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# A new class of tobacco chitinases homologous to bacterial exo-chitinases displays antifungal activity

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## Summary

A novel chitinase gene of tobacco was isolated and characterized by DNA sequence analysis of a genomic clone and a cDNA clone. Comparative sequence analysis of both clones showed an identity of 94%. The proteins encoded by these sequences do not correspond to any of the previously characterized plant chitinases of classes I–IV and are designated as class V chitinases. Comparison of the chitinase class V peptide sequence with sequences in the Swiss Protein databank revealed significant sequence similarity with bacterial exo-chitinases from *Bacillus circulans*, *Serratia marcescens* and *Streptomyces plicatus*. It was demonstrated that class V chitinase gene expression is induced after treatment of tobacco with different forms of stress, like TMV-infection, ethylene treatment, wounding or ultraviolet irradiation.

Two related chitinase class V proteins of 41 and 43 kDa were purified from Samsun NN tobacco leaves inoculated with tobacco mosaic virus. The proteins were purified by Chelating Superose chromatography and gel filtration. *In vitro* assays demonstrated that class V chitinases have endo-chitinase activity and exhibit antifungal activity toward *Trichoderma viride* and *Alternaria radicina*. In addition, it was shown that class V chitinase acts synergistically with tobacco class I  $\beta$ -1,3-glucanase against *Fusarium solani* germlings.

## Introduction

Chitinases are commonly found in a wide range of organisms including bacteria, fungi, higher plants, insects, crustaceans, and some vertebrates. A major role of chitinases of fungi, crustaceans, and insects is modification of the organism's structural wall component chitin. Plant chitinases are considered to play a major role in defense mechanisms against fungal pathogens (Boller, 1988; Bowles, 1990; Linthorst, 1991). Four classes (I–IV) of plant endo-chitinases have been described based on amino acid sequence features (Collinge *et al.*, 1993; Mikkelsen *et al.*, 1992; Shinshi *et al.*, 1990). Class I chitinases are enzymes with an N-terminal cysteine-rich domain and a highly conserved main structure. Class II chitinases lack the N-terminal cysteine-rich domain but have high amino acid sequence identity to the main structure of class I chitinases. Class III chitinases show no sequence similarity to enzymes in class I or II. Class IV chitinases contain a cysteine-rich domain and a main structure with 41–47% amino acid sequence identity to the main structure of class I chitinases. The class I and II enzymes are serologically related, while class I and IV enzymes are serologically distinguishable. The tobacco chitinases identified to date can be divided into three classes. The tobacco class I chitinases are localized in the plant vacuoles and inhibit the growth of many fungi *in vitro* by causing lysis of hyphal tips (Mauch *et al.*, 1988; Sela-Buurlage *et al.*, 1993). The combination of class I chitinases and class I  $\beta$ -1,3-glucanases inhibit synergistically fungal growth (Sela-Buurlage *et al.*, 1993). In contrast, the extracellular class II chitinases of tobacco, which are very homologous to class I enzymes, are not antifungal. Class III chitinases show no sequence similarity to enzymes in class I or II (Lawton *et al.*, 1992). It is not yet known whether class III chitinases exhibit fungal growth-inhibiting activities *in vitro*. Recently, a tobacco chitin-binding protein of 20 kDa (CBP20) was shown to possess low endo-chitinase activity and was able to inhibit fungal growth in synergy with either class I  $\beta$ -1,3-glucanases or class I chitinases (Ponstein *et al.*, 1994). The pathogen- and wound-inducible CBP20 protein was classified as an intracellular (class I) PR-4 type protein. Class IV chitinases have been reported for sugar beet, bean and rape (Collinge *et al.*, 1993; Mikkelsen *et al.*, 1992).

Chitinases, like various other pathogenesis-related (PR) proteins, have been shown to be developmentally

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regulated in several tissues including roots, lower leaves, and flowers of tobacco and *Arabidopsis* (Lotan *et al.*, 1989; Memelink *et al.*, 1990; Neale *et al.*, 1990; Samac *et al.*, 1990).

The extent to which chitinases and other PR-proteins contribute to pathogen resistance is being studied using genetic and molecular techniques (Lamb *et al.*, 1992). Reports in the literature support the hypothesis that chitinases play an important role in defense against fungal pathogens. Transgenic tobacco plants constitutively expressing a bean class I chitinase gene showed an enhanced resistance to the fungal root pathogen *Rhizoctonia solani* (Brogliè *et al.*, 1991). Recently, partial resistance against *Fusarium* in tomato was demonstrated by the simultaneous expression of a class I chitinase and a class I  $\beta$ -1,3-glucanase gene from tobacco (Melchers *et al.*, 1993b). From a large number of literature reports it becomes clear that more plant proteins involved in plant defense will be characterized in future studies. For example, among the six different sets of mRNAs (originally named cluster-A to cluster-F) induced by tobacco mosaic virus (TMV) infection of tobacco, the cluster-A type has not been characterized (Hooft van Huijsduijnen *et al.*, 1986).

Here, we report the isolation and sequence analysis of both a cDNA clone and genomic clone, which encode a new class of chitinase proteins. The class V chitinase proteins were shown to contain sequences homologous to exo-chitinases of bacteria. The pattern of class V chitinase gene expression in tobacco was analyzed under different forms of stress (TMV infection, ethylene, wounding, UV light). Furthermore, we describe the purification and characterization of class V chitinase proteins from tobacco and measurements of their inhibitory effects on the growth of several plant pathogenic fungi *in vitro*.

