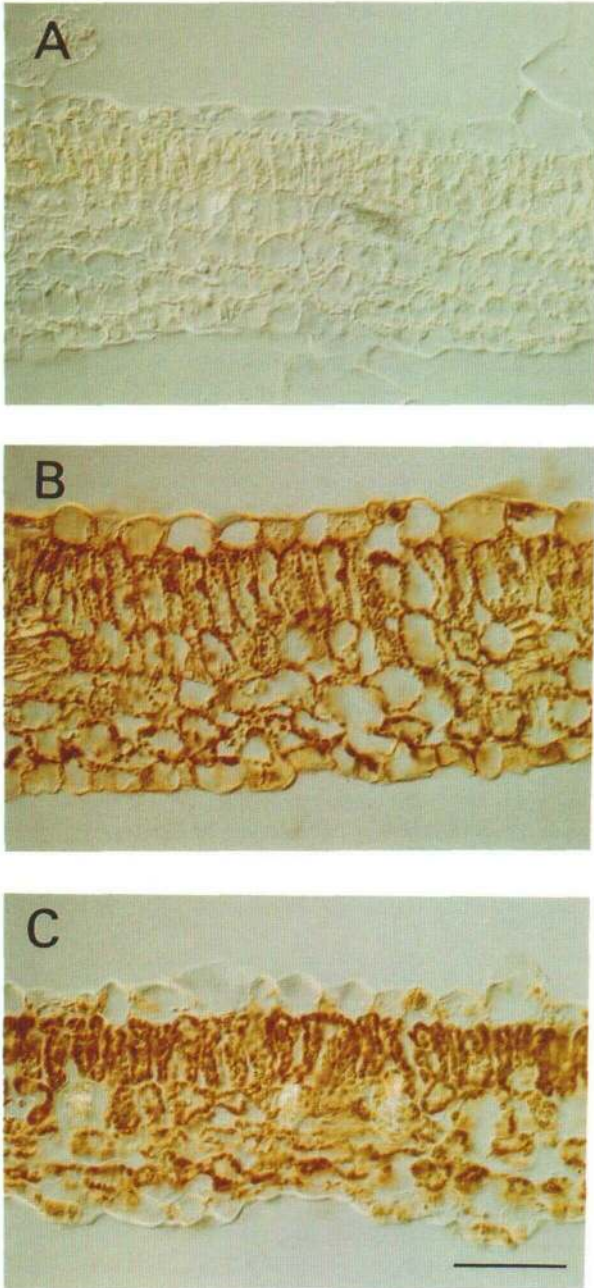
Figure 5. Immunohistochemical Localization of PR-1 Protein in Treated and Untreated Leaves.

Leaves (5-cm long) were either treated with 0.2 μmol m^{-2} sec^{-1} UV-B for 22 hr or left untreated. Sections of the leaves were then immunodressed using antibodies raised against PR-1 and RbcS.

(A) Control leaves immunodressed with antibodies raised against PR-1.
(B) UV-B–treated leaves immunodressed with antibodies raised against PR-1.
(C) UV-B–treated leaves immunodressed with antibodies raised against RbcS.
The bar in **(C)** = 50 μm.

accumulation was not cell specific, and PR-1 protein was found at high levels throughout the leaf section after treatment with UV-B (Figure 5B). Untreated leaves showed no reaction (Figure 5A).

Effects of Visible Light on UV-B Induction of Acidic-Type PR-1 Accumulation

UV-B has been reported to damage DNA in plant cells, resulting in the formation of cyclobutane-type pyrimidine dimers (Quaite et al., 1992). This type of DNA damage in plants can be repaired by simultaneous or subsequent irradiation with light of 370 to 450 nm, or with white light including these wavelengths, in a process known as photoreactivation (Pang and Hays, 1991; Batschauer, 1993). Some UV-induced plant responses are reversed by irradiation with 370- to 450-nm or white light; such responses include the induction of the synthesis of the isoflavonoid coumestrol in bean leaves (Beggs et al., 1985) and damage to Arabidopsis seedlings (Harlow et al., 1994). These responses may involve damaged DNA in the form of cyclobutane-type pyrimidine dimers. To determine whether the effect of UV-B irradiation on the accumulation of acidic-type PR-1 protein was via photoreversible DNA damage, leaves were irradiated simultaneously with UV-B (1 μmol m^{-2} sec^{-1}) and high levels of visible light (800 μmol m^{-2} sec^{-1}), or with UV-B or visible light alone. Figure 6 shows that irradiation with UV-B and visible light induced acidic-type PR-1 accumulation. This suggests that PR-1 accumulation induced by UV-B is not mediated by a photoreactivated type of DNA damage.
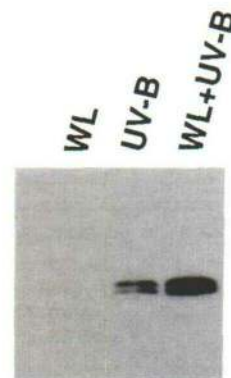

