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Differential Regulation of β-1,3-Glucanase Messenger RNAs in Response to Pathogen Infection

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ABSTRACT

The acidic, extracellular, glucan endo-1,3-β-glucosidases (EC 3.2.1.39; β-1,3-glucanases), pathogenesis-related proteins-2, -N, and -O (*i.e.* PR-2, PR-N, and PR-O) were purified from *Nicotiana tabacum* (tobacco) and their partial amino acid sequences determined. Based on these data, complementary DNA (cDNA) clones encoding the proteins were isolated. Additional cDNAs were isolated that encoded proteins approximately 90% identical with PR-2, PR-N, and PR-O. Although the proteins encoded by these cDNAs have not been identified, their deduced amino acid sequences have slightly basic or neutral calculated isoelectric points, as well as carboxy-terminal extensions. These physical characteristics are shared by the vacuolar form of β-1,3-glucanase and other vacuolar localized analogs of PR proteins, suggesting that the unidentified proteins may be similarly localized. A preliminary evolutionary model that separates the β-1,3-glucanase gene family from tobacco into at least five distinct subfamilies is proposed. The expression of β-1,3-glucanase messenger RNAs (mRNAs) in response to infection by tobacco mosaic virus was examined. Messages for the acidic glucanases were induced similarly to the mRNAs for other PR proteins. However, the basic glucanase showed a different response, suggesting that different isoforms are differentially regulated by tobacco mosaic virus infection at the mRNA level.

Plants draw on a large repertoire of defense responses when infected by pathogens. The best studied of these responses is the synthesis of new proteins that can have direct or indirect action on the course of pathogenesis. These proteins include enzymes involved in phenylpropanoid and flavonoid metabolism (reviewed in ref. 14), peroxidases (13) β-1,3 glucanases and chitinases (1), hydroxyproline-rich glycoproteins (25), and a diverse group of acidic, extracellular proteins known collectively as PR[1] proteins (reviewed in ref. 4). Among the proteins having known enzymatic function, the β-1,3-glucanases are particularly interesting because they are hormonally and developmentally regulated in uninfected plants (7, 18) and are thought to protect plants from fungal infection (reviewed in ref. 3).

A role of β-1,3-glucanases in plant defense is suggested by the observation that β-1,3-glucanases and endochitinases are coordinately induced as part of the hypersensitive response to pathogen infection (17, 28). β-1,3-Glucans are important structural components of fungal cell walls (reviewed in ref. 29) and *in vitro* evidence shows that β-1,3-glucanase in combination with chitinase has a direct fungicidal action on some phytopathogenic fungi (16). These enzymes could, therefore, act directly by inhibiting growth of invading fungal hyphae. β-1,3-Glucanases also may act indirectly by releasing from fungal cell walls elicitors that can stimulate phytoalexin accumulation in the host plant (*e.g.* see ref. 12).

In healthy plants, β-1,3-glucanases accumulate to high concentrations (up to 4% of soluble protein) in lower leaves and roots (7). These enzymes also can be found in specialized tissue types such as the leaf epidermis (11) and in stylar tissues of the flower (15). In cultured tobacco cells, basic β-1,3-glucanases and their mRNAs are down-regulated by combinations of the growth hormones auxin and cytokinin (7, 18) and are induced both in culture and in intact plants by the stress hormone ethylene (8).

Investigating the complex developmental, hormonal, and disease-related regulation of the β-1,3-glucanases is complicated by the finding that the enzyme exists in several isoforms that differ in physical properties, enzyme activity, antigenicity, and cellular compartmentation. At least three major classes of the enzyme have been identified in tobacco (21). The class I enzymes include three related isoforms, characterized by basic pI values, that accumulate predominantly in the vacuole (11, 27). In healthy plants, they are expressed primarily in epidermal cells and accumulate to high levels in mesophyll cells after ethylene treatment (11). The class II enzymes, which include the pathogenesis-related proteins PR-2, -N, and -O, are acidic proteins found in the extracellular compartment of leaves (10, 27). These proteins are not found in healthy tissues but accumulate to high levels in response to pathogen infection and treatment with certain chemical inducers such as aspirin (reviewed in ref. 4). To date, one class III enzyme has been identified, PR-Q′ (9, 21), an acidic protein that also accumulates in the apoplast when infected with a pathogen.

cDNA clones have been isolated for the class I and III enzymes (21, 24), but the lack of cDNAs for the class II enzymes has limited detailed studies of their gene structure and regulation. Here, we describe the isolation and character-

[1] Abbreviations: PR, pathogenesis related; TMV, tobacco mosaic virus; ICF, intercellular fluid; bp, base pair(s); pI, isoelectric point; ORF, open reading frame.

ization of cDNAs encoding the class II β-1,3-glucanases PR-2, -N, and -O, as well as cDNAs encoding two novel glucanases closely related to these PR proteins. Studies of the regulation of these genes by a pathogen show that the class I glucanase is regulated at the mRNA level differently from classes II and III.