## Results

### *Molecular characterization of a novel plant chitinase gene*

The cluster-A mRNA was reported to be strongly induced by TMV infection of *Nicotiana tabacum* cv. Samsun NN (Hooft van Huijsduijnen *et al.*, 1986). Screening of a lambda ZAP cDNA library of TMV-infected Samsun NN tobacco plants with a partial cluster-A cDNA clone as probe resulted in the isolation of 11 positively hybridizing clones. The trivial name cluster-A was changed to *chi-V*. Restriction enzyme analysis and (partial) sequence analysis suggested that all *chi-V* cDNA clones were identical. The entire nucleotide sequence of a nearly complete cDNA clone (cA-3) was elucidated and is shown in Figure 1.

A genomic library of *N. tabacum* was screened using the *chi-V* cDNA clone as a probe. Three recombinant lambda phages, each containing different restriction fragments hybridizing to the *chi-V* cDNA, were purified (Figure 2). A genomic clone, highly homologous to clone *chi-V*, was selected by a polymerase chain reaction using specific oligonucleotide primers corresponding to the 5'-terminal part of the coding region and 3' terminal noncoding region of the cDNA. Only one of the three phages (i.e. clone 59) showed a very strong amplification of specific sequences with these primers and was selected for further analysis. Apparently, the identity of two other phages (clones 56 and 66) to the *chi-V* cDNA sequence was not sufficient for a successful DNA amplification with the specific primers used. The complete nucleotide sequence of the *chi-V* gene, including the deduced primary structure of the encoded protein and sequences of the 5'-flanking and 3'-flanking regions of the gene, is shown in Figure 1.

Comparison of the cDNA clone with the corresponding tobacco gene revealed that these sequences share a high degree of identity (94 %) (Figure 1). Within the 5'-terminal upstream region a 'CAAT' motif (position 391) and 'TATAAT' motif (position 428) were found. In the 3' terminal noncoding region a potential polyadenylation signal (5'-AATAAA-3') was present at position 2962.

The coding region of the *chi-V* gene is contained in three exons and yields a precursor protein of 377 amino acids. The Chi-V preprotein contains a putative signal peptide of 25 amino acids possibly involved in transport across the membrane of the endoplasmic reticulum and four potential N-linked glycosylation sites (N-X-S/T). The predicted mature protein has a calculated molecular weight of 39 033 Da and a calculated isoelectric point of  $pI = 9.8$ . The mature protein predicted from the cDNA sequence contains one additional amino acid and has a calculated molecular weight of 39 229 Da.

The intervening sequences of 952 and 349 bp in length are present after codons 151 and 364, respectively. The number of genes encoding Chi-V-like proteins in tobacco was determined previously by hybridization of a genomic Southern blot with the cDNA clone (Bol, 1988). The pattern of bands hybridizing to the probe under stringent conditions shows that there are probably four different members in the *chi-V* gene family (Bol, 1988).

To determine the induction of *chi-V* gene expression, tobacco plants were treated under different stress conditions and mRNA accumulation was analyzed on a Northern blot. Samples of different treatments were taken 3 days after inoculation with TMV, 2 days after wounding, 1 day after ethephon treatment, and 1 day after UV light irradiation. At these time points maximal expression of the PR-genes (PR-1 to PR-5) has been reported previously by Brederode *et al.* (1991), and was used here to study

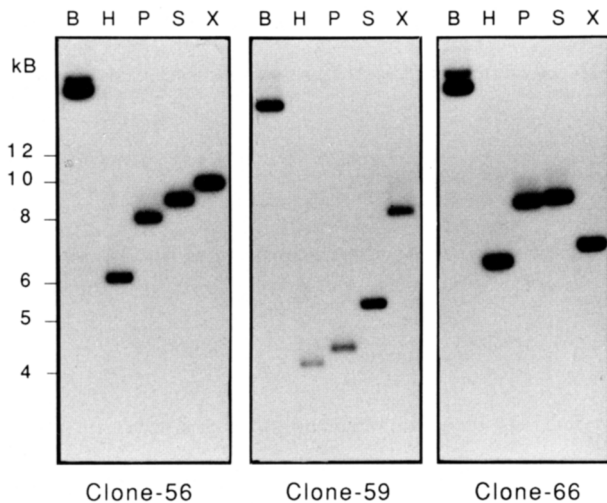
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a
b AAATAAAAAGCCCGGTAGACAGTAAACTCAAACTCTCAAACCTTTAACTCGAAGTCACAAATTAATAAGCTATCAACCTTCAATTTAATGGCTAATCTGTCACTCTTTTCGCC 480
c M A N S V T L F A
d
a
b ATTATTTCTCATGTTTCTCCTCCAGCACTAGTTTGACAAATAGCCAAAATGTT---AAGGGAGGATACTGGTTAAGGACAGTGGATTAGCATTAACAACATAGATTCAACACTT 600
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d
a
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d
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d
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c Q L G F H G L D L D W E Y P L S A A D M T N L G
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d I M
a
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d Y N
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d I
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d P D
a
b GACTTATAACAGAATTAGGGATTATATAGTGGAGATCGGCCACGACTGTGTATAACGCTACCATTTGTTGGAGATTATTGTTACTCTGGTAGTAATGGATTAGCTATGATATACTCA 2400
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d Q
a
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d S
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c M K
d
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TGTTTTTCGATATTATTCTCTGTTTTCAATTAATAA 3159

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**Figure 1.** Nucleotide sequence of the *chi-V* gene (clone 59) and of the homologous cDNA clone (cA-3).

The complete nucleotide and amino acid sequence of the genomic *chi-V* clone are shown in line b and line c, respectively. Above the nucleotide sequence only the nucleotides which are different in the cDNA clone are indicated in line a. Similarly, below the Chi-V protein sequence only the amino acids which are different in the cDNA sequence are indicated in line d. The amino acid sequences determined by sequencing the protein are underlined. The putative cleavage site of the signal peptide, between residues Ser (S) and Gln (Q), is indicated by an arrow. The putative transcriptional regulatory signals are underlined in line b. The boundaries of the cDNA sequence (line a) are indicated with a broken line (—).



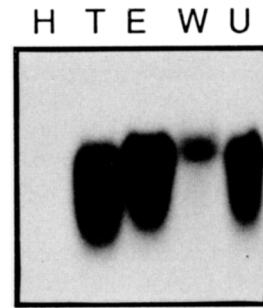
**Figure 2.** Southern blot analysis of three genomic clones containing sequences homologous to the tobacco *chi-V* gene.

Three genomic clones of tobacco (clone-56; 59; 66) were purified and digested with either *Bam*HI, *Hind*III, *Pst*I, *Sst*I or *Xba*I, lanes 1–5 respectively, and electrophoresed on a 0.8% agarose gel. The gel was blotted and hybridized with the *chi-V* cDNA and washed at 65°C in 0.1 × SSPE, 0.1% SDS.

*chi-V* gene expression. Figure 3 shows that in non-stressed tobacco leaves there is no detectable expression of the *chi-V* gene. TMV infection of tobacco leaves induces the expression of the *chi-V* gene to high levels (Figure 3, lane T). The treatment of leaves with ethephon, to produce ethylene, or UV light resulted both in a high increase of *chi-V* mRNA (Figure 3, lanes E and U). In contrast, a relatively low induction of *chi-V* expression was found after wounding of the leaves. Together, these results indicate that *chi-V* can be classified as a novel pathogenesis-related gene of tobacco.

#### Homology of tobacco Chi-V protein to bacterial chitinases from *Serratia marcescens* and *Bacillus circulans*

A search for similar proteins in the protein database revealed homology of Chi-V to the bacterial chitinases ChiA and ChiB from *Bacillus circulans* and *Serratia marcescens*, respectively. Although the overall identity of tobacco Chi-V to ChiA (31%) and ChiB (26%) is low, specific regions are highly conserved within all three amino acid sequences, as shown in Figure 4. In addition, homology was found with chitinase proteins from *Streptomyces plicatus*, *Brugia malayi* (nematode), and a secretory protein from *Mus musculus* (mouse). No structural homology was found with the four distinct classes of plant chitinases (Collinge *et al.*, 1993; Lawton *et al.*, 1992).



**Figure 3.** Northern blot analysis of the tobacco *chi-V* gene.

Accumulation of *chi-V* mRNAs upon different forms of stress. Equal amounts of total RNA isolated from nonstressed healthy tobacco leaves (H) or from leaves infected with TMV (T), sprayed with ethephon (E), wounded by cutting (W) or irradiated with UV light (U) were hybridized to a *chi-V* cDNA probe.

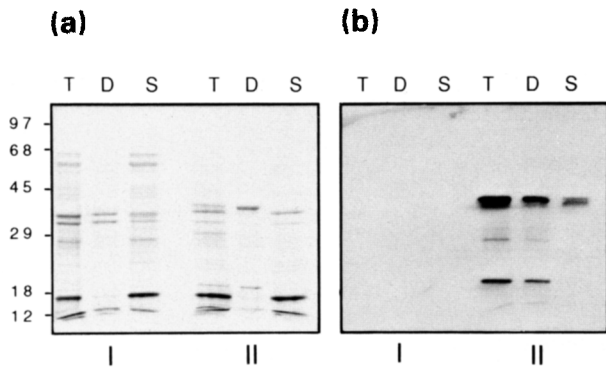
#### Isolation and characterization of Chi-V proteins from tobacco

The *chi-V* cDNA was placed under the control of the lambda  $p_R$  and  $p_L$  promoters (Schauder *et al.*, 1987), in order to overexpress the gene in *Escherichia coli*. Different protein fractions of induced *E. coli* cells including, total (T), debris (D), and soluble (S) fractions, were analyzed. Figure 5(a-II) shows the overproduction of a 40 kDa Chi-V protein in *E. coli* strain KA1092. After disrupting the cells by sonification, Chi-V protein aggregates were isolated by centrifugation with the cell debris (Figure 5a-II, lane D). Chi-V proteins produced in *E. coli* were purified and used for the generation of antibodies (see Experimental procedures). Figure 5(b) shows a Western blot analysis of the protein gel shown in Figure 5(a), and demonstrates that the isolated antibodies are specifically directed against the Chi-V protein. A strong signal for Chi-V is found in the total fraction (lane T) and cell debris fraction (Figure 5b-II, lane D) of induced *E. coli* cells which contained the *chi-V* expression construct. The weak signal found in lane S indicates that a minor amount of the Chi-V protein is present in the soluble *E. coli* protein fraction.

The purification of the Chi-V protein from TMV-infected tobacco leaves was monitored by immunodetection. The protein appeared to absorb to a cation-exchanger S-Sepharose indicating a basic nature of the protein. No cross-reacting proteins were observed in the flow-through of the S-Sepharose column (data not shown). Chi-V was eluted from the S-Sepharose column in the presence of 75–140 mM NaCl. Close examination of the SDS–polyacrylamide gels and immunoblots revealed that two proteins with molecular weights of 41 and 43 kDa, cross-reacted to the antiserum. Apparently, both proteins were related.

The presence of potential N-linked glycosylation sites deduced from both the cDNA sequence (3-sites) and the





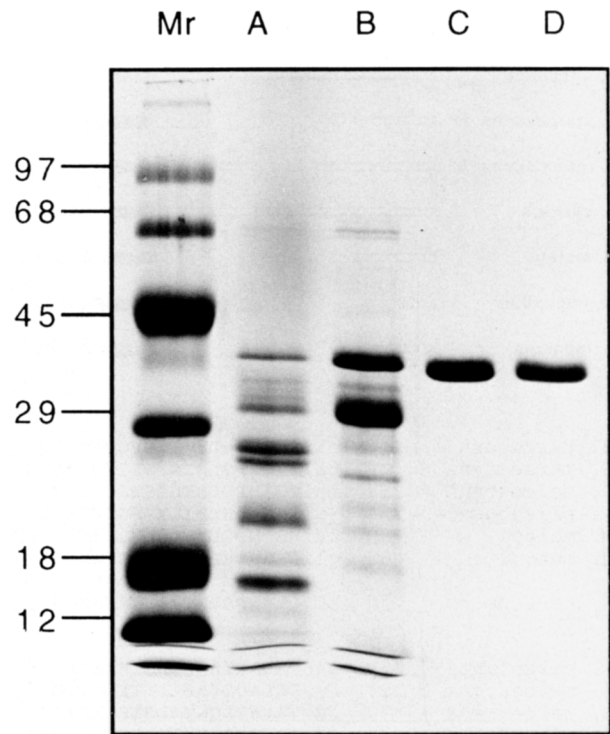
**Figure 5.** Overproduction of Chi-V in *E. coli*.

