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Xylanase, a Novel Elicitor of Pathogenesis-Related Proteins in Tobacco, Uses a Non-Ethylene Pathway for Induction[1]

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ABSTRACT

Antisera to acidic isoforms of pathogenesis-related proteins were used to measure the induction of these proteins in tobacco (*Nicotiana tabacum*) leaves. Endo-(1-4)-β-xylanase purified from culture filtrates of *Trichoderma viride* was a strong elicitor of pathogenesis-related protein synthesis in tobacco leaves. The synthesis of these proteins was localized to tissue at the area of enzyme application. The inhibitors of ethylene biosynthesis and ethylene action, 1-aminoethoxyvinylglycine and silver thiosulfate, inhibited accumulation of pathogenesis-related proteins induced by tobacco mosaic virus and α-aminobutyric acid, but did not inhibit elicitation by xylanase. Likewise, the induction of these proteins by the tobacco pathogen *Pseudomonas syringae* pv. *tabaci* was not affected by the inhibitors of ethylene biosynthesis and action. The leaf response to tobacco mosaic virus and α-aminobutyric acid was dependent on light in normal and photosynthetically incompetent leaves. In contrast, the response of leaves to xylanase was independent of light. Tobacco mosaic virus and α-aminobutyric acid induced concerted accumulation of pathogenesis-related proteins. However, xylanase elicited the accumulation of only a subset of these proteins. Specifically, the plant (1-3)-β-glucanases, which are normally a part of the concerted response, were underrepresented. These experiments have revealed the presence of a novel ethylene-independent pathway for pathogenesis-related protein induction that is activated by xylanase.

The *de novo* synthesis of PR[2] proteins is a ubiquitous reaction of monocot (10, 23, 33) and dicot plants (2, 15) to pathogen attack. Originally characterized in relation to the hypersensitive response induced by TMV in tobacco plants containing the N gene (30), they appear to be part of a nonspecific host response to pathogens (22). The PR proteins of tobacco consist of a few major groups that are coordinately regulated in response to infection by viruses, bacteria and fungi (2). The enzymatic functions of two groups of PR proteins have been described. One group, consisting of acidic and basic PR proteins, has (1-3)-β-glucanase activity (15); the acidic polypeptides of this group are PR-2, N and O. A second group consisting of two basic and two acidic proteins has endochitinase activity; the acidic polypeptides of this group are PR-P and Q (17). Both hydrolases can digest the main cell wall components of some bacterial and fungal pathogens (2, 20). A third group consisting of an undetermined number of basic and at least three acidic proteins (PR-1a, 1b, and 1c) has no known biological function.

Ethylene is thought to be the natural mediator of PR protein accumulation (9, 25, 28, 32) as has been found for other defense functions. Physiological stresses that are associated with ethylene, such as flowering (12) and aberrant hormonal levels (8, 21), induce synthesis of PR proteins. Exogenous application of ethephon has the same effect (29). A plethora of abiotic elicitors can induce synthesis of PR proteins; among them are polyacrylic acid, salicylic acid and some amino acid derivatives (1). Because many of these chemicals (except salicylic acid) trigger ethylene biosynthesis, chemical elicitation of PR protein accumulation may be via ethylene. Recently, an endoxylanase has been purified from culture filtrates of *Trichoderma viride* and has been identified as the ethylene-inducing factor in Cellulysin (a commercial cellulase-containing product) (13). Here we show that this enzyme induces synthesis of PR proteins when applied directly to tobacco leaves. In contrast to other effective treatments, induction by xylanase was found to be independent of ethylene.

MATERIALS AND METHODS

Plant Material

Nicotiana tabacum cv. 'Samsun NN' plants were grown in the greenhouse, in 18 h day, 26 °C and 6 h night, 22 °C diurnal cycles. All experiments were conducted in the greenhouse on young potted plants with three to four leaves at least 10 cm long. A Chl-less cytoplasmic mutant, isolated as described (11), was maintained as a chimera by grafting onto normal plants.

Preparation of Sera

Intercellular fluid extracts were prepared from TMV-infected tobacco leaves (1). The extracts were fractionated on 12.5% native PAGE and the protein bands were excised and directly homogenized in PBS buffer (10 mM $NaHPO_4$ (pH7.4), 150 mM NaCl, and 3 mM KCl) containing 0.1% SDS. The homogenate was emulsified with an equal volume of complete Freund's adjuvant. The mixture (4 mL) containing about 100 μg protein was injected intradermally into a 2- to 3-month-old rabbit. Boost injections (after 30, 45, and 60 d) were

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[2] Abbreviations: PR, pathogenesis-related; AVG, 1-aminoethoxyvinylglycine; STS, silver thiosulfate; TMV, tobacco mosaic virus.

performed in a similar manner with incomplete Freund's adjuvant. The sera were stored at −20 °C.

Induction of PR Proteins in Leaves

Induction by pathogens was carried out in the following manner.

Viral Induction

TMV-U2 strain was applied at an inoculum density to yield 200 lesions/leaf (24). Leaves were harvested after the appearance of lesions, 48 h later.

Bacterial Induction

Pseudomonas syringae pv. tabaci at a concentration of 5×10^4 absorbance units (measured at 550 nm), in 10 mM phosphate buffer pH 6.0, was injected (approximately 100 $\mu L/cm^2$) into the extracellular leaf spaces using a syringe with the needle removed. Leaves were harvested 24 h later.