MATERIALS AND METHODS

Plant Materials and Virus Infection

Greenhouse-grown tobacco, *Nicotiana tabacum* cv Xanthi.nc, plants were infected when 8 weeks old by gently rubbing the leaves with a suspension of the common strain (U1) of TMV (0.5 μg/mL) in a solution of 10 mM sodium phosphate (pH 7) containing carborundum. Mock-inoculated control plants were treated with buffer and carborundum only.

Purification and Amino Acid Sequencing of PR-2, PR-N, and PR-O Proteins

The ICF of TMV-infected leaves was isolated, concentrated, and fractionated as described previously (20). ICF proteins were then separated by chromatography on DEAE-Sephacel. Fraction 3 contained PR-2, PR-N, and several minor contaminants. Fraction 6 contained a mixture of PR-R major, PR-R minor, PR-P, PR-Q, PR-O, and several minor contaminants. PR-2 and PR-N were purified from fraction 3 by anion exchange HPLC using a Brownlee Aquapore AX-300 column (2.1 × 100 mm) eluted with a linear gradient of 10 to 350 mM ammonium acetate, pH 7. PR-N eluted at 37 min (115 mM ammonium acetate) and PR-2 eluted at 50 min (290 mM ammonium acetate). PR-O was purified from fraction 6 using a reverse-phase Vydac phenyl column (4.6 × 250 mm). Protein was eluted with a linear gradient of 20 of 80% acetonitrile:isopropanol (1:1) in 0.1% TFA.

Partial amino acid sequences of PR-2, PR-N, and PR-O were determined from tryptic peptides, generated by digesting the reduced and alkylated purified proteins with trypsin in 0.1 M Tris-HCl, pH 8.5, for 24 h at 37°C at an enzyme:substrate ratio of 1:50. The peptides were isolated by HPLC on a Vydac C18 column and eluted with a linear gradient of 0 to 60% acetonitrile:isopropanol (1:1) in 0.1% TFA. Automated Edman degradations were performed by using the Applied Biosystems 470A gas-phase sequencer. Phenylthiohydantoin amino acids were identified by using an Applied Biosystems 120A phenylthiohydantoin analyzer. Further details of the protein purification and sequencing are available on request.

Isolation of cDNA Clones

The construction of a cDNA library from tobacco leaves 5 d after TMV infection has been described (20). Duplicate plaque lifts were hybridized and washed as described (5). Inserts from candidate clones were amplified by the polymerase chain reaction (23) using the GeneAmp kit from Perkin-Elmer/CETUS according to the manufacturer's instructions. The oligonucleotide primers flanked the cloning site in λ-