Induced extracts of *E. coli* KA1092 control cells (panel I), and of strain KA1092 containing the *chi-V* expression construct (panel II), were analyzed. (a) On a 12.5% SDS-polyacrylamide gel the protein profile of different samples, total fraction (T), cell debris (D) and soluble fraction (S), is shown.

(b) Immunoblot analysis of an identical protein gel with specific detection of Chi-V proteins.

Superose chromatography was successfully used after activation with  $Zn^{2+}$  ions. About 20% of the total amount of protein was retarded by the activated matrix and appeared to consist mainly of Chi-V proteins. A second passage through the Chelating Superose column resulted in an almost pure Chi-V protein preparation (Figure 6, lane C). The proteins were purified to homogeneity by subsequent gel filtration chromatography (Figure 6, lane D).

A mixture of both 41 and 43 kDa Chi-V protein was separated on a 12.5% SDS-polyacrylamide gel and electroblotted to a PVDF membrane. Upon Edman degradation, the N-terminus appeared to be blocked and no sequence information was obtained. However, the presence of a unique site susceptible to acid hydrolysis enabled us to determine an internal sequence: P-V-N-H-V/I-S-G-S-D-(G)-I-N-A-?-I-Q. The amino acid preceding this sequence is most probably an Asp (D) residue since the digestion applied is specific for Asp-Pro bonds (Landon, 1977). Additional sequence information was obtained after cleaving Chi-V with hydroxylamine. This resulted in the formation of two peptides of about the same molecular weight which after separation on SDS-polyacrylamide gel were transferred to a PVDF membrane. Sequencing of the upper band yielded the following sequences: G-L-N-Y-P-V-E-S-V-A-R-N-L-N-(W), and S-H-A-Q-L-F-D-P-V-N-H-V/I-S-G-S-D-G-I-N-A-W-I-Q-A-G-V. These peptide sequences showed complete identity to the amino acid sequence deduced from the cDNA clone cA-3 (Figure 1). The peptide sequence, corresponding to codon position 233, showed that two amino acids (V/I) were present at this position in a 1:1 ratio. Possibly two highly identical Chi-V proteins were co-purified and differ at least for one amino acid position.



**Figure 6.** Purification of the Chi-V protein from TMV-infected tobacco leaves.

SDS-polyacrylamide gel shows the protein profile of different purified fractions. A, sample of the desalted crude leaf extract; B, protein pool after S-Sepharose cation exchange chromatography; C, protein pool after Chelating Superose column; D, purified Chi-V after gel filtration chromatography. The lane Mr shows prestained markers with the molecular weights indicated.

#### *Enzyme activities and cellular localization of the Chi-V protein*

The homology of the tobacco Chi-V protein with bacterial (exo)chitinases suggested that Chi-V might be able to catalyze the hydrolysis of chitin, a  $\beta$ -1,4-linked polymer of N-acetyl-D-glucosamine. To test this hypothesis purified Chi-V of tobacco was assayed for chitinase activity. Using a chitinase assay, with tritiated chitin as a substrate (Molano *et al.*, 1977), Chi-V had a specific activity of  $40 \pm 6$  c.p.m.  $\mu\text{g}^{-1}$  protein, which is 250- to 500-fold lower than the specific activities of the tobacco class I chitinases (Table 1) (Legrand *et al.*, 1987; Sela-Buurlage *et al.*, 1993). However, chitinase activity of Chi-V measured by a colorimetric assay, using a soluble dye-labeled chitin substrate (Wirth and Wolf, 1990), was twofold higher than that of the class I chitinase (Table 1). Testing for additional enzyme activities indicated that Chi-V possesses no detectable exo-chitinase activity (Table 1), chitobiase,  $\beta$ -1,3-glucanase or lysozyme activity. We propose that Chi-V represents a novel chitinase class V, with no sequence similarity to the

**Table 1.** Chitinase activities of Chi-V proteins

Protein		Chitinase activities (expressed per $\mu\text{g}$ )				
		$^3\text{H}$ -chitin assay <sup>a</sup>	CM-chitin assay <sup>b</sup>	ODu	exo-chitinase assay <sup>c</sup>	nmol
PR-3a (PR P)	class II	1180 $\pm$ 57 c.p.m.	36.2 $\pm$ 1.1	ODu	< 0.05	nmol
PR-3b (PR Q)	class II	1087 $\pm$ 35 c.p.m.	30.9 $\pm$ 1.9	ODu	< 0.05	nmol
32 kDa (Chi-I)	class I	11471 $\pm$ 347 c.p.m.	1.0 $\pm$ 0.03	ODu	< 0.05	nmol
Chi-V, chitinase	class V	40 $\pm$ 6 c.p.m.	2.1 $\pm$ 0.03	ODu	< 0.05	nmol
exo-chitinase <sup>d</sup>		746 $\pm$ 60 c.p.m.	7.0 $\pm$ 0.34	ODu	450 $\pm$ 0.1	nmol

<sup>a</sup>Tritiated-chitin substrate (Molano *et al.*, 1977).

<sup>b</sup>Soluble dye-labeled chitin substrate (CM-chitin), Remazol Brilliant Violet bound to carboxymethyl-chitin (Wirth and Wolf, 1990).

<sup>c</sup>*p*-nitrophenyl liberation assay, substrate *p*-nitrophenyl chitobiose (Roberts and Selitrennikoff, 1988).

<sup>d</sup>Exo-chitinase from *S. griseus* (Sigma C-1650)

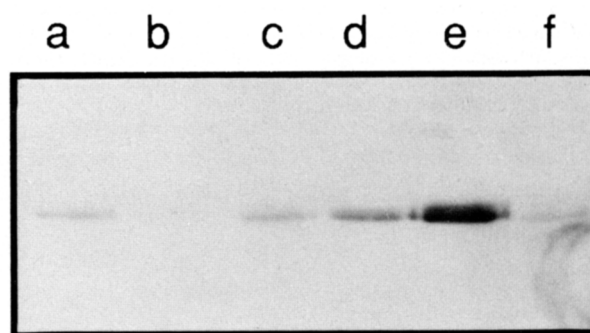
All assays were performed at 37°C and pH 6.0. Incubation time 30 min. The specific activity is expressed as c.p.m. liberated, blue substrate liberated (measured as an increase of OD550), and nmol *p*-nitrophenyl liberated per 30 min, respectively.

four classes of plant chitinases described previously (Collinge *et al.*, 1993; Shinshi *et al.*, 1990).

The cellular localization of the class V chitinase (Chi-V) was analyzed using different leaf extracts of healthy and TMV-infected tobacco plants, including total leaf fraction (Figure 7, lane a/d), leaf material from which the extracellular fluid (EF) was removed (Figure 7, lane b/e), and the EF-fraction (Figure 7, lane c/f). Western analysis indicated that Chi-V proteins are predominantly located intracellularly, as shown in Figure 7. A strong signal for Chi-V was found in fractions d and e of TMV-infected leaves. In the extracellular fluid of TMV-infected leaves relatively low levels of Chi-V protein were found (lane f). Moreover, in healthy tobacco leaves a weak signal for Chi-V was present in the total leaf fraction (lane a) and in the EF (lane c). This result suggests that possibly an extracellular homolog of Chi-V is present in tobacco.

#### *In vitro* antifungal activity of class V chitinases

In previous reports it has been demonstrated that class I chitinases (Chi-I) and class I  $\beta$ -1,3-glucanases (Glu-I) inhibit fungal growth *in vitro* (Mauch *et al.*, 1988; Sela-Buurlage *et al.*, 1993). Both hydrolytic enzymes have been implicated to play an important role in the defense mechanisms of plants against invading fungi. The antifungal activity of Chi-V was investigated by testing *in vitro* the lysis activity of this protein alone or in combination with Chi-I or Glu-I on different fungi. Chi-V caused lysis and complete growth inhibition of *Trichoderma viride* at a

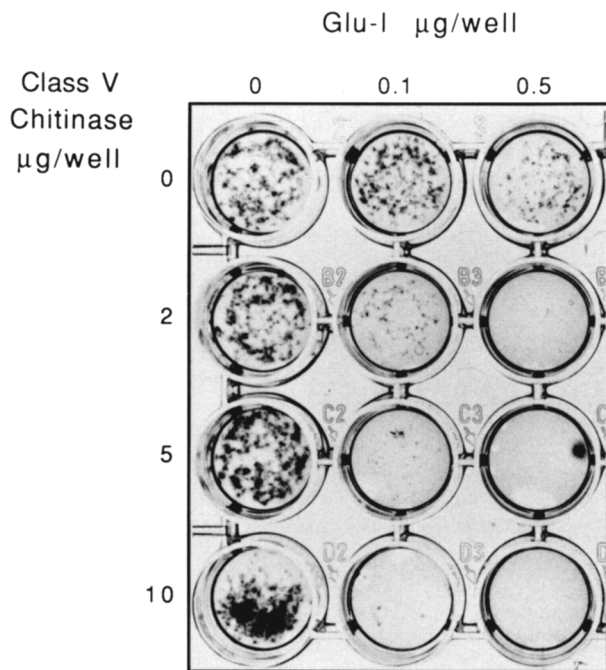


**Figure 7.** Cellular localization of Chi-V.

The antiserum to the Chi-V protein was used to determine the cellular localization of tobacco Chi-V. Healthy noninfected (lanes a,b and c) and TMV-infected (lanes d,e and f) tobacco plants were analyzed. Samples of a total protein fraction (lanes a and d), leaf fraction without extracellular washing fluid (lanes b and e), and EF-fraction (lanes c and f), were separated on a 12.5% SDS-gel and electroblotted to a PVDF membrane, and used for detection of Chi-V proteins.

concentration of 2  $\mu\text{g}$  per well. On the phytopathogenic fungus *Alternaria radicina* Chi-V significantly inhibited fungal growth at 5–10  $\mu\text{g}$  per well, while at high concentrations (100  $\mu\text{g}$  per well) no effect was found on *Fusarium solani*. However, Chi-V exhibited a potent antifungal effect on *F.solani* in synergy with the class I  $\beta$ -1,3-glucanases. The synergistic action of these proteins caused both lysis of germlings and growth inhibition of *F.solani* (Figure 8). No synergistic activity against *F.solani* was observed between Chi-V and Chi-I proteins. The Chi-V protein exhibited no effect on the phytopathogenic fungi *Septoria*





**Figure 8.** Growth inhibition of *Fusarium solani* by the synergistic activity of Chi-V and class I  $\beta$ -1,3-glucanase proteins. The effect of Chi-V on fungal growth *in vitro* was tested by the addition of Chi-V to pregerminated spores of *Fusarium solani* either alone or in combination with class I  $\beta$ -1,3-glucanases. Amounts of protein (in  $\mu\text{g}$ ) per well are indicated. After 3 days incubation at 20°C the fungal mycelia was visualized by staining with lactophenol cottonblue.

