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Pathogenesis-Related Acidic β-1,3-Glucanase Genes of Tobacco Are Regulated by Both Stress and Developmental Signals

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Three pathogenesis-related (PR) proteins of tobacco are acidic isoforms of β-1,3-glucanase (PR-2a, -2b, -2c). We have cloned and sequenced a partial cDNA clone (λFJ1) corresponding to one of the PR-2 β-1,3-glucanases. A small gene family encodes the PR-2 proteins in tobacco, and similar genes are present in a number of plant species. We analyzed the stress and developmental regulation of the tobacco PR-2 β-1,3-glucanases by using northern and western analyses and a new technique to assay enzymatic activity. Stress caused by both thiamine and tobacco mosaic virus (TMV) infection resulted in a dramatic increase in the levels of PR-2 mRNA, protein, and enzyme activities. The increased PR-2 gene expression in upper uninoculated leaves of plants infected with TMV also suggests a role in systemic acquired resistance. During floral development, a number of β-1,3-glucanase activities were observed in all flower tissues. However, PR-2 polypeptides were observed only in sepal tissue. In contrast, an mRNA that hybridized to the PR-2 cDNA was present in stigma/style tissue and the sepals. Primer extension analysis confirmed the identity of the PR-2 mRNA in sepals, but indicated that the β-1,3-glucanase gene expressed in the stigma/style of flowers was distinct from the PR-2 genes. The induction of PR-2 protein synthesis by both stress and developmental signals was accompanied by a corresponding increase in the steady-state levels of PR-2 mRNA, suggesting that PR-2 gene expression is regulated, in part, at the level of mRNA accumulation.

Additional keywords: flowering, *Nicotiana tabacum*.

β-1,3-Glucanases or laminarinases (EC 3.2.1.39) hydrolyze β-1,3-glucans such as laminarin and are found in bacteria, fungi, algae, higher plants, and some invertebrates (Bull and Chesters 1966). Plant β-1,3-glucanases and chitinases represent potential antifungal hydrolases that act synergistically to inhibit fungal growth *in vitro* (Mauch *et al.* 1988). In addition, β-1,3-glucanases release glycosidic fragments that could act as elicitors of host defense mechanisms (Keen and Yoshikawa 1983; Hahn *et al.* 1989). In higher plants, β-1,3-glucanase activity increases in response to pathogen infection or hormonal treatments. Several studies suggest that plant β-1,3-glucanases may be components of a general defense mechanism against pathogen invasion in a number of different plant species (Benhamou *et al.* 1989; Jondle *et al.* 1989; Joosten and De Wit 1989; Kauffmann *et al.* 1987; Kombrink and Hahlbrock 1986; Kombrink *et al.* 1988; Mauch *et al.* 1988; Meins and Ahl 1989; Vögeli *et al.* 1988).

In *Nicotiana tabacum* L. 'Xanthi-nc' and 'Samsun NN,' tobacco mosaic virus infection results in a hypersensitive response characterized by the formation of necrotic lesions at the site of virus entry and the restriction of virus spread from the virus entry site. Associated with the hypersensitive response is the protection of adjacent tissues against subsequent infection (Ross 1961). Plant proteins correlated with resistance can be grouped into several categories such as: proteins able to degrade fungal and bacterial wall polymers (chitinases, β-1,3-glucanases and lysozymes); proteins that act as inhibitors of microbial enzymes; proteins with agglutinating properties such as lectins; and cell-wall-associated proteins such as hydroxyproline-rich glycoproteins and glycine-rich proteins (Boller 1985; Hahn *et al.* 1989; van Kan *et al.* 1988; Varner and Lin 1989). Some of these proteins are also included in a group called the pathogenesis-related proteins (PR or b proteins). PR proteins are host-encoded polypeptides synthesized after infection by diverse pathogens or after treatment with various chemicals or plant hormones. Originally, PR proteins were described as extracellular acidic polypeptides (low isoelectric point). However, basic isoforms are now known to exist. PR proteins have been classified into five families based on their structure and function. A nomenclature for PR proteins has been proposed by van Loon and co-workers (1987) (for reviews see Carr and Klessig 1989; Cutt and Klessig, in press; Fritig *et al.* 1990). To date, complementary DNA (cDNA) and genomic DNA clones have been reported for three of the tobacco PR-protein families: PR-P and -Q (chitinases; Payne *et al.* 1990); PR-R, or -S, (a thaumatinlike protein; Payne *et al.* 1988); and PR-1a, -1b, -1c (Cornelissen *et al.* 1987; Pfitzner and Goodman 1987; Matsuoka *et al.* 1987; Cutt *et al.* 1988).

A recent study by Kauffmann *et al.* (1987) demonstrated that three acidic, serologically related (Fortin *et al.* 1985) tobacco PR proteins PR-2a (b_4 or 2), -2b (b_5 or N) and -2c (b_{6b} or O) have β-1,3-glucanase activity. A fourth serologically related protein that is basic and vacuolar was shown also to have β-1,3-glucanase activity (Kauffmann

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et al. 1987). Partial amino acid sequence analyses of the basic (vacuolar) and acidic (extracellular) glucanases confirmed that they were related (Van den Bulcke *et al.* 1989). Both vacuolar and extracellular forms of glucanases have also been demonstrated in bean (Mauch and Staehelin 1989). The nucleotide sequences of cDNAs have been reported for a hormonally regulated, basic glucanase from *N. tabacum* (Shinshi *et al.* 1988), for a basic glucanase from *N. plumbaginifolia* Viv. (De Loose *et al.* 1988), for two barley glucanases (Fincher *et al.* 1986; Hoj *et al.* 1989), and for a soybean elicitor-releasing β-1,3-glucanase (Takeuchi *et al.* 1990).

We are interested in the mechanisms that govern the selective expression of PR genes because of the association of these genes with disease resistance and their reported expression during normal plant development (Lotan *et al.* 1989; Memelink *et al.* 1990). We report on the isolation and sequence of a partial cDNA clone encoding an acidic, extracellular form of a PR β-1,3-glucanase from tobacco. The cDNA clone was used to characterize the structure of the acidic glucanase genes and examine their expression in stressed tissue and during normal floral development in tobacco. Furthermore, we have analyzed the enzymatic activities of the acidic isoforms of β-1,3-glucanases present in tobacco by a new polyacrylamide gel assay (Côté *et al.* 1989).

MATERIALS AND METHODS

Plant material and PR gene induction. Plants were grown under greenhouse conditions (Parent and Asselin 1984). Fully mature nonsenescent leaves of *N. tabacum* 'Xanthi-nc' were used for experiments when 6–8 wk old. *Lycopersicon esculentum* Miller 'CR-864', *Cucumis sativus* L. 'SMR-18,' *Phaseolus vulgaris*, and *Hordeum vulgare* L. 'Léger' were put in the dark for 2 days before they were harvested for DNA extraction. Induction of PR gene expression was done either by inoculating tobacco with the U_1 or U_2 strains (Siegel and Wildman 1954) of purified tobacco mosaic virus (TMV) (1.5 μg/ml) or by floating leaf disks (4 cm^2) on a 1 mM solution of thiamine-HCl for 3 days at room temperature (Asselin *et al.* 1985).

