Plant Physiol. (1994) 104: 109–118

A Novel Pathogen- and Wound-Inducible Tobacco (*Nicotiana tabacum*) Protein with Antifungal Activity

Anne S. Ponstein*, Sandra A. Bres-Vloemans, Marianne B. Sela-Buurlage, Peter J.M. van den Elzen, Leo S. Melchers, and Ben J.C. Cornelissen[1]

MOGEN International NV, 2333 CB Leiden, The Netherlands

A novel pathogen- and wound-inducible antifungal protein of 20 kD was purified from tobacco (*Nicotiana tabacum*) Samsun NN leaves inoculated with tobacco mosaic virus (TMV). The protein, designated CBP20, was purified by chitin-affinity chromatography and gel filtration. In vitro assays demonstrated that CBP20 exhibits antifungal activity toward *Trichoderma viride* and *Fusarium solani* by causing lysis of the germ tubes and/or growth inhibition. In addition it was shown that CBP20 acts synergistically with a tobacco class I chitinase against *F. solani* and with a tobacco class I β-1,3-glucanase against *F. solani* and *Alternaria radicina*. Analysis of the protein and corresponding cDNAs revealed that CBP20 contains an N-terminal chitin-binding domain that is present also in the class I chitinases of tobacco, the putative wound-induced (WIN) proteins of potato, WIN1 and WIN2, and several plant lectins. The C-terminal domain of CBP20 showed high identity with tobacco pathogenesis-related (PR) proteins, PR-4a and PR-4b, tomato PR-P2, and potato WIN1 and WIN2. CBP20 is synthesized as a prepro-protein, which is processed into the mature protein by the removal of an N-terminal signal peptide and a C-terminal propeptide, most likely involved in the vacuolar targeting of the protein. The intracellular localization of CBP20 and its induction upon TMV infection and wounding indicate that CBP20 is the first class I PR-4 type protein purified.

In plants resistance against pathogens can be induced by a variety of biotic and abiotic elicitors (Sequeira, 1983). For example, infection of tobacco with a necrotizing strain of TMV leads to the induction of resistance against a broad range of pathogens including fungi, bacteria, and viruses. Resistance is induced both locally around the site of infection and systemically in noninfected parts of the plant. Concomitant with resistance, the synthesis of a large number of proteins including the so-called PR proteins is induced. The PR proteins form a group of proteins that are pathogen induced in the infected parts of the plant, although they may be synthesized in other parts of the plant constitutively or during specific developmental stages (Van Loon, 1990). PR proteins have been classified into five different groups (reviewed by Bol et al., 1990; Linthorst, 1991). Most groups can be subdivided into two or more classes. Generally, class I proteins are localized in the vacuole of the plant cell, whereas class II proteins are present extracellularly. The class I and class II proteins are related both structurally and immunologically but differ in their induction patterns (Brederode et al., 1991; Ward et al., 1991).

Two of the five groups of PR proteins have known enzymic functions: the PR-2 group consists of β-1,3-glucanases (Kauffmann et al., 1987) and the PR-3 proteins exhibit chitinase activity (Legrand et al., 1987). A third class of PR-2 proteins (Payne et al., 1990b) and two new classes of PR-3 proteins (Lawton et al., 1992; Collinge et al., 1993) have been described recently. It has been shown that chitinases and β-1,3-glucanases from various plant sources are able to inhibit fungal growth in vitro (Mauch et al., 1988). This is also true for the class I chitinases and β-1,3-glucanases from tobacco. However, both class II hydrolases seem to lack detectable amounts of antifungal activity in in vitro assays (Sela-Buurlage et al., 1993). As yet the biochemical basis for the difference in the antifungal effect of class I and class II proteins is not known. In the case of the tobacco PR-3 polypeptides the main structural difference is the presence in the class I proteins of a chitin-binding domain and a hinge region that are both absent in the mature class II proteins (Linthorst et al., 1990b; Shinshi et al., 1990; Linthorst, 1991; Collinge et al., 1993). Class III chitinases seem to lack antifungal activity as well (Vogelsang and Barz, 1993). Chitin-binding proteins, such as hevein (Van Parijs et al., 1991), stinging nettle lectin (Broekaert et al., 1989), and some antimicrobial peptides purified from *Amaranthus caudatus* seed (Broekaert et al., 1992), have been shown to exhibit antifungal activity. These chitin-binding proteins all lack detectable levels of chitinase activity. In contrast to the lectins mentioned above, wheat germ agglutinin, consisting of four hevein domains in tandem (Raikhel and Wilkins, 1987), was reported to lack antifungal activity (Schlumbaum et al., 1986; Chrispeels and Raikhel, 1991).