Figure 6. Induction of Acidic-Type PR-1 Protein by UV-B in the Presence of White Light.

Detached leaves were irradiated with 1 μmol m^{-2} sec^{-1} UV-B (UV-B; in a dark room), 1 μmol m^{-2} sec^{-1} UV-B supplemented with 800 μmol m^{-2} sec^{-1} daylight (WL+UV-B; in a greenhouse), or daylight alone (WL; in a greenhouse) for 22 hr with an interim 10-hr dark period. Protein was extracted from the leaves, and 30-μg aliquots from each sample were fractionated using SDS-PAGE and immunoblotted with the anti–PR-1 antibody.
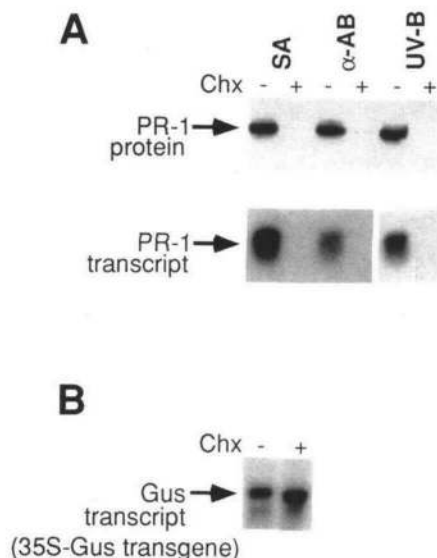
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Figure 7. Chx Inhibits PR-1 Transcript Accumulation.

Detached leaves were pretreated with 0.1 mM Chx for 3 hr (+) or left untreated (−) before being treated with 0.2 μmol m^{-2} sec^{-1} UV-B, 5 mM α-aminobutyric acid (α-AB), or 1 mM SA for 22 hr.

(A) Protein and RNA gel blots of PR-1 accumulation. Total RNA was extracted from the leaves, and 20-μg aliquots from each sample were fractionated in agarose–formaldehyde denaturing gels and hybridized with a probe to PR-1M. Protein was extracted from the leaves, and 30-μg aliquots from each sample were fractionated using SDS-PAGE and immunoblotted with the anti–PR-1 antibody.

(B) RNA gel blot of a CaMV 35S–*Gus* construct in transgenic plants. Total RNA was extracted from leaves of transgenic plants, fractionated as described in **(A)**, and hybridized with a probe specific to *Gus*.

Requirement for de Novo Protein Synthesis for PR-1 Induction

The phytochrome-mediated light induction of some plant nuclear genes has been shown to require cytoplasmic protein synthesis and is blocked by cycloheximide (Chx; Lam et al., 1989). To determine whether the induction of PR-1 by UV-B also required cytoplasmic protein synthesis, leaves were treated with Chx before irradiation with UV-B. PR-1 expression induced by UV-B was blocked completely by the Chx treatment, as was the expression of PR-1 induced by α-aminobutyric acid and SA (Figure 7A). In contrast, the levels of expression of a cauliflower mosaic virus (CaMV) 35S–β-glucuronidase (*Gus*) construct in transgenic plants increased after Chx treatment (Figure 7B). This Chx inducibility of a CaMV 35S promoter has been reported previously (Lam et al., 1989) and suggests that the inhibition of PR-1 expression observed is specific and not due to a general malfunction in transcription.