Construction and screening of the cDNA library. The λgt11 cDNA library was prepared from membrane-bound polysomal poly(A)$^+$ RNA isolated from Xanthi-nc plants infected with TMV (Cutt *et al.* 1988). The λgt11 library was screened by using an antiserum against TMV-infected Xanthi-nc tobacco intracellular fluid extract (Fortin *et al.* 1985). Because the antiserum recognized a number of extracellular proteins found in the intracellular fluid, we used a screening procedure with radiolabeled antigen as a probe for identifying the glucanase clones (Chao *et al.* 1989). Polyacrylamide gel purified PR-2a and -2b proteins were radiolabeled with (^{125}I) by the lactoperoxidase method and desalted over a Bio-Rad P-30 spin column (Bio-Rad Laboratories, Richmond, CA). One positive clone (λFJ1) was obtained with this procedure.

Plasmids and DNA sequencing. The λgt11 *Eco*RI cDNA inserts were isolated from the phage and subcloned into the pUC118 and pUC119 vectors (Viera and Messing 1987). Single-stranded DNA was prepared and then sequenced with the U.S. Biochemical sequenase kit (U.S. Biochemical Corp., Cleveland, OH).

DNA and RNA preparation. Plant genomic DNA was isolated according to Dellaporta *et al.* (1983) with an additional cesium chloride gradient purification step in the protocol. RNA was isolated according to Verwoerd *et al.* (1989).

Nitrocellulose filter hybridizations. The Southern blot and phage plaque lifts were hybridized in 6.6× SSPE (1× SSPE is 0.15 M NaCl, 10 mM sodium phosphate, 1mM EDTA, pH 7.4), 30% formamide at 42° C, and washed under the same conditions for low stringency. Northern blots were hybridized in 5× SSPE, 50% formamide at 42° C, and washed in 0.1× SSPE and 0.1% sodium dodecyl sulfate at 50° C.

Primer extension analysis. The primer extension protocol has been described by Dunsmuir *et al.* (1987). A 36-mer oligonucleotide 5′-GGAATTCC **AGCATTGAAGACATTT GTTTCTGGATAG**-3′ complementary (28 boldface nucleotides) to the PR-2 mRNA was annealed to total RNA at

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1    A GTA TGC TAT GGA AAA ATT GCC AAC AAT
1      Val Cys Tyr Gly Lys Ile Ala Asn Asn

29 TTA CCA TCA GAC CAA GAT GTT ATA AAC CTA
10 Leu Pro Ser Asp Gln Asp Val Ile Asn Leu

59 TAC AAT GCT AAT GGC ATC AAA AGA ATG AGA
20 Tyr Asn Ala Asn Gly Ile Lys Arg Met Arg

89 ATA TAC TAT CCA GAA ACA AAT GTC TTC AAT
30 Ile Tyr Tyr Pro Glu Thr Asn Val Phe Asn

119 GCT CTC AAG GGA AGT AAC ATT GAG ATC ATT
40 Ala Leu Lys Gly Ser Asn Ile Glu Ile Ile

149 CTC GAT GTC CCA AAT CAA GAT CTT GAA TCC
50 Leu Asp Val Pro Asn Gln Asp Leu Glu Ser

179 CTT ACG GAT CCT TCA AGA GCC AAT GGA TGG
60 Leu Thr Asp Pro Ser Arg Ala Asn Gly Trp

209 GTC CAA GAT AAC ATA ATA AAT CAT TTT CCA
70 Val Gln Asp Asn Ile Ile Asn His Phe Pro

239 GAT GTT AAA TTT AAA TAT ATA GCT GTT GGA
80 Asp Val Lys Phe Lys Tyr Ile Ala Val Gly

269 AAT GAA GTA TCT CCT ACA AAT AAT GGT CAA
90 Asn Glu Val Ser Pro Thr Asn Asn Gly Gln

299 TAT GCA CCA TTT GTT GGT CCT GCC ATG CAA
100 Tyr Ala Pro Phe Val Gly Pro Ala Met Gln

329 AAT GTG TAC AAT GCA TTA GCA GCA GCA GGG
110 Asn Val Tyr Asn Ala Leu Ala Ala Ala Gly

359 TTG CAA GAT CAA ATC AAG GTT TCA ACT GCA
120 Leu Gln Asp Gln Ile Lys Val Ser Thr Ala

389 ACA TAT TCA GGG CTC TTA GCA AAC ACC TAC
130 Thr Tyr Ser Gly Leu Leu Ala Asn Thr Tyr

419 CCA CCT AAA GAT AGT ATT
140 Pro Pro Lys Asp Ser Ile
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Fig. 1. λFJ1 cDNA sequence. The nucleotide sequence is shown with the deduced amino acid sequence below. Both are numbered from the beginning of the clone. Standard three-letter abbreviations for amino acids are used.

42° C for the extension reaction.

Intracellular fluid protein extraction and total protein extracts. Intracellular fluid was extracted from leaf disks by infiltration with 50 mM Tris-HCl, pH 7.0, as described by Parent and Asselin (1984). For total protein extracts, tissues were ground in liquid nitrogen and homogenized in 1 vol of extraction buffer (50 mM Tris-HCl, pH 7.0, 1 mM phenylmethylsulfonyl fluoride). The homogenates were clarified by centrifugation (10,000 × *g*, 10 min, 4° C). Protein concentration was determined with the Bio-Rad protein assay reagent.

PAGE, western analyses, and glucanase enzymatic assay. Protein analyses were performed by PAGE (polyacrylamide gel electrophoresis) in 15% gels under native conditions (Davis 1964). This system uses a pH 8.9 separation gel and is designed to separate neutral or acidic native proteins. Electrophoresis was performed at 20 mA for 1.5 hr. Proteins PR-2a and -2b were eluted from gel strips by incubation overnight in 10 ml of distilled water under gentle shaking at 4° C. The recovered eluate was filtered through a 0.22-μm membrane filter, frozen, lyophilized, and subsequently resuspended in distilled water. For western blotting, proteins were transferred to nitrocellulose paper and immunoblotted with PR-2b (PR-N) specific antiserum (Kauffmann *et al.* 1987; Benhamou *et al.* 1989). For the β-1,3-glucanase assay, laminarin (Sigma L-9634; Sigma Chemical Co., St. Louis, MO) was incorporated into the separation gel at a final concentration of 2 mg/ml. After electrophoresis, the gel was incubated for 45 min in 200 ml of 50 mM sodium acetate buffer, pH 5.0, at 37° C. After incubation, the gel was immersed in aniline blue fluorochrome (sirofluor; 35 mg/ml in 100 mM glycine-NaOH, pH 11.5; Côté *et al.* 1990) for at least 15 min. Lytic zones were visualized by using a long-wave UV transilluminator (C-62; U. V. P. Technology, Rancho Cordova, CA). Gels were photographed (Polaroid Type 57) for 30 sec with UV-HAZE and O2 orange photography filters (Côté *et al.* 1989, in press).

RESULTS

Isolation and sequence of an acidic β-1,3-glucanase cDNA clone. To isolate a cDNA clone that encodes an acidic extracellular β-1,3-glucanase, we screened a TMV-infected *N. tabacum* 'Xanthi-nc' λgt11 cDNA library with antiserum made against proteins extracted from the intracellular fluid of leaves infected with TMV. Because this antiserum reacts with several proteins in the intracellular fluid, we used a radiolabeled antigen overlay method for the screening procedure to obtain glucanase-specific clones (see Methods). One positive clone (λFJ1) that contained a partial cDNA clone of 436 nucleotides was identified. The nucleotide sequence and deduced amino acid sequence are shown in Figure 1. Comparison of this deduced amino acid sequence to the partial amino acid sequences of three tobacco acidic glucanases (98% identity with the PR[36] peptide fragments; Van den Bulcke *et al.* 1989) indicated that our clone encoded an acidic β-1,3-glucanase. Peptide and cDNA sequences of the three acidic isoforms provided to us by E. Ward, G. Payne, and K. Sharkey (personal communication) show that λFJ1 is most closely related to PR-2c (PR-O). In contrast, the λFJ1 cDNA has 62.5% identity with the basic prepro-β-1,3-glucanase cDNA isolated by Shinshi *et al.* (1988) at the nucleotide level and 47.9% identity at the amino acid sequence level. Based on these analyses, we concluded that λFJ1 corresponds to the amino-terminal domain of the PR-2c protein.