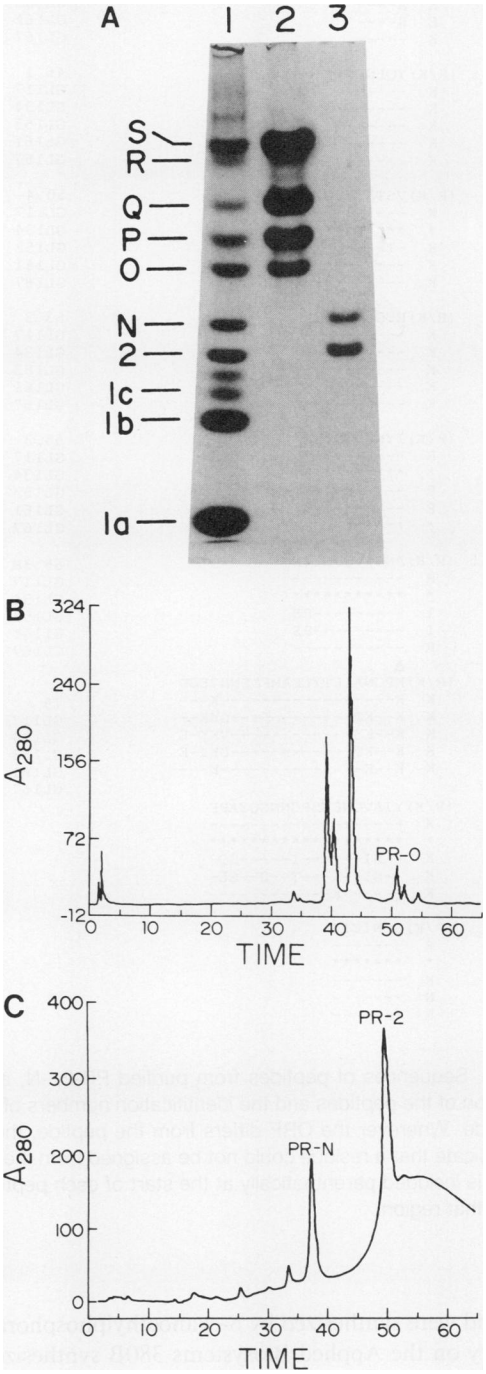

Figure 1. A, Native PAGE analysis of total ICF extracted from Xanthi tobacco (lane 1), fraction 6 from ICF, from which PR-O was purified (lane 2), and fraction 3, from which PR-2 and -N were purified (lane 3). B, Elution profile showing purification of PR-O by HPLC. C, Elution profile showing purification of PR-2 and -N by HPLC.