*lycopersici*, *Rhizoctonia solani* and *Phytophthora infestans* (data not shown).

## Discussion

To learn more about the possible involvement of pathogenesis-related proteins in plant defense, it is important to increase our knowledge about the different proteins that accumulate in stressed tissue. In this study, a novel chitinase protein (class V), named Chi-V, was isolated and characterized in detail. The molecular structure of the *chi-V* gene (previously cluster-A) has been determined, the enzymatic and antifungal properties of the encoded protein have been assayed, and expression characteristics of the gene both in healthy plants and in response to various stress treatments have been characterized.

### *The Chi-V protein shares homology with bacterial chitinases*

The molecular structure of the *chi-V* gene was determined by isolation and DNA sequence analysis of a genomic clone and a cDNA clone. Comparative sequence analysis revealed that the isolated *chi-V* cDNA and *chi-V* gene

shared 94% identity. The Chi-V proteins encoded by these sequences do not correspond to any of the previously characterized PR-proteins. Interestingly, by comparing the Chi-V peptide sequence with sequences in the Swiss Prot databank (release 23), we found significant sequence similarity with bacterial chitinases from *B. circulans*, *S. marcescens* and *S. plicatus*. The chitinases from *S. marcescens* have been shown to inhibit efficiently the growth of chitin containing fungi and to be useful as a biocontrol agent (Ordentlich *et al.*, 1988). For example, tobacco plants expressing the ChiA protein from *S. marcescens* showed improved resistance against the soil pathogen *R. solani* (Logemann *et al.*, 1993). In conclusion, the homology of Chi-V with bacterial chitinases and with chitinases of nematode origin might reflect a possible biological function of this plant protein.

### *The chi-V gene encodes a novel endo-chitinase*

Infection of tobacco plants with TMV results in the accumulation of high amounts of Chi-V proteins ( $2 \mu\text{g g}^{-1}$  fresh leaf). The basic nature of these proteins and the characteristic property to bind to a Chelating Sepharose column was used to purify Chi-V to homogeneity. Amino acid sequence analysis of an internal part of Chi-V, revealed that two closely related proteins were isolated which differ at least at one amino acid position. Chi-V was found to be a glycosylated protein which corroborates with the presence of either three or four putative glycosylation sites in the protein sequence encoded by the cDNA and genomic clone, respectively. Protein sequencing revealed that the Chi-V protein characterized in this study corresponds to the product encoded by the *chi-V* cDNA clone (cA-3). Localization studies indicated that this protein accumulates intracellularly in TMV-infected leaves. Within each family of tobacco PR-proteins (PR-1 to PR-5), both intracellular and extracellular isoforms have been identified. Western blot analysis of healthy tobacco leaves indicated that there is possibly also an extracellular homolog of Chi-V that is constitutively expressed at a low level in tobacco.

Analysis of the enzymatic properties demonstrated that Chi-V has endo-chitinase activity whereas the homologous bacterial chitinases from *B. circulans*, *S. marcescens* and *S. plicatus* are exo-chitinases. A number of assays were used to measure both endo- and exo-chitinase activity. The Chi-V protein lacks detectable exo-chitinase activity, like the tobacco class I and class II hydrolases. The specific endo-chitinase activity of tobacco Chi-V is about 25- and 250-fold lower compared with the activity of the class II and class I chitinases of tobacco, respectively, when tested on tritiated-chitin. However, using an alternative chitin substrate (carboxymethyl-

chitin) completely different activities were found for chitinases of class I, II and V. The different specific activities observed are most probably explained by the difference in solubility of the substrate used. Tritiated chitin, prepared by reacylation of chitosan, is an insoluble substrate, while the alternative chitin substrate is highly soluble in water. The differences between the chitinase enzymes tested may reflect distinct *in vivo* functions, rather than a difference in potency to hydrolyse the same chitin substrate.

Four classes of plant chitinases have been proposed based on the primary structure (Collinge *et al.*, 1993; Shinshi *et al.*, 1990). We propose that the Chi-V protein represents a class V chitinase, which lacks sequence identity with the previously described class I–IV enzymes and contains sequence identity to bacterial exo-chitinases.

#### *The Chi-V protein participates in the plant defense response*

Differential expression of intracellular and extracellular PR-proteins has been observed during plant development and in response to various exogenous stimuli (Brederode *et al.*, 1991; Memelink *et al.*, 1990; Ori *et al.*, 1990).

We have characterized the expression pattern of the *chi-V* gene in tobacco in response to different stress conditions. The *chi-V* gene is highly induced by infection with TMV, which confirmed an earlier report by Hooft van Huijsdijnen *et al.* (1986). Ethephon treatment strongly induced the expression of *chi-V*, similar to the level observed for the different class I PR-genes. In addition, wounding of tobacco leaves results in enhanced synthesis of *chi-V* mRNA, although to a much lesser extent than in TMV infected or ethephon-treated leaves. The expression level upon induction with UV light is comparable with the response of *chi-V* to TMV. In summary, the expression pattern of *chi-V* to different forms of stress is similar to the response of the class I PR-genes (Brederode *et al.*, 1991; Ponstein *et al.*, 1994). This observation suggests that Chi-V can be classified as an intracellular class I PR-gene. As reported for nearly all the vacuolar PR-proteins (PR-2 to PR-5), Chi-V most likely contains a carboxyl-terminal propeptide which directs the protein to the vacuole (Melchers *et al.*, 1993a).

Here we present evidence that the purified Chi-V proteins, either alone or in synergy with class I  $\beta$ -1,3-glucanases, inhibit the growth of *F. solani* and *A. radicina*, pathogens of bean and carrot, respectively. The fact that Chi-V accumulates in induced plants and inhibits the growth of *F. solani* and *A. radicina*, suggests a possible role for Chi-V in plant defense to these and possibly other fungi. In tobacco the majority of Chi-V is located intra-

cellularly. Interestingly, all the intracellular (class I) PR-proteins known to date, have been shown to possess direct *in vitro* antifungal activity against one or more fungi. The extracellular (class II) proteins which lack fungal growth-inhibiting activity might function in a more indirect way, as proposed previously by Mauch and Staehelin (1989) for chitinases and  $\beta$ -1,3-glucanases. Future studies will have to prove whether in pathogen-infected plants the Chi-V proteins actually contribute to the plant defense mechanism. The overexpression of the gene encoding Chi-V in transgenic plants, alone or together with the constitutive production of other antifungal proteins, may substantiate its role in plant defense.

## Experimental procedures

### *Synthesis and analysis of cDNA libraries*

Isolation of poly(A)<sup>+</sup>-RNA from TMV-infected tobacco, the synthesis of cDNA and the construction of a cDNA library, unidirectionally cloned in the lambda vector Uni-ZAP XR, according to the manufacturer's instructions, was done as described (Stratagene, La Jolla, CA). Recombinant phages were screened using a <sup>32</sup>P-labeled partial cDNA clone (450 bp insert) as a probe (Hooft van Huijsdijnen *et al.*, 1986). After rescue of the cDNA-containing pBluescript plasmids from the isolated lambda phage by co-infection with helper phage R408 (Stratagene), the cDNA insert of clone cA-3 was subcloned in M13 derivatives and sequenced using the M13 primer and a primer based on the cDNA sequence obtained with the M13 primer. Alternatively, the cDNA was directly sequenced from denatured plasmid DNA, using T3 or T7 primers (Promega, Madison, WI; Chen and Seeburg, 1985).

### *Polymerase chain reaction*

The genomic *chi-V* clone highly homologous to the *chi-V* cDNA clone cA-3 was selected by a polymerase chain reaction using specific oligonucleotide primers corresponding to the 5'-end coding region (5'-TCTCCGCAACTAGTTTGCAC-3') and 3'-end noncoding region (5'-GTCATAGTTCACATATCTGTTGCC-3') of *chi-V* cDNA. The amplification of genomic DNA was done during 30 cycles of sequential incubations at 95°C for 1 min, 55°C for 1 min and 72°C for 3 min, in a 100  $\mu$ l reaction mixture, containing 100 ng of genomic DNA, 0.2  $\mu$ M of the specific primers listed above, and 2 units of *Thermus aquaticus* DNA polymerase (Perkin-Elmer Cetus, Gouda, The Netherlands).

In order to clone the *chi-V* cDNA into a bacterial expression vector, DNA amplification was performed during 35 cycles of sequential incubations at 95°C for 0.5 min, 50°C for 2 min and 72°C for 1.5 min, in a 50  $\mu$ l reaction mixture, containing 1 ng of *chi-V* DNA (cA-3 clone), 0.2  $\mu$ M of T7 primer, 0.2  $\mu$ M of primer p1 (5'-GTTTCCTTCTCCATGGAAGTACTAGTTTGCAC-3') and 2 units of *Thermus aquaticus* DNA polymerase. Part of the reaction product (4%) was electrophoresed in an 0.8% agarose gel, to confirm the synthesis of a DNA fragment with the correct size. The remainder of the PCR DNA products was precipitated with ethanol and washed to remove excess of salts.

### *Cloning of the chi-V gene into a bacterial expression vector*

After digestion of the PCR DNA with restriction enzymes *Nco*I and *Xho*I, electrophoresis and isolation of the fragment from agarose gel, it was ligated into an intermediate *Nco*I/*Xho*I-digested vector (pGV84), followed by transformation of *E. coli* DH5 $\alpha$ . The *Nco*I/*Xho*I fragment from positive clones resulting from this transformation was subsequently ligated into a *Nco*I/*Sal*I-digested bacterial expression vector (pJLA602, Medac, Hamburg, Germany) and used to transform *E. coli* DH5 $\alpha$ . Positive transformants, were screened by hybridization to the labeled *chi*-V insert and the presence of the correct size and orientation of the insert was determined by restriction enzyme analysis. The resulting plasmid, pJLA-A53 contains the *chi*-V open reading frame downstream of a tandem arrangement of the repressible  $p_R$  and  $p_L$  promoters of phage lambda and a highly efficient *E. coli* translational initiation region. Finally, plasmid pJLA-A53 was used to transform protease(-) *E. coli* strain KA1092.

### *Expression in E. coli and immunization*

Expression of the *chi*-V cDNA gene was induced in KA1092 by switching cultures grown for 3 h at 30°C to a waterbath at 42°C and incubating for another 3 h. Bacteria were collected by centrifugation and dissolved in 1/25 vol. of sample buffer (25 mM Tris pH6.8, 192 mM glycine, 6 M urea, 2.5% SDS, 10% glycerol, 5%  $\beta$ -mercaptoethanol). After heating for 3 min at 100°C, the samples were electrophoresed in SDS-polyacrylamide gel (Laemmli, 1970) and proteins were visualized by staining with Coomassie Brilliant Blue. Similar gels were stained with ice-cold 1 M KCl and the regions of the gel containing the overproduced 40 kDa proteins were cut out. After washing in water, the acrylamide was ground and mixed with Freund's complete immunization medium, upon which a suspension with about 200  $\mu$ g Chi-V was injected subcutaneously into rabbits. After three additional immunizations at 2-week intervals with similar suspensions in Freund's incomplete medium, 50 ml blood were collected from the rabbits and the serum fraction was tested for antibodies directed against the Chi-V protein.

### *Protein purification*

Proteins were extracted from tobacco leaves, 7 days after infection with TMV and desalted by passage through a G-25 column, equilibrated in 40 mM NaOAc, pH 5.2 (Woloshuk *et al.*, 1991). The desalted protein solution was left overnight on ice before centrifugation for 50 min at 20 000 *g*. The resulting supernatant was loaded on to a S-Sepharose (fast flow, Pharmacia) column (5  $\times$  5 cm) equilibrated in 40 mM NaOAc, pH 5.2. Adsorbed proteins were eluted with a linear salt gradient from 0 to 0.3 M NaCl in the above buffer. Every third fraction was analyzed by immunoblotting and the Chi-V-containing fractions were pooled and dialysed to 50 mM Tris/HCl pH 8.0, containing 150 mM NaCl. This solution was passed through a Chelating Superose column (HR 10/2; Pharmacia) activated by a slightly acidic solution (50 ml) of 0.1 M ZnCl<sub>2</sub> (pH=5.4). The column was extensively washed with water (500 ml) and then equilibrated in the above Tris buffer prior to use. The Chi-V-containing pool was loaded in several runs each containing 3–4 mg protein. The retarded protein peak was pooled and again subjected to Chelating Superose chromatography. Although, a tighter binding was observed after activation of the Chelating Superose column

with Cu<sup>2+</sup> ions, purification under these conditions was less effective (data not shown). The final purification step consisted of gel filtration through a Superdex-75 column (HR 10/30; Pharmacia) equilibrated in 50 mM KPi buffer, pH 7.0, containing 200 mM NaCl. Gel filtration was carried out at a flow rate of 0.5 ml min<sup>-1</sup> and fractions of 0.5 ml were collected. The fractions were analyzed by SDS-polyacrylamide gelelectrophoresis and immunoblotting. The Superdex-75 column was calibrated with BSA (68 kDa), carbonic anhydrase (29 kDa) and cytochrome C (12.5 kDa). On a nondenaturing polyacrylamide gel Chi-V showed an apparent native molecular weight of 28 kDa.

### *SDS-gel electrophoresis, immunoblotting and protein sequence analysis*

Protein samples were separated on 12.5% SDS-polyacrylamide gels as described by Laemmli (1970). Immunoblotting was performed as described by Ponstein *et al.* (1994). For immunodetection the antiserum to Chi-V protein was used in a 1:5000 dilution.

For sequencing purposes 0.4 mg protein was separated as described by Moos *et al.* (1988) and electroblotted on to PVDF membrane as described by Matsudaira (1987). Proteins were visualized by Coomassie Brilliant Blue R-250 staining (Matsudaira, 1987). Since the protein was N-terminally blocked it was acid hydrolysed on the PVDF membrane as described by Landon (1977). The digest (consisting of two peptides) was sequenced by Eurosequence, Groningen, The Netherlands, using Edman degradation on an Applied Biosystems 477A protein sequencer. Cleavage with hydroxylamine was performed as described by Aitken *et al.* (1989). About 200  $\mu$ g purified protein was used for hydroxylamine cleavage.

### *Enzyme assays*

Chitinase activity measurements were routinely carried out as described by Molano *et al.* (1977). Alternative assays like the CM-chitin assay was essentially done as described by Wirth and Wolf (1990). For the CM-chitin assay proteins were diluted in a total of 200  $\mu$ l 0.1 M sodium acetate buffer (pH 5.2). The reaction was started by the addition of 100  $\mu$ l substrate (2 g l<sup>-1</sup> carboxymethyl-chitin-RBV). After a 30 min incubation at 37°C the reaction was terminated by the addition of 100  $\mu$ l 1.0 M HCl. Samples were cooled on ice and spun for 10 min in an Eppendorf centrifuge. The absorbance of 200  $\mu$ l supernatant solution was measured in a microtiter plate (350  $\mu$ l cavities) at 550 nm. The exo-chitinase (p-nitrophenyl liberation assay; Roberts and Selitrennikoff, 1988),  $\beta$ -1,3-glucanase, and lysozyme assays were performed as described previously (Ponstein *et al.*, 1994). Chitobiase activity was measured according to Roberts and Cabib (1982) with modifications as described by Legrand *et al.* (1987).

### *RNA blot and DNA blot analysis*

Total RNA from nonstressed or stressed tobacco was extracted from frozen leaf tissue by homogenization in extraction buffer (1 M Tris-HCl, 0.1 M LiCl, 10 mM EDTA, 1% SDS, pH 9.0). The homogenate was extracted with phenol and chloroform and the RNA was precipitated with 2 M LiCl. The RNA was electrophoresed in 1.5% agarose gels after glyoxylation and blotted to nylon membranes (Genescreen, New England Nuclear or

Hybond-N, Amersham). A genomic DNA library of Samsun NN tobacco in Charon 35 was screened for the presence of *chi-V* sequences using  $^{32}\text{P}$ -labeled cDNA inserts (Cornelissen *et al.*, 1987). Hybridization of tobacco nucleic acids was performed with the  $^{32}\text{P}$ -labeled cDNA insert of *chi-V*, in  $5 \times \text{SSC}$ , 2% SDS, 50% formamide,  $100 \mu\text{g ml}^{-1}$  herring sperm DNA at  $42^\circ\text{C}$  ( $1 \times \text{SSC}$  contains 150 mM NaCl, 15 mM sodium citrate). Northern blots were finally washed in  $0.4 \times \text{SSC}$ , 0.1% SDS at  $50^\circ\text{C}$  and autoradiographed.

#### Antifungal assays

*In vitro* testing of tobacco proteins (41–43 kDa Chi-V, 32 kDa Chi-I, 33 kDa Glu-I) for antifungal activity on *Fusarium solani*, *Alternaria radicina*, *Trichoderma viride*, *Septoria lycopersici* and *Phytophthora infestans*, was performed as described previously (Sela-Buurlage *et al.*, 1993; Woloshuk *et al.*, 1991).

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