Chemical Induction

Accumulation of PR proteins was induced by spraying leaves until run-off with a solution of 1 mM α-aminobutyric acid (D,L-2-amino-*n*-butyric acid) containing 0.01% Tween 20. Leaves were harvested 24 h later. Purified (1-4)-β-xylanase, a gift from Drs. J. F. D. Dean and J. D. Anderson, was prepared as a solution of 10 ng/mL in 10 mM phosphate buffer (pH 6.0), and was injected as above. Leaves were harvested 24 h later. With all inducers, incubation of the leaves for an additional 24 h caused marginal increase in the amount of PR proteins detected. Leaf discs were excised from treated sites and frozen at −70 °C. The discs were homogenized directly in PBS for nondenaturing electrophoresis gel-fractionation or in SDS sample buffer for denaturing electrophoresis (16).

Measurements of Ethylene

Treated leaves, attached to the plants, were enclosed in stoppered 400-mL glass bottles for 22 h. Samples of air were withdrawn by syringe and injected into a gas chromatograph (Vega series 2, Carlo-Erba). The injector was set at 120 °C, the alumina column at 70 °C, and the flame ionization detector was set at 150 °C. The carrier gas was nitrogen. Each bottle was sampled 5 to 10 times and experiments were run in duplicate. Inhibition of ethylene biosynthesis was achieved by injecting leaves with a solution of 0.1 mM AVG (approximately 100 $\mu L/cm^2$). Ethylene action was inhibited by spraying plants with a solution of 50 μM STS containing 0.01% Tween 20. The inhibitors were applied together with the various inducers. In the case of TMV infection, AVG was applied on the second day after inoculation, 24 h prior to harvest. Lesion size and number were not affected by this treatment.

Isolation of Basic (1-3)-β-Glucanase and Chitinase

The basic counterparts of (1-3)-β-glucanase and chitinase were isolated according to Felix and Meins (8) and Boller *et al.* (3), respectively.

Electrophoresis and Immunoblotting

SDS-PAGE was carried out with 10 μg of total leaf extract as described (16). The same system was used for native gels without SDS. Immunoblots were processed in the following manner: SDS or native PAGE fractionated proteins were electrophoretically transferred to nitrocellulose sheets (27) and blocked for 1 h with 5% skim milk in PBS containing 0.1% Triton X-100 (quenching buffer). Diluted rabbit antiserum was added for a 2-h incubation, and the nitrocellulose was rinsed four times, 5 min each, with PBS containing 0.1% Triton X-100. ^{125}I-Protein A (affinity purified Amersham, 0.1 mCi/mL) in quenching buffer was then added, and the blots were incubated for an additional 2 h. The sheets were washed as above, dried and autoradiographed.

RESULTS

Immunoblot Analysis of PR proteins Induction by TMV

Nicotiana tabacum plants containing the N genetic locus exhibit a hypersensitive response to TMV infection and produce local lesions. A group of acidic polypeptides are secreted into the extracellular spaces of leaves during lesion formation (25). We prepared polyclonal antibodies specific for three classes of acidic PR proteins from PAGE-purified extracellular extracts of TMV-infected leaves (Fig. 1A, lane 2). Each set of antibodies revealed two to three immunoreactive bands on immunoblots of native PAGE in which TMV-treated extracellular leaf extracts were fractionated (Fig. 1A, lanes 4, 6, and 8). Untreated leaves had a very low level of PR proteins (Fig. 1A, lanes 1, 3, 5, and 7). When the extracts of TMV-treated leaves were fractionated on denaturing SDS-PAGE the polypeptides of each PR protein class migrated at the following apparent molecular masses; PR-1a,1b,1c at 19.5 kD; PR-2,N,O at 34 kD and PR-P,Q at 25 kD (Fig. 1B) (15, 17, 23). When all three antisera were applied simultaneously to the immunoblot (Fig. 1B, lane 4), the concerted appearance of the three PR proteins families, as a result of TMV induction, was apparent.

Specificity of Antisera to PR proteins Acidic Isoforms

PR protein classes have acidic and basic counterparts that appear to cross react immunologically (15, 17). It is important to distinguish between them because readily detectable constitutive levels of mRNA and enzymatic activity of both the basic chitinase and basic (1-3)-β-glucanases are present in tobacco leaves (22, 28, 31). Therefore, we tested our antisera for immunospecificity. When the extracellular leaf extract of TMV-infected leaves was fractionated on SDS-PAGE, a 34 kD polypeptide reacted to PR-2,N,O antisera (Fig. 2A, lane 2). However, purified basic (1-3)-β-glucanase migrated more slowly (35 kD) (15) and reacted very poorly to this antisera (Fig. 2A, lanes 3 and 4). When the extracellular leaf extract of TMV-infected leaves was fractionated on SDS-PAGE, a 25 kD polypeptide reacted to PR-P,Q antisera (Fig. 2B, lane 2). However, affinity-purified basic chitinase migrated with a molecular mass of 34 kD (17) and contained a 22 kD contaminant (Fig. 2B, lane 3). Neither polypeptide reacted with PR-P,Q antisera (Fig. 2B, lane 4). We conclude that both antisera

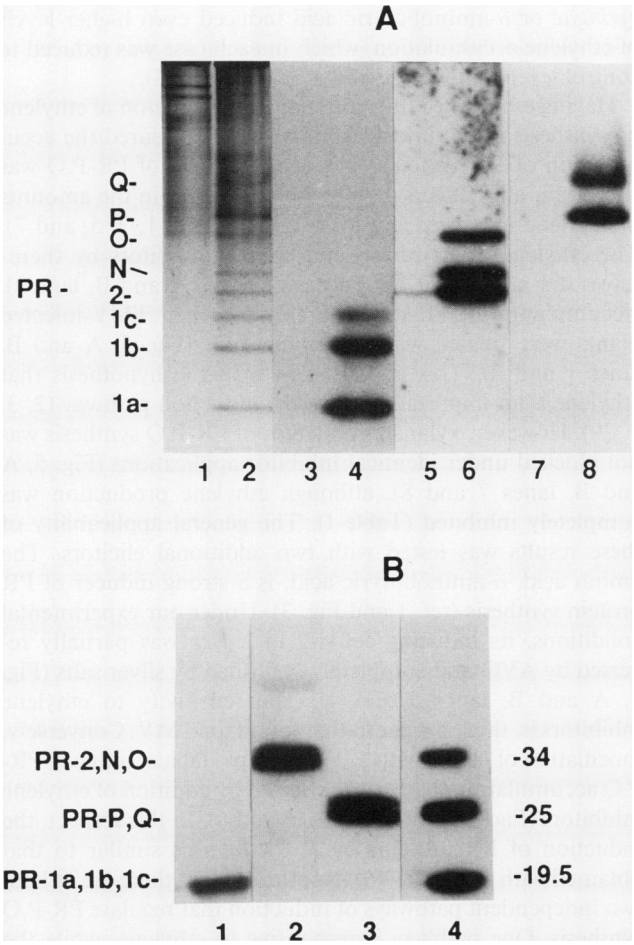

Figure 1. TMV-induction of PR proteins in tobacco leaves. (A) Native polyacrylamide-gel fractionation of leaf extracellular proteins. Odd-numbered lanes are protein extracts from mock-inoculated leaves; even-numbered lanes are from TMV-infected leaves. Lanes 1 and 2 are coomassie brilliant blue stain and lanes 3–8 are autoradiographs of immunoblots of these lanes treated with the following antibodies: lanes 3 and 4, anti-PR-1a,1b,1c; lanes 5 and 6, anti-PR-2,N,O; lanes 7 and 8, anti-PR-P,Q. (B) Autoradiograph of immunoblot of denaturing polyacrylamide gel-fractionated protein extracts from TMV-infected leaves treated with the following antibodies: Lane 1, anti-PR-1a,1b,1c; lane 2, anti-PR-2,N,O; lane 3, anti-PR-P,Q; lane 4, a mixture of all three antibody classes.

are specific for the acidic PR protein families. Basic PR-1 class proteins have not been isolated. Thus, the antisera to PR-1a,1b,1c could not be tested.

Induction of PR-P,Q by α-Aminobutyric Acid, *P. syringae* pv. tabaci and Xylanase

Amino acid analogs have been shown to elicit the accumulation of PR proteins in tobacco leaf discs (1). We found that in intact tobacco leaves application of 1 to 5 mM α-aminobutyric acid gave comparable levels of elicitation of PR-P,Q as achieved by TMV (Fig. 3). Higher concentrations of this elicitor caused marked necrosis and could not be tested. *P. syringae* pv. tabaci, a natural pathogen of tobacco, has been shown to elicit basic chitinase and basic (1-3)-β-glucanase (22). We extended those observations to include the induction of the acidic chitinase, PR-P,Q. Inoculation with *P. syringae* induced PR-P,Q to levels comparable to TMV infection (Fig. 3) but did not cause visible necrosis in the leaf tissue within the 24-h induction period.

Purified (1-4)-β-endoxylanase from *T. viridi* cultures has been shown to elicit ethylene production in tobacco leaf discs (13). Since ethylene has been correlated with induction of PR proteins, we tested the ability of xylanase to induce the accumulation of PR proteins. Figure 4 shows that 50 pg of xylanase were effective in eliciting PR-P,Q accumulation (Fig. 3 and Fig. 4, lane 2). Maximal elicitation, compared with that obtained by TMV infection, was achieved with 1 to 5 ng of enzyme (Fig. 3 and Fig. 4, lanes 6 and 8). Inactivation of the enzymatic activity by boiling (13) eliminated induction of PR proteins (data not shown). TMV induces systemic accumulation of PR proteins (2, 24, 30, 31). However, as seen in Figure 4 (lanes 1, 3, 5, and 7), leaf tissue directly adjacent to the sites treated with xylanase (within 2–3 mm) showed no accumulation of PR proteins. The observed localized effect of PR-P,Q induction is inconsistent with the hypothesis that ethylene is involved in the induction pathway used by xylanase.

To further explore the possibility of alternate induction pathways, we chose amounts for each inducer, as described in "Materials and Methods," that did not cause necrosis, but induced accumulation of PR proteins to levels similar to that achieved by TMV after 48 h (Fig. 3).

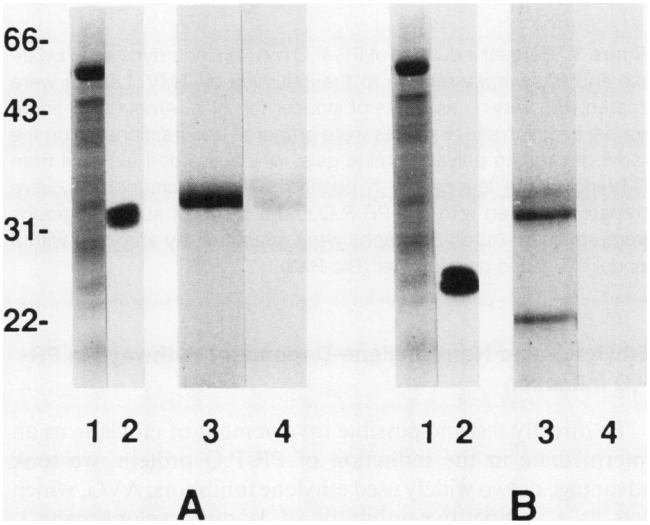

Figure 2. Specificity of antisera to acidic forms of (1-3)-β-glucanase and chitinase. A: Lane 1, Coomassie brilliant blue stain of SDS-PAGE fractionated extracellular leaf extracts from TMV-infected leaves (10 μg). Lane 2, immunoblot of lane 1 developed with anti-PR-2,N,O. Lane 3, Coomassie brilliant blue stain of SDS-PAGE fractionated purified basic (1-3)-β-glucanase (10 μg). Lane 4, immunoblot of lane 3 developed with anti-PR-2,N,O. B: Lane 1, Coomassie stain of SDS-PAGE fractionated extracellular leaf extracts from TMV-infected leaves (10 μg). Lane 2, immunoblot of lane 1 developed with anti-PR-P,Q. Lane 3, Coomassie brilliant blue stain of SDS-PAGE fractionated purified basic chitinase (10 μg). Lane 4, immunoblot of lane 3 developed with anti-PR-P,Q.

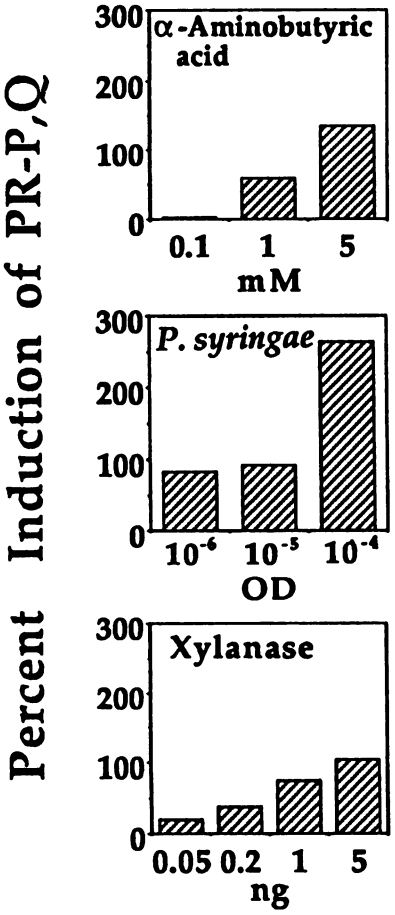

Figure 3. Percent induction of PR-P,Q by α-aminobutyric acid, xylanase and *P. syringae* relative to the induction by TMV. Leaves were treated with varying amounts of inducer for 24 h. Amounts in figure are per injection site. Extracts were prepared and fractionated on the same denaturing polyacrylamide gels, which included extracts from TMV-inoculated leaves (48 h postinoculation). Immunoblots were prepared, reacted with anti-PR-P,Q and a series of autoradiographs were obtained. Autoradiographs were quantitatively analyzed with a model 620 video densiometer (Bio-Rad).

Ethylene- and Non-Ethylene-Dependent Pathways in PR-P,Q Induction

To directly test the possible involvement of ethylene as an intermediate in the induction of PR-P,Q protein, we took advantage of two widely used ethylene inhibitors: AVG, which acts as a competitive inhibitor of 1-aminocyclopropane-1-carboxylic acid synthase activity, a key enzyme in the ethylene biosynthesis pathway; and STS, which inhibits ethylene action (34). Experiments involving ethylene evolution have been carried out on leaf discs (6, 13). We wished to confirm the biosynthesis of ethylene and its inhibition in our experimental system, *i.e.* the intact plant. Xylanase induced a nine-fold increase in ethylene evolution when compared with non-treated leaves and greater than a three-fold increase compared with mock-injected leaves (Table I). This increase was abolished in the presence of AVG. Application of TMV, *P. syringae* or α-aminobutyric acid induced even higher levels of ethylene accumulation, which in each case was reduced to control levels in the presence of AVG (Table I).

Having established the induction and inhibition of ethylene biosynthesis in the intact plant, we then measured the accumulation of PR proteins. The accumulation of PR-P,Q was similar for all inducers despite the differences in the amounts of ethylene measured (Fig. 5, A and B, lanes 1, 3, 5, and 7). The ethylene, biosynthesis and action, inhibitors by themselves did not induce PR proteins (Fig. 5, A and B, lane 9). Accumulation of PR-P,Q was blocked when TMV-infected plants were treated with either inhibitor (Fig. 5, A and B, lanes 1 and 2). This is consistent with the hypothesis that ethylene is an important part of the induction pathway (2, 3, 9, 29). However, xylanase induction of PR-P,Q synthesis was not blocked under identical inhibitor applications (Fig. 5, A and B, lanes 7 and 8), although ethylene production was completely inhibited (Table I). The general applicability of these results was tested with two additional elicitors. The amino acid, α-aminobutyric acid, is a strong inducer of PR protein synthesis (ref. 1 and Fig. 3). Under our experimental conditions, its inducing activity in leaves was partially reversed by AVG and completely abolished by silver salts (Fig. 5, A and B, lanes 3 and 4). The sensitivity to ethylene inhibitors is, thus, similar to that found for TMV. Conversely, inoculation of leaves with *P. syringae* pv. tabaci, induced PR-P,Q accumulation that was unaffected by addition of ethylene inhibitors (Fig. 5, A and B, lanes 5 and 6). In this respect, the induction of PR proteins by *P. syringae* is similar to that obtained with xylanase. We hypothesize that there are at least two independent pathways of induction that regulate PR-P,Q synthesis. One pathway is responsive to ethylene, while the other is not.

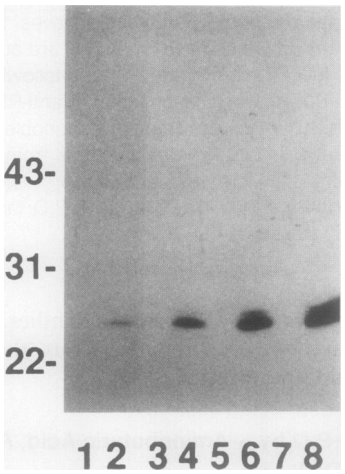

Figure 4. Induction of PR-P,Q by xylanase. Autoradiograph of immunoblot of denaturing polyacrylamide gel-fractionated protein extracts reacted with anti-PR-P,Q. Even-numbered lanes are extracts from plants treated with the following amounts per injection site of xylanase: lane 2, 50 pg; lane 4, 200 pg; lane 6, 1000 pg; lane 8, 5000 pg. Odd-numbered lanes contain extracts of leaf tissue adjacent to the xylanase-treated zones (within 2–3 mm).

Table I. *Ethylene Evolution by Intact Leaves*

Of the total area of leaves (10 cm long, approximately 3 g fresh weight) 75% was treated with inducers alone, or inducers and AVG. Control was nontreated intact leaves. Another control was injection of a 10 mM phosphate buffer at pH 6.0. The amounts of inducers were as indicated in "Materials and Methods." Each leaf was sampled 5 to 10 times and experiments were conducted in duplicate.

Treatment	Ethylene Evolution (μL/22 h/leaf)	
	Without AVG	With AVG
Control	3.08	ND[a]
Buffer phosphate	5.50	ND
Xylanase	18.06	4.88
P. syringae	22.80	1.96
α-Aminobutyric acid	69.53	5.55
TMV	33.12	2.09

[a] ND = not detected.

Light Dependence of PR-P,Q Induction in Normal and Mutant Tobacco Leaves

Light and the presence of green photosynthetically competent tissue have been shown to be necessary for PR protein accumulation induced by diverse chemical elicitors (1). Therefore, we wished to test whether both pathways induction of PR proteins required light. Potted plants were treated with xylanase, *P. syringae,* or α-aminobutyric acid, and the plants were either kept in the light or transferred to the dark for 48 h. All treatments induced the accumulation of PR-P,Q in the light (Fig. 6, lanes L), but only xylanase and *P. syringae* did so in the dark (Fig. 6, panels 1 and 2, lanes D). Thus, the inductive pathway for the latter two elicitors does not require light. In order to ascertain whether the light requirement in the case of α-aminobutyric acid indicates a requirement for photosynthesis, a Chl-less albino cytoplasmic mutant (11) was treated with α-aminobutyric acid under similar light regimes. The results in Figure 6 (panel 4) show that the photosynthetically incompetent mutant reacted like a normal plant with respect to synthesis of PR proteins. It was capable of supporting PR-P,Q accumulation induced by α-aminobutyric acid only in the light, yet in the absence of photosynthesis.

Differential Induction of PR Proteins

Concomitant production of all classes of PR proteins is the general pattern of induction observed in pathogen and elicitor-stressed tobacco plants. Having determined that two pathways for PR-P,Q induction exist, we examined the induction of two additional groups of PR proteins using elicitors of each pathway. The two additional classes monitored were the PR-1 group of 19.5 kD proteins of unknown biological function and the PR-2,N,O group of 34 kD proteins, which are (1-3)-β-glucanases (15). Plants were treated with xylanase, *P. syringae,* or α-aminobutyric acid, and the leaf extracts were simultaneously examined for the presence of all three PR classes. The results in Figure 7 show that α-aminobutyric acid induced PR proteins in concert (Fig. 7, lanes 3 and 4) similar to that seen for TMV (Fig. 1B, lane 4) (1, 24, 29, 30). However,

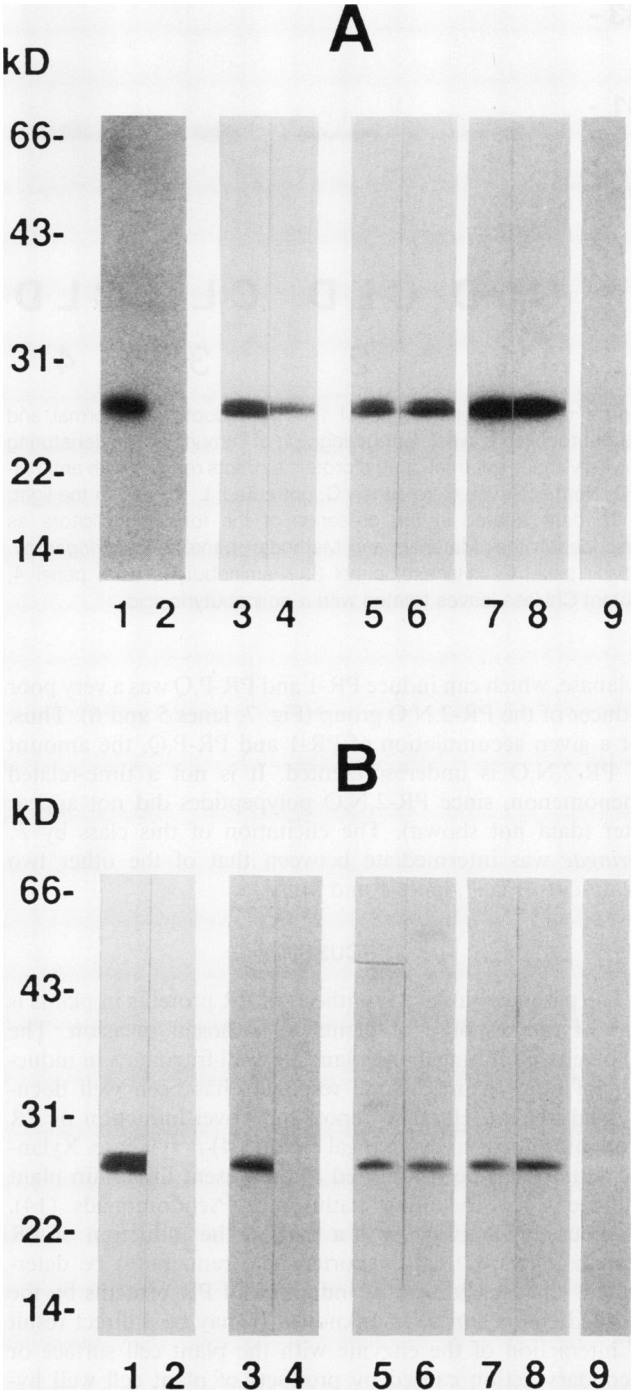

Figure 5. Effect of ethylene inhibitors on PR proteins induction. Autoradiograph of immunoblot of denaturing polyacrylamide gel-fractionated protein extracts reacted with anti-PR-P,Q. Odd-numbered lanes are extracts from leaves treated with elicitor. Even-numbered lanes are the same treatments with the addition of inhibitor as described in the "Materials and Methods." Treatments were: lanes 1 and 2, TMV; lanes 3 and 4, α-aminobutyric acid; lanes 5 and 6, *P. syringae*; lanes 7 and 8, xylanase; lane 9, inhibitor only. A, Inhibitor was AVG. B, Inhibitor was STS.

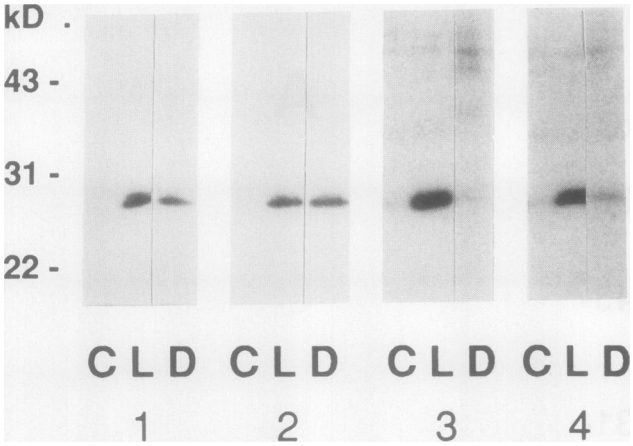

Figure 6. Light dependence of PR-P,Q induction in normal and mutant tobacco leaves. Autoradiograph of immunoblot of denaturing polyacrylamide gel-fractionated protein extracts reacted with anti-PR-P,Q. Normal leaves were either: C, untreated; L, treated in the light; or D, dark treated in the presence of the following elicitors as described in the "Materials and Methods:" panel 1, *P. syringae* pv. tabaci; panel 2, xylanase; panel 3, α-aminobutyric acid; panel 4, mutant Chl-less leaves treated with α-aminobutyric acid.

xylanase, which can induce PR-1 and PR-P,Q was a very poor inducer of the PR-2,N,O group (Fig. 7, lanes 5 and 6). Thus, for a given accumulation of PR-1 and PR-P,Q, the amount of PR-2,N,O is underrepresented. It is not a time-related phenomenon, since PR-2,N,O polypeptides did not appear later (data not shown). The elicitation of this class by *P. syringae* was intermediate between that of the other two treatments (Fig. 7, lanes 1 and 2).

DISCUSSION

The pathogen-induced synthesis of PR proteins in plants is part of the response of plants to pathogen invasion. The involvement of fungal and plant cell wall fragments in induction of other plant defense responses has been well documented (4, 26). Here we report the novel induction of PR proteins in tobacco by a fungal endo-(1-4)-β-xylanase. Xylanase activity has been reported to be present in certain plant pathogens (5) including pathogenic Pseudomonads (14). Whether this activity plays a part in the induction of PR proteins by *P. syringae* reported here remains to be determined. The mechanism of induction of PR proteins by the purified endoxylanase is unknown. It may be a direct result of interaction of the enzyme with the plant cell surface or secondary action caused by products of plant cell wall hydrolysis released by xylanase. Putative hydrolytic products must be either short lived or not translocated in order to account for the localized response observed. A straight chain of (1-4)-β-linked D-xylopyranosyl residues has been isolated from the holocellulosic polysaccharides of the midrib of tobacco leaves (6). It could serve as a plausible substrate for xylanase if it is present in the smaller vascular elements of the leaves, which were included in the injection sites. However, in this respect, the application of xylose, or xylan-based oligomers did not elicit PR proteins accumulation (T Lotan, unpublished data).

Cell wall fragments of *Colletotrichum lagenarium* can induce chitinase activity in melon plants. This activity is significantly inhibited by metabolic blockers of ethylene biosynthesis (25). In contrast, ethylene was considered to be a concomitant symptom, not signal, for chitinase and (1-3)-β-glucanase synthesis in pea seedlings treated with elicitor (chitosan) or inoculated with *Fusarium solani* (19). Nonetheless, ethylene treatment of the seedlings can by itself coordinately induce both enzymatic activities (32). The apparent discrepancy in a large number of published observations between different plant species can be resolved if multiple inductive pathways for the induction of PR proteins exist in other plants as they do in tobacco. Thus, the expression of PR-1 and PR-P,Q will show ethylene control when induced by TMV and α-aminobutyric acid. However, ethylene will appear to be a concomitant symptom together with the accumulation of PR proteins when plants are induced by xylanase or by *P. syringae*. The use of ethylene inhibitors does not distinguish between the possibility that ethylene and xylanase-based induction of PR proteins are two points on one common pathway rather than independent pathways. However, the obvious lack of (1-3)-β-glucanase induction by xylanase, in contrast to the coordinate induction by TMV and α-aminobutyric acid, suggests that independent parallel inductive pathways rather than consecutive inductive pathways are in operation. Indeed, the segregated spatial accumulation of PR proteins from different gene families documented in floral structures (18) lends credence to their complex and independent control.

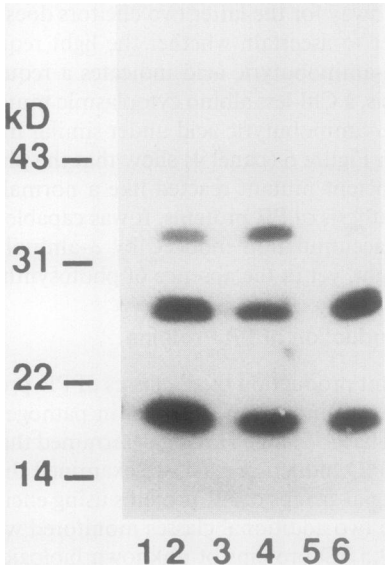

Figure 7. Differential induction of PR proteins. Autoradiograph of immunoblot of denaturing polyacrylamide gel fractionated protein extracts reacted with a mixture of anti-PR-1a,1b,1c, anti-PR-2,N,O, and anti PR-P,Q. Odd-numbered lanes are from untreated leaves. Even-numbered lanes are from leaves treated with the following elicitors as described in "Materials and Methods": lane 2, *P. syringae*; lane 4, α-aminobutyric acid; lane 6, xylanase.

The light dependency of PR induction first documented by Asselin *et al.* (1) has been shown here to be limited to the ethylene-dependent pathway. Hence, the accumulation of PR proteins, a defense-related phenomenon, is not limited to conditions of daylight. Although we have shown that photosynthesis is not involved, it remains to be seen what photoreceptor mediates this response. Our preliminary observations show that, together with the induction of PR proteins by xylanase, the sesquiterpenoid, capsidiol, also accumulates in treated intact leaves (A Sharon, unpublished data). Interestingly, an extracellular protein from *Phytophthora parasitica* var nicotianae was shown to be associated with induction of sesquiterpenoid phytoalexins in tobacco cell cultures (7). In that case it was observed that a (1-4)-β-endoxylanase activity co-purified with the phytoalexin-inducing protein. Thus, it appears that in tobacco, two defense-related plant responses, induction of PR proteins and accumulation of phytoalexins, share common features of regulation.

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LITERATURE CITED

1. **Asselin A J, Grenier J, Cote F** (1985) Light-influenced extracellular accumulation of b (pathogenesis-related) proteins in *Nicotiana* green tissue induced by various chemicals or prolonged floating on water. Can J Bot **63**: 1276–1283
2. **Bol JF, van Kan JAL** (1988) The synthesis and possible functions of virus-induced proteins in plants. Microbiol Sci **5**: 47–52
3. **Boller T, Gehri A, Mauch F, Vogeli U** (1983) Chitinase in bean leaves: induction by ethylene purification, properties and possible function. Planta **157**: 22–31
4. **Darvill AG, Albersheim P** (1984) Phytoalexins and their elicitors—a defense against microbial infection in plants. Annu Rev Plant Pathol **35**: 243–275
5. **Dean JFD, Gamble HR, Anderson JD** (1989) The ethylene biosynthesis-inducing xylanase: Its induction in *Trichoderma viride* and certain plant pathogens. Phytopathology **79**: 1071–1078
6. **Eda S, Ohnishi A, Kato K** (1976) Xylan isolated from the stalk of *Nicotiana tabacum*. Agric Biol Chem **40**: 359–364
7. **Farmer EE, Helgeson JP** (1987) An extracellular protein from *Phytophthora parasitica* var nicotianae is associated with stress metabolite accumulation in tobacco callus. Plant Physiol **85**: 733–740
8. **Felix G, Meins F Jr** (1985) Purification immunoassay and characterization of an abundant cytokinin regulated polypeptide in cultured tobacco cells. Planta **164**: 423–428
9. **Felix G, Meins F Jr** (1987) Ethylene regulation of β-1,3-glucanase in tobacco. Planta **172**: 386–392
10. **Fink W, Liefland M, Mendgen K** (1988) Chitinases and β-1,3-glucanases in the apoplastic compartment of oat leaves (*Avena sativa* L.). Plant Physiol **88**: 270–275
11. **Fluhr R, Aviv D, Galun E, Edelman M** (1985) Efficient induction and selection of chloroplast-encoded antibiotic resistant mutants in *Nicotiana*. Proc Natl Acad Sci USA **82**: 1485–1489
12. **Fraser RSS** (1987) Evidence for the occurrence of the "pathogenesis-related" proteins in leaves of healthy tobacco plants during flowering. Physiol Plant Pathol **19**: 69–76
13. **Fuchs Y, Saxena A, Gamble HR, Anderson JD** (1989) Ethylene biosynthesis inducing protein from cellulysin is an endoxylanase. Plant Physiol **89**: 138–143
14. **Gross DC, Cody YS** (1985) Mechanisms of plant pathogenesis by *Pseudomonas species*. Can J Microbiol **31**: 403–410
15. **Kauffmann S, Legrand M, Geoffroy P, Fritig B** (1987) Biological function of 'pathogenesis-related' proteins: four PR proteins of tobacco have 1,3-β-glucanase activity. EMBO J **6**: 3209–3212
16. **Laemmeli UK** (1970) Cleavage of structural proteins during the assembly of the head of a bacteriophage T4. Nature **227**: 680–685
17. **Legrand M, Kauffmann S, Geoffrey P, Fritig B** (1987) Biological function of pathogenesis-related proteins: four tobacco pathogenesis-related proteins are chitinases. Proc Natl Acad Sci USA **84**: 6750–6754
18. **Lotan T, Ori N, Fluhr R** (1989) Pathogenesis-related proteins are developmentally regulated in tobacco flowers. Plant Cell **1**: 881–887
19. **Mauch F, Hadwiger LA, Boller T** (1984) Ethylene: Symptom, not signal for the induction of chitinase and β-1,3-glucanase in pea pods by pathogens and elicitors. Plant Physiol **76**: 607–611
20. **Mauch F, Mauch-Mani B, Boller T** (1988) Antifungal hydrolases in pea tissue. II Inhibition of fungal growth by combinations of chitinase and β-1,3-glucanase. Plant Physiol **88**: 936–942
21. **Memelink J, Hoge JHC, Schilperoort RA** (1987) Cytokinin stress changes the developmental regulation of several defence-related genes in tobacco. EMBO J **6**: 3579–3583
22. **Meins F Jr, Ahl P** (1989) Induction of chitinase and β-1,3-glucanase in tobacco plants infected with *Pseudomonas* tabacci and *Phytophtora parasitica* var. nicotianae. Plant Sci **61**: 155–161
23. **Nasser W, de Tapia M, Kauffmann S, Montaser-Koahsari S, Burkard G** (1988) Identification and characterization of maize pathogenesis-related proteins: four maize PR proteins are chitinases. Plant Mol Biol **11**: 529–538
24. **Parent J-G, Asselin A** (1983) Detection of pathogenesis-related proteins (PR a or b) and of other proteins in the intercellular fluid of hypersensitive plants infected with tobacco mosaic virus. Can J Bot **62**: 564–569
25. **Roby D, Toppan A, Esquerre-Tugaye MT** (1986) Cell surfaces in plant-microorganism interactions. VI. Elicitors of ethylene from *Colletotrichum lagenarium* trigger chitinase activity in melon plants. Plant Physiol **81**: 228–233
26. **Ryan CA** (1987) Oligosaccharide signalling in plants. Annu Rev Cell Biol **3**: 295–317
27. **Towbin H, Staehlin T, Gordon J** (1979) Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc Natl Acad Sci USA **76**: 4340–4354
28. **Van den bulcke M, Bauw G, Castresna C, Van Montagu M, Vandekerckhove J** (1989) Characterization of vacuolar and extracellular β-(1,3)-glucanases of tobacco: evidence for strictly compartmentalized plant defense system. Proc Natl Acad Sci USA **86**: 2673–2677
29. **Van Loon LC, Antoniw JF** (1982) Comparison of the effects of salicylic acid and ethephon with virus-induced hypersensitivity and acquired resistance in tobacco. Neth J Plant Pathol **88**: 237–256
30. **Van Loon LC, van Kammen A** (1970) Polyacrylamide disc electrophoresis of the soluble leaf proteins from *N. tabacum* var. 'Samsun' and 'Samsun NN.' Virology **40**: 199–211
31. **Vogeli-Lange R, Hansen-Gehri A, Boller T, Meins F Jr** (1988) Induction of the defense-related glucanohydrolases, β-1,3-glucanase and chitinase, by tobacco mosaic virus infection of tobacco leaves. Plant Sci **54**: 171–176
32. **Vogeli U, Meins F Jr, Boller T** (1988) Co-ordinated regulation of chitinase and β-1,3-glucanase in bean leaves. Planta **174**: 364–372
33. **White RF, Rybicki EP, Von Wechmar MB, Dekker JL, Antoniw JF** (1987) Detection of PR1-type proteins in *Amaranthaceae, Chenopodiaceae, Graminae* and *Solanacae* by immunoelectroblotting. J Gen Virol **68**: 2043–2048
34. **Yang SF** (1985) Biosynthesis and action of ethylene. HortScience **20**: 41–45