In this paper we report the purification of a chitin-binding protein of 20 kD from tobacco (*Nicotiana tabacum*) Samsun NN. Structural and immunological data indicate that this protein belongs to the PR-4 group. The polypeptide is located intracellularly like all tobacco class I proteins. The pathogen- and stress-induction pattern of CBP20 mRNA resembles the induction pattern of class I PR proteins rather than the pattern

[1] Present address: Institute for Molecular Cell Biology, BioCentrum Amsterdam, Kruislaan 318, 1098 SM Amsterdam, The Netherlands.

* Corresponding author; fax 31–71–221471.

Abbreviations: CBP20, 20-kD chitin-binding protein; CTPP, C-terminal propeptide; EF, extracellular fluid; ODU, optical density units; PR, pathogenesis related; PVDF, polyvinylidene difluoride; TMV, tobacco mosaic virus; WIN, wound induced.

of the class II PR proteins. Consequently, CBP20 was classified as a class I PR-4 protein. The purified protein was shown to inhibit growth of several fungi and to act synergistically with both a tobacco class I chitinase and a class I β-1,3-glucanase.

MATERIALS AND METHODS

Biological Materials

Tobacco (*Nicotiana tabacum* cv Samsun NN) was grown at 24°C in an artificially illuminated room (12,000 lux at plant height) with a 16-h photoperiod.

Purification of the Class I Chitinases and CBP20

Proteins were extracted from tobacco leaves 7 d after infection with TMV (Woloshuk et al., 1991). The protein extract was desalted by passage through a G-25 column and partly separated by cation-exchange chromatography as described by Woloshuk et al. (1991). Fractions containing chitinase activity were pooled and concentrated by ultrafiltration through a YM10 (Amicon) membrane (molecular mass cutoff 10 kD). The concentrated solution was brought to 20 mM $NaHCO_3$. The pH of the protein solution was adjusted to 8.3 by the addition of 1 M NaOH, and the proteins were further dialyzed to 20 mM $NaHCO_3$ (pH 8.3). Chitin-affinity purification was performed essentially as described by Broekaert et al. (1988). Proteins were allowed to adsorb to a matrix (50 mL) of regenerated chitin (Molano et al., 1977) equilibrated in 20 mM $NaHCO_3$ for 1 h at 4°C under continuous stirring. A column was poured, and unbound proteins were washed off by passage of 100 mL of 20 mM $NaHCO_3$ (pH 8.3). The column was further washed with 100 mL of 20 mM Na acetate (pH 5.2), and bound proteins were eluted by the passage of 20 mM acetic acid (pH 3.5). Fractions of about 4 mL each were collected at a flow rate of 1 mL min^{-1}.

Fractions containing chitin-binding proteins were further purified by gel filtration chromatography. The chitin eluate was concentrated (to about 1 mL) and loaded in several runs onto a Superdex-75 column (HR 10/30; Pharmacia) equilibrated in 50 mM $KHPO_4$ buffer (pH 7.0), containing 0.2 M NaCl. Gel filtration was carried out at 0.5 mL min^{-1} in the same buffer, and fractions (0.5 mL) were collected. The gel filtration column was calibrated with BSA (68 kD), carbonic anhydrase (29 kD), and Cyt *c* reductase (12.5 kD). Two protein peaks at apparent molecular masses of 15 to 15.5 and 8 kD, containing the class I chitinases and CBP20, respectively, were obtained. The two peaks were pooled separately and rechromatographed (usually twice). The basic chitinases were purified as previously described (Sela-Buurlage et al., 1993).

