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# Isolation of complementary DNA clones encoding pathogenesis-related proteins P and Q, two acidic chitinases from tobacco

(plant defense gene/polymerase chain reaction/cDNA cloning/*Nicotiana tabacum*)

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ABSTRACT **Complementary DNA clones encoding two isoforms of the acidic endochitinase (chitinase, EC 3.2.1.14) from tobacco were isolated. Comparison of amino acid sequences deduced from the cDNA clones and the sequence of peptides derived from purified proteins show that these clones encode the pathogenesis-related proteins PR-P and PR-Q. The cDNA inserts were not homologous to either the bacterial form of chitinase or the form from cucumber but shared significant homology to the basic form of chitinase from tobacco and bean. The acidic isoforms of tobacco chitinase did not contain the amino-terminal, cysteine-rich "hevein" domain found in the basic isoforms, indicating that this domain, which binds chitin, is not essential for chitinolytic activity. The accumulation of mRNA for the pathogenesis-related proteins PR-1, PR-R, PR-P, and PR-Q in Xanthi.nc tobacco leaves following infection with tobacco mosaic virus was measured by primer extension. The results indicate that the induction of these proteins during the local necrotic lesion response to the virus is coordinated at the mRNA level.**

Plants respond to pathogen infection by producing a number of proteins believed to be important in protecting them from the deleterious effects of the pathogen (1). These include enzymes involved in the process of lignification and the synthesis of antibiotics (i.e., phytoalexins) as well as enzymes capable of hydrolyzing structural components of the pathogen. Of particular interest is endochitinase (chitinase, EC 3.2.1.14), an enzyme that can hydrolyze chitin, a major component of the cell wall of some phytopathogenic fungi (2). A direct role for chitinase in the plant's defense against fungal infection has been suggested (3), and this hypothesis is supported by the observation that the basic chitinases of bean in combination with the basic form of $\beta$-1,3-glucanase have pronounced fungicidal activity *in vitro* (4).

Several different forms of chitinase have been found in plants. Two basic isoforms have been identified in tobacco, which are induced in parts of the plant infected with viruses, bacteria, and fungi under conditions in which a local necrotic lesion forms (2, 5, 6). These proteins appear to be localized primarily in the central vacuole of cells (2, 7). Recently, 2 of the 10 pathogenesis-related (PR) proteins from tobacco, PR-P and PR-Q, and a PR protein from cucumber, have been shown to have endochitinase activity as well (8, 9). The acidic enzymes are found in the extracellular fluid from leaves, suggesting that they are localized in the apoplastic compartment. This localization is consistent with a defense function for the acidic chitinases since fungi usually penetrate plants by growing in the space between cells (2).

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In this report, we describe the isolation and characterization of cDNA clones encoding two acidic isoforms of tobacco chitinase.¶ The cDNA clones were isolated, in part, by a method, based on the polymerase-catalyzed chain reaction (PCR) (10), that permits the amplification and cloning of 5′ ends of cDNAs directly from libraries. Our results show that these cDNA clones encode the PR proteins PR-P and PR-Q and that there is substantial homology between the acidic and basic chitinases (11) of tobacco, but no significant homology to either the bacterial chitinases (12, 13) or the acidic cucumber chitinase (14). Following infection of leaves with tobacco mosaic virus (TMV), there is a coordinated induction of the mRNAs for these acidic chitinases and two structurally unrelated PR proteins, PR-1 and PR-R.

## MATERIALS AND METHODS

**Plant Materials and Virus Infection.** Plants of *Nicotiana tabacum* cv. Xanthi.nc were grown in a greenhouse and infected when 8 weeks old by gently rubbing the leaves with a suspension of the common strain (U1) of TMV (0.5 $\mu$g/ml) in a solution of 10 mM sodium phosphate (pH 7.0) containing carborundum. Mock-inoculated plants were treated with buffer and carborundum only. Leaves used for RNA and protein isolation were harvested 5 and 7 days after infection, respectively.