Characterization of the glucanase gene family. Partial amino acid analyses of three acidic β-1,3-glucanase polypeptides from tobacco, corresponding to the PR-2 proteins, indicated that these proteins were encoded by separate, but related genes (Fortin *et al.* 1985; Kauffmann *et al.* 1987; Van den Bulcke *et al.* 1989). We investigated the complexity of the glucanase gene family by Southern analysis of genomic DNA isolated from tobacco, cucumber, tomato, bean, and barley. The Southern blot was probed with the λFJ1 insert and the result is shown in Figure 2. Under low stringency conditions, six to seven bands were detected in tobacco. Multiple bands were also observed in the other species tested. The Southern analysis

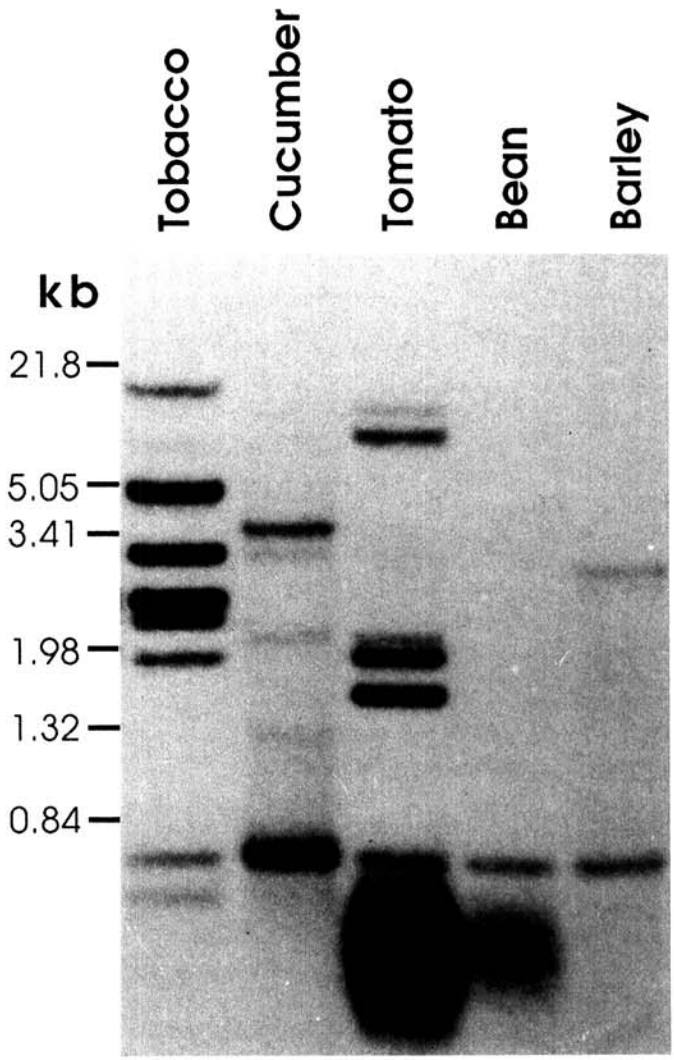

Fig. 2. Southern blot of plant genomic DNA. Ten micrograms of tobacco, cucumber, tomato, bean, and barley DNA were digested with *Eco*R1, separated by gel electrophoresis on a 1.0% agarose gel, blotted, and hybridized with the λFJ1 cDNA clone. Size markers are in kilobases (kb).

suggests that β-1,3-glucanase genes are present in a number of plant species and that tobacco contains a small family of PR-2 β-1,3-glucanase genes.

Regulation of acidic glucanase genes by thiamine. Thiamine is a known chemical inducer of PR-2 protein synthesis (Asselin *et al.* 1985). However, little is known about the regulation of PR-2 genes by thiamine. We investigated the mechanisms underlying PR-2 gene expression by examining the kinetics of accumulation of PR-2 mRNAs and proteins after thiamine induction. The northern blot analysis in Figure 3A shows a low level of glucanase mRNA in untreated or water-treated leaf disks. The presence of PR-2 mRNA in untreated leaf tissue was most likely related to the developmental stage of the plants. We have observed the accumulation of PR-2 β-1,3-glucanase mRNA in the leaves of tobacco plants just before the onset of flowering (J. R. Cutt, F. Côté, and D. F. Klessig; unpublished data). The leaf disks used in this experiment were harvested from plants approaching the flowering stage. The steady-state level of PR-2 mRNA rapidly increased within 6 hr after thiamine treatment and continued to increase until the induction was terminated at 72 hr. The hybridization and wash conditions we used in our northern analyses with the λFJ1 cDNA probe should specifically detect the acidic PR-2a, 2b, -2c mRNAs, but not the basic β-1,3-glucanase isoforms.

PR-2a, -2b, and -2c proteins were first detected in a western blot analysis of the intracellular fluid extracts at