Purification of PR-4a,b

The proteins that did not bind to the S-Sepharose column (see above) were dialyzed to 20 mM Tris-HCl (pH 8.0) and allowed to flow through a Q-Sepharose column equilibrated in the same buffer (Woloshuk et al., 1991). The flow through was collected, concentrated, and dialyzed to 25 mM diethanolamine-HCl (pH 9.7). The resulting protein solution was loaded onto a Mono P column (Pharmacia) equilibrated in the same buffer. Bound proteins were eluted with a decreasing pH gradient produced by a 10% solution of Polybuffer 96 (pH 6.0). PR-4a,b (formerly called PR-r_1,r_2) was readily eluted as deduced from the electrophoretic pattern of the protein fractions on 10% native gels. The PR-4a,b- containing fractions were concentrated and passed through the Superdex-75 gel filtration column as described above. PR-4a,b-containing fractions were pooled and used for antifungal assays.

Protein Analysis

EFs were isolated as described by De Wit and Spikman (1982). "−EF" and "total" fractions were prepared as described by Melchers et al. (1993). Protein concentrations were determined according to the method of Bradford (1976) with BSA as the standard.

Chitinase activity measurements were carried out with dye-labeled CM-chitin (Wirth and Wolf, 1990). Mixtures of substrate (100 μL of a 2 mg mL^{-1} solution per assay) in 50 mM $KHPO_4$ buffer (pH 6.4) and appropriate amounts of enzyme were incubated at 37°C for 30 min in a final volume of 200 μL. The reaction was terminated by the addition of 100 μL of 1 M HCl, causing precipitation of the nondegraded substrate. The reaction vials were cooled on ice for 10 min and centrifuged (5 min in a Eppendorf centrifuge). The resulting supernatant (200 μL) was pipetted into a microtiter dish (96 wells), and A_{540} was read. The *A* (expressed in ODU) was taken as a measure for enzyme activity. Lysozyme activity measurements were carried out in 50 mM $KHPO_4$ buffer (pH 6.0) as described by Selsted and Martinez (1980).

Electrophoretic analysis was performed by the use of 10% native or 12.5% SDS-polyacrylamide gels (Laemmli, 1970). Gels were either stained with Coomassie brilliant blue G-250 (Neuhoff et al., 1988) or transferred to nitrocellulose filters to allow for immunological detection. The transfer buffer consisted of 48 mM Tris, 39 mM Gly, 20% (v/v) methanol, and 0.0375% (w/v) SDS. Immunodetection was performed according to the enhanced chemiluminescence western blotting protocol provided by Amersham. The antiserum to the PR-4a,b analog from tomato (PR-P2) was kindly provided by Matthieu Joosten (Wageningen, The Netherlands; Joosten et al., 1990). The antisera against PR-3a from tobacco (Linthorst et al., 1990b) and PR-P2 from tomato were produced in rabbits. The antisera were diluted 1:5000 and 1:1000, respectively.

Protein Sequence Determinations

Purified CBP20 was used to determine the amino acid sequence of the mature protein. Internal sequences were obtained after digestion of 10 μg with *N*-chlorosuccinimide/urea (Lischwe and Ochs, 1982) or V_8 protease (Boehringer Mannheim; Cleveland et al., 1977). Trypsin digestion was carried out on 40 μg of purified CBP20 as described earlier (Yokosawa and Ishii, 1979; Ishii et al., 1983; Kumazaki et al., 1986).

To obtain the amino acid sequences, digested protein sam-

ples were separated on 17.5% SDS-polyacrylamide gels as described by Moos et al. (1988) and electroblotted to PVDF membranes according to the method of Matsudaira (1987). Proteins were visualized by Coomassie brilliant blue R-250 staining (Matsudaira, 1987). Protein bands of interest were cut out and sequenced by Eurosequence (Groningen, The Netherlands), using Edman degradation on an Applied Biosystems 477A protein sequencer.

Antifungal Assays

Fusarium solani, *Trichoderma viride*, and *Alternaria radicina* were maintained and in vitro assays performed as described by Sela-Buurlage et al. (1993).