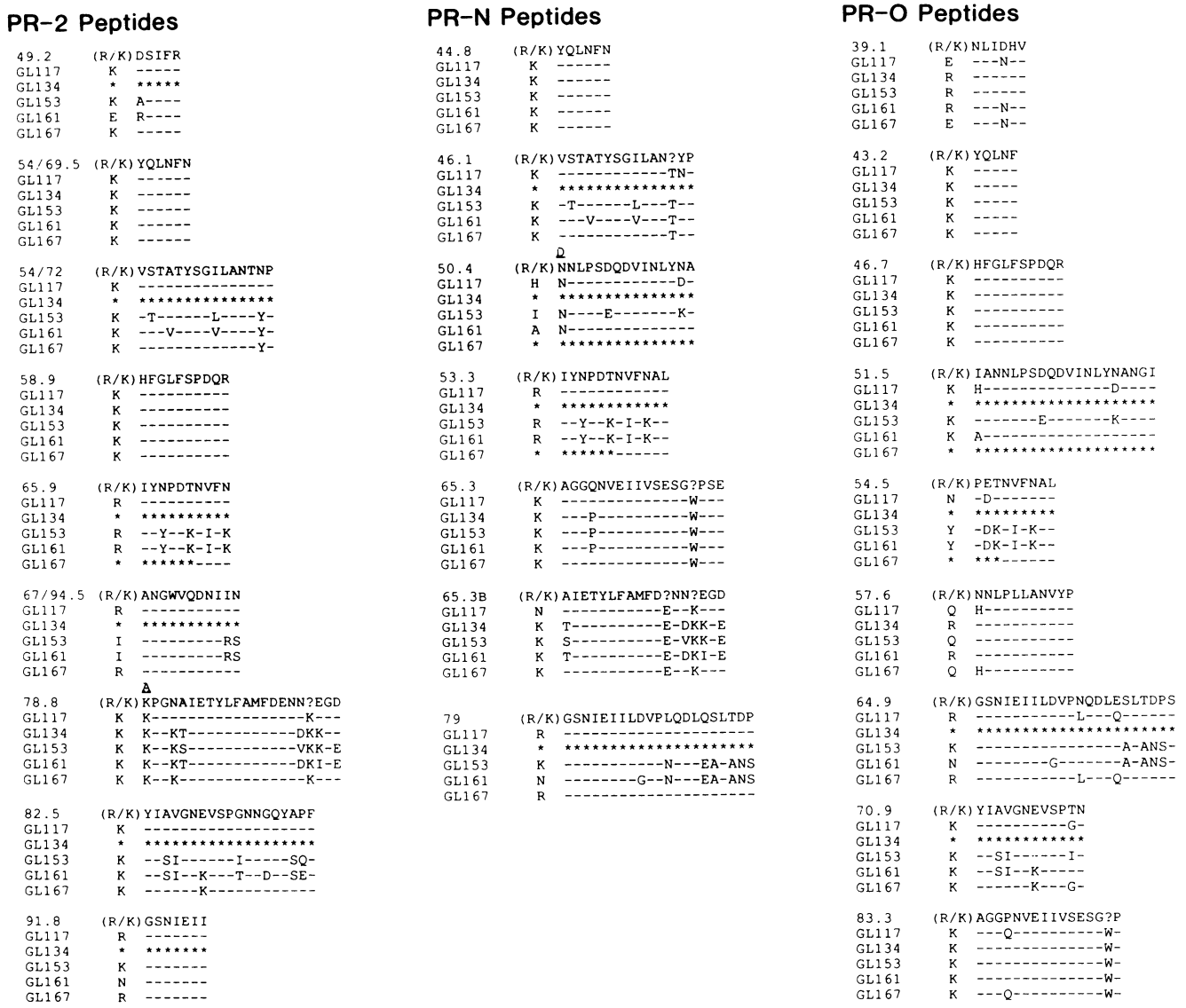

Figure 2. Sequences of peptides from purified PR-2, -N, and -O aligned with translations of ORFs from the isolated cDNAs. The numerical designation of the peptides and the identification numbers of the clones are at the left of each column. Hyphens indicate identity of the ORF with the peptide. Wherever the ORF differs from the peptide, the single letter code for the amino acid encoded at that position is given. Question marks indicate that a residue could not be assigned from the peptide-sequencing data at that position. The arginine or lysine inferred from tryptic cleavage is included parenthetically at the start of each peptide sequence. Asterisks indicate that the ORF of the particular clone did not extend as far as that region.

OngC and were synthesized by β-cyanoethylphosphoramidite chemistry on the Applied Biosystems 380B synthesizer. The amplified cDNA inserts were subcloned and sequenced[2] as described before (20). For each cDNA insert, two independent plasmid subclones were completely sequenced in order to check for possible mutations caused by the polymerase chain reaction amplification.

[2] The nucleotide sequence data reported in this paper will appear in the EMBL, GenBank, and DDBJ Nucleotide Sequence Databases under accession numbers M60460–M60464.

DNA and RNA Blot Analysis

Genomic DNA was isolated by standard methods (2). DNA that had been digested with various restriction enzymes was separated by agarose gel electrophoresis and alkaline blotted to nylon membrane (GeneScreen Plus, New England Nuclear) as described previously (22). Total RNA from TMV-infected tobacco leaves was separated by formadehyde agarose gel electrophoresis and blotted to nylon membrane as described before (2). Equal loading of the lanes was confirmed by ethidium bromide staining. The blots were hybridized and

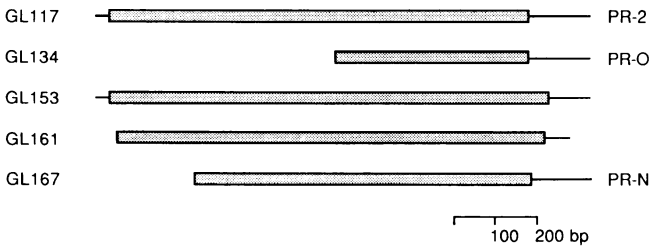

Figure 3. Schematic diagram depicting the cDNA inserts isolated. The stippled boxes are ORFs. The thin lines indicate 5′- and 3′-untranslated regions.

washed using conditions described previously (5). Probes were radiolabeled by the random-priming method (6).

RESULTS

Purification and Partial Amino Acid Sequence of PR-2, PR-N, and PR-O

PR-2, PR-N, and PR-O were purified from the ICF of *N. tabacum* cv Xanthi.nc leaves infected with TMV. The proteins contained in the ICF were separated by chromatography on DEAE-Sephacel. One fraction (fraction 6) contained PR-O and a second fraction (fraction 3) contained PR-2 and -N (Fig. 1A). PR-O was purified from the other proteins in fraction 6 by HPLC using a reverse-phase phenyl column (Fig. 1B). PR-2 and PR-N were purified by HPLC anion exchange chromatography (Fig. 1C). The identity of the purified proteins was confirmed by mobility on native PAGE relative to PR protein standards (data not shown). The partial amino acid sequences of the proteins, obtained by Edman degradation of purified tryptic peptides, are shown in Fig. 2.

Isolation of cDNA Clones Encoding PR-2, PR-N, and PR-O

The amino acid sequence MFDENN, which occurred in both PR-2 and PR-N, was used to design a 16-fold degenerate, 17-base oligodeoxyribonucleotide probe with the sequence: 5′-ATGTT^{C}/$_{T}$GA^{C}/$_{T}$GA^{A}/$_{G}$AA^{C}/$_{T}$AA-3′. This probe was used to screen a cDNA library constructed with mRNA isolated from TMV-infected leaf tissue. In a screen of approximately 300,000 plaques of this library, 32 positively hybridizing clones were isolated. Two of the clones, designated GL103 and GL117, were identical in sequence and appeared to be β-1,3-glucanases based on dot matrix comparison to the basic form of β-1,3-glucanase (data not shown). Two additional rounds of isolation using either a 125-bp *Pst*I fragment from the middle portion of GL117 or a 212-bp *Taq*I-*Pvu*I fragment from the 5′ end of GL117 as a probe yielded 35 additional positive plaques. Based on partial DNA sequence, the clones were grouped into five types, which are represented schematically in Figure 3. When possible, two independent cDNA clones from each type were selected and completely sequenced. The compiled DNA-sequencing data is shown in Figure 4.

