

Analysis of Acidic and Basic Chitinases from Tobacco and Petunia and Their Constitutive Expression in Transgenic Tobacco

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cDNA clones of messenger RNAs for acidic and basic chitinases were isolated from libraries of tobacco mosaic virus-infected Samsun NN tobacco and petunia. The tobacco cDNA clones for acidic chitinase fell into two different groups, whereas all petunia cDNA clones had the same sequence. Also, tobacco genomic clones were isolated and one was characterized. This genomic clone, corresponding to one of the cDNA clones, showed that this acidic chitinase gene contains two introns. The amino acid sequences of the acidic chitinases from tobacco, as deduced from the cDNA clones, fully agreed with partial sequences derived from peptides obtained from purified tobacco-derived pathogenesis-related proteins PR-P and PR-Q. The deduced amino acid sequences showed that PR-P and PR-Q are 93 and 78%,

respectively, identical to the petunia enzyme. All deduced chitinase sequences indicated the presence of an NH₂-terminal, highly hydrophobic signal peptide. In addition, the polysaccharide-binding domain present at the NH₂-terminus of basic chitinases from mature tobacco is not present in these acidic chitinases. Furthermore, the complete coding sequence for the petunia chitinase, constructed downstream of the cauliflower mosaic virus 35S promoter, was used to transform tobacco. The resulting chimeric gene was constitutively expressed, and the petunia enzyme was targeted to the extracellular fluid. In contrast, a basic chitinase of tobacco, expressed from a chimeric gene, was found in total leaf extracts but not in preparations of extracellular fluid.

Additional keywords: extracellular chitinase, *Nicotiana tabacum*, *Petunia × hybrida*, protein targeting, vacuolar chitinase.

Plants infected by pathogens such as viruses, fungi, or bacteria sometimes respond in a hypersensitive way by forming localized necrotic lesions. Lesion formation is established by a lignification of the tissue surrounding the infection site, which in many cases provides a physical barrier against the systemic spread of the pathogen. Furthermore, upon infection with such a necrotizing pathogen, the plant may acquire resistance to a subsequent pathogenic attack by a fungus, bacterium, or virus (Gianinazzi 1983). Induction of this resistance is accompanied by the *de novo* synthesis of proteins that before infection are present in the plant in low amounts, if at all. Among these proteins is the group of so-called pathogenesis-related (PR) proteins, which can be subdivided into several classes of serologically related proteins (for reviews see Fritig *et al.* 1989; Van Loon 1989; Bol *et al.* 1990). PR proteins, first described in tobacco, were characterized initially as being extracellular (Parent and Asselin 1984) and relatively protease-

resistant and having low pI values (Van Loon 1985). However, recently it was found that serologically related proteins with similar enzymatic activities exist intracellularly, possibly accumulating in the central vacuole (Van den Bulcke *et al.* 1989; Mauch and Staehelin 1989). In tobacco these intracellular proteins are mostly basic (Kauffmann *et al.* 1987; Legrand *et al.* 1987).

The concomitant induction of resistance and the synthesis of PR proteins have led to speculations about a role for these proteins in the resistance to pathogens (Fritig *et al.* 1989; Van Loon 1989; Bol *et al.* 1990). Several proteins induced by tobacco mosaic virus (TMV), notably PR-1 and a glycine-rich protein (GRP), are also highly inducible by salicylate (Hooft van Huijsduijnen *et al.* 1986a). The fact that salicylate treatment also induces virus resistance (White 1979; Hooft van Huijsduijnen *et al.* 1986a) suggests a function of these proteins in the acquired resistance of tobacco to virus infection. Furthermore, analysis of cDNA clones has indicated amino acid sequence similarity between PR-S (according to the newly proposed nomenclature PR-5 [Van Loon *et al.* 1987]) and a protein from maize, which is an inhibitor of digestive enzymes α -amylase and protease of certain insects (Cornelissen *et al.* 1986; Richardson *et al.* 1987), suggesting a function of PR-S in the resistance to insect attack. However, studies with transgenic plants constitutively expressing PR-1, GRP, or PR-S have failed to show any direct involvement in the resistance to viruses or insects (Linthorst *et al.* 1989; Cutt *et al.* 1989; Linthorst *et al.* 1990).