Screening of a cDNA Library and DNA Sequence Analysis

A tobacco cDNA library, prepared from polyadenylated RNA isolated from TMV-infected Samsun NN tobacco leaves, was made using a ZAP-cDNA synthesis kit (Stratagene catalog Nos. 200400 and 200401) and was kindly provided by Dr. Huub J.M. Linthorst (Linthorst et al., 1991). DNA (1 μg) isolated from the tobacco λZAP library was used in a PCR with oligonucleotides LS20 (5′-CAGCTATGACCATGATTACG-3′) and LS46 [5′-CTCGAATTCGG(A/T)CCIACIGG(A/T)CC(G/A)TA(G/A)AAAGCIGTCCA-3′] to amplify a partial CBP20 cDNA fragment. The PCR product was cloned after digestion with *Eco*RI into a pBS vector to yield clone pMOG684. From this clone a specific CBP20 probe (187 bp) was amplified in a PCR using the oligonucleotides LS48 (5′-CTCGAATTCGGCACGAGGATCCTCTATTTC-3′) and LS49 (5′-CTCGAATTCCACTGCACTGGC TTTGGCAGC-3′). Recombinant DNA procedures were performed as described by Maniatis et al. (1982). The nucleotide sequence of the different cDNA clones was determined using the double-stranded DNA sequencing method (Chen and Seeburg, 1985).

Northern and Southern Blot Analysis

Tobacco genomic DNA (10 μg) was digested with either *Sst*I, *Pst*I, *Hin*dIII, or *Eco*RI restriction endonucleases, and fragments were separated on a 1% agarose gel and transferred onto Hybond N^+ membranes. Hybridization was performed with either the ^{32}P-labeled cDNA of clone *cbp*20–44 or PR-4a, and the membranes were washed in 0.5× SSC containing 0.1% SDS at 55°C. Total RNA was isolated from the leaves of healthy Samsun NN tobacco plants and from stressed plants using phenol extraction and LiCl precipitation (Verwoerd et al., 1989). RNA (15 μg), denatured by glyoxal treatment, was separated on a 1.5% agarose gel, blotted, and cross-linked onto a GeneScreen membrane, and hybridized to a ^{32}P-labeled insert of clone *cbp*20–44. The northern blot was washed in 0.1× SSC at 65°C.

RESULTS

Identification and Characterization of a Novel Chitin-Binding Protein

Seven days after inoculation of Samsun NN tobacco plants with TMV, proteins were extracted from the infected leaves. The leaf extract was passed through a Sephadex G-25 column (Fig. 1, lane A) and a cation-exchange column (Fig. 1, lane B). Chitin-binding proteins were isolated by the subsequent use of affinity chromatography. Proteins were allowed to bind to insoluble chitin at pH 8.3 at 4°C. Bound proteins were eluted by lowering the pH of the mobile phase to 3.5. Three proteins of 20, 32, and 34 kD were present in the eluate (Fig. 1, lane C). The 32- and 34-kD proteins were identified as the two class I chitinases that are known to be induced in tobacco upon infection with TMV (Legrand et al., 1987) and known to reversibly bind to chitin (Broekaert et al., 1988; Sela-Buurlage et al., 1993). The third protein of about 20 kD appeared to be a novel chitin-binding protein. This protein (referred to as CBP20) was purified to homogeneity by three rounds of gel-filtration chromatography. The class I chitinases eluted at an apparent molecular mass of 15 to 15.5 kD, and CBP20 eluted at an apparent molecular mass of 8 kD. Usually, baseline separation occurred after the second passage. The CBP20-containing fractions resulting after the third passage were pooled and appeared to be electrophoretically pure (Fig. 1, lane E). Usually 100 μg of pure CBP20 was obtained from about 400 g of TMV-infected tobacco leaves (containing about 100 mg of protein). In extracts of healthy tobacco leaves no chitin-binding proteins were detected (data not shown).