The complete sequence of the GL117 insert was 1204 bp

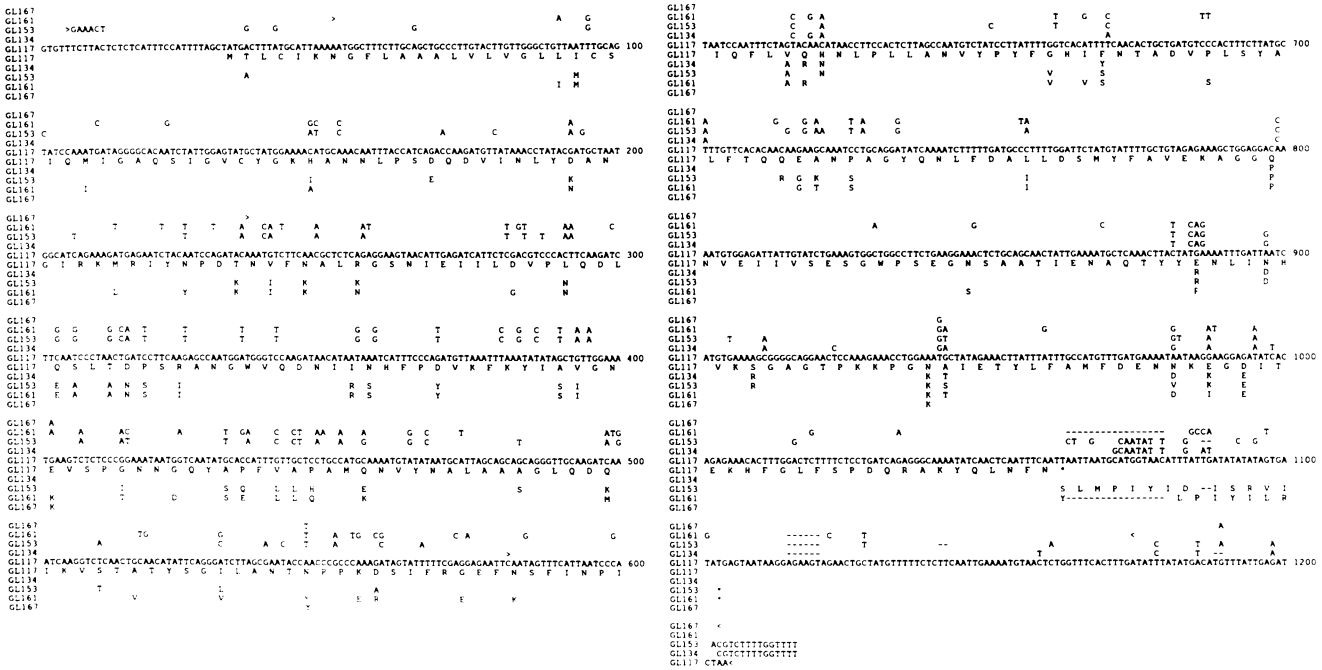

Figure 4. DNA sequences of the cDNA inserts. The complete sequence and single letter amino acid translation for GL117 are shown on the central lines. Above the GL117 sequence, only the positions at which the other clones differ in sequence are indicated. Similarly, below the translation of the GL117 sequence, only the positions at which the amino acid sequence differs are shown. Dashes indicate regions of deleted sequence compared with GL117. The symbols ">" and "<" indicate the termini of cDNA inserts.

in length and encoded a predicted protein of 343 amino acids. Based on homology to other β-1,3-glucanases, GL117 encoded a pre-β-1,3-glucanase with a 29-amino acid N-terminal signal peptide that presumably is processed to give a mature protein of M_r 34,704 with a predicted pI of 4.7. The deduced amino acid sequence from GL117 was compared with the peptide sequence data from the purified acidic glucanases (Fig. 2). GL117 had no mismatches to the PR-2 data, four mismatches to PR-N (two of which were within the peptides and two of which were inferred from tryptic cleavage at arginine or lysine residues), and 11 mismatches to PR-O (nine internal plus two inferred). Based on this comparison, we concluded that the GL117 encoded the PR-2 protein.