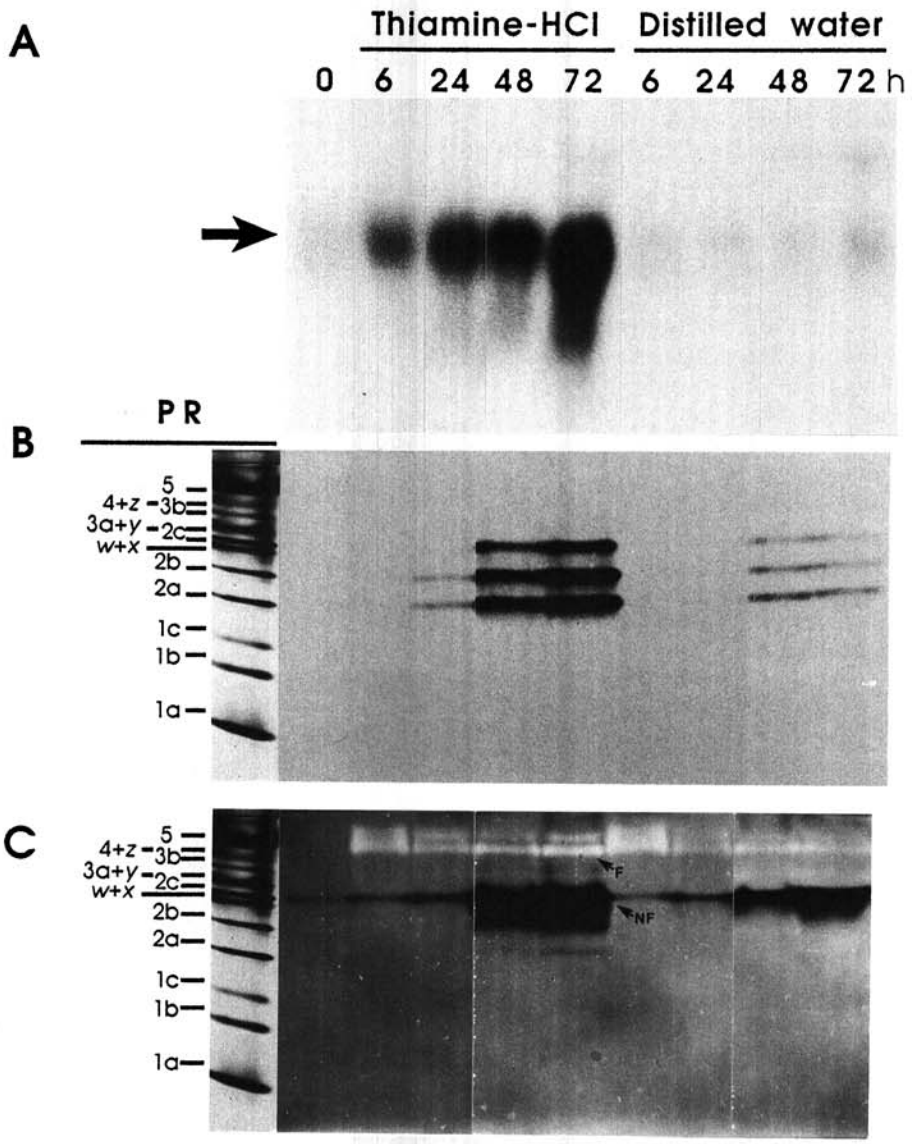

Fig. 3. Steady-state glucanase levels at different times after thiamine or water treatment of leaves. **A,** Northern blot analysis. Ten micrograms total RNA was separated by gel electrophoresis, blotted, and hybridized with the λFJ1 cDNA clone. The arrow indicates the glucanase mRNA transcript. **B,** Western blot analysis of intracellular fluid extracts (6 μl) using PR-2 antisera. A silver-stained protein profile of intracellular extract from thiamine-treated tissues (72 hr) with the PR protein nomenclature is depicted in the first lane. Letters w, x, y, and z refer to unnamed bands in van Loon's nomenclature (1987) and correspond respectively to b_{6a} (peroxidase), b_{6c} (lysozyme-chitinase), b_{7a} (peroxidase) and b_{8a}. **C,** β-1,3-Glucanase activities observed in intracellular fluid extracts. PR protein markers are in the first lane. The arrows indicate typical nonfluorescent and fluorescent lysis zones.

24 hr after thiamine treatment (Fig. 3B). PR-2 proteins accumulated to high levels in the intracellular fluid in a manner consistent with the observed rise in steady-state levels of PR-2 mRNA. A low level of PR-2 proteins was also detected in water-treated leaf disks (Asselin *et al.* 1985). Thus, the observed parallel accumulation of PR-2 proteins and PR-2 mRNA suggested that PR-2 gene expression is regulated, in part, at the level of mRNA accumulation.

Because the functional product encoded by these genes is an enzyme, we also examined the glucanase activities in the intracellular fluid extracts used in the preceding experiment. The assay was based on visualization of glucanase activities after protein separation of intracellular fluid extracts by native PAGE (see Methods). Two types of β-1,3-glucanase activity could be detected by this assay. The first type yields a dark, nonfluorescent zone corresponding to extensive hydrolysis of the laminarin substrate embedded in the gel matrix. The second type yields an intense fluorescent zone. The increased fluorescence of the latter type could be attributable to a ternary complex that involves the fluorochrome interacting with the enzyme and the carbohydrate, or to the interaction of fluorochrome with partially hydrolyzed laminarin. Because the fluorescent zones become nonfluorescent lysis zones with increasing amounts of enzyme or prolonged incubation, we suspect that the high fluorescence is related to incomplete hydrolysis of laminarin (Côté *et al.*, in press).

A low level of one species of the nonfluorescent-type activity was detected in untreated leaf disks that comigrated with the PR-2c polypeptide (Fig. 3C). The level of PR-2c activity increased dramatically after thiamine treatment and to a lesser extent after water treatment. Two species of nonfluorescent-type glucanase activity that corresponded to the PR-2a and PR-2b proteins (Côté *et al.* 1989) were also detected by 48 hr after thiamine treatment, but at lower levels than PR-2c. The higher specific activity reported for PR-2c (PR-2a and PR-2b are 260- and 57-fold less active, respectively, than PR-2c; Kauffmann *et al.* 1987) could account for the differences observed between this assay and the western analysis and for the relatively high levels of PR-2c activity detected after water treatment. In addition to the PR-2a, -2b, and -2c activities, fluorescent-type activities of slower mobility were present in water- and thiamine-treated (Fig. 3C), as well as in TMV- and mock-inoculated plants (data not shown). These activities were most prominent at 6 hr after treatment began.

Induction of glucanase genes after TMV infection. Previous studies have shown that the level of the acidic glucanase proteins PR-2a, -2b, and -2c increased in Xanthi-nc plants infected with TMV (Parent and Asselin 1984). We analyzed RNA at 0, 24, 48, 72, and 96 hr after TMV inoculation to determine if steady-state levels of PR-2 mRNA also increased. mRNA that hybridized to the cDNA probe was present in TMV-inoculated leaves but not present in healthy tissue as shown in the northern analysis represented in Figure 4A. Low levels of this mRNA were detected as early as 24 hr after virus inoculation (Fig. 4A inset; long exposure of the same autoradiogram shown in 4A) and continued to accumulate to a high steady-state level by 96 hr.

In Xanthi-nc tobacco plants infected with TMV, PR proteins are synthesized first in the inoculated leaf and later throughout the plant in association with an induced form of resistance to subsequent pathogen challenge (known as systemic acquired resistance; Ross 1961). Therefore, we determined whether the synthesis of acidic PR-2 proteins in the upper, uninoculated leaves also could be regulated at the level of mRNA accumulation. Low levels of PR-2 mRNA were detected by 48 hr (Fig. 4B inset; long exposure of the same autoradiogram shown in 4B) and steadily increased until the last time examined at 168 hr after TMV inoculation (Fig. 4B). Thus, the level of regulation of the PR-2 genes appears to be similar in thiamine-treated plants