Because CBP20 bound to chitin, it seemed obvious to assay this protein for chitinase activity. To this end we used the chitinase assay described by Wirth and Wolf (1990). Some activity was found to be associated with CBP20: 0.03 to 0.05 ODU μg^{-1} of protein (data not shown). However, this activity is extremely low compared to the activity measured for the class I chitinases (approximately 3 ODU μg^{-1}). Because some chitinases exert lysozyme activity, we tested CBP20 for such an activity as well. Activity measurements indicated that

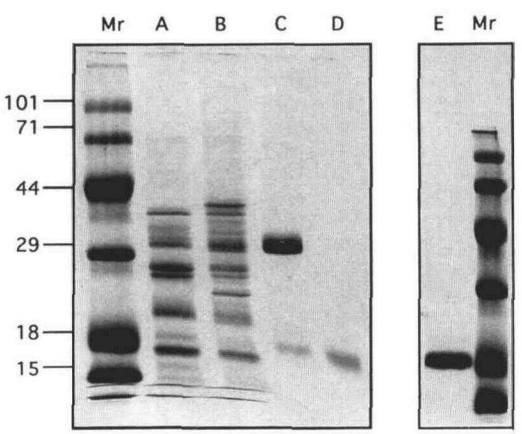

Figure 1. Purification of CBP20. Protein samples were taken at different stages in the purification procedure of CBP20 and analyzed on 12.5% SDS-polyacrylamide gels. A sample of the crude desalted leaf extract is shown in lane A. Protein pools obtained after S-Sepharose cation-exchange chromatography, chitin-affinity chromatography, and gel-filtration chromatography are shown in lanes B, C, and D, respectively. In lane E, 3 μg of purified protein was loaded. The lanes indicated by Mr show prestained markers. The corresponding molecular masses in kD are as indicated.

CBP20 exhibited some lysozyme activity: 0.01 ODU μg^{-1} of protein. This specific activity is in the same range as the lysozyme activity associated with the class I chitinases but about 1000-fold lower than the specific activity for hen egg lysozyme measured under the same reaction conditions.

CBP20 Inhibits Fungal Growth in Vitro

The antifungal activity associated with several chitin-binding proteins prompted us to test the effect of CBP20 on fungal growth. To this end 24-well microtiter plates were used (Sela-Buurlage et al., 1993). Spores of either *T. viride*, *F. solani*, or *A. radicina* were pipetted onto potato dextrose agar and allowed to germinate for 6 to 16 h. Purified and filter-sterilized protein solutions were added to the pregerminated spores. The ability to lyse the fungus was studied 1 h after the addition of protein. Growth inhibition was scored 2 to 3 d later. *T. viride* appeared to be the most sensitive fungus. Almost complete lysis of the germ tubes was observed immediately after the addition of small amounts (1 μg/well = 6.7 $\mu g\ mL^{-1}$) of CBP20 (data not shown). Consequently, growth was severely inhibited (data not shown). Hyphal tips of *F. solani* showed no lysis in the presence of up to 10 μg of CBP20 per well, although swelling of the hyphal tips was observed microscopically (data not shown). However, some effect of purified CBP20 (1 μg/well) was observed on the growth of *F. solani* (Fig. 2). The addition of CBP20 (up to 5 μg/well) to spores of *A. radicina* did not result in lysis either. Unlike *F. solani*, growth of *A. radicina* was not affected in the presence of CBP20 (data not shown).

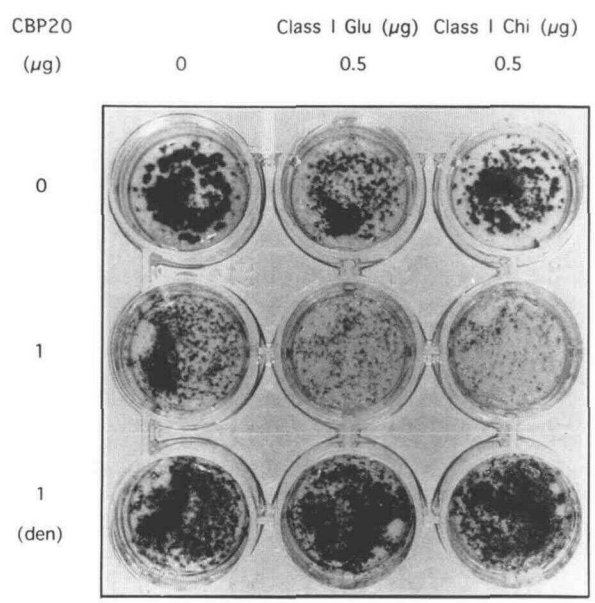

Figure 2. Antifungal activity of CBP20. The effect of purified CBP20 and combinations of CBP20 with a class β-1,3-glucanase (Glu) or a class I chitinase (Chi) on the in vitro growth of *F. solani*. Amounts of protein (in μg) per well are indicated. The effect of the protein combinations should be compared to the effect of the individual proteins (left column and top row) and heat-denatured (den) control samples (bottom row).