Involvement of ROS in PR-1 Induction

Irradiation of animal cells with UV results in the induction of a large number of genes (Ronai et al., 1990). ROS have been shown to serve as messengers in the UV induction of one of these genes (Devary et al., 1992). To examine the involvement of ROS in PR-1 induction by UV-B irradiation, leaves were treated with the antioxidants N-acetyl-L-cysteine (NAC) and pyrrolidine dithiocarbamate (PDTC) before irradiation with UV-B. NAC is a nontoxic chemical widely used in vivo and in vitro as an antioxidant. It can directly scavenge oxidants and increases intracellular glutathione levels (Aruoma et al., 1989; Roederer et al., 1990). PDTC is a dithiocarbamate that may act either as a chelator of metals, thus protecting the cell from ROS, or, directly, by scavenging ROS (Zanocco et al., 1989; Meyer et al., 1993). Figures 8A and 8B show that treatment with NAC and PDTC inhibited the induction of PR-1 synthesis by UV-B. The effective concentrations required for inhibition were found to be in the same range as those used in experiments in mammalian systems (Devary et al., 1992; Schreck et al., 1992; Meyer et al., 1993). Scanning protein gel blots from three different experiments of at least three leaves for each treatment showed that pretreatment with NAC or PDTC reduced the amount of PR-1 protein accumulating after UV-B irradiation by 70% ($\pm$16.5, SE) and 67% ($\pm$9.6, SE), respectively. However, induction of PR-1 by α-aminobutyric acid was not significantly affected by PDTC and NAC (Figures 8A and 8B). Similarly, NAC had no significant effect on the SA induction of PR-1 (Figure 8A). Interestingly, treatments with NAC and PDTC alone were found to induce low levels of PR-1 synthesis (Figures 8A and 8B). This has also been reported in other systems in which a gene whose induction is normally attenuated by a ROS scavenger is induced slightly by treatment with the scavenger alone (Devary et al., 1992).

To test directly whether ROS are able to induce the synthesis of acidic-type PR-1, leaves were treated with the photodynamic ROS inducer 4,5,6,7-tetrachloro-2′,4′,5′,7′-tetra-iodofluoroscein (rose bengal). Rose bengal is a water-soluble xanthene dye that forms singlet oxygen ($^1O_2$) upon irradiation with light of the appropriate wavelengths (Knox and Dodge, 1984). Figure 9 shows that rose bengal treatment induced acidic-type PR-1 accumulation and that this induction was inhibited by NAC. In the plant, $^1O_2$ is rapidly and spontaneously converted into other ROS, and thus ROS involved in this pathway need not necessarily be $^1O_2$.

Active oxygen species are a by-product of photosynthetic reactions, which are efficiently eliminated under normal physiological conditions. However, perturbations of electron transport, such as those that can be caused by UV-B irradiation damage to the photosynthetic apparatus (Jansen et al., 1993), may lead to the production of ROS (Bowler et al., 1992). To examine whether ROS produced during photosynthesis are involved in the UV-B–induced accumulation of acidic-type PR-1 protein, leaves from albino mutants, which lack both chlorophyll and mature chloroplasts (Fluhr et al., 1985), were irradiated with UV-B. High levels of PR-1 protein accumulation, similar
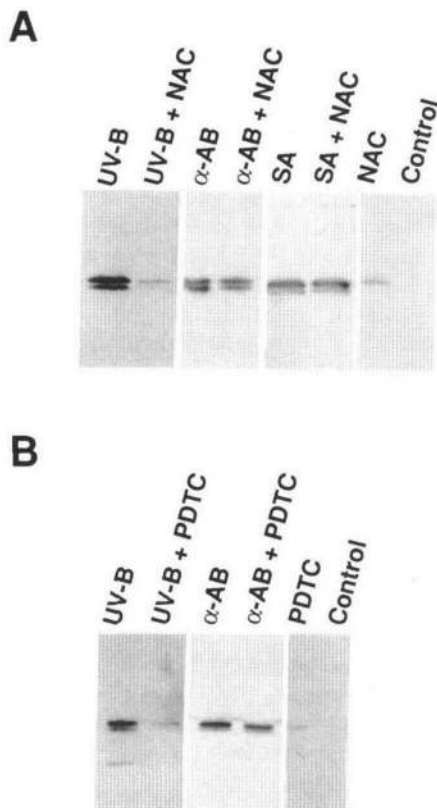
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Figure 8. Inhibition of UV-B–Induced PR-1 Accumulation by Antioxidants.