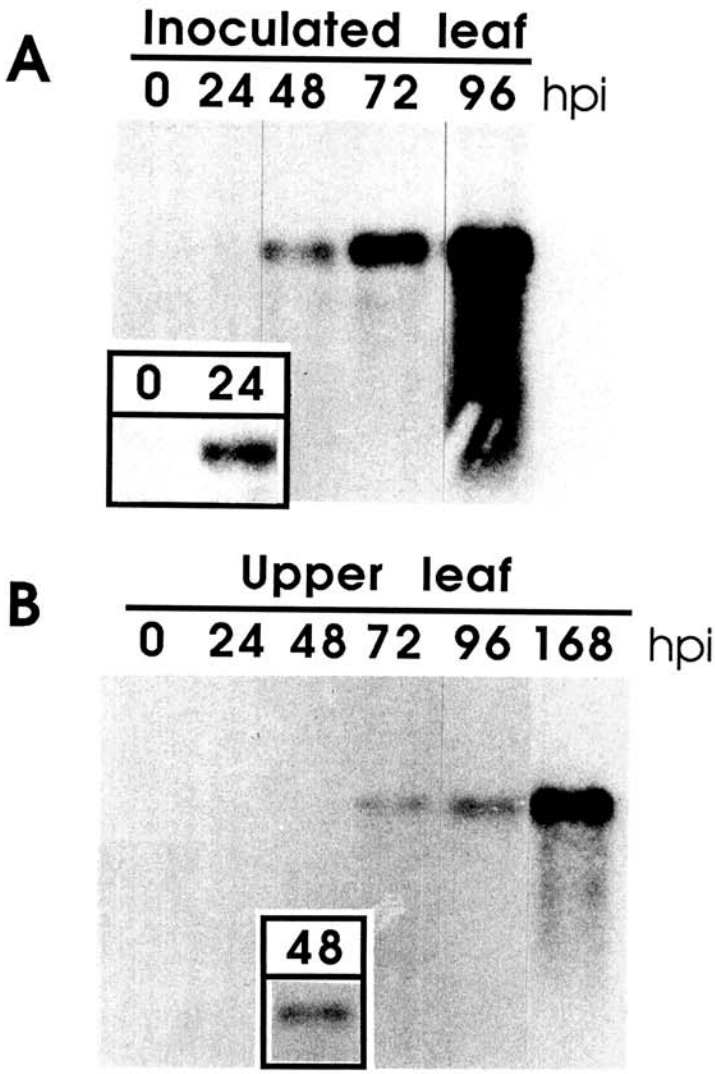

Fig. 4. Time course of mRNA accumulation in tobacco plants infected with tobacco mosaic virus (TMV). Northern blot analyses of total RNA extracted at various times after TMV inoculation (hours postinoculation: hpi) of *Nicotiana tabacum* 'Xanthi-nc'. Twenty micrograms of RNA per lane was separated by gel electrophoresis, blotted, and hybridized with the λFJ1 cDNA clone. **A,** RNA extracted from the lower leaves of plants directly inoculated with TMV. The inset represents a longer exposure of the 0 and 24 hpi time points to demonstrate the presence of glucanase mRNA at 24 hpi. **B,** RNA extracted from the upper leaves of plants inoculated with TMV from the lower leaves. The inset represents a longer exposure of the 0 and 48 hpi time points to demonstrate the presence of glucanase mRNA at 48 hpi.

and in both the inoculated and uninoculated leaves of plants infected with TMV.

Expression of glucanases during floral development. Lotan and co-workers (1989) demonstrated that a β-1,3-glucanase was one of three classes of PR proteins that accumulated in floral structures of tobacco. They observed a 41-kDa protein in stigma/style tissue that was serologically related to the PR-2 proteins (35, 36, and 37 kDa). They suggested that this protein species was another form of the PR-2 proteins whose different mobility in PAGE was attributable to posttranslational processing.

We analyzed floral tissues at different stages of development for the presence of glucanase mRNA, protein, and enzyme activity. These data are presented in Figure 5A–C. Glucanase mRNA, detected in sepal, stigma/style, and ovary tissues at anthesis, comigrated with the mRNA synthesized in tissue infected with TMV (Fig. 5A). The steady levels of glucanase mRNA increased dramatically in sepals from anthesis to 3 wk after anthesis. No glucanase mRNA was detected at anthesis in the stamen (Fig. 5A) or petal tissues, nor after anthesis in the placenta, pericarp, or developing seeds (data not shown).

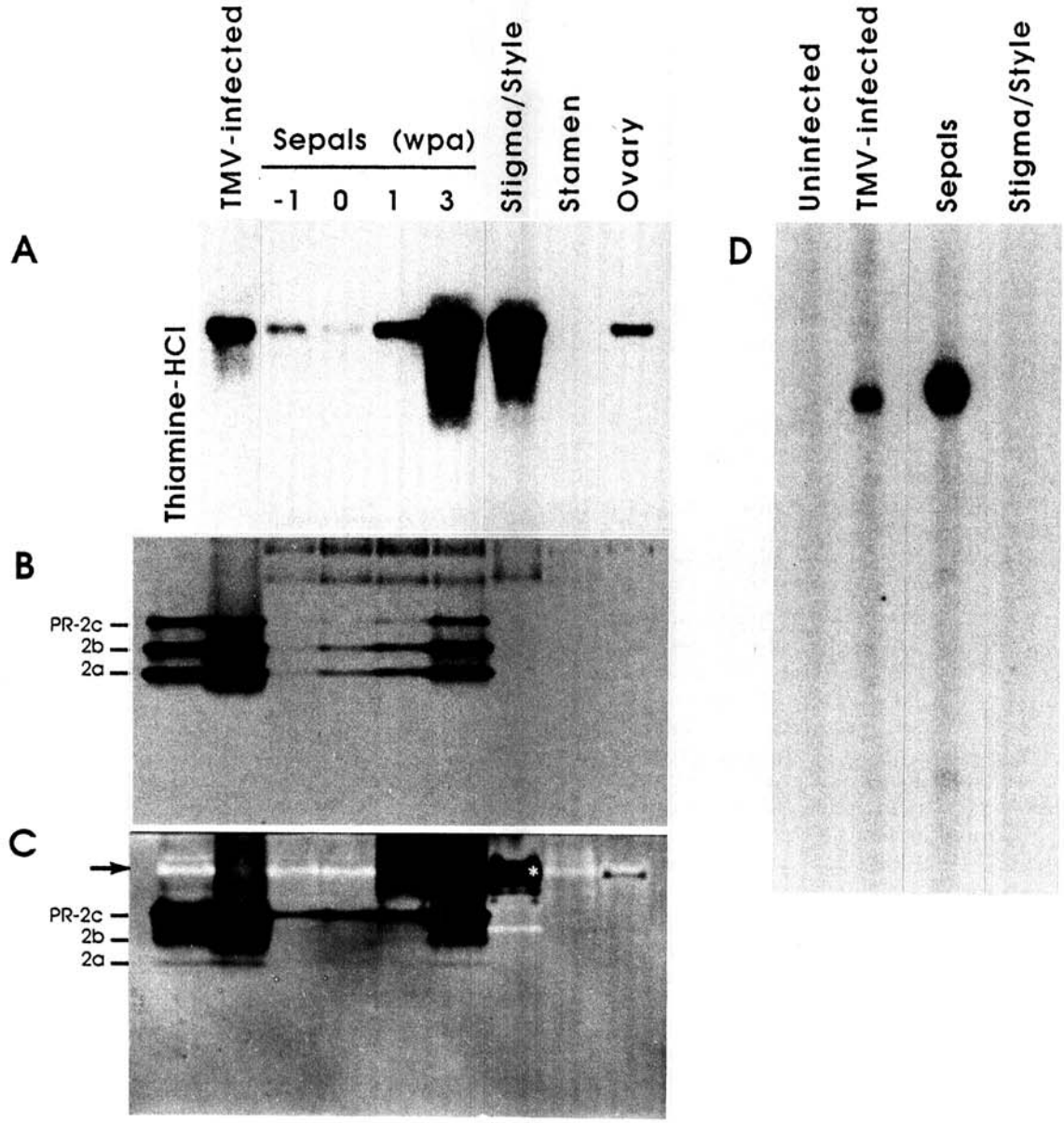

Fig. 5. Tissue-specific expression of β-1,3-glucanases in flowers. Tobacco flowers were dissected into separate organs at different times over the course of development for the determination of glucanase RNA, protein, and enzyme activity levels. Times are in weeks postanthesis (wpa). Stigma/style, stamen, and ovary tissues were harvested at anthesis (0 wpa). **A,** Northern blot analysis. Ten micrograms total RNA per lane was separated by gel electrophoresis, blotted, and hybridized with the λFJ1 cDNA clone. RNA from leaves infected with tobacco mosaic virus (TMV) was used as a control. **B,** Western blot analysis of 10 μg of total protein per lane using PR-2 antisera. Intracellular fluid protein extracts from TMV-infected and thiamine-treated leaves were used as positive controls. **C,** β-1,3-Glucanase activities observed in total protein extracts. The arrow shows the increasing sepal fluorescent-type activity and the asterisk shows the stigma/style fluorescent-type glucanase activity. **D,** Primer extension analysis. Ten micrograms of total RNA was annealed to a PR-2 specific oligonucleotide and the products of the primer extension reaction were separated on a 6% sequencing gel.

In contrast, western analysis revealed PR-2a, -2b, and -2c proteins in only the sepal tissue, where they accumulated to high levels over the course of development (Fig. 5B). Furthermore, additional serologically related polypeptide species, which migrated slower in the gel, were observed in the sepal, stigma/style, stamen, and ovary tissues (from whole tissue extracts) but were not present in TMV- or thiamine-induced leaf tissue (from intracellular fluid extracts). One of these two proteins seems to have the same electrophoretic mobility in native gel as a newly described glucanase PR-Q′ (Fritig *et al.* 1990). The polypeptide species observed in the stigma/style tissue may correspond to the 41-kDa protein reported by Lotan *et al.* (1989). The corresponding increase in both PR-2 protein and mRNA levels in sepals suggests that the mechanisms controlling PR-2 gene expression are probably similar during the development of sepals and in stressed leaves.

A number of glucanase enzyme activities were seen in floral tissues. PR-2c enzyme activity was present in sepals and increased from 1 wk before anthesis up to 3 wk after anthesis (Fig. 5C). Glucanase activities corresponding to PR-2a, -2b were detected in sepals only at 3 wk after anthesis. In addition, we observed a fluorescent-type activity in sepals (indicated by an arrow) that migrated slower than the PR-2 proteins. This activity increased after anthesis, as is evident by the transition of the band from a high fluorescent zone to a dark lysis zone. The stamen, ovary, and, moreover, stigma/style tissues contained only fluorescent-type activities while nonfluorescent-type activities corresponding to the PR-2a, -2b, and -2c proteins were absent as expected from the western analysis. One of the fluorescent-type glucanase activities in the stigma/style (indicated by an asterisk) was present at high enough levels to produce a nonfluorescent zone in the gel assay; when less stigma/style protein extract was analyzed in the gel assay this appeared as a fluorescent-type activity (data not shown).

In light of the high level of glucanase mRNA detected by our PR-2 cDNA probe but in the absence of PR-2 proteins and activities in the stigma/style, we considered the possibility that the expression of another related glucanase gene could be responsible for these observations and those of Lotan *et al.* (1989). To test this possibility, we performed a primer extension analysis on RNA from uninfected leaf tissue, TMV-infected leaf tissue, and uninfected sepal and stigma/style tissues with a PR-2-specific oligonucleotide (Fig. 5D). Primer extension products corresponding to the PR-2 mRNA were detected in sepals and leaves infected with TMV but not in stigma/style tissue or uninfected leaves. These results suggest that the glucanase mRNA and enzymatic activities observed in stigma/style are attributable to the expression of another class of glucanase genes related to the PR-2 family.

DISCUSSION

The structure of the acidic PR β-1,3-glucanase genes. Extracellularly located β-1,3-glucanases represent one of several classes of β-1,3-glucanase enzymes that have been identified and characterized in higher plants. In this paper, we observed the isolation and nucleotide sequence of a partial cDNA clone from tobacco encoding an acidic, extracellular β-1,3-glucanase isoform. Comparison with partial amino acid sequences for the three acidic PR β-1,3-glucanase proteins (Van den Bulcke *et al.* 1989; J. Ryals and E. Ward, personal communication) indicate that this clone corresponds to the amino-terminal domain of the PR-2c (PR-O) protein. The existence of different acidic β-1,3-glucanase isoforms and the Southern blot analysis both suggest that the acidic β-1,3-glucanases are encoded by a small gene family. Moreover, the Southern blot analysis of different plant species suggests these genes, and perhaps their enzymatic function, have been evolutionarily conserved. Finally, these results also confirm that the acidic PR β-1,3-glucanase genes are related to, but distinct from, the basic PR β-1,3-glucanase gene previously isolated from tobacco (Shinshi *et al.* 1988).

Control of PR-2 gene expression by stress. Asselin *et al.* (1985) previously reported on the induction of PR-2 protein synthesis in tobacco leaves by the chemical agent thiamine. We have demonstrated that after thiamine treatment (or salicylic acid treatment, data not shown) the steady-state levels of PR-2 mRNA increase in parallel with the accumulation of PR-2 proteins. Furthermore, steady-state levels of PR-2 mRNA increased in both the inoculated and uninoculated leaves of plants infected with TMV in which PR-2 protein synthesis was also induced; this is consistent with the findings of Memelink *et al.* (1990) who demonstrated an accumulation of acidic β-1,3-glucanase mRNA in TMV-inoculated leaves of Samsun NN, 6 days after infection. These data indicate that the primary mechanism governing the selective expression of PR-2 genes is either transcriptional activation or mRNA stability. The isolation of the PR-2 gene promoter sequences (R. E. Dewey, J. R. Cutt, and D. F. Klessig; unpublished results) should permit us to examine the *cis*- and *trans*-acting factors involved in PR-2 gene expression. The PR-2 genes of tobacco therefore appear to be regulated in a similar manner as the PR-1 genes after stress (Carr *et al.* 1985).

PR-2 proteins are synthesized during normal plant development. PR protein synthesis has been characterized primarily in the leaves of plants in association with environmental stress. The correlation of PR gene expression with disease resistance suggests they may function as plant defense genes. Fraser (1981) provided strong evidence for the developmental regulation of PR proteins (PR-1 and possibly PR-2 proteins) in the leaves of healthy tobacco plants during flowering. Fraser's results and those of Lotan *et al.* (1989) and Memelink *et al.* (1990) on the floral-specific expression of PR genes and the synthesis of a protein serologically related to PR-2 proteins (Lotan *et al.* 1989) suggest that PR genes play a role in normal development.

In our studies the expression of the PR-2 genes and other acidic glucanases in flowers has been characterized. PR-2 mRNA, proteins, and the corresponding enzyme activities were found in the sepal tissue (Fig. 5), a tissue specificity not previously reported by Lotan *et al.* (1989) or detected by Memelink *et al.* (1990). We have also observed the same pattern of increasing expression of the PR-1 genes during sepal development (J. R. Cutt, D. C. Dixon, and D. F. Klessig; unpublished results). One possibility is that expression of the two PR-gene families in the sepal might be developmentally regulated; perhaps in

the case of the sepal, they are controlled by the programmed senescence of this tissue after maturation of the inflorescence. Another alternative is that these genes may have been induced by a cryptic pathogenic infection. However, our observations that the PR-1, but not PR-2, genes are expressed during early seed development (J. R. Cutt, D. C. Dixon, and D. F. Klessig, unpublished data) strongly suggest that expression of PR-2 and PR-1 genes in the flower is controlled by tissue-specific developmental factors.

Our studies also indicated that a β-1,3-glucanase mRNA was present in the stigma/style and ovary tissues as well as the sepal tissue. However, while the PR-2 proteins were detected in the sepal, they were not seen in the stigma/style or ovary tissues. A second anomaly was that although proteins serologically related to but migrating slower than the PR-2 proteins were identified in the sepal, ovary, stigma/style, and stamen tissues, the type of glucanase activities of these proteins were different than those shown by the PR-2 proteins. The identity of these proteins is thus unknown; however, one of the two proteins detected in the stigma/style by the western analysis may be the 41-kDa glycoprotein identified by Lotan *et al.* (1989). Finally, the primer extension analysis suggested that the β-1,3-glucanase mRNA that we detected in the stigma/style was not encoded by one of the PR-2 genes. This conclusion would also be in accordance with the differential localization of the 41-kDa (intracellular) and PR-2 (extracellular) proteins. The presence of this glucanase in stigma/style tissue could be related to pollen tube formation during the fertilization process; however, the significance of this observation will require further characterization of the gene encoding this novel glucanase isoform and its enzymatic activity.

The possible functions of the PR-2 proteins. The rapid induction of PR-2 genes in inoculated leaves as well as in upper uninoculated leaves of Xanthi-nc plants infected with TMV suggests that β-1,3-glucanase activity could be a factor involved in the hypersensitive response and systemic acquired resistance toward viral infections. Callose, a β-1,3-glucan, which is known to form plugs in phloem and to accumulate between plasmalemma and the cell wall in response to the stress of infection has been implicated in the restriction of viral spread. Therefore, β-1,3-glucanase activity might influence the course of viral infections. However, Kearney and Wu (1984) were unable to correlate the levels of β-1,3-glucanase activity with lesion size or virus spread in plants infected with TMV and concluded that the activity performs no direct role in viral spread. The ability of glucanases to inhibit fungal growth *in vitro* (Mauch *et al.* 1988) and the localization of host glucanase proteins on the cell surface of invading fungi (Benhamou *et al.* 1989) suggest that these proteins function in defending the host against fungal pathogens. This might be accomplished by inhibition of fungal growth through destruction of its cell wall and/or by release of glucan-elicitor fragments. We suspect the β-1,3-glucanase genes are induced after viral infection as one component of a broad, generalized defense mechanism to pathogen attack. Their association with systemic acquired resistance would be part of a protective mechanism toward subsequent pathogen challenges. Indeed, enhanced resistant to a challenge infection by *Phytophthora parasitica* Dastur var. *nicotiana* (Breda de Haan) Tucker, a fungus with a high proportion of β-1,3-glucans and cellulose, was demonstrated in tobacco preinoculated with TMV (Bonnet *et al.* 1986). Therefore, β-1,3-glucanases are likely to be part of a basic resistance to pathogen invasion and not necessarily related to incompatible host–viral interactions. Ultrastructural immunocytological localization of PR-2 glucanases in relation to invading fungal pathogens and the construction of transgenic plants that constitutively express the PR-2 genes or inhibit their activity will be required to examine this hypothesis.

Three of the five families of PR proteins (PR-1, -2, and -P/-Q) have now been identified in the sepal tissue of tobacco (this report; Lotan *et al.* 1989; Trudel *et al.* 1989). The occurrence of PR proteins in the sepal tissue suggests a specific biological function during the normal development of the flower. We can only speculate whether this role is in the actual development of this tissue or in defending the developing embryo against pathogen invasion. Hence, future investigations should focus on the function of these PR genes in the flower and the expression of other PR gene families in the inflorescence. It will be interesting to know if the regulatory networks activated by environmental stress are similar to those functioning during normal development.

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LITERATURE CITED

Asselin, A., Grenier, J., and Côté, 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.

Benhamou, N., Grenier, J., Asselin, A., and Legrand, M. 1989. Immunogold localization of β-1,3-glucanase in two plants infected by vascular wilt fungi. Plant Cell 1:1209-1221.

Boller, T. 1985. Induction of hydrolases as a defense reaction against pathogens. Pages 247-262 in: Cellular and Molecular Biology of Plant Stress. J. L. Key and T. Kosuge, eds. Allan R. Liss, New York.

Bonnet, P., Poupet, A., Abad, P., Venard, P., and Cardin, L. 1986. Induction de nécroses foliaires, de protéines b et de résistance dans les interactions tabac-*Phytophthora.* Agronomie 6 (Paris):829-837.

Bull, A. T., and Chesters, C. G. C. 1966. The biochemistry of laminarin and nature of laminarinase. Adv. Enzymol. 28:325-364.

Carr, J. P., Dixon D. C., and Klessig D. F. 1985. Synthesis of pathogenesis-related proteins in tobacco is regulated at the level of mRNA accumulation and occurs on membrane-bound polysomes. Proc. Natl. Acad. Sci. USA 82:7999-8003.

Carr, J. P., and Klessig, D. F. 1989. The pathogenesis-related proteins of plants. Pages 69-109 in: Genetic Engineering: Principles and Methods, Vol. 11. J. K. Setlow, ed. Plenum Publishing Co., New York.

Chao, S., Chao, L., and Chao, J. 1989. Enhanced specificity in immunoscreening of expression cDNA clones using radiolabeled antigen overlay.

BioTechniques 7:68-72.
Cornelissen, B. J. C., Horowitz, J. van Kan, J. A. L., Golberg, R. B., and Bol, J. F. 1987. Structure of tobacco genes encoding pathogenesis-related proteins from the PR-1 group. Nucleic Acids Res. 15:6799-6811.
Côté, F., El Ouakfaoui, S., and Asselin, A. β-glucanases acting on various β-1,3 and β-1,4-glucans can be detected after native and denaturing polyacrylamide gel electrophoresis. Electrophoresis. In press.
Côté, F., Letarte, J., Grenier, J., Trudel, J., and Asselin, A. 1989. Detection of β-1,3-glucanase activity after native polyacrylamide gel electrophoresis: Application to tobacco pathogenesis-related proteins. Electrophoresis 10:527-529.
Cutt, J. R., Dixon, D. C., Carr, J. P., and Klessig, D. F. 1988. Isolation and nucleotide sequence of cDNA clones for the pathogenesis-related proteins PR1a, PR1b and PR1c of *Nicotiana tabacum* cv. Xanthi-nc induced by TMV infection. Nucleic Acids Res. 16:9861.
Cutt, J. R., and Klessig, D. F. Pathogenesis-related proteins. In: Plant Gene Research. F. Meins, Jr. and T. Boller, eds. Springer-Verlag, Wein, New York. In press.
Davis, B. J. 1964. Disc electrophoresis. II. Method and application to human serum proteins. Ann. NY Acad. Sci. 121:404-427.
Dellaporta, S. L., Wood, J., and Hicks, J. B. 1983. A plant DNA minipreparation: Version II. Plant Mol. Biol. Rep. 1:19.
De Loose, M., Alliotte, T., Gheysen, G., Genetello, C., Gielen, J., Soetaert, P., Van Montagu, M., and Inzé, D. 1988. Primary structure of a hormonally regulated β-glucanase of *Nicotiana plumbaginifolia*. Gene 70:13-23.
Dunsmuir, P., Bond, D., Lee, K., Gidoni, D., and Townsend, J. 1987. The expression of introduced genes in regenerated plants. Pages 45-59 in: Plant Molecular Biology Manual, Vol. 9. S. Gelvin and R. Schilperoort, eds. Plenum Publishing Co., New York.
Fincher, G. B., Lock, P. A., Morgan, M. M., Lingelbach, K., Wettenhall, R. E. H., Mercer, J. F. B., Brandt, A., and Thomsen, K. K. 1986. Primary structure of the (1-3, 1-4)-β-D-glucan 4-glucanohydrolase from barley aleurone. Proc. Natl. Acad. Sci. USA 83:2081-2085.
Fortin, M. G., Parent, J. G., and Asselin, A. 1985. Comparative study of two groups of b proteins (pathogenesis-related) from the intercellular fluid of *Nicotiana* leaf tissues infected by tobacco mosaic virus. Can. J. Bot. 63:932-937.
Fraser, R. S. S. 1981. Evidence for the occurrence of the "pathogenesis-related" proteins in leaves of healthy tobacco plants during flowering. Physiol. Plant Pathol. 19:69-76.
Fritig, B., Kauffmann, S., Rouster, J., Dumas, B., Geoffroy, P., Kopp, M., and Legrand, M. 1990. Defense proteins, glycanhydrolases and oligosaccharide signals in plant–virus interactions. Pages 375-394 in: Recognition and Response in Plant–Virus Interactions. NATO ASI Series Vol. H41. R. S. S. Fraser, ed. Springer-Verlag, Berlin.
Hahn, M. G., Bucheli, D., Cervone, F., Doares, S. H., O'Neill, R. A., Darvill, A., and Albersheim, P. 1989. The roles of cell wall constituents in plant–pathogen interactions. Pages 131-181 in: Plant-Microbe Interactions, Vol, 3. E. Nester and T. Kosuge, eds. McGraw-Hill Publishing Co., New York.
Hoj, P. B., Hartman, D. J., Morrice, N. A., Doan, D. N. P., and Fincher, G. B. 1989. Purification of (1-3)-β-glucan endohydrolase isoenzyme II from germinated barley and determination of its primary structure from a cDNA clone. Plant Mol. Biol. 13:31-42.
Jondle, D. J., Coors, J. G., and Duke, S. H. 1989. Maize leaf β-1,3-glucanase activity in relation to resistance to *Exserohilum turcicum*. Can. J. Bot. 67:263-266.
Joosten, M. H. A. J., and De Wit, P. J. G. M. 1989. Identification of several pathogenesis-related proteins in tomato leaves inoculated with *Cladosporium fulvum* (syn. *Fulvia* Fulva) as 1,3-β-glucanases and chitinases. Plant Physiol. 89:945-951.
Kauffmann, S., Legrand, M., Geoffroy, P., and Fritig, B. 1987. Biological function of pathogenesis-related proteins. Four PR proteins have β-(1-3) glucanase activity. EMBO J. 6:3209-3212.
Kearney, C. M., and Wu, J. H. 1984. β-1,3-glucanase and the spread of tobacco mosaic virus in *Nicotiana* and *Phaseolus*. Can. J. Bot. 62:1984-1988.
Keen, N. T., and Yoshikawa, M. 1983. β-1,3-Endoglucanase from soybean releases elicitor-active carbohydrates from fungus cell walls. Plant Physiol. 71:460-465.
Kombrink, E., and Halhbrock, K. 1986. Responses of cultured parsley cells to elicitors from phytopathogenic fungi. Plant Physiol. 81:216-221.
Kombrink, E., Schroder, M., and Halhbrock, K. 1988. Several "pathogenesis-related" proteins in potato are 1,3-β-glucanases and chitinases. Proc. Natl. Acad. Sci. USA 85:782-786.
Lotan, T., Ori, N., and Fluhr, R. 1989. Pathogenesis-related proteins are developmentally regulated in tobacco flowers. Plant Cell 1:881-887.
Matsuoka, M., Yamamoto, N., Kano-Murakami, Y., Tanaka, Y., Ozeki, Y., Hirano, H., Kagawa, H., Oshima, M., and Ohashi, Y. 1987. Classification and structural comparison of full-length cDNAs for pathogenesis-related proteins. Plant Physiol. 85:942-946.
Mauch, F., Mauch-Mani, B., and 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.
Mauch, F., and Staehelin, L. A. 1989. Functional implications of the subcellular localization of ethylene-induced chitinase and β-1,3-glucanase in bean leaves. Plant Cell 1:447-457.
Meins, F., Jr., and Ahl, P. 1989. Induction of chitinase and β-1,3-glucanase in tobacco plants infected with *Pseudomonas tabaci* and *Phytophthora parasitica* var. *nicotianae*. Plant Sci. 61:155-161.
Memelink, J., Linthorst, H. J. M., Schilperoort, R. A., Hoge, J. H. C. 1990. Tobacco genes encoding acidic and basic isoforms of pathogenesis-related proteins display different expression patterns. Plant Mol. Biol. 14:119-126.
Parent, J. G., and Asselin, A. 1984. Detection of pathogenesis-related proteins (PR or b) and other proteins in the intercellular fluid of hypersensitive plants infected with tobacco mosaic virus. Can. J. Bot. 62:546-569.
Payne, G., Ahl, P., Moyer, M., Harper, A., Beck, J., Meins, F., Jr., and Ryals, J. 1990. Isolation of complementary DNA clones encoding pathogenesis-related proteins P and Q, two acidic chitinases from tobacco. Proc. Natl. Acad. Sci. USA 87:98-102.
Payne, G., Middlesteadt, W., Williams, S., Desai, N., Parks, T. D., Dincher, S., Carnes, M., and Ryals, J. 1988. Isolation and nucleotide sequence of a novel cDNA clone encoding the major form of pathogenesis-related protein R. Plant Mol. Biol. 11:223-224.
Pfitzner, U. M., and Goodman, H. M. 1987. Isolation and characterization of cDNA clones encoding pathogenesis-related proteins from tobacco mosaic virus infected tobacco plants. Nucleic Acids Res. 15:4449-4465.
Ross, A. F. 1961. Systemic acquired resistance induced by localized virus infections in plants. Virology 14:340-358.
Shinshi, H., Wenzler, H., Neuhaus, J. M., Felix, G., Hofsteenge, J., and Meins, F., Jr. 1988. Evidence for N- and C-terminal processing of a plant defense-related enzyme: Primary structure of tobacco prepro-β-1,3-glucanase. Proc. Natl. Acad. Sci. USA 85:5541-5545.
Siegel, A., and Wildman, S. G. 1954. Some natural relationships among strains of tobacco mosaic virus. Phytopathology 44:277-282.
Takeuchi, Y., Yoshikawa, M., Takeba, K., Shibata, D., and Horino, O. 1990. Molecular cloning and ethylene induction of mRNA encoding a phytoalexin elicitor-releasing factor, β-1,3-endoglucanase, in soybean. Plant Physiol. 93:673-682.
Trudel, J., Audy, P., and Asselin, A. 1989. Electrophoretic forms of chitinase activity in Xanthi-nc tobacco, healthy and infected with tobacco mosaic virus. Mol. Plant-Microbe Interact. 2:315-324.
Van den Bulcke, M., Bauw, G., Castresana, C., Van Montagu, M., and Vandekerkhove, J. 1989. Characterization of vacuolar and extracellular β-1,3-glucanases of tobacco: Evidence for a strictly compartmentalized plant defense system. Proc. Natl. Acad. Sci. USA 86:2673-2677.
van Kan, V. A. L., Cornelissen, B. J. C., and Bol, J. F. 1988. A virus-inducible tobacco gene encoding a glycine-rich protein shares putative regulatory elements with the ribulose biphosphate carboxylase small subunits gene. Mol. Plant-Microbe Interact. 1:107-113.
van Loon, L. C., Gerritsen, Y. A. M., and Ritter, C. E. 1987. Identification, purification, and characterization of pathogenesis-related proteins from virus-infected Samsun NN tobacco leaves. Plant Mol. Biol. 9:593-609.
Varner, J. E., and Lin, L. S. 1989. Plant cell wall architecture. Cell 56:231-239.
Verwoerd, T. C., Dekker, B. M. M., and Hoekema, A. 1989. A small scale procedure for the rapid isolation of plant RNAs. Nucleic Acids Res. 17:2362.
Viera, J., and Messing, J. 1987. Production of single-stranded plasmid DNA. Pages 3-11 in: Methods in Enzymology, Vol. 153. R. Wu and L. Grossman, eds. Academic Press, San Diego.
Vögeli, U., Meins, F., Jr., and Boller, T. 1988. Co-ordinated regulation of chitinase and β-1,3-glucanase in bean leaves. Planta 174:364-372.