Because chitin-binding proteins are known to act synergistically with chitinases (Broekaert et al., 1989; Hejgaard et al., 1992) and chitinases and β-1,3-glucanases are known to act synergistically as well (Mauch et al., 1988; Sela-Buurlage et al., 1993), we studied the effect of CBP20 in combination with these two enzymes. The addition of 0.5 μg of class I β-1,3-glucanase alone resulted in a small amount of lysis (<5%) and some growth inhibition in the case of *F. solani* (Fig. 2). The combination of 1 μg of CBP20 and 0.5 μg of β-1,3-glucanase showed a strong lysing activity (about 70%). Also, a severe effect on growth was visible (Fig. 2). From these data we conclude that CBP20 and the class I β-1,3-glucanase act synergistically. Addition of 0.5 μg of class I chitinase alone did not cause lysis of the germ tubes or interfere with the growth of *F. solani* (Fig. 2). The combination of 1 μg of CBP20 and 0.5 μg of class I chitinase did not cause lysis either but inhibited the growth of *F. solani* in a synergistic manner (Fig. 2). The effect of the latter combination was even more potent than the combination of CBP20 and the β-1,3-glucanase.

The addition of 0.5 μg/well of class I β-1,3-glucanase alone inhibited growth of *A. radicina* substantially, whereas 0.1 μg of class I β-1,3-glucanase was ineffective in inhibiting fungal growth. Combinations of CBP20 (5 μg/well) and β-1,3-glucanase (0.1 μg/well) inhibited the growth of *A. radicina* (about 35%), resulting in more condensed growing mycelia (data not shown). Apparently, both proteins acted synergistically. Combining 5 μg of CBP20 with a class I chitinase (0.5 μg/well) did not result in enhanced growth inhibition compared to the effect of the class I chitinase alone. Thus, in the case of *A. radicina* no synergism was observed between CBP20 and the class I chitinase. In all cases heat inactivation of the protein mixtures eliminated their antifungal effect.

CBP20 Is C-Terminally Processed

To further characterize CBP20, amino acid sequences were determined.The protein was separated on a 12.5% SDS-polyacrylamide gel and electroblotted to a PVDF membrane to allow for N-terminal sequencing. However, no sequence data became available, probably due to the presence of a modified Gln residue interfering with Edman degradation (see below). An internal sequence was obtained after digesting CBP20 with the Glu-C-specific endoproteinase (V_8 protease) from *Staphylococcus aureus* (Cleveland et al., 1977). The major reaction product was a 17-kD peptide that gave the following amino acid sequence (CBP-PEP1): Tyr-(Ala/Gly)-Ser-Pro-Ser-Gln-Gly-X-Gln-Ser-Gln-(Arg)-Ser-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Ala-Gln-Asn. From the specificity of the protease it can be predicted that a Glu residue precedes the first given amino acid residue. The amino acid indicated as X is most likely a Cys residue, because no special care was taken to alkylate the Cys residues prior to sequence analysis. The amino acid residues given in parentheses were not characterized unequivocally.

A second internal amino acid sequence was obtained after chemical digestion of CBP20 with *N*-chlorosuccinimide (Lischwe and Ochs, 1982). A band of 9 to 11 kD was used for the sequence determination (CBP-PEP2): Thr-Ala-Phe-Tyr-Gly-Pro-Val-Gly-Pro-(Pro/Arg)-Gly-Arg-Asp-Ser-X-Gly-Lys-(Gly)-Leu-?-Val-Thr-Asn. Because *N*-chlorosucci-

nimide cleaves at the C terminus of Trp residues, it is assumed that this amino acid precedes the given sequence. The amino acid indