

Molecular characterization of four chitinase cDNAs obtained from *Cladosporium fulvum*-infected tomato

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Abstract

Complementary DNA clones encoding acidic and basic isoforms of tomato chitinases were isolated from *Cladosporium fulvum*-infected leaves. The clones were sequenced and found to encode the 30 kDa basic intracellular and the 26 and 27 kDa acidic extracellular tomato chitinases previously purified (M.H.A.J. Joosten *et al.*, in preparation). A fourth truncated cDNA which appears to encode an extracellular chitinase with 82% amino acid similarity to the 30 kDa intracellular chitinase was also isolated. Characterization of the clones revealed that the 30 kDa basic intracellular protein is a class I chitinase and that the 26 and 27 kDa acidic extracellular proteins which have 85% peptide sequence similarity are class II chitinases. The characterized cDNA clones represent four from a family of at least six tomato chitinases. Southern blot analysis indicated that, with the exception of the 30 kDa basic intracellular chitinase, the tomato chitinases are encoded by one or two genes. Northern blot analysis showed that the mRNA encoding the 26 kDa acidic extracellular chitinase is induced more rapidly during an incompatible *C. fulvum*-tomato interaction than during a compatible interaction. This difference in timing of mRNA induction was not observed for the 30 kDa basic intracellular chitinase.

Introduction

Plants respond to pathogenic attack by the activation of several defence mechanisms [17]. These include the synthesis of antimicrobial phytoalexins [9], the deposition of lignin, callose and phe-

nolic compounds [15, 30] and the accumulation of several defence-related proteins. Among these proteins is a set of proteins termed pathogenesis-related (PR) proteins [18]. These have been studied intensively in tobacco in recent years because their induction is correlated to the acquisition of

The nucleotide sequence data reported will appear in the EMBL, GenBank and DDBJ Nucleotide Sequence Databases under the accession numbers Z15138 (Chi14), Z15139 (Chi17), Z15140 (Chi9) and Z15141 (Chi3).

systemic resistance [4, 18]. Five PR protein groups have been identified in tobacco and within each group both acidic and basic proteins exist [32].

In tomato, after infection with *Cladosporium fulvum*, several PR proteins have been shown to accumulate [13, 14]. As in tobacco, they can also be classed into five groups [14, 19, 31, 34]. Among these are the proteins from group 3, the chitinases. These proteins catalyse the hydrolysis of chitin, a component of the cell walls of most filamentous fungi (with the exception of the Oomycetes). Purified chitinase preparations and combinations of chitinase and 1,3- β -glucanases have been shown to inhibit fungal growth *in vitro* [21, 27]. Furthermore, lysis of fungal hyphae *in vivo* correlates with an increase in plant chitinase activity [24]. It has been demonstrated that the breakdown products of fungal cell walls may act as elicitors of additional plant defence responses [1, 16]. In addition, chitinases have been shown to accumulate around fungal hyphae in infected tomato root tissue [3]. The role of plant chitinases in defence has been further highlighted by the recent observation that transgenic plants with elevated levels of chitinase expression are more resistant to fungal pathogens [5]. All these data stress the importance of plant chitinases and have prompted much research on these proteins.

In this paper we present the complete cDNA sequences of three tomato chitinases corresponding to previously purified 26 and 27 kDa acidic extracellular and 30 kDa basic intracellular proteins (M.H.A.J. Joosten *et al.*, in preparation). We also present the sequence of an incomplete cDNA clone which encodes a fourth tomato chitinase sharing 82% amino acid similarity to the 30 kDa basic intracellular chitinase. The precursor protein encoded by the latter cDNA lacks a C-terminal extension suggesting that it is an extracellular protein. We also show that the mRNAs encoding the intracellular and extracellular chitinases are differentially expressed in the leaves of *C. fulvum*-infected tomato and that, with the exception of the 30 kDa chitinase, they are each encoded by one or two genes.

Materials and methods

Plants, fungi and inoculations

Near-isogenic lines of tomato cultivar Money-maker carrying resistance genes Cf4 of Cf5 were grown as described by de Wit and Flach [8].

Cladosporium fulvum (Cooke) (syn. *Fulvia fulva* [Cooke] Cif) was subcultured on PDA, and conidial suspensions were used to inoculate entire composite leaflets of tomato plants as described [7].

Tomato line Cf4 susceptible to *C. fulvum* race 4 and resistant to race 5, whereas line Cf5 is resistant to race 4 and susceptible to race 5.

At various days after inoculation, four leaves were harvested randomly from a set of inoculated plants and of these, all leaflets were taken for RNA isolation.

Protein sequencing

Sequencing of the purified 30 and 32 kDa chitinases by the Edman degradation method was carried out by Leiden University, Netherlands. In addition, tryptic fragments of the purified 26, 27, 30 and 32 kDa chitinases (Joosten *et al.*, in preparation) were sequenced by Plant Genetic Systems, Gent, Belgium.

RNA isolation and northern blotting

RNA was isolated from liquid nitrogen-frozen leaf tissue by homogenizing in guanidine-HCl buffer (8 M guanidine-HCl, 20 mM MES, 20 mM EDTA, 50 mM 2-mercaptoethanol pH 7.0) followed by phenol/chloroform (1:1 v/v) and chloroform extraction steps. The RNA was precipitated overnight using 2 M LiCl. Samples of 25 μ g were denatured and electrophoresed in 1.5% (w/v) agarose gels containing 2.2% (v/v) formaldehyde. The gel was blotted onto Hybond N⁺ membranes (Amersham Nederland BV) according to Sambrook *et al.* [25].