GL148 and GL167, 673 and 966 bp in length, respectively, were identical over their overlapping region and thus represented independent isolates of the same mRNA. The ORF in the longer clone, GL167, began at a position aligning with amino acid 69 of the ORF in GL117 (Fig. 4). The deduced amino acid sequence of the GL167 ORF had two mismatches to PR-2, nine mismatches to PR-O (seven internal and two inferred), and no mismatches to PR-N (Fig. 2). We concluded, therefore, that these clones encoded PR-N. However, these cDNAs were not full-length because the deduced proteins were considerably smaller than purified PR-N, which was nearly identical in size to PR-2 (data not shown).

GL134 had an insert 627 bp in length encoding an ORF that began at a position corresponding to codon 184 of the GL117 ORF (Fig. 4). The deduced protein encoded in GL134 had four mismatches to PR-2, five mismatches to PR-N, but matched all the residues for PR-O (Fig. 2). It was concluded that GL134 encoded a protein corresponding to PR-O.

Isolation of cDNA Clones for Novel β-1,3-Glucanases

Three additional cDNA clones were found that did not match the clones for either PR-2, -N, or -O. GL135 and GL161, with lengths of 1022 and 1099 bp, respectively, were identical in the region shared by the two clones. The longer clone, GL161, had an ORF that started at a position aligning with codon 8 of the PR-2 signal peptide (Fig. 4). In addition, the carboxy-terminus of the predicted protein from GL161 extended eight amino acids past the conserved carboxy-terminus of PR-2, -N, and -O (Fig. 4). The mature protein from GL161, lacking the signal peptide, would have a predicted mol wt of 35,977 and a calculated pI of 7.0. Comparison of the DNA sequence of GL161 to the PR-2, -N, and -O clones revealed that the deduced protein had many mismatches to the peptide sequence data for all three acidic glucanases (Fig. 4).

GL153 was a unique 1201-bp clone with an ORF of 356 codons that apparently encoded a full-length pre-β-1,3-glucanase (Fig. 3). As in the GL161 clone, the predicted protein extended past the C-terminus of PR-2, -N, and -O, in this case by 13 amino acids. If we assume cleavage of a predicted signal peptide at the position found in other glucanases, the mature protein from GL153 would have a mol wt of 36,545 and a pI of 8.4. GL153 also had numerous mismatches to the peptides from PR-2, -N, and -O (Fig. 4).

Induction of β-1,3-Glucanase mRNAs by TMV Infection

To examine the steady-state mRNA levels for the acidic and basic glucanases in response to TMV infection, Northern blots of total RNA isolated from tobacco leaves at various times after TMV infection were probed with radiolabeled glucanase cDNA inserts. To differentiate the mRNAs, the blots were washed under stringent conditions (65°C, 75 mM Na^+). As a control to detect possible cross-hybridization, *in vitro* synthesized RNA from each clone was added to the blots (data not shown). It should be noted that, unless high stringency conditions were used, the probes would cross-hybridize on Northern blots.

In TMV-infected leaves, PR-Q′ and PR-2 mRNAs increased to detectable levels within 2 d after infection (Fig. 5).

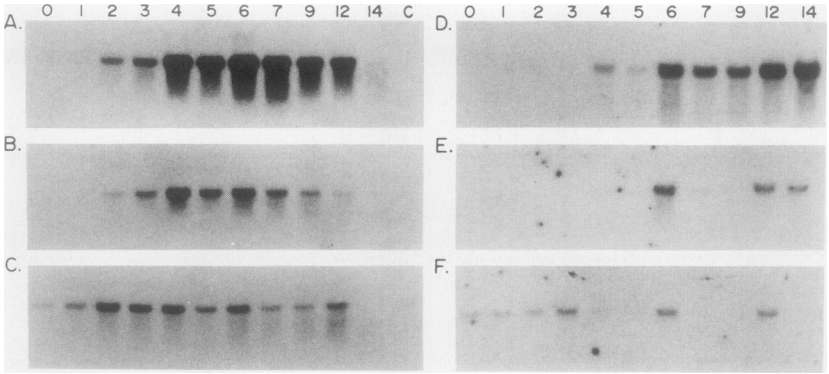

Figure 5. Expression of the three classes of glucanase in response to pathogen infection. Autoradiograms of Northern blots of RNA from tobacco plants collected at various times (in days) after infection with TMV, probed with cDNAs for PR-2 (A and D), PR-Q′ (B and E), and basic glucanase (C and F). Primary, infected leaf samples (A to C) were prepared from leaves onto which the virus was inoculated. Three plants were collected at each time point, and three leaves were inoculated on each plant. Secondary, systemic leaf samples (D to F) were prepared from the leaf immediately acropetal to the three inoculated leaves. The lanes labeled "C" contain RNA from leaves mock inoculated with buffer and carborundum, collected 7 d after treatment. The probes were full-length cDNA inserts derived from GL117 (PR-2), GL5b-12 (PR-Q′; 21), and pGLN17 (basic glucanase, constructed from pGL31 and pGL36; 24).

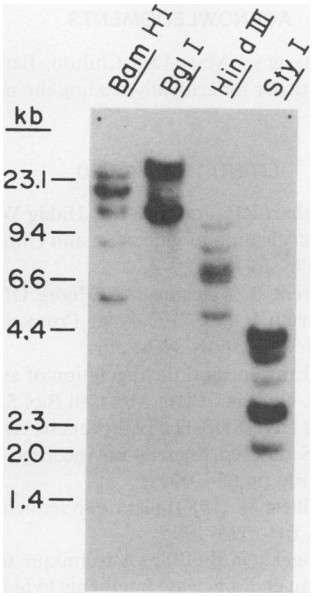

Figure 6. Autoradiogram of a Southern hybridization of genomic Xanthi tobacco DNA, digested with the restriction enzymes indicated and probed with the GL117 insert.

Both messages peaked at approximately 4 to 6 d after inoculation. The amount of PR-Q′ mRNA decreased dramatically between d 9 and d 12 and returned to basal level at 14 d after infection. PR-2 mRNA remained relatively abundant through d 12 and decreased to a low level after 14 d. RNA encoding the basic form of glucanase, on the other hand, increased within 1 d of inoculation, reaching a peak approximately 10-fold greater than its basal level by d 2. The basic glucanase mRNA remained significantly elevated until d 12.

To test whether the mRNAs for the different glucanases were systemically induced, RNA was prepared from the uninfected leaves immediately acropetal to the TMV-inoculated leaves. PR-2-related mRNA was induced from a low level in uninfected tissue 4 d after inoculation (Fig. 5). The mRNA level increased to a maximum after 6 d and remained near this high level for at least 8 more d.

PR-Q′ mRNA was induced in a fashion qualitatively similar to PR-2, although at a lower level. A small amount was detectable at 4 d, increasing to a maximum at 6 d and remaining high through d 14, except for a decrease at d 7 and d 9 that paralleled a similar decrease in the PR-2 mRNA level. In contrast, the mRNA for the basic form of glucanase had a relatively high level at d 0 and increased approximately two- to threefold within 3 d after inoculation. The message level then decreased at d 4 and appeared to fluctuate for the remainder of the experiment, with high levels at d 6 and d 12, and low levels at d 5, d 7, d 9, and d 14.

Complexity of the PR-2, -N, -O Family

To assess the number of genes encoding acidic glucanases in tobacco, a genomic Southern blot was probed with GL117 insert (Fig. 6). Tobacco genomic DNA was digested with several restriction enzymes that lacked sites in all the cDNAs described above. The pattern of bands hybridizing to a PR-2 probe under high stringency shows that six to eight discrete, closely related sequences are found in the amphidiploid tobacco genome.

DISCUSSION

In this study, cDNAs encoding the extracellular β-1,3-glucanases, PR-2, PR-N, and PR-O, as well as two closely related β-1,3-glucanases, were isolated. Comparisons of DNA and predicted protein sequences among these and previously isolated cDNA clones encoding the basic isoform of β-1,3-glucanase (24) and the glucanase PR-Q′ (21) revealed several interesting observations. Within the group comprising PR-2, -N, and -O, PR-2 and PR-N are extremely close in sequence, having 99% amino acid identity and greater than 99% DNA sequence similarity. PR-O, on the other hand, is only 93% identical with PR-2 and -N in amino acid sequence and 94% identical in DNA sequence. GL153 and GL161 are 89% identical with each other in amino acid sequence and 93% in DNA sequence. These clones are 89 to 90% identical with PR-2/-N at the DNA level and 82 to 84% identical with PR-2/-N at the amino acid level. PR-O is approximately equally related to PR-2/-N, to GL153, and to GL161, having an average amino acid identity of 92% and an average DNA sequence identity of 94% to both groups. All five clones described here are 57% identical in DNA sequence and approximately 54% identical in amino acid sequence with the acidic glucanase PR-Q′. The clones are approximately 55% identical in DNA sequence and 51% identical in amino acid sequence with the basic, ethylene-inducible β-1,3-glucanase. These results imply that β-1,3-glucanases in tobacco are encoded by at least five related groups of genes: the basic, vacuolar, ethylene-inducible glucanase genes, the PR-2 and PR-N glucanase genes, the PR-O glucanase gene(s), genes encoding previously uncharacterized basic or neutral glucanases very closely related to PR-O, and the gene(s) encoding glucanase PR-Q′ (21), a tobacco analog of the elicitor-releasing glucanase from soybean (26).

A speculative scheme for the evolution of the β-1,3-glucanases in tobacco is illustrated in Figure 7. This model predicts that the PR protein glucanases and "PR-like" glucanases

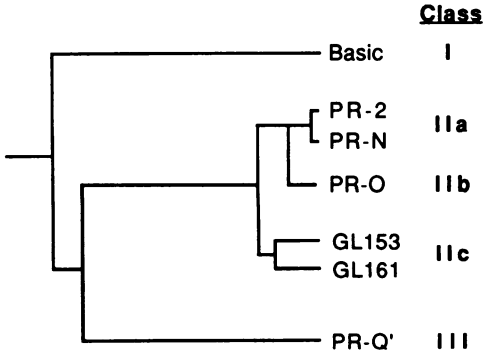

Figure 7. A possible evolutionary tree for the β-1,3-glucanases. Approximate distances were calculated by the unweighted pair-group method with arithmetic mean (19).

diverged more recently than did the others. In the suggested nomenclature for differentiating the different glucanases, class I refers to the basic, vacuolar forms, class II refers to the acidic, extracellular glucanases, and class III refers to the acidic, extracellular glucanases similar to PR-Q′ (21). We suggest that the class II glucanases can be further divided into class IIa comprising the nearly identical proteins PR-2 and PR-N, class IIb comprising PR-O, which "links" PR-2/-N to GL153 and GL161, and class IIc comprising the proteins encoded in GL153 and GL161. Although the GL153 and GL161 proteins may not be acidic or extracellular, their high degree of relatedness to class IIb dictates that they fall into class II.

Van den Bulcke *et al.* (27) previously purified three acidic β-1,3-glucanase proteins from tobacco, which they characterized by partial amino acid sequence and designated PR-35, PR-36, and PR-37. Comparison of their amino acid sequence data to the predicted translation products of the acidic glucanase cDNA clones described in this study shows that PR-36 corresponds to PR-2 and PR-37 corresponds to PR-O. We previously showed that PR-35 corresponds to the β-1,3-glucanase, PR-Q′ (21).

Our data concerning mRNA accumulation in response to TMV infection indicate that the expression of class II and class III genes is coordinately regulated, in both primary (infected) and secondary (uninfected) tissue. Their induction kinetics in both tissue types are qualitatively similar to those seen for the mRNAs encoding other PR proteins (20; ER Ward, SC Williams, SS Dincher, JA Ryals, unpublished observations). Thus, pathogen infection appears to coordinately induce at the mRNA level all the major PR protein classes for which nucleic acid probes are available. The mRNA for the class I glucanase, on the other hand, is induced with significantly different kinetics in inoculated leaves and is only weakly induced in systemic tissue of an infected plant. It appears, therefore, to be regulated differently from the PR proteins.

In vitro studies of purified β-1,3-glucanases have shown that different classes can have dramatically different activities against defined substrates (10). These differences may reflect distinct *in vivo* functions, rather than simply varying potency of identical function. The diversity of structure and regulation among the β-1,3-glucanase family that we describe here may further indicate such diversity of function.

NOTE ADDED IN PROOF

Since submission of this paper, two reports of related β-1,3-glucanase clones from tobacco have appeared. Ori *et al.* (1990, EMBO J **9:** 3429–3436) described two cDNAs encoding a class of glucanases expressed specifically in the stylar matrix. One of these, designated Sp41a, is 80% identical to PR-2, 82% identical to PR-O, 88% identical to GL153, and 85% identical to GL161. Linthorst *et al.* (1990, Proc Natl Acad Sci USA **87:** 8756–8760) described several cDNAs and a genomic clone encoding acidic β-1,3-glucanases expressed in TMV-infected leaves of tobacco cv Samsun NN. One of their unidentified clones, designated cl32, is identical to our clone GL134. Another, designated cl37, is identical to GL153.

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