(A) Leaves were pretreated with NAC (30 mM) for 1 hr or left untreated and then treated with 0.2 μmol m^{-2} sec^{-1} UV-B, 1 mM SA, or 5 mM α-aminobutyric acid (α-AB) for another 22 hr. An additional treatment was with NAC alone. Control indicates no treatment. Protein was extracted from the leaves, and 30-μg aliquots from each sample were fractionated using SDS-PAGE and blotted with the anti–PR-1 antibody. **(B)** Leaves were pretreated with PDTC (100 μM) for 1 hr or left untreated and then treated with 0.2 μmol m^{-2} sec^{-1} UV-B or 5 mM α-aminobutyric acid (α-AB) for another 22 hr. An additional treatment was with PDTC alone. Control indicates no treatment. Protein extraction, fractionation, and blotting were as given in **(A)**.

to levels detected in normal leaves, were observed in the leaves without chlorophyll (Figure 10). The higher basal level detected in control albino leaves was reported previously (Lotan and Fluhr, 1990). Thus, it appears that ROS involved in the signal transduction pathway between UV-B and PR-1 are not generated during photosynthesis.

DISCUSSION

Despite its possible harmful ecological effects, little is known about the mechanisms by which plants perceive solar UV-B and the signal transduction mechanisms by which UV-B

activates gene expression. In this study, we have begun to characterize the effect of UV-B on the accumulation of a plant defense gene, PR-1, and have examined some of the possible components of the signal transduction cascade between UV-B irradiation and PR-1 protein accumulation.

We show that UV-B irradiation resulted in increases in PR-1 mRNA and protein levels in tobacco. Furthermore, UV-B induction of PR-1 protein accumulation in tobacco leaves was localized to the irradiated areas, and the protein was not found systemically in untreated parts of the leaf. Induction was fluence dependent, and the fluences required for UV-B induction of acidic-type PR-1 corresponded to the effective fluences reported for other UV-B–induced responses, such as cotyledon curling in *Brassica napus* (Wilson and Greenberg, 1993), flavonoid accumulation in rye leaves (Tevini et al., 1991), and anthocyanin synthesis in carrot (Takeda and Abe, 1992). The decrease in response to UV-B at high fluences seen here is also characteristic of other responses (Hashimoto et al., 1991; Takeda and Abe, 1992; Wilson and Greenberg, 1993) and may be a result of damage to the leaf. However, the UV-B–induced accumulation of PR-1 differs from that of the flavonoids in that high levels of PR-1 proteins are present in all leaf cells after UV-B irradiation and are, unlike the flavonoids, not confined to the epidermal layer of cells. The differences between tissue specificities of PR-1 and flavonoids may suggest the involvement of different transduction pathways or an epidermal-specific restriction to flavonoid synthesis or accumulation. Our results also show that the induction of PR-1 by UV-B is not photoreversible, unlike the UV-B/UV-C induction of isoflavonoids (Beggs et al., 1985). Thus, photoreversible DNA damage is probably not involved in the signal pathway between UV-B
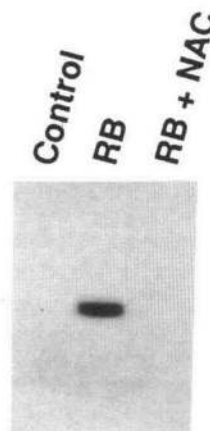

Figure 9. Induction of Acidic-Type PR-1 by Rose Bengal.

Leaves were pretreated with NAC (30 mM) alone for 1 hr in the light and then with 40 mM rose bengal (RB) for 1 hr in the dark or left untreated (Control) before being transferred to the light again for 22 hr. Protein was extracted from the leaves, and 30-μg aliquots from each sample were fractionated using SDS-PAGE and immunoblotted with the anti–PR-1 antibody.
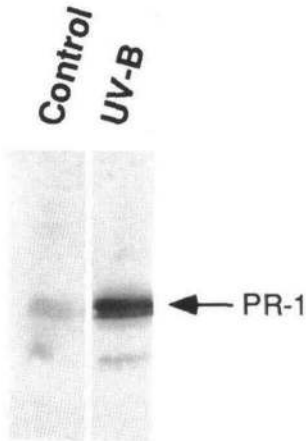
Figure 10. UV-B Induction of PR-1 in Albino Leaves.

Protein was extracted from albino leaves that had been irradiated with 0.2 µmol m^{-2} sec^{-1} UV-B or left untreated. Aliquots (30 µg) from each sample were separated using SDS-PAGE and immunoblotted with the anti–PR-1 antibody.

and PR-1. These results also imply that different pathways are induced by UV-B in plants.

Flavonoids and anthocyanins have been shown to accumulate in the epidermis and absorb UV-B radiation, preventing damage to the underlying cells (Beggs et al., 1986; Li et al., 1993). However, the protection from UV-B irradiation afforded by flavonoids is often incomplete, and UV-B is able to penetrate the epidermis to the underlying tissue (Tevini et al., 1991; Day et al., 1993), where it may induce further protective responses. It is possible that PR-1 protein induced by UV-B has a role in protecting cells from the damaging effects of UV-B irradiation. As yet, however, no enzymatic function has been found for the PR-1 gene family. Transgenic tobacco plants that constitutively express high levels of PR-1 protein show an increased protection against oomycete pathogens (Alexander et al., 1993) but not against tobacco mosaic virus or alfalfa mosaic virus (Cutt et al., 1989; Linthorst et al., 1989).

We have also started to elucidate some of the components of the signal transduction cascade between UV-B and PR-1. UV-C (200 to 280 nm) irradiation has been shown to cause increases in ROS in plant cells, for example, in cultured rose cells (Murphy and Huerta, 1990). ROS are released by elicitor-treated tobacco and soybean cells; it has been suggested that they play a direct role in defense as antimicrobial agents (Baker et al., 1993; Legendre et al., 1993). In addition, ROS have been shown to be involved in the induction of PR-1 by SA (Chen et al., 1993).

Here we have demonstrated that UV-B induction of a plant response also involves ROS (Figure 11). We used the antioxidants NAC and PDTC, which have been well characterized in animal cells. NAC has been used to suppress the UV-C–mediated induction of c-jun (Devary et al., 1992), whereas PDTC has been shown to attenuate the H$_2$O$_2$-induced activation of

the transcription factor NF-κB (Schreck et al., 1992). Both NAC and PDTC inhibited the induction of acidic-type PR-1 accumulation by UV-B. Furthermore, induction of PR-1 accumulation could be directly induced by the ROS generator rose bengal, and this induction could also be blocked by NAC.

We have also looked at a possible source for the ROS involved in UV-B–induced PR-1 accumulation. Using albino plants, we have shown that photosynthesis is not a requirement for the UV-B induction of PR-1 accumulation. Hence, ROS involved in the signal transduction cascade between UV-B irradiation and PR-1 induction are probably not a by-product of photosynthesis.

Our results show that SA and UV-B induction of PR-1 mRNA are both blocked by Chx, implying that there is either a requirement for de novo cytoplasmic protein synthesis or a labile protein element involved in the signal transduction pathways from both of these elicitors. This result is consistent with those of Uknes et al. (1993), who showed that Chx blocks SA induction of PR-1. Similarly, Chx has been shown to inhibit the phytochrome-mediated light induction of some plant nuclear genes (Lam et al., 1989).

In contrast to the induction by UV-B, induction of acidic-type PR-1 accumulation by α-aminobutyric acid and SA was unaffected by the antioxidants. This result appears to contradict those of Chen et al. (1993), who reported that SA in tobacco leaves can bind a catalase and inactivate it, resulting in an increase in levels of H$_2$O$_2$ (a ROS). They showed that increasing H$_2$O$_2$ levels, either directly or by elevating SA, caused an increase in PR-1 accumulation. It is possible that SA induces PR-1 expression via more than one pathway, only one of which requires ROS (Figure 11). Chen et al. (1993) did not report testing the effect of antioxidants that remove or inactivate ROS and thus try to block SA induction of PR-1. Therefore, the 50% increase in H$_2$O$_2$ levels after SA treatment of tobacco leaves may be ancillary to SA-induced PR-1 accumulation. Thus, in the presence of ROS scavengers, SA is still able to cause the
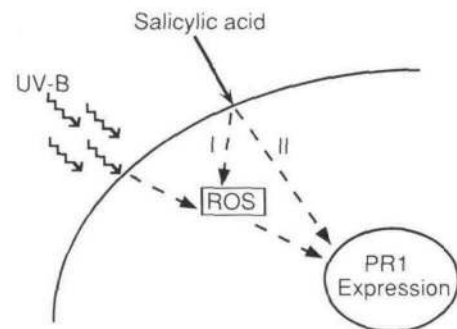

Figure 11. A Scheme for the Signal Transduction Pathways Induced by UV-B and SA.

UV-B induction of PR-1 accumulation requires ROS, whereas SA induction of PR-1 accumulation may be via both ROS-requiring (path I; Chen et al., 1993) and non-ROS–requiring (path II; this study) pathways.

accumulation of PR-1 protein, unlike UV-B, which has an absolute requirement for ROS to induce PR-1 (Figure 11). Further evidence for the existence of non-ROS–requiring pathways for SA comes from studies of systemic induction of PR gene expression in tobacco. There it has been shown that even very slight increases in SA levels in systemic tissue can induce PR gene expression via a pathway that was suggested not to involve H_2O_2 (Vernooij et al., 1994).

This work highlights similarities between the UV-induced defense responses of plants and animals, with a requirement for ROS in both. We are now investigating whether other steps in the animal UV induction pathways are conserved in plants. It would also be of interest to determine whether there is a ROS requirement for other UV-B–induced genes in plants.

METHODS

Plant Material and Treatments

Nicotiana tabacum cv Samsun NN plants were grown in a greenhouse under a regime of 18 hr of light at 26°C and 6 hr of dark at 22°C. Treatments were performed on excised leaves taken from 8- to 10-week-old plants with three or four leaves of at least 10 cm in length. Experiments with chemical elicitors were performed in a greenhouse, and UV treatments were, unless otherwise indicated, performed in a dark room at a constant 24°C. All of the experiments were started between 9 and 11 AM.

The elicitors α-aminobutyric acid (DL-α-2-amino-n-butyric acid; 5 mM) and rose bengal (40 mM) and the antioxidants N-acetyl-L-cysteine (30 mM) and pyrrolidine dithiocarbamate (100 μM) were brushed onto the leaves (2 mL per leaf). Salicylic acid (1.0 mM) was imbibed by the leaves. The protein synthesis inhibitor cycloheximide (0.1 mM) was brushed onto the detached leaves and then imbibed by the leaves during the course of the experiment. UV-B irradiation was supplied by two Rayonet RPR-3000Å lamps (Southern New England Ultraviolet Company, Hamden, CT). Photon fluxes were adjusted by varying the distance of the leaves from the light source. Fluence was measured using an integrating quantum/radiometer/photometer (Licor, Lincoln, NE) with the pyranometer sensor calibrated for UV-B. During shading experiments, leaves were protected from irradiation by light-opaque foil.

RNA Gel Blot Analysis

Total RNA was extracted from leaf discs using Tri Reagent (Molecular Research Center, Cincinnati, OH) according to the manufacturer's instructions. Aliquots (20 μg) of RNA were denatured, electrophoresed through 1% formaldehyde gels, and blotted as described by Sambrook et al. (1989). Hybridizations were performed at a stringency of 50% formamide at 42°C, and filters were washed at a maximum stringency of 0.2 × SSC (1 × SSC is 0.15 M NaCl, 0.015 M sodium citrate), 0.1% SDS at 65°C.

Immunoblotting and Antibodies

SDS-PAGE and immunoblotting were performed as described by Raz and Fluhr (1993). Native PAGE was performed using the same system without SDS. The antibodies raised against acidic-type pathogenesis-related protein PR-1 and acidic chitinase (also known as PR-P and PR-Q) and their specificities have been described (Lotan and Fluhr, 1990). Antibodies raised against basic-type PR-1 were raised to PRB-1b overexpressed in Escherichia coli using glutathione S-transferase fusion polypeptide (Smith and Johnson, 1988). Antibodies raised against the small subunit of ribulose bisphosphate carboxylase were a gift from A. Vainstain (Faculty of Agriculture, Rehovot, Israel).

Immunohistochemical Staining

Small sections of leaf were fixed in 5% formaldehyde, 5% acetic acid, 63% ethanol for 24 hr at room temperature. After dehydration with ethanol, the leaves were embedded in paraffin. Transverse sections of 15-μm thickness were cut using a microtome and mounted on poly-L-lysine–treated slides. Immunohistochemical staining of the sections was performed using a universal anti-rabbit kit (SIH918-B) from Sigma according to the manufacturer's instructions. Sections were incubated with a 1:200 dilution of primary antibody, and the peroxidase substrate used was 3,3′-diaminobenzidine tetrahydrochloride.

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