**Purification and Amino Acid Sequencing of PR-P and PR-Q Proteins.** The intercellular-fluid fraction of TMV-infected leaves was prepared as described (15) and then concentrated and fractionated on an Ultragel AcA54 column. Fractions judged to contain PR proteins by electrophoresis on 10% polyacrylamide gels (16) were pooled and concentrated. This mixture was then separated by chromatography on DEAE-Sephacel. Fraction 6 (see Fig. 3*A*), which contained a mixture of PR-R major, PR-R minor, PR-P, PR-Q, PR-O, and several minor contaminants, was collected and further purified by HPLC with a reverse-phase phenyl column (see Fig. 3*B*). PR-P and PR-Q proteins coeluted as a single peak, which was collected and further purified by HPLC with a Brownlee Labs AX-300 HPLC ion-exchange column (2.1 mm × 10 cm). The proteins were eluted from the column with a linear gradient of 10–250 mM ammonium acetate (pH 7.0). PR-P and PR-Q eluted in two distinct peaks at ≈75 mM and ≈95 mM ammonium acetate, respectively (see Fig. 3*C*).

**Isolation of Peptides and Determination of Amino Acid Sequences.** Partial amino acid sequences of PR-P and PR-Q were determined from either CNBr peptides, tryptic peptides, or the amino terminus of the protein following an *in situ*

Abbreviations: TMV, tobacco mosaic virus; PCR, polymerase chain reaction; PTH, phenylthiohydantoin; PR, pathogenesis-related.

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¶The sequence reported in this paper has been deposited in the GenBank data base (accession nos. M29868 and M29869).

deblocking procedure. Automated Edman degradations were performed by using the Applied Biosystems 470A gas-phase sequencer. Phenylthiohydantoin (PTH) amino acids were identified by using an Applied Biosystems 120A PTH analyzer. Details of the protein purification, peptide purification, and protein sequencing are available upon request.

**Isolation of cDNA Clones Encoding PR-P and PR-Q.** Poly(A)$^+$ RNA was isolated essentially as described by Lagrimini *et al.* (17) from TMV-infected tobacco leaves. cDNA was synthesized as described (18) and cloned into the *Eco*RI site of the λOngC cloning vector (Stratagene). Putative clones of PR-P and PR-Q were identified by plating the cDNA library and screening duplicate filters with a labeled cDNA probe, pCHN50, encoding the basic chitinase from tobacco (11). The filters were hybridized and washed under low-stringency conditions (125 mM NaCl/1% SDS/40 mM sodium phosphate, pH 7.2/1 mM EDTA at 50°C), and positive plaques were isolated. The plaques were further screened by washing at a higher stringency (same buffer at 65°C), and clones that no longer hybridized or hybridized only weakly to the probe were purified. DNA was isolated from the phage, and the cDNA insert was subcloned into the Bluescript plasmid (Stratagene). DNA sequences were determined by dideoxy sequencing with double-stranded templates (19). PCR reactions were carried out with the GeneAmp kit from Perkin–Elmer/Cetus as recommended by the manufacturer. Oligonucleotides were synthesized by using β-cyanoethylphosphoramidite chemistry on the Applied Biosystems 380A DNA synthesizer.

**Primer Extension Mapping.** Treated leaves from three plants were harvested, pooled, and frozen in liquid nitrogen; total RNA was extracted as described (17) at various times after inoculation. Primer extension reactions were carried out on total RNA as described (14) by using oligonucleotides with the following sequences: 5′-CAGCAGCTATGAATGCAT-3′ for PR-P and PR-Q; 5′-ATAGTCTTGTTGAGAGTT-3′ for PR-1a, PR-1b, and PR-1c; 5′-GTAGGTGCATTGGTTGAC-3′ for PR-R major and PR-R minor.

## RESULTS

**Isolation of cDNA Clones Encoding Acidic Chitinase.** A strategy to isolate cDNA clones encoding the acidic isoforms of chitinase was designed based on the observation that the acidic and basic forms of tobacco chitinase have identical amino acids in ≈65% of the positions near the carboxyl ends of the polypeptides (11, 20). Plaques were identified by their ability to bind a tobacco basic cDNA probe under low-stringency hybridization conditions and then were screened for their inability to bind the probe under high-stringency hybridization conditions. Approximately 300,000 plaques of a cDNA library prepared from RNA isolated from TMV-infected leaves were screened with a labeled cDNA probe encoding the basic form of tobacco chitinase. Eleven plaques were isolated and then subjected to high-stringency hybridization. Three of the partially purified clones either lost the ability to hybridize with the probe or showed a much weaker binding. After partial DNA sequencing of the cDNA inserts, one of the three, λcht15, showed a significant nucleotide sequence similarity to the basic chitinase by dot matrix analysis. The 964-base-pair (bp) cDNA insert from this clone was completely sequenced and found to encode a protein with ≈65% identity to the basic chitinase and ≈87% identity to the carboxyl-terminal polypeptide previously reported (Fig. 1), suggesting that a cDNA encoding the acidic chitinase had been isolated. Nevertheless, the dot-matrix analysis indicated that the clone was truncated at the 5′ end relative to the basic chitinase cDNA. This was confirmed by determining the length of the 5′ end of the mRNA by using a primer extension analysis. An oligonucleotide primer (GP35) was

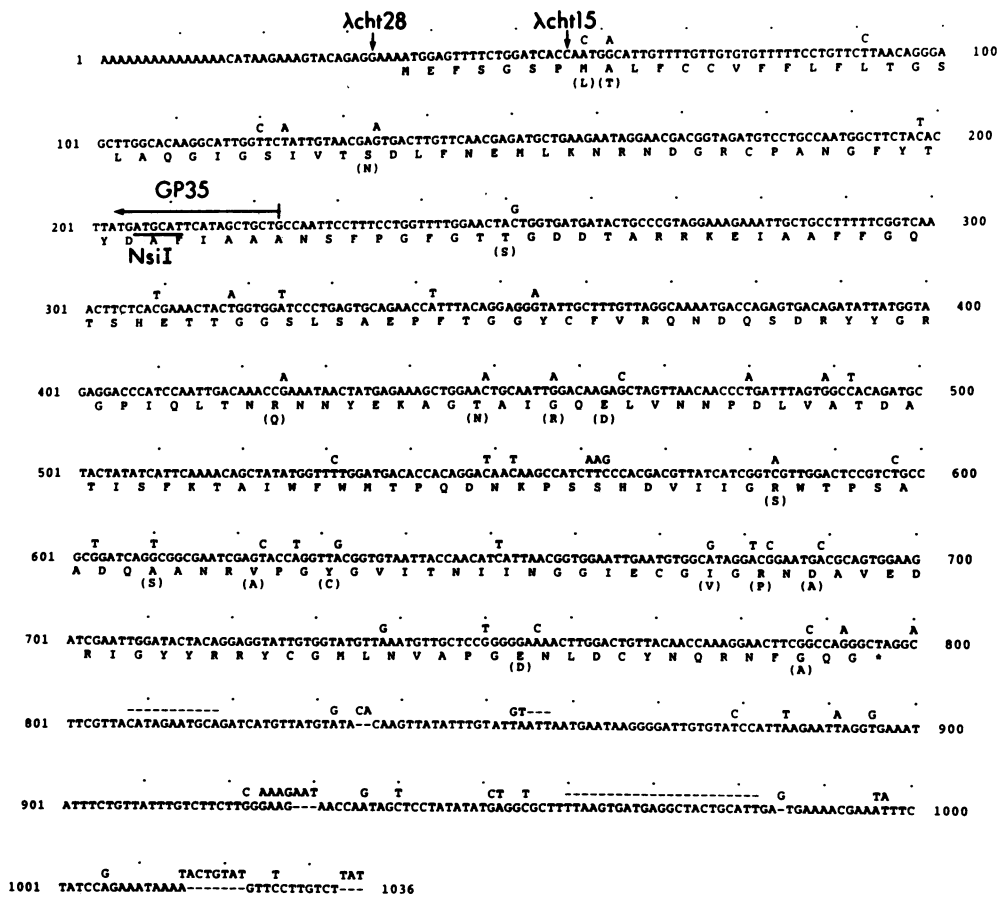


FIG. 1. The DNA sequence of the PR-Q cDNA is shown with the protein translation below it. This sequence is a composite derived from the two cDNA clones, λcht15 and λcht24 as well as sequences from the 5′ end of cDNAs cloned by PCR amplification. The nucleotide sequence of PR-P is shown on the line above the PR-Q sequence, with differences indicated. Differences in the deduced protein sequence are indicated below the PR-P protein sequence in parentheses. The PR-P sequence was derived by sequencing two independent cDNA clones, λcht28 and λcht21. Deletions or insertions in the 3′ end of the clone are designated by dashes. The 5′ end of the λcht15 and λcht28 cDNAs are indicated with vertical arrows. The location of the GP35 primer and the *Nsi* I site used in the PCR experiment are also indicated.

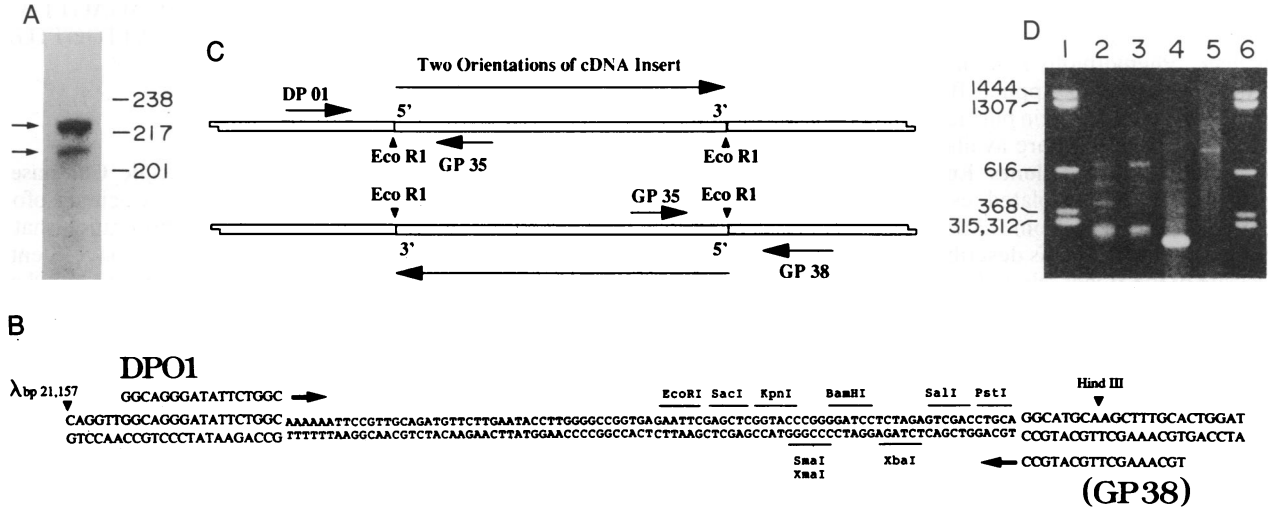


FIG. 2. PCR cloning of the 5′ end of PR-P and PR-Q. (*A*) Total RNA extracted from TMV-infected tobacco leaves was used in a primer extension assay to determine the length of the PR-P/Q mRNA. The primer used, GP35 (5′-CAGCAGCTATGAATGCAT-3′), is complementary to positions 206–223 of the PR-Q cDNA sequence shown in Fig. 3 and contains an *Nsi* I site. Two major transcripts are indicated by arrows, which correspond to a length of about 210 ± 3 bp and 220 ± 3 bp. (*B*) DNA sequence of the λOngC cloning vector with the positions of two oligonucleotide primers, DP01 and GP38. (*C*) The PCR amplification strategy is diagrammed. In one orientation a combination of DP01 and GP35 would amplify the 5′ end of the clone, and in the reverse orientation a combination of GP38 and GP35 would amplify the 5′ end. (*D*) PCR products from the amplification of a cDNA library with either DP01 and GP35 (lane 2) or GP35 and GP38 (lane 3) were separated on a 1.5% agarose gel. Control amplifications were performed by using the λcht15 clone with either DP01 and GP35 (lane 4) or GP35 and GP38 (lane 5). Lanes 1 and 6, molecular size standards (in bp; pBR322 digested with *Taq* I).

synthesized that was complementary to nucleotides 162–179 of λcht15 (positions 205–223 in Fig. 1) and that contained an internal *Nsi* I site. This primer was labeled at its 5′ end and used to extend RNA prepared from leaves infected with TMV. Two transcripts were detected that were about 220 ± 3 bp and 210 ± 3 bp long (Fig. 2*A*), indicating that the λcht15 clone was about 45–55 bp short of the full-length mRNA.

A method based on the PCR was developed to isolate the 5′ end of the acidic chitinase cDNA, which involved determining the length of the mRNA and then amplifying and cloning the 5′ ends directly from the cDNA library. The 5′ end of the acidic chitinase mRNA had been determined to be 210–220 bases from the GP35 priming site and 45–55 bases longer than the cDNA insert in λcht15. This primer was used in combination with either of two other primers, DP01 or GP38 (Fig. 2*B*), to amplify the 5′ end of the cDNA. The use of two primers in separate reactions provided for amplification of the 5′ end of the insert when the cDNA is cloned in either orientation (Fig. 2*C*). A diffuse band, 40–50 bases longer than the λcht15 control (Fig. 2*D*, lane 4), resulted when either GP35 and DP01 (Fig. 2*D*, lane 2) or GP35 and GP38 (Fig. 2*D*, lane 3) were used to prime the reaction. A negative control in which purified λcht15 was amplified by using primers for the wrong orientation did not produce a band of the expected size (Fig. 2*D*, lane 5). The diffuse band probably resulted from the amplification of a population of molecules with differing 5′ ends. The amplified DNA was purified, digested with *Nsi* I and *Eco*RI, and cloned into pBluescript. The inserts from positive transformants were sequenced and compared to the insert in λcht15, and from this analysis two types of 5′ ends were identified. One type of clone was identical to the λcht15 insert but extended beyond the 5′ end of λcht15, and a second type was very similar to λcht15 but had several nucleotide substitutions. The longest insert extended 56 bp beyond the end of the λcht15 clone.

The 3′ end of the second type of acidic chitinase was isolated by rescreening the cDNA library with a probe from the 5′ end of the chitinase cDNA. Eight positively hybridizing clones were isolated, and the DNA sequence of the inserts was determined. Five clones contained inserts with a sequence that matched the insert in λcht15, and the composite DNA sequence is shown in Fig. 1. Three other clones, including λcht28, contained a sequence that was 95% identical to the λcht15 sequence and that composite sequence is included as the upper sequence in Fig. 1. It was concluded

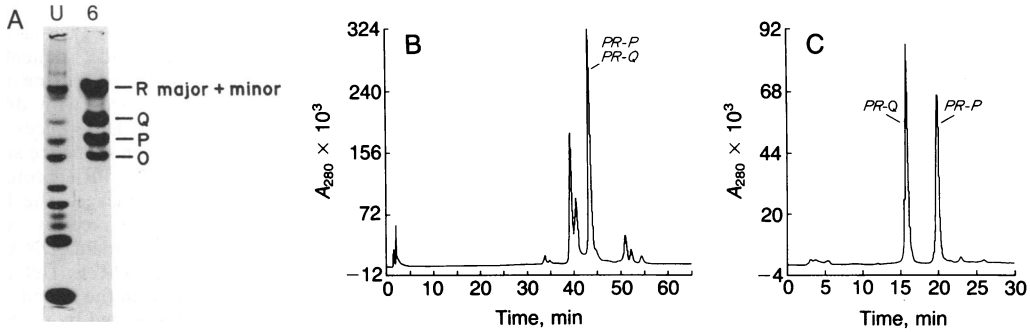


FIG. 3. Isolation and protein sequence of PR-P and PR-Q. (*A*) Nondenaturing 10% polyacrylamide gel of intercellular fluid from TMV-infected *N. tabacum* before fractionation (U) or fraction 6 (6) from the first ion-exchange chromatography purification step. (*B*) Fraction 6 was further purified by reverse-phase HPLC on a phenyl column. (*C*) The peak containing PR-P and PR-Q was collected, and PR-P and PR-Q were separated from each other by HPLC using ion-exchange chromatography.

from these experiments that cDNA clones encoding two closely related isoforms of chitinase had been isolated, but at this point it was not possible to determine if they encoded the PR-P and PR-Q proteins.

**Purification of PR-P and PR-Q Proteins and Determination of Amino Acid Sequences.** To establish the identity of the proteins encoded by λcht15 and λcht28, the PR-P and PR-Q proteins were purified to homogeneity (Fig. 3), and the amino acid sequence was determined from either the amino terminus of the mature protein, cyanogen bromide-cleaved protein, or tryptic peptides. The results of the amino acid sequencing for PR-P are summarized in Fig. 4. When the sequence encoded by λcht28 was compared to the determined sequence, there was agreement at every residue; however, when the sequence encoded by the λcht15 was compared, there were five residues that differed.

Similarly, the results of the protein sequencing of the PR-Q protein are summarized in Fig. 5. In this case, there was complete agreement between the experimentally determined sequence and the sequence from the λcht15 clone. When the deduced protein sequence from λcht28 was compared to the protein sequence there were four residues that differ. Two of the residues were experimentally determined, and two were inferred based on the cleavage by trypsin at either an arginine or a lysine residue. There was a fifth difference between the two clones in the deduced sequence at the amino terminus; however, it was not possible to confirm this residue by amino acid sequencing. This residue may be a modified or glycosylated serine that could not be identified as a PTH amino acid. It was concluded from these results that the protein encoded by the λcht15 clone corresponds to the PR-Q protein and the protein encoded by the λcht28 clone corresponds to the PR-P protein.

Both the PR-P and PR-Q cDNA clones encode a protein of 253-amino acid residues. When compared to the amino acid sequence derived from the purified protein, it is apparent that a 24-amino acid signal peptide is removed to leave a mature protein of 229 amino acids. The calculated molecular mass of PR-P is 24,859 Da, whereas that of PR-Q is 25,033 Da; the charges on PR-P and PR-Q at pH 7.0 are −4.9 and −3.8, respectively.

**Homology to Known Chitinases.** Three structurally distinct types of chitinases have been reported, one from bacteria (12, 13) and two from plants (11, 15, 21). The DNA sequence for PR-Q was compared to each of these chitinases and also used to search the data bases for homologous proteins. There was no structural homology found between the PR-Q sequence and either the bacterial chitinase or the chitinase from cucumbers. However, there was significant homology to the basic chitinase from tobacco and the ethylene-inducible chitinase from bean. A dot-matrix comparison of the DNA sequence for the tobacco chitinase and PR-Q is shown in Fig. 6. The sequence similarity between the two genes begins just after the signal peptide of the acidic chitinase and at about residue 68 of the mature basic isoform of chitinase (Fig. 7). The basic chitinase contains an amino-terminal, cysteine-rich, "hevein" domain of about 40 amino acids, which is shared among several plant lectins, including wheat germ agglutinin, hevein, and two wound-inducible genes from potato (Win1 and Win2), as has been noted by Lucas *et al.* (25) and Stanford *et al.* (22). It has been postulated that this domain is involved in binding oligosaccharides, and it is clear that it is missing in the acidic chitinase molecule.

PR-P

| | |
|---|---|
| amino terminus | (Q)GIGSIVT**N**DLFNEML |
| CHT28 | - ---------- |
| CHT15 | - -------**S**------- |
| CNBR | RNDGR?PANGF |
| CHT28 | -----C----- |
| CHT15 | -----C----- |
| Tryptic 46.4 | (R/K)GPIQLTN**Q**NNYEK |
| CHT28 | R ---------- |
| CHT15 | R -------**R**----- |
| Tryptic 64.7 | (R/K)QDLVNNPDLVATDATI |
| CHT28 | R ---------- |
| CHT15 | G -**E**---------- |
| Tryptic 60.2 | (R/K)Y?GMLNVAPGDNLD?YNQ(R/K) |
| CHT28 | R -C----------C--- R |
| CHT15 | R -C--------**E**---C--- R |

FIG. 4. The amino acid sequence of purified PR-P was determined from either the amino terminus or various peptides. Inferred residues based on the method of cleavage are shown in parentheses. The deduced amino acid sequence for two cDNA clones, λcht28 (CHT28) and λcht15 (CHT15), are shown under the amino acid sequence; agreement is indicated by a dash. Residues that serve to differentiate between the two proteins are boldfaced.

PR-Q

| | |
|---|---|
| amino terminus | (Q)GIGSIVT?DLFNEML |
| CHT28 | - -------N------- |
| CHT15 | - -------S------- |
| CNBR | (M)LKNRNDGR?PANGFYTYDAFIA |
| CHT28 | - --------C---------- |
| CHT15 | - --------C---------- |
| Tryptic 74.2 | (R/K)?PANGFYTYDAF |
| CHT28 | R C---------- |
| CHT15 | R C---------- |
| Tryptic 44.7 | (R/K)GPIQLTN(R/K) |
| CHT28 | R ------- Q |
| CHT15 | R ------- R |
| Tryptic 38.6 | (R/K)WTPSAADQAAN(R/K) |
| CHT28 | S --------S-- R |
| CHT15 | R ---------- R |
| Tryptic 37.5 | (R/K)IGYY(R/K) |
| CHT28 | R ---- R |
| CHT15 | R ---- R |
| Tryptic 50.3 | (R/K)YCGMLNVAPGENLDCYN |
| CHT28 | R ----------D------ |
| CHT15 | R ---------- |

FIG. 5. The amino acid sequence of purified PR-Q was determined from either the amino terminus or various peptides and was compared to the deduced amino acid sequence of λcht28 (CHT28) and λcht15 (CHT15) as described in Fig. 4.

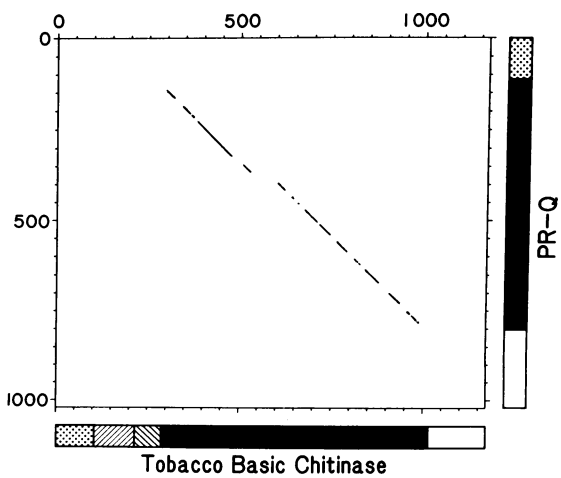


FIG. 6. Sequence similarity of PR-P and PR-Q to the basic chitinase from tobacco. The cDNA sequence of PR-Q (ordinate) was compared to the cDNA sequence of the basic chitinase from tobacco (abscissa) by using a dot-matrix computer analysis. The structure of the cDNA is represented by the large rectangle. ■, Coding sequence of the mature protein; □, 3′ noncoding sequence; ▨, signal peptide and 5′ nontranslated sequence; ▨, hevein domain; ▧, spacer peptide.

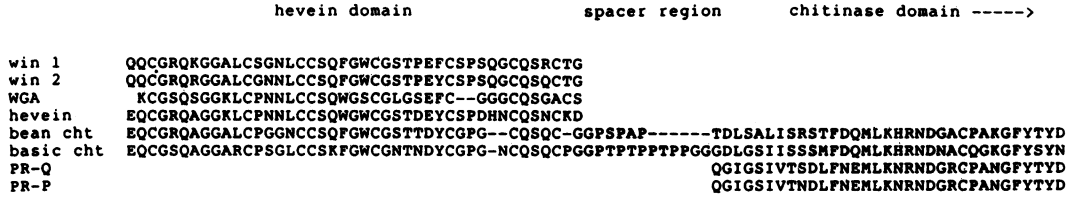


FIG. 7. The amino acid sequence of Win1 (amino acids 26–68) and Win2 (amino acids 26–68) (ref. 22), wheat germ agglutinin (WGA; amino acid 88–127; ref. 23), hevein (amino acids 1–43; ref. 24), the ethylene-inducible chitinase from bean (bean cht; amino acids 1–79; ref. 21), the basic chitinase from tobacco (basic cht; amino acids 1–87; ref. 11), the mature PR-P protein (amino acids 25–57), and the mature PR-Q protein (amino acids 25–57) are aligned to maximize sequence identity.

**Coordinate Induction of PR-P, PR-Q, PR-1, and PR-R mRNA Accumulation by TMV Infection.** At least 10 different PR proteins accumulate in tobacco leaves following pathogen infection (26). However, the coordinate regulation of the mRNAs encoding these proteins has not been clearly demonstrated. Therefore, we compared the accumulation of mRNAs for PR-P, PR-Q, PR-1, and PR-R in leaves following infection with TMV. Leaves were infected at day 0 with a suspension of TMV, samples were taken at various times after infection, and total RNA was extracted. The accumulation of RNA for the various genes was determined by primer extension in which an oligonucleotide for PR-1a, PR-1b, and PR-1c; an oligonucleotide for PR-R major and PR-R minor; and an oligonucleotide for PR-P and PR-Q was used to prime the extension reaction. All of the messages exhibited a similar pattern of induction. There were low levels of message at day 0, and the levels increased with time after infection to give a maximum between days 5 and 7 (data not shown).

## DISCUSSION

Higher plants produce several structurally distinct chitinases (2). Based on a comparison of amino acid sequences, we have proposed that these enzymes fall into at least three structural groups (28). Class I chitinases are structurally homologous, basic proteins, primarily located in the vacuole, that contain two domains. The amino-terminal cysteine-rich hevein domain of ≈40 amino acids is shared by several other proteins, such as certain lectins, wheat germ agglutinin, hevein, and the proteins encoded by the Win1 and Win2 wound-inducible potato genes (22, 25). This domain, which could possibly serve as an oligosaccharide-binding site, is linked by a variable glycine- and proline-rich "spacer" to the catalytic domain of the enzyme. The ethylene-inducible chitinase from bean and the basic tobacco chitinase are class I enzymes. Class II chitinases have amino acid sequences similar to the catalytic domain of class I enzymes but are acidic proteins that lack the hevein domain and spacer. Finally, class III chitinases, which include the acidic chitinase of cucumber (14), lack the hevein domain and do not have structural homology to either class I or class II enzymes. Class II and class III chitinases are located in the extracellular compartment (2, 27).

According to this classification, proteins PR-P and PR-Q are class II chitinases. Recently, a class III chitinase has been also isolated from tobacco (G.P. and J.R., unpublished results); therefore, it appears that all three classes can be present in the same plant. The functional significance of the structural differences among the proteins is not fully understood, but these differences apparently play a role in the differential compartmentalization of the proteins.

It should be noted that the original cDNA, λcht15, contained a truncated molecule, as is often a problem in the isolation of cDNA clones. The 5′ end of the λcht15 cDNA and a related cDNA were amplified and cloned directly from the cDNA library using a PCR-based procedure. Compared to the conventional method of rescreening and repurifying clones that may or may not contain the 5′ end of the cDNA, this procedure was extremely rapid and should be applicable to the isolation of most cDNAs.

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