Thus far, the only PR proteins for which an enzymatic activity is known are the acidic chitinases PR-P and PR-Q (PR-3a and PR-3b, respectively [Van Loon *et al.* 1987])

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Nucleotide and/or amino acid sequence data has been submitted to EMBL/GenBank with the following accession numbers: tobacco acidic chitinase PR-Q, X51425; tobacco acidic chitinase PR-P, X51426; and petunia acidic chitinase, X51427.

and their basic counterparts (Legrand *et al.* 1987; Shinshi *et al.* 1987) and the acidic β -1,3-glucanases PR-2, PR-N, and PR-O (PR-2a, PR-2b, and PR-2c, respectively [Van Loon *et al.* 1987]) and their basic equivalents (Kauffmann *et al.* 1987; Felix and Meins 1985). These proteins are capable of hydrolyzing the polysaccharides chitin and β -1,3-glucan *in vitro*. Both polysaccharides are present in the cell walls of many fungi (Wessels and Sietsma 1981). It has indeed been shown that chitinases (Schlumbaum *et al.* 1986) and combinations of chitinase and β -1,3-glucanase (Mauch *et al.* 1988) strongly inhibit the growth of a number of fungi on agar plates. However, whether these pathogen-induced enzymes also play a role in the resistance to fungal attack *in planta* is still not known. In addition, it should be noted that genes encoding basic chitinases and β -1,3-glucanases are constitutively expressed in tobacco roots (Memelink *et al.* 1990), and that the chitinases PR-P and PR-Q and a protein serologically related to the acidic glucanases are synthesized in specific flower organs during flower development (Lotan *et al.* 1989). As a next step in a series of experiments aimed at unraveling the biological function of PR proteins, we have isolated and characterized cDNA and genomic clones encoding several chitinases.

MATERIALS AND METHODS

Synthesis and analysis of cDNA libraries. Polyadenylated RNA was isolated from leaves of Samsun NN tobacco 4 days after infection with TMV and from leaves of *Petunia* \times *hybrida* Hort. Vilm., inbred line R27. The tobacco cDNA library was made according to the lambda ZAP-cDNA synthesis and cloning system of Stratagene (La Jolla, CA). In short, tobacco cDNA was made using a primer containing oligo(dT) and an *Xho*I site for the synthesis of the first strand. Second strand DNA was made using RNase H and *Escherichia coli* DNA polymerase I. Following T4 DNA polymerase incubation, ligation to *Eco*RI adapters, and restriction with *Xho*I, the cDNA was ligated to lambda DNA arms. The petunia cDNA was made with oligo(dT) as the first strand primer and with RNase H and DNA polymerase I being used for the second strand (Gubler and Hoffman 1983). After T4 DNA polymerase incubation, ligation to *Eco*RI linkers, and restriction with *Eco*RI, the cDNA was ligated into the unique *Eco*RI site of lambda gt11 DNA (Young and Davis 1983). After packaging, recombinant bacteriophages were screened for the presence of chitinase-DNA-specific sequences using ³²P-labeled cDNA from tobacco cDNA clones PROB30 and PROB3 that corresponded to acidic and basic chitinases, respectively (Hooft van Huijsduijnen *et al.* 1987). Positive clones hybridizing to each of the probes were isolated, and the cDNA inserts, or restriction fragments thereof, were subcloned into M13 derivatives tg130 and tg131 (Kieny *et al.* 1983).

Dideoxy sequencing (Sanger *et al.* 1977) was performed using a universal M13 DNA primer or with synthetic chitinase-DNA-specific oligonucleotides. To obtain a full-length coding sequence for the basic chitinase of tobacco, two synthetic complementary oligonucleotides, containing the missing five codons at the 5' end of the coding region,

were annealed and ligated to a nearly full-length cDNA clone for basic chitinase. The resulting clone (Tbch1) contained a full-length coding region as determined by sequencing.

Analysis of genomic sequences. A genomic DNA library in lambda Charon 35 of Samsun NN tobacco was screened for the presence of acidic chitinase DNA sequences using PROB30 DNA as a probe (Cornelissen *et al.* 1987). Chitinase-specific sequences were obtained from the phage DNAs by serial transcription with *Taq*-DNA polymerase (Saïke *et al.* 1988) using synthetic oligonucleotide primers with sequences based on the sequence of tobacco chitinase cDNA clones Tach1 and Tach3. The resulting DNA was subsequently cloned for sequencing.

Sequence analysis of peptides. PR proteins P and Q were purified from tobacco leaves infected with TMV (Legrand *et al.* 1987) and cleaved with *N*-chlorosuccinimide (Lischwe and Ochs 1982). The cleavage products were electrophoresed in a denaturing polyacrylamide gel and transferred to a polyvinylidene difluoride membrane. The regions of the membrane containing cleavage products of approximately 7 kDa were cut out and sequenced using an Applied Biosystems pulse-liquid sequencer (Model 477A) coupled on-line to a PTH analyzer (Aberdeen Amino Acid Sequencing Facility, Aberdeen, U.K.).

Transformation of tobacco and analysis of transgenic plants. The complete chitinase coding region of both tobacco cDNA clone Tbch1 and petunia cDNA clone Pach1 was tailored onto a *Bam*HI fragment, which subsequently was cloned into *Bam*HI-linearized vector pMOG181. On an *Eco*RI-*Hind*III fragment, this vector contains an expression cassette consisting of the cauliflower mosaic virus 35S promoter with a double enhancer, a unique *Bam*HI site, and the nopaline synthase transcription terminator. The expression units of the newly obtained constructs with the chitinase coding sequences in the right orientation behind the 35S promoter were cloned into the binary vector pMOG23 (Sijmons *et al.* 1990). These constructs were subsequently used to transform tobacco leaf disks, upon which plants from kanamycin-resistant shoots were regenerated (Linthorst *et al.* 1989). RNA and protein blot analyses were used to assay the expression of transgenes in these plants (Linthorst *et al.* 1989). Protein from the extracellular fluid was isolated as described by Parent and Asselin (1984).

RESULTS

Analysis of cDNA clones. Previously, we reported that differential hybridization of a cDNA library of TMV-infected Samsun NN tobacco resulted in the isolation of cDNA clones corresponding to several classes of PR proteins (Hooft van Huijsduijnen *et al.* 1986b). Upon sequencing, two classes of these clones represented by PROB30 and PROB3 were found to correspond to acidic and basic chitinases, respectively (Hooft van Huijsduijnen *et al.* 1987; Shinshi *et al.* 1987). These cDNA clones were not full-length. Here we used PROB30 and PROB3 to screen a new cDNA library of tobacco. In addition, a cDNA library of petunia was screened with PROB30 as a probe.

From the tobacco and petunia cDNA clones selected with PROB30, five of each were characterized by sequence

analysis. The tobacco cDNA clones could be divided into two groups. Group 1, represented by clones Tach1 and Tach12, corresponded to PROB30; the clones in group 2 were represented by clone Tach3. All petunia cDNA clones had the same sequence. The clones that were sequenced contained the complete open reading frames for acidic chitinases.

Figure 1 shows the nucleotide sequences of each group. The sequence of tobacco chitinase cDNA clones of group 1 (clones Tach1 and Tach12) is given. The differences in the other tobacco sequence (clone Tach3) are indicated above the sequence of Tach1 and Tach12, and differences in the petunia sequence (clones Pach1 and Pach5) are indicated below it. Also shown are the initiation and termination triplets of the longest open reading frames. At the 3' termini, the positions that were found to be alternative polyadenylation sites in the respective clones of each group being characterized are indicated.

Figure 2 shows the complete amino acid sequence of the tobacco acidic chitinase encoded by clone Tach1. Differences in the tobacco acidic chitinase encoded by clone Tach3 are given above this sequence; differences in the petunia chitinase (clone Pach1) are shown below this sequence. Both tobacco enzymes are 253 amino acid residues long, whereas the petunia chitinase contains 254 amino acid residues.

Screening of the tobacco cDNA library with PROB3 yielded a nearly full-length clone. A comparison showed

that this clone was identical to clone pCHN50, which encodes a basic chitinase of tobacco and which was isolated by Shinshi *et al.* (1987). Because our clone lacked the leader sequence of the mRNA and the first 13 nucleotides of the open reading frame, sequence information on the highly similar tobacco basic chitinase encoded by clone pCHN48 (J. M. Neuhaus, personal communication; Shinshi *et al.* 1990) was used to complete the coding sequence by ligation of a synthetic restriction fragment. The clone thus obtained was designated Tbch1.

Analysis of genomic clones. A cDNA library of Samsun NN tobacco was screened (Cornelissen *et al.* 1987) with the insert of PROB30 as a probe. The recombinant phages that were obtained could be divided into three groups as a result of restriction enzyme analyses of phage DNA. Based on the nucleotide sequence of cDNA clone Tach1, synthetic oligonucleotides were synthesized colinearly with nucleotides 1 to 22 and complementary to nucleotides 830 to 854 of clone Tach1. These oligonucleotides were used in a polymerase chain reaction (PCR) with DNA of the genomic clones CHA1, CHA16, and CHA18, representing the three groups. The PCR resulted in the synthesis of DNA fragments 200 to 400 nucleotides longer than expected from the cDNA sequences, suggesting the presence of introns in the genomic DNA. To investigate this further, the 1,200-nucleotide fragment that resulted from the PCR and synthesized on CHA18 DNA was cloned and sequenced.

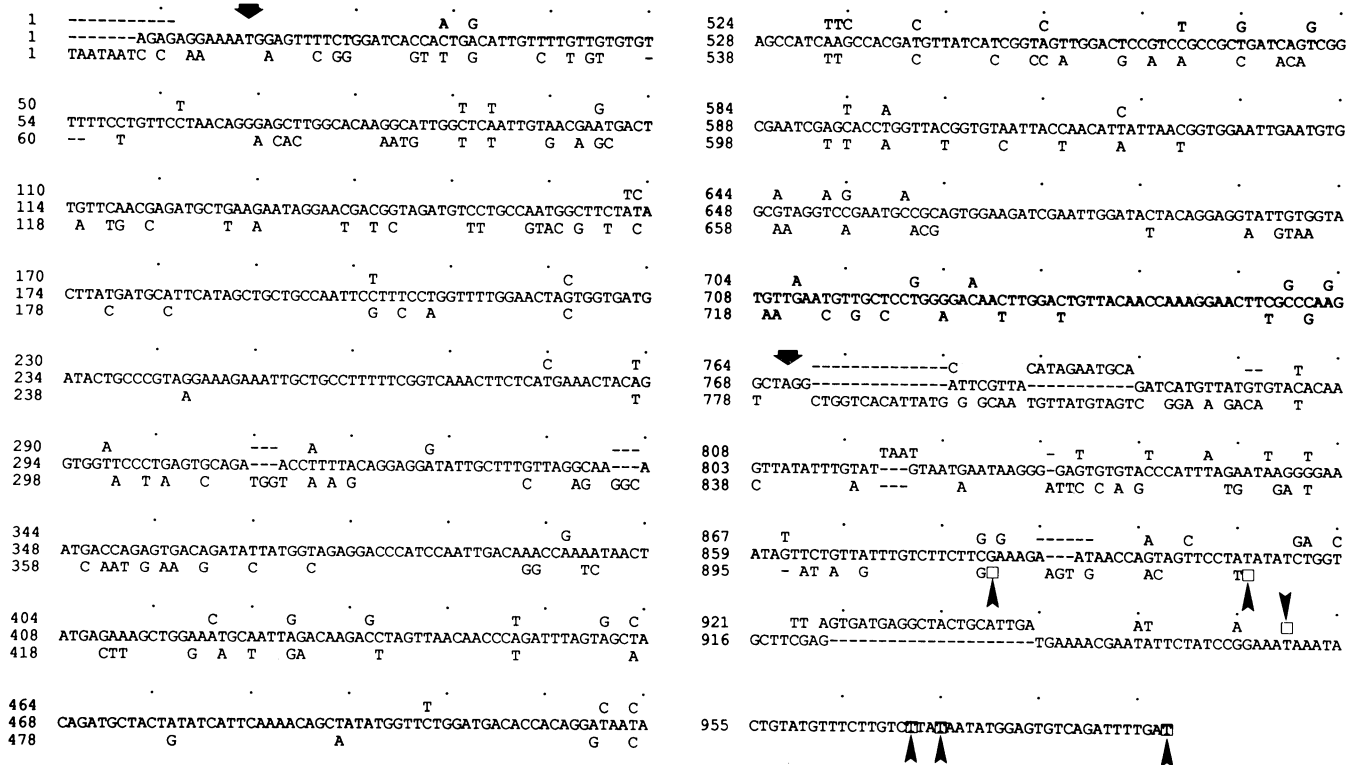


Fig. 1. Nucleotide sequences of cDNA clones for tobacco and petunia acidic chitinases. The sequence of tobacco cDNA clone Tach1 is given, supplemented with 24 extra 3' terminal nucleotides of Tach12. Indicated above are the differences in the sequence of tobacco clone Tach3, and indicated below are the differences in the sequence of petunia clone Pach1 (supplemented with 10 extra 5' terminal nucleotides of petunia cDNA clone Pach5). Gaps (dashes) were introduced for optimal alignment. The positions of the initiation and termination triplets of the largest open reading frames are given (fat arrows). The blocked nucleotides indicate the positions of alternative polyadenylation sites in different clones (thin arrows).

			▼			
1	MA			S		F
1	MEFSGSPLTLFCCVFFLFLTGSLAQGIGSIVTNDLNFNEMLKNRNDGRCPANGFYTYDAFI					
1	K W V A -SF V		T NV	S DQ		A F VR
61		T				
61	AAANSFPGFGTSGDDTARRKEIAAFFGQTSHETTGGSLA-AEPFTGGYCFVR-QNDQSDR					
60		T	K		T PDG YA	L EG QMGNG
119		R	T G E			
119	YYGRGPIQLTNQNNYEKAGNAIRQDLVNNPDLVATDATISFKTAIWFWMTPQDNKPS SHD					
120		G S DL	K E		V	G C
179	R	A V		I R D		
179	VIIGSWTPSAADQSANRAPGYGVITNI INGGIECGVGPNAAVEDRIGYYRRYCGMLNVAP					
180	T R	T V		K Q R		NV SIM
239	E	G				253
239	GNLDCYNQRNFAQG					253
240		EV				254

Fig. 2. Amino acid sequences of chitinase proteins from tobacco and petunia. The protein sequence deduced from cDNA clone Tach1 is given. Above and below this sequence are given the differences in the amino acid residues as deduced from tobacco cDNA clone Tach3 and petunia cDNA clone Pach1, respectively. Gaps (dashes) were introduced for optimal alignment. Numbers refer to amino acid residue positions in the corresponding proteins. The arrow between the alanine (A) and glutamine (Q) residues indicates the putative signal peptide cleavage site.

PR-P **WTPSAADQSANRAPGYGVITNI INGGIE-GVGPNAAVE--IGY**

PR-Q **WTPSAADQAAN-VPGY**

Fig. 3. Amino acid sequences of peptides obtained after cleavage of tobacco chitinases PR-P and PR-Q. Bold letters indicate unambiguously characterized amino acid residues; thin letters give the most probable amino acid residues, and dashes indicate residues that could not be positively identified.

The primary structure was identical to the insert of Tach1, except for the presence of a G residue in the coding sequence, corresponding to the A residue at position 606 in Figure 1. Furthermore, the genomic sequence contains two introns (sequence not shown). The first intron is located between nucleotides 293 and 294 in the sequence of Tach1 (Fig. 1) and has a length of 195 base pairs. The second intron was 184 base pairs long and is located between nucleotides 396 and 397. The splice donor and acceptor sites of both introns agree fully with the splice consensus sequence.

Sequence of peptides from PR-P and PR-Q. PR proteins P and Q were purified from tobacco leaves infected with TMV and cleaved with *N*-chlorosuccinimide. Upon separation by electrophoresis, peptides of approximately 7 kDa were transferred to polyvinylidene difluoride membranes and sequenced by automated Edman degradation. The amino acid sequences that were obtained are shown in Figure 3. From PR-P, a stretch of 43 residues was obtained, whereas 16 residues were obtained from the PR-Q peptide. Since *N*-chlorosuccinimide cleaves after tryptophan residues, the first residue in both peptides must be trypto-

phan. The PR-P and PR-Q peptide sequences appeared to differ in two residues, which made it possible to identify the tobacco cDNA clones. The PR-P sequence was completely identical to the deduced amino acid sequence in cDNA clone Tach1 and genomic clone CHA18; the PR-Q sequence was identical to the one in clone Tach3.

Similarities to other proteins. The deduced amino acid sequences of the proteins encoded by cDNA clones Tach1, Tach3, and Pach5 were compared with each other (see Fig. 2) and with the sequence of the basic chitinase deduced from cDNA clone Tbch1. The identity percentage based on the number of identical amino acid residues in similar positions between the two tobacco acidic chitinases was 93%, between the petunia chitinase and the enzyme encoded by Tach1 was 79%, and between the petunia enzyme and that encoded by Tach3 was 77%. All three proteins had a highly hydrophobic NH₂-terminal region of approximately 20 amino acid residues. Between the two tobacco proteins this region contained only two different amino acids, but the petunia chitinase had eight differences in comparison to the tobacco enzymes in the corresponding NH₂-terminal part.

The amino acid sequence similarity between the acidic (253 residues) and basic (324 residues) chitinases of tobacco was only 57% (see Fig. 4A). Figure 4B shows a schematic representation of the acidic and basic chitinases. The NH₂-terminal hydrophobic region of 24 residues of the basic chitinase as deduced from the sequence of cDNA clones (data not shown, Shinshi *et al.* 1987) is not present in the mature protein (Shinshi *et al.* 1987) and apparently functions as a signal peptide. The schematic comparison shows

that the region immediately following the signal peptide in the basic enzyme is not present in the acidic proteins. This region of approximately 50 amino acid residues somewhat resembles each of the four similar chitin-binding domains of wheat germ agglutinin (Wright *et al.* 1984; Lucas *et al.* 1985).

Expression of chitinases in transgenic tobacco. cDNA clones Pach1 and Tbch1 were used to construct chimeric genes encoding petunia acidic chitinase and tobacco basic chitinase, respectively, under the control of the constitutive cauliflower mosaic virus 35S promoter. After transformation of tobacco and regeneration of transgenic plants, 10 independent plants of each series of transformants (Pach-transformed and Tbch-transformed plants) were analyzed for the expression of the chimeric gene. Plants showing the highest expression levels were used for the extraction of total protein and of extracellular protein.

Figure 5 shows a western blot of a denaturing polyacrylamide gel electrophoresed with proteins of the different extracts and probed with an antiserum against PR-Q. This antiserum reacted with the acidic chitinases PR-Q, PR-

P, and the petunia chitinase, as well as with the two basic chitinases of tobacco, Ch.32 and Ch.34 (Legrand *et al.* 1987). As can be seen in lanes 1 and 8 of Figure 5, total protein extracts of noninfected control plants contain small amounts of the acidic and basic chitinases. However, when compared to TMV-induced control plants (lane 4), it is evident that this low level reflects only some basal expression of the chitinase genes in plants under the growing conditions used. The band in lane 2 of Figure 5, containing the total protein extract of the Pach transformant, shows the relatively weak expression of the petunia chitinase, which migrated slightly slower than did tobacco chitinase PR-Q. Apparently, this petunia enzyme was efficiently transported outside the cell (lane 3).

Lane 6 of Figure 5, containing a total protein extract of the Tbch1 transformant, shows an increased amount of a protein comigrating with the faster one of two basic chitinase bands (Ch.32, compare lane 4). The absence of a band in this position in lane 7 shows that this chitinase is not present in the extracellular fluid. This is in agreement with the finding that the basic chitinases induced in non-transformed control plants by TMV infection accumulated intracellularly (Fig. 5, lane 5; Van Loon and Gerritsen 1989).

DISCUSSION

We have isolated and characterized cDNA clones with complete coding regions for chitinases from tobacco and petunia. The acidic chitinases encoded by cDNA clones Tach1 and Tach3 corresponded to PR-P and PR-Q of Samsun NN tobacco, respectively, as was shown by protein sequence analysis. Sequence analyses of genomic clone CHA18 showed that it corresponded to the cDNA sequence encoding PR-P. However, there was a difference in one nucleotide when comparing this genomic clone to the three PR-P cDNA clones characterized (a G instead of A residue at position 606 in Fig. 1). This difference resulted in an amino acid change (cysteine instead of tyrosine at position

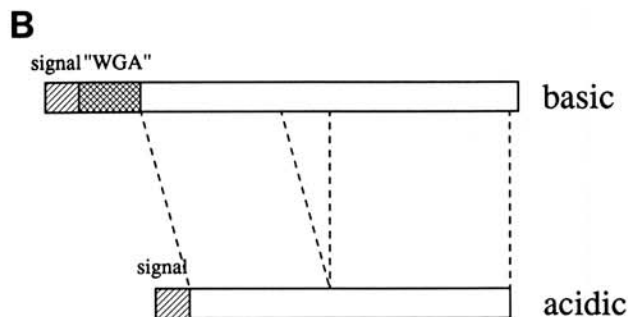


Fig. 4. A, Similarity between acidic chitinase PR-P, as deduced from clone Tach1, and the basic chitinase encoded by cDNA clone Tbch1. The first five residues in the basic chitinase sequence were derived from a cDNA sequence encoding a highly similar basic chitinase from tobacco. Identical residues are indicated by bars between the sequences. Gaps (dashes) were introduced for optimal alignment. The arrow indicates the cleavage site between the signal peptide and the mature basic chitinase. **B,** Schematic representation of the similarity between the two sequences. The signal peptide of the basic chitinase and putative signal peptide of the acidic chitinase are indicated by hatched blocks. The wheat germ agglutinin ("WGA") homologous region of approximately 50 residues at the NH₂-terminus of the mature basic chitinase is indicated by cross-hatching. Between the two proteins (represented by blocks), regions with high amino acid sequence similarity are indicated by connecting lines

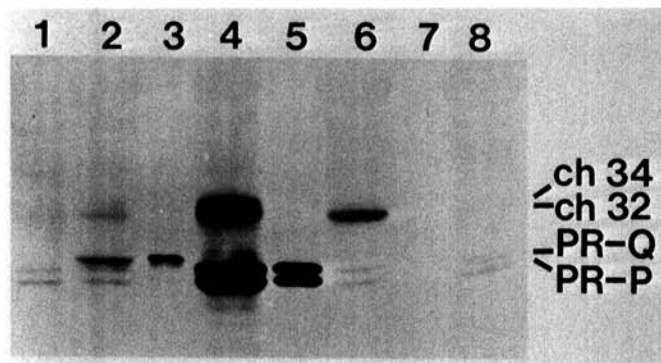


Fig. 5. Protein blot showing bands of chitinases present in nontransformed tobacco before (lanes 1 and 8) and after induction with tobacco mosaic virus (lanes 4 and 5), and in tobacco transformed with genes constitutively expressing petunia acidic chitinase (lanes 2 and 3) or tobacco basic chitinase (lanes 6 and 7). Both total leaf protein extracts (lanes 1, 2, 4, 6, and 8) and extracellular protein (lanes 3, 5, and 7) were electrophoresed and blotted. The blots were incubated with antisera against purified tobacco chitinase PR-Q. The positions of the basic chitinases Ch.34 and Ch.32 and the acidic chitinases PR-Q and PR-P are indicated.

199 in Fig. 2) and may reflect the presence of two genes in the tobacco genome encoding PR-P proteins differing in only one amino acid residue. Misincorporation of a nucleotide during PCR or cDNA synthesis was very unlikely since Payne *et al.* (1990) have found the G residue in several cDNA clones encoding PR-P from Xanthi-nc tobacco. The only other differences between the Xanthi-nc and Samsun NN cDNA clones were a G residue at position 832, where there was a T residue in the Xanthi-nc sequence, and an extra C residue at position 920 in the Samsun NN cDNA clones. No differences were found between the two PR-Q cDNA sequences from Samsun NN and Xanthi-nc.

Clones from a petunia cDNA library were isolated using a clone encoding an acidic tobacco chitinase as a probe. These petunia cDNA clones encoded an acidic protein highly similar to the two acidic chitinases of tobacco. All three proteins had a highly hydrophobic NH₂-terminal region, which probably functions as a signal peptide. This peptide was cleaved between the conserved alanine and glutamine residues at positions 24–25 in both tobacco proteins, as was shown by Payne *et al.* (1990). After cleavage of the signal peptide, the mature tobacco chitinases PR-P and PR-Q and the petunia chitinase had molecular masses of 24.9, 25.0, and 25.1 kDa and net charges at neutral pH of –5, –4, and –3, respectively.

The similarity between amino acid sequences of basic and acidic chitinases is known (Hooft van Huijsdijnen *et al.* 1987) and has been further extended here. However, the acidic chitinases appear to lack the cysteine-rich domain that is present at the NH₂-terminus of the mature basic enzymes (Lucas *et al.* 1985). This domain is similar in amino acid sequence to corresponding blocks at the NH₂-termini of basic chitinases of bean (Broglie *et al.* 1986) and potato (Gaynor 1988) and of wound-induced proteins of potato (Stanford *et al.* 1989). Such a domain has also been found in oligosaccharide-binding wheat germ agglutinins (Wright *et al.* 1984) and other lectins (Chapot *et al.* 1986).

Upon expression in transgenic tobacco, the tobacco basic chitinase accumulated intracellularly. The enzyme is probably correctly targeted into the central vacuole (Van den Bulcke *et al.* 1989; Mauch and Staehelin 1989). The petunia acidic chitinase, constitutively expressed from a chimeric gene in tobacco, accumulated in the extracellular fluid, similar to PR-P and PR-Q after induction. In petunia infected with the necrosis-causing alfalfa mosaic virus strain YSMV, a serologically related protein was induced, which comigrated with the product of the chimeric Pach gene (results not shown). Also, in petunia this protein was targeted to the extracellular fluid, indicating that tobacco correctly recognizes the putative petunia targeting signals. Whether signals for targeting, either into the extracellular spaces or to subcellular locations, are present in the hydrophobic leader sequences, cysteine-rich domains, COOH-terminal extensions, or in other parts of the proteins can now be studied in transgenic plants expressing chimeric proteins in which different regions of acidic and basic chitinases are combined. In addition, transgenic plants constitutively expressing acidic and basic chitinase will allow the putative function of these enzymes in a defense of the plant against pathogen attack to be investigated.

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