DNA isolation and Southern blotting

DNA was isolated from freeze-dried leaf tissue of tomato line Cf5 as described previously [31]. After RNase treatment, samples of 2.5 μ g were digested with either *Eco* RI, *Hind* III, *Bam* HI or *Eco* RV in a final volume of 250 μ l. The restricted DNA was electrophoresed in 0.8% agarose gels, depurinated and then denatured before being transferred to Hybond N⁺ membranes by vacuum blotting using a Millipore vacuum blotter (Millipore BV, Netherlands).

Probe synthesis and hybridizations

Radio-labelled DNA probes were prepared according to the oligolabelling procedure of Hodgson and Fisk [12]. RNA and DNA blots were hybridized at 65 °C in 5 $\times$ SSC, 5 $\times$ Denhardt's solution, 0.5% SDS containing 100 μ g/ml denatured calf thymus DNA. The membranes were washed at 65 °C in 0.2 $\times$ SSC, 0.5% SDS.

As a control for even loading and RNA integrity on the northern blots, a 1.8 kb *Eco* RI-*Bam* HI fragment of the *Arabidopsis thaliana* actin gene [22], kindly provided by Dr R.J. Ferl (University of Florida, Gainesville, USA), was labelled and hybridized under low stringency conditions, at 60 °C in 2 $\times$ SSC, 0.5% SDS.

Screening of cDNA libraries

The cDNA libraries used for hybridization screening were described in van Kan *et al.* [31]. Heterologous screening, using the tobacco PR-3b clone kindly provided by Dr H.J.M. Linthorst (Leiden University, Netherlands), was carried out as in van Kan *et al.* [31] at 60 °C in 2 $\times$ SSC, 0.5% SDS. Homologous screening was carried out at 65 °C in 0.5 $\times$ SSC, 0.2% SDS. Screening by PCR was performed using a primer complementary to the T3 promoter upstream of the 5' end of the cDNA insert in pBluescript II SK(–) (Stratagene, La Jolla, CA), and a 20-mer primer derived from a sequence within the Chi14

clone. 1 μ g DNA isolated from the λ ZAP cDNA library, 0.2 μ M T3 primer, 1 μ M Chi14 primer and 2.5 units *Taq* DNA polymerase were employed and the reaction conditions were as described previously [19].

Cloning and sequencing procedures

λ ZAP clones identified by the screening were subcloned (according to Stratagene's protocol) by *in vivo* excision of pBluescript II SK(–) following infection of *Escherichia coli* cells with R408 helper phage. All other cloning procedures were performed according to Sambrook *et al.* [25]. Plasmid DNA was sequenced using the dideoxy chain-termination method [26], using α -³⁵S-dATP label and T3, T7 or SP6 promoter primers (Promega, Madison, WI).

Results

Isolation and characterization of chitinase cDNA clones

A λ ZAP cDNA library, made on mRNA isolated from *C. fulvum*-infected tomato leaves 11 days after inoculation, was screened using a full-length cDNA clone encoding the tobacco acidic chitinase, PR-3b. Eighteen positive plaques were obtained and purified, and the inserts were sequenced partially from their 3' ends. This analysis indicated that they were derived from four distinct chitinase mRNAs. The four types of chitinase mRNAs were represented by eleven (Chi3), two (Chi9), one (Chi17) and one (Chi14) clone respectively. Three other clones contained very long poly (A) tails which made 3'-end sequencing difficult; these clones were not analysed further.

Several of the clones corresponding to chitinase type, Chi3, were found upon further sequencing to contain a complete open reading frame (ORF) of 253 amino acids. The assembled sequence of Chi3 type cDNA clones is presented in Fig. 1. It encodes a protein with a calculated

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1  GGAAAAATGAAGTTCAATATTGTATCAGCGGTTGCACGTCTTGTCTCTTTTCTTGTTCCTAACAGGTACTTTAGCACA
   M K F N I V S P V A L S G L F F L F L T G T L A Q
81  AAATGCCGGTTCCATTGTAAACGCGGAATTGTTGGAACAAATGCTGAGTTTATAGGAACAATGACGCATGTCCTGCCAAAG
   N A G S I V T R E L F E Q M L S F R N N D A C P A K G
161 GATTCTACACTTATGATGCATTATAGCTGCAGCCAATTCGTTTCAGGTTTTGTTACTGCTGCTGATGATACTGCACGT
   F Y T Y D A F I A A A N S F P G F G T A G D D T A R
241 AAGAAGGAAATTGCTGCCTTTTTCGGTCAAACATCTCAGGAACTAATGGTGGTAGTGACGGAACATTCACTGGAGGATA
   K K E I A A F F G Q T S H E T N G G S A G T F T G G Y
321 TTGCTTTGTTAAGCAAATAGAGCAGTCAGACAGATACTATGGCAGAGGAGCTATCCAATTGACAGACCAATCTAACTAG
   C F V K Q I E Q S D R Y Y G R G F I Q L T H Q S N Y E
401 AACGAGCTGGACAAGCTATTGGTCTTGGACAAGAGTTAGTGAACAACCTGATTAGTTGCGACAGATCCTATAATATCA
   R A G Q G I G V G Q E L V N N P D L V A T D P I I S
481 TTCAAAACAGCAATATGGTTCTGGATGACAGAACAGGATAATAAACCATCATGCCACAACGTTATCATGGACAATGGAC
   F K T A I W F W M T E Q D N K P S C H N V I I G Q W T
561 GGCATCCCCTAAAGATACAGCAGCTAATAGAGTTCCAGGGTACGGTGTGATTACCAACATCATTAACGGTCAATTTGAAT
   P S P K D T A A N R V P G Y G V I T N I I N G Q F E C
641 GTGGGATGGGTCCAAATACTGCAGCGGAAAGTCGAATTGGATTTTACAGGAGGTATTGTGATGTTGAATGTTCTGACT
   G M G P N T A A E S R I G F Y R R Y C G M L N V P T
721 GGTGAAAATTTGGACTGTAAACATCAAAAGAACTTCGCCCGGGCTAAGCGCTTTTATTATATATAGAGAGAGAATGCA
   G E N L D C N N Q K N F A Q G
801 AATTATGTTTATGTATTACGTTGTGAAGTCTATAAGTTATATTGGATGTAATCAATAAGGGGATTCTGTATGCCCATTT
   V
881 AGAAAAATGGAAGTTTATTTTCAGAAATACTAAAGTTATATGATTTTGATCTTTTGTATATA
   V

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Fig. 1. DNA and protein sequence of the 26 kDa chitinase as determined from Chi3-type cDNA. In the DNA sequence, the start and stop codons are underlined. Polyadenylation sites observed in different cDNA clones are indicated by closed triangles above the DNA sequence. In the protein sequence, the putative signal peptide cleavage site is indicated by an arrow. The underlined amino acids correspond to the amino acid sequences of tryptic fragments of the 26 kDa chitinase.

isoelectric point (pI) of 5.7. The ORF of this chitinase completely matches the amino acid sequences of two tryptic fragments, with a total of 23 residues (underlined in Fig. 1), of the acidic extracellular 26 kDa chitinase purified and partially sequenced by Joosten *et al.* [14, and manuscript in preparation].

The remaining chitinase clones identified in the heterologous screening, were found to be partial clones encoding chitinase type, Chi17, chitinase type, Chi9, and chitinase type, Chi14, respectively. The cDNA insert of the longer of the clones encoding Chi9 and the insert of the Chi17 clone were used to stringently screen the λ ZAP library thus facilitating the isolation of cDNAs containing the complete ORFs of Chi9 and Chi17 respectively. The sequence of these complete clones is presented in Figures 2 and 3.

Chi17 contains an ORF of 247 amino acids and encodes a protein with a calculated pI of 4.3. The amino acid sequences of three tryptic fragments of the 27 kDa acidic extracellular chitinase

(Joosten *et al.*, in preparation), with a total of 15 residues (underlined in Fig. 2), are present in the ORF of Chi17, confirming that Chi17 encodes this 27 kDa chitinase.

The Chi9 sequence contains an ORF of 322 amino acids and completely matches the amino acid sequence of three tryptic fragments, with a total of 40 residues (underlined in Fig. 3), of the basic intracellular 30 kDa tomato chitinase (Joosten *et al.*, in preparation). N-terminal sequencing of the 30 kDa chitinase confirmed the predicted signal peptide cleavage site between the alanine and glutamic acid residues at positions 22 and 23. Following the signal peptide, a cysteine-rich region of ca. 40 amino acids is present. This region is thought to be a chitin-binding domain and is also found in the tobacco basic chitinases and in other proteins such as hevein and wheat germ agglutinin [20, 28, 33, 35]. The cysteine-rich domain is separated from the catalytic domain by a spacer region which is rich in glycine and proline residues. Comparison to the spacer regions of

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1  GTTTTGGAGATCAGAAATGCTATTATGTTGTGTCCTTTTGTGTTCTTAACGGGAAGCTTTGCAGAGATGTTGGTACAA
   M V L C C V F L L F L T G S F A Q D V G T I
                                     ▲
81  TTGTGACAAAGTGACTTGTTCACGAGATGCTGAAGAATCGAAACGATGATAGATGTCCTGCCAAGGGCTTCTACACTTAT
   V T S D L F N E M L K N R N D D R C P A K G F Y T Y

161 GATGCATTTCATAGCTGCAGCGCAATTCATTTCAGGTTTGGTACTACGGTGATGATACTGCGCGTAAGAAGGAAATTGC
   D A F I A A A N S F P G F G T T G D D T A R K K E I A

241 TGCCTTTTTCGGTCAAACCTTCTCATGAAACTACTGCTGCTAGCCTAAGTGTGATGCTCCATTTCGCGGAGGATATTGTT
   A F F G Q T S H E T T G G S L S A D G P F A G G Y C F

321 TTGTTAGGGAAGGCAATCAAATGGGAAGTGGATTTTATGGCAGAGGACCTATCCAATTGACAGGGCAATCTAACTATGAC
   V R E G N Q M G S G F Y G R G P I Q L T G Q S N Y D

401 TTAGCTGGGCAAGCAATTGGACAAGACTTAGTTAAACAACCGCTGACTTAGTAGCTACAGATGCAACTGTATCTTTTAAAC
   L A G Q A I G Q D L V N N P D L V A T D A T V S F K T

481 AGCAATATGTTCTGATGACCGCAGCAAGGCAATAAGCCATCATGCCAGGATGTTATCACAGGGCAATGGACGCCATCAG
   A I W F W M T A Q G N K P S C H D V I T G Q W T P S A

561 CCGCGGATGCATCAGCAATAGACAACCGCTACCGTGTCTATTACAAACATAATTAACGCTGGAATTGAATGTGCGCAA
   A D A S A N R Q P G Y G V I T N I I N G G I E C G K

641 GGACAGAAATCCACAAGTGGAGGATCGGATTGGATTCTACAGAAGGTATTGTACGATACTGAATGTTGCTCCCGGGACAA
   G Q N P Q V E D R I G F Y R R Y C T I L N V A P G D N

721 CCTTGATTGCTACGACCAGAGGAACCTTGTCTGAGGCCTAGCTTTGTGTTTTAGTCATGATGCTCTGAATGAATAAGGG
   L D C Y D Q R N F A E A

801 ATTTTGGATGCCCATTTGGAATATGTAGTTTCTTATTTTGTCTACACAAATAAGAGTTTCTTTTATCTTATAACATAATA

881 TACGACATGTACTATTATCTCATGGATATTTTAATTTCGGCTTTGTTTA

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Fig. 2. DNA and protein sequence of the 27 kDa chitinase as determined from Chi17-type cDNA. In the DNA sequence, the start and stop codons are underlined. The closed triangle indicates the polyadenylation site. In the protein sequence, the putative signal peptide cleavage site is indicated by an arrow. The underlined amino acids correspond to the amino acid sequences of tryptic fragments of the 27 kDa chitinase.

two tobacco basic chitinases, indicates that two potentially hydroxylated proline residues are present [29]. Prolyl hydroxylation, if present, might be important in the modulation of chitinase activity or specificity. The structural characteristics of the Chi9-encoded protein place it in the category of class I chitinases [28].

When the Chi14 cDNA, which contains only the 3' end of a chitinase ORF, was used to screen the λ ZAP library, two positive plaques were obtained. However these were both shorter than the truncated Chi14 cDNA and also lacked the 5' end of the ORF. Furthermore, when attempts to isolate additional upstream sequences by PCR reactions on the cDNA library, using a λ ZAP-specific primer and a Chi14-specific primer complementary to the ORF, were performed, no

fragments were amplified (data not shown). These results probably reflect the low abundance of this chitinase mRNA in *C. fulvum*-infected tomato leaves. The sequence of the truncated Chi14 clone is presented in Fig. 4. The ORF encoded by Chi 14 has an excess of basic residues and is more homologous to the 30 kDa chitinase (82%) than to the 26 and 27 kDa chitinases (74%, see Fig. 5). The amino acid sequence of four internal tryptic peptides of the purified 32 kDa chitinase (Joosten *et al.*, in preparation) do not correspond perfectly to the ORF encoded by the Chi14 clone. Out of a total of 25 residues, only 20 are identical (data not shown). Therefore it can be concluded that clone Chi14 does not correspond to the mRNA for the purified 32 kDa chitinase.

A comparison of the deduced protein se-

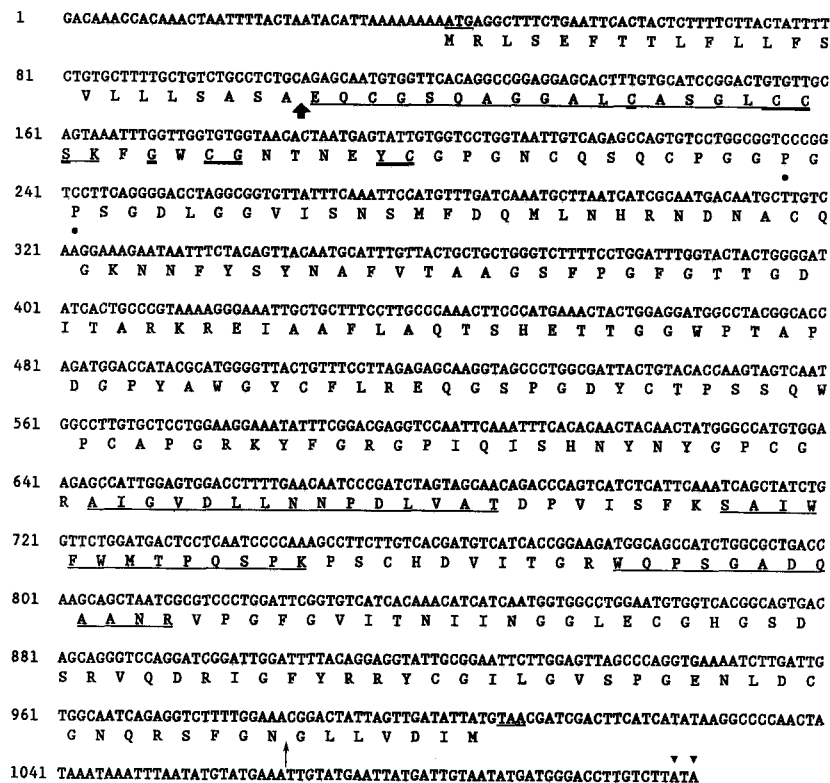


Fig. 3. DNA and protein sequence of the 30 kDa chitinase as determined from Chi9-type cDNA. In the DNA sequence, start and stop codons are underlined. Closed triangles indicate polyadenylation sites in different cDNA clones. In the protein sequence, the signal peptide cleavage site is indicated by a thick arrow. The putative C-terminal processing site is indicated by a thin arrow. The underlined amino acids correspond to the amino acid sequences of tryptic fragments of the 30 kDa chitinase and to its N-terminal peptide sequence. The strictly conserved residues of the 'chitin-binding' domain are indicated by double underlining. Circles under the protein sequence indicate residues which are hydroxylated in a homologous tobacco chitinase [28].

quences of the four tomato chitinases isolated in this study is presented in Fig. 5. A short C-terminal extension shown to be responsible for the targeting of basic chitinase A of tobacco to the vacuole [23] is present only in the precursor protein of the 30 kDa chitinase. This extension, which is probably cleaved off after the tripeptide sequence, FGN, at position 315, is not present in the precursor proteins of the three other tomato chitinases examined here. The 26 and 27 kDa acidic extracellular chitinases are 85% similar. Figure 5 shows that they both lack the 47 amino acid sequence present in the 30 kDa basic chitinase which includes the cysteine-rich 'chitin-binding' domain followed by a glycine- and proline-rich spacer region. Furthermore, they both lack a stretch of 15 amino acids which is

conserved in both Chi14 and the 30 kDa basic chitinase. This is analogous to the situation in tobacco where a similar stretch of amino acids has also been shown to be absent in acidic chitinases, PR-P and PR-Q, compared to their basic counterparts. Based on the structural characteristics of the 26 and 27 kDa proteins, they can be considered as class II chitinases.

Genomic organization of tomato chitinase genes

In order to determine the complexity of the gene families encoding the chitinases, Southern blot hybridization analysis was carried out on genomic DNA isolated from tomato genotype Cf5. The DNA was digested with *Eco* RI or *Hind* III, elec-

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1  TTTTATCAGAAAATATTAGTAGTCTTATTAGTAAAAATTTATTTGAGAGAATACTTGTGCATGGAACGATGCTGTTG
   L S Q N I S S L I S K N L F E R I L V H R N D R A C
81  GGTGGGAAAGGTTTCTATACCTATGAAGCTTTTATAACAGCAACTAAAAACATTGTGCTGCTTTGGAACTACTGGTGATA
   G A K G F Y T Y E A F I T A T K T F A A F G T T G D T
161 TAATACTCGTAACAAGGAAATGCTGCTTTTGGCTCAAACTTCTCATGAAACTACGGGTGGATGGGCAACAGCAGCA
   N T R N K E I A A F L A Q T S H E T T G G W A T A P D
241 ATGACCATATTTCATGGGATATTGCTACAAACAGGGAACAGGAAGTCCTGGAGATTACTGTGCTTCAAGTCAGCAGTG
   G P Y S W G Y C Y N R E Q G S P G D Y C A S S Q Q W
321 CCTTGCTGCTGGGCAAAAGTATTTTGGTCGAGTCCTATCGAAATTCATACAACATAATTATGGGGCAGCAGGGA
   P C A P G K K Y F G R G P I Q I S Y N Y N Y G A A G S
401 TGCAATAGGTGTAATCTGTAAACAACCCAGATTAGTAGCCAATGATGAGTAGTGTCTTCAAAACAGCGTGTGG
   A I G V N L L N N P D L V A N D A V V S F K T A L W F
481 TCTGGATGACAGCACAACAGGCAAAACCATCGGCACAGATGTCATTACTGGAAGATGGAGTCCCTCTGTTGCTGATTG
   W M T A Q Q P K P S A H D V I T G R W S P S V A D S
561 GCGCCTGGCGCGTCCCGGGCTTTGGTGAATCACTAACATCATCAATGGTGAATGGAATGTAACAGCGGTTCAAATG
   A P G R V P G F G V I T N I I N G G M E C N S G S N A
641 ACTAATGGATAACAGAAATGGGTTCTATAGGAGATATGTCAAATTTGGGAGTTGATCCTGGTAATAATTGGACTGT
   L M D N R I G F Y R R Y C Q I L G V D P G N N L D C A
721 CTAACTAAAGGCCATTGGGTAAGGCAACTTAATTAATTTTACTAGAAATAAGTTGCTGCTTAAATTATATCTGTGA
   N Q R P F G
801 CCATATTAGTACGTGACACATTAATAAGGTTTGGTCCATCTAAACTGTGTATTAGTTGCTTTGTTCTATAATGGAA
   ^
881 TTCCATTTTGTACGCGAGGCGAAATAAAGTTAAAGTTTATGCAATTA

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Fig. 4. DNA and protein sequence as determined from the truncated Chi14 cDNA. The stop codon is underlined. The polyadenylation site is indicated by a closed arrow.

trophoresed and then blotted onto nylon membranes. Identical blots were hybridized with the cDNA inserts corresponding to the four different chitinases isolated in this study. Figure 6 shows the hybridization patterns obtained. The Chi3 cDNA insert, corresponding to the 26 kDa acidic chitinase, hybridized to single bands of 4.8 and 3.2 kb in the lanes containing DNA digested with *Eco* RI and *Hind* III, respectively (Fig. 6A). It also hybridized to single bands in lanes containing *Bam* HI- and *Eco* RV-digested DNA (data not shown). These results indicate that the gene encoding the 26 kDa acidic chitinase is present in a single copy in the tomato genome.

When the Chi17 cDNA insert, corresponding to the 27 kDa acidic chitinase was used as probe (Fig. 6C), single bands of 10 kb and 4.2 kb were obtained in the *Eco* RI-digested DNA and *Hind* III-digested DNA, respectively. Single bands were also observed in lanes containing *Bam* HI- and *Eco* RV-digested DNA (data not shown). Upon longer exposure, weak cross-hybridization with the Chi3 gene was observed (data not shown). The hybridization patterns ob-

tained indicate that the 27 kDa chitinase is coded for by a single copy gene.

The Chi14 cDNA hybridized strongly to a 4.5 kb band in the lane containing *Eco* RI-digested DNA (Fig. 6D) and with less intensity to a band of 13 kb in the lane containing DNA digested with *Hind* III. Upon longer exposure, two additional faint bands of 7.5 and 4.3 kb were also present in this lane (data not shown). The Chi14 cDNA also hybridized, strongly and with equal intensity, to two bands of 3.7 and 2.7 kb in a lane of *Eco* RV-digested DNA (data not shown). The Chi14 cDNA clone does not contain *Eco* RI or *Eco* RV restriction sites. From the hybridization patterns obtained it is not clear whether this cDNA is encoded by a single gene containing an intron with restriction sites, or whether it is encoded by two genes.

When a 700 bp *Eco* RV-*Xho* I fragment of the Chi9 cDNA (lacking the chitin-binding coding sequences) was used to probe tomato genomic DNA, very complex hybridization patterns were obtained (Fig. 6B). Five major bands (7.4, a doublet of 4.9, 2.9 and 2.7 kb) and 6 minor bands (of

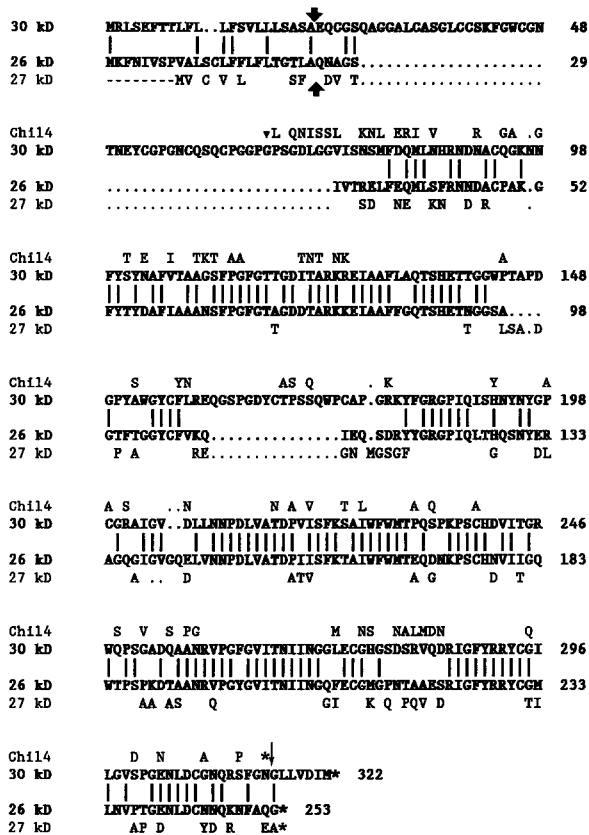


Fig. 5. Comparison of tomato chitinases. The amino acid sequences of the 30 kDa and 26 kDa chitinases are presented, in bold, in the two central lines, such that optimal alignment is achieved. Above the sequence of the 30 kDa chitinase, only the differences in the sequence deduced from the truncated Chi14 cDNA are indicated. Below the sequence of the 26 kDa chitinase, only the differences in the sequence of the 27 kDa chitinase are indicated. Gaps introduced to optimize the alignment are indicated by dots. Putative signal peptide and C-terminal extension cleavage sites are indicated by arrows. A closed triangle indicates the start of the truncated Chi14 chitinase sequence. The start of the 27 kDa chitinase is preceded by dashes. The translation termination codons are indicated by asterisks.

ca. 8.0, 6.1, 6.0, 2.3, 1.2, and 0.9 kb) are visible in the lane containing *Eco* RI-digested DNA. An equally complex hybridization pattern is visible in the lane containing *Hind* III-restricted DNA. Two strong bands of 5.3 and 2.6 kb and seven weak bands (13, a doublet of 7.6, 6.2, 3.8, 2.4 and 2.1 kb) are present in this lane. The complex patterns observed with this clone, which corresponds to the 30 kDa intracellular chitinase, are not ex-

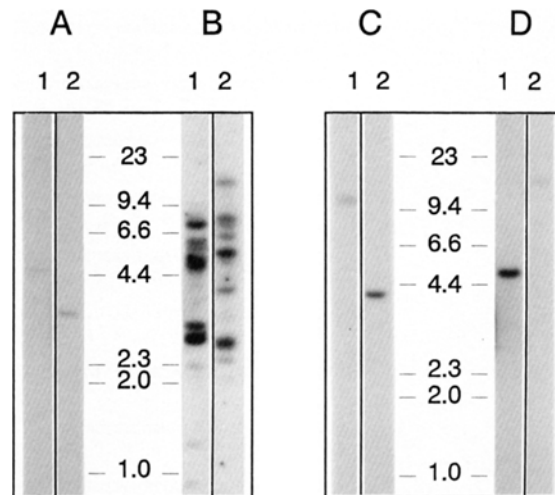


Fig. 6. Southern blot analysis of the tomato chitinase genes. Tomato DNA was digested with *Eco* RI (lane 1) or *Hind* III (lane 2) and electrophoresed in a 0.8% agarose gel. The gel was blotted and identical blots were probed with (A) the full-length cDNA insert encoding the 26 kDa chitinase, (B) a 700 bp fragment of the 30 kDa chitinase cDNA downstream of the chitin-binding domain, (C) a full-length cDNA fragment encoding the 27 kDa chitinase, and (D) the entire Chi14 cDNA insert.

plained by cross-hybridization to sequences encoding the other three chitinases identified in this study. Furthermore, they cannot be attributed to the presence of internal restriction sites in Chi9 since the cDNA fragment used as probe lacked a *Hind* III restriction site and contained only one *Eco* RI site. It is possible that the Chi9 probe used hybridized to other chitinase genes or pseudogenes present in the tomato genome. The presence of introns in the gene encoding the Chi9 cDNA would also contribute to the pattern observed. Since an identical hybridization pattern was observed (data not shown) when the entire Chi9 cDNA (which includes the sequence encoding the chitin-binding domain) was employed, it is possible to conclude that sequences homologous to the chitin-binding domain of Chi9 are not present in many copies in the tomato genome. This was confirmed when a restriction fragment containing only the chitin-binding domain of Chi9 was used as probe (data not shown).

Accumulation of acidic and basic chitinase mRNAs upon infection with Cladosporium fulvum

Northern blots containing total RNA, isolated from uninoculated tomato leaves and from *C. fulvum*-infected tomato leaves undergoing either an incompatible (Cf4/5) or a compatible (Cf5/5) interaction at 2, 3, 4, 6, 8, 10 and 14 days after inoculation, were examined for expression of the 26 kDa acidic (Fig. 7A) and the 30 kDa basic chitinases (Fig. 7B). Under the stringent conditions employed, cross-hybridization between the two cDNAs did not occur. It is possible, however, that chitinase transcripts highly homologous to the 30 kDa chitinase mRNA, encoded by a gene family (see above), are detected when a probe for the latter is used. Under the conditions used, the 26 kDa chitinase probe does not detect other transcripts, because it is encoded by a single gene (see above). As a control for even loading and RNA integrity, the same blot was rehybridized with a heterologous constitutive probe, the *Ara-*

bidopsis thaliana actin gene [22]. This control hybridization indicated even loading, but showed substantial mRNA degradation in samples collected 6 days after inoculation, particularly in the incompatible interaction Cf4/5, and less so in the compatible interaction Cf5/5 (data not shown).

It can be seen, in Fig. 7, that both types of chitinase transcripts are induced by inoculation with *C. fulvum* but that they show different expression patterns. The mRNA of the acidic extracellular 26 kDa chitinase accumulates in both types of interactions (Fig. 7A). Maximal expression of this transcript occurs in the incompatible interaction between 4 and 8 days after inoculation (note that the sample at 6 days after inoculation has undergone substantial degradation), and then declines to almost background levels. In the compatible interaction this mRNA increases very abruptly at 8 days after inoculation to reach maximum levels, which remain high throughout the rest of the infection cycle.

The accumulation of the mRNA encoding the basic intracellular 30 kDa chitinase shows a different pattern (Fig. 7B). There is a transient increase with similar kinetics in both interactions. The mRNA level is slightly increased 2 days after inoculation with the fungus, progressively accumulating to a maximum level between 4 and 8 days after inoculation during both types of interaction, subsequently decreasing to background levels. Very little transcript is detectable 10–14 days after inoculation.

Expression patterns consistent with that of the 26 kDa extracellular chitinase gene, during *C. fulvum* infection, have also been observed for the tomato genes encoding an acidic extracellular glucanase and other extracellular tomato PR proteins [19, 31]. Similarly the expression pattern of the intracellular 30 kDa chitinase gene appears to be typical of tomato intracellular PR genes during *C. fulvum* infection [31].

Discussion

This molecular study of tomato chitinases indicates the presence of at least six expressed chiti-

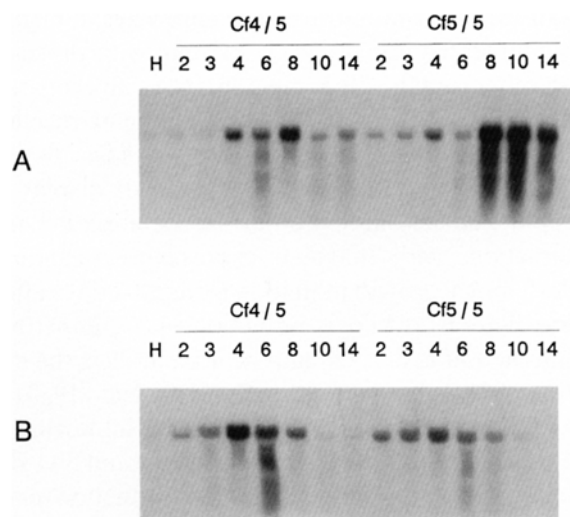


Fig. 7. Northern blot analysis of tomato chitinases. Total leaf RNA was isolated from healthy Cf5 plants (H), from an incompatible interaction (between tomato genotype Cf4 and *C. fulvum* race 5) and from a compatible interaction (between tomato genotype Cf5 and *C. fulvum* race 5), at 2, 3, 4, 6, 8, 10 and 14 days after inoculation, as indicated. Blots containing equal amounts of RNA from each sample were probed with the labelled full-length cDNA inserts of the 26 kDa acidic extracellular chitinase (panel A) or the 30 kDa basic intracellular chitinase (panel B).

nase genes in tomato. These include the 26 and 27 kDa acidic extracellular proteins for which we have isolated complete cDNA sequences and obtained peptide sequence data. We have also isolated a cDNA corresponding to the basic intracellular 30 kDa chitinase and a truncated cDNA, Chi14, corresponding to a fourth chitinase as yet unidentified but with a predicted basic isoelectric point and 82% peptide sequence similarity to the 30 kDa chitinase. The protein deduced from the Chi14 sequence does not correspond to a 32 kDa basic chitinase purified from leaf homogenates (Joosten *et al.*, in preparation) nor does it correspond to the predicted protein encoded by a partially sequenced ethephon-inducible tomato chitinase gene [10]. Considering the absence of a carboxy-terminal extension that has been shown to be responsible for vacuolar targeting [2, 23], and assuming that the predicted Chi14 protein is synthesized as a precursor containing a hydrophobic N-terminal signal peptide, we would expect the mature protein to have an extracellular location. However, as already mentioned, such a protein has not been detected in apoplastic fluids by Joosten *et al.* (unpublished data), possibly due to its low abundance.

A cDNA clone corresponding to the 32 kDa basic intracellular chitinase was not obtained during the screening of the cDNA library, possibly due to the low homology to the tobacco chitinase used as a probe. However, the N-terminal sequence and internal tryptic peptides of the 32 kDa chitinase clearly revealed a high degree of similarity to the 30 kDa chitinase (37 residues out of 43 identical; unpublished data), including the presence of a cysteine-rich chitin-binding domain. Thus, the 30 kDa and 32 kDa chitinases have all the structural characteristics of a class I chitinase. Their structure suggests that they are multifunctional proteins containing the N-terminal cysteine-rich chitin-binding domain separated from a conserved chitinolytic domain by a variable spacer region. The 30 kDa chitinase shows 83–95% sequence similarity to bean and tobacco class I chitinases respectively [6, 11].

The acidic extracellular 26 and 27 kDa chitinases share 85% peptide sequence similarity and

show 85–91% similarity to the class II chitinases of tobacco, PR-P and PR-Q [20].

The Southern blot analysis of tomato chitinase genes indicated that the acidic extracellular 26 and 27 kDa chitinases are each encoded by a single gene in tomato. The Chi14 chitinase could be encoded by one or two genes but since only one cDNA of this chitinase could be detected in the cDNA library, it is uncertain whether more than one gene is expressed. A complex hybridization pattern was obtained for the 30 kDa basic intracellular chitinase. This complexity was not caused by the presence of large copy numbers of the chitin-binding domain, but rather due to cross-hybridization to other functional chitinase genes (such as those encoding the 32 kDa basic intracellular chitinase and the ethephon-induced chitinase [10]) or to pseudogenes present in the tomato genome. The presence of introns with appropriate restriction sites, in the basic chitinase gene(s) could also contribute to the complex band patterns observed on the Southern blot.

The expression patterns of mRNAs encoding extracellular and intracellular chitinases in *C. fulvum*-infected tomato are consistent with those of all other tomato PR protein mRNAs investigated so far [19, 31]. All mRNAs encoding extracellular PR proteins (P2, P4, P6, 35 kDa β -1,3-glucanase and the 26 kDa chitinase) display a rapid transient induction in the incompatible interaction, with maximal expression coinciding with fungal growth inhibition (around 6 days after inoculation), whereas induction in a compatible interaction is delayed and rather parallels the extent of fungal growth throughout the leaf [19, 31]. On the contrary, mRNAs encoding intracellular PR proteins (35 kDa β -1,3-glucanase and 30 kDa chitinase) show identical timing of induction in an incompatible and a compatible interaction. For the 35 kDa intracellular β -1,3-glucanase mRNA the increase is transient in an incompatible interaction (as we observed here for the 30 kDa chitinase for both types of interaction), but it remains very high in a compatible interaction [31]. The induction kinetics, however, point to coordinate regulation of extracellular PR proteins on one side and intracellular PR proteins on the other side.

The northern blot analysis performed here is not informative with regard to the location in the leaf where expression is induced most rapidly. Possibly, in an incompatible interaction, high accumulation of PR protein mRNA occurs in a limited number of plant cells surrounding the fungal penetration site, whereas the expression might be more diffuse throughout the leaf during a compatible interaction. However, such a differential, strictly localized accumulation of PR proteins in incompatible interactions has not been observed [36]. There seemed to be no significant difference between compatible and incompatible interactions with regard to the location of chitinases and β -1,3-glucanases. However, it should be considered that in this study, due to problems with fixation of extracellular proteins, mainly intracellular proteins could be observed [36].

Is there a role of extracellular and intracellular PR proteins in the defense of tomato vs. *C. fulvum*? In a compatible interaction, this fungus grows exclusively in the intercellular space without damaging the host cells. In such an interaction there is no contact between fungal cells and host intracellular components. Apparently *C. fulvum* is not harmed by the extracellular PR proteins secreted in vast amounts into the intercellular space. However, it is possible that *C. fulvum* is sensitive to intracellular PR proteins released from the cell during hypersensitive cell death occurring in an incompatible interaction. Such a model would require that intracellular PR proteins are induced at very early stages of infection, in order to accumulate in the vacuoles of mesophyll cells before the event of HR, and are subsequently released during HR from the cell into the neighbouring apoplast. The release of chitinase and other hydrolytic PR proteins during HR could contribute directly to the inhibition of fungal growth by hydrolysing fungal cell wall material [21, 24, 27, 34] and indirectly by releasing oligosaccharides which could, in turn, act as elicitors of other defence responses in the plant [1, 16]. Clearly, the intracellular chitinase mRNA expression has been induced early enough to accumulate sufficient enzyme to propose such a role, as the mRNA level is maximal around 4 days

after inoculation, which coincides with the activation of HR during the incompatible interaction.

The observed synchronous induction of extracellular PR protein mRNAs on the one hand, and intracellular PR protein mRNAs on the other, might be indicative of shared signalling pathways. Some of these pathways might be connected with ethylene or salicylate, potent inducers of intracellular and extracellular PR proteins of tobacco, respectively. Investigating this in more detail might give indications that such signalling also occurs in tomato upon inoculation with *C. fulvum*. Work is now in progress to study the induction and expression of all the known tomato PR proteins for which we have isolated cDNAs, in response to ethylene and salicylate. This may increase our understanding of the role of PR protein genes in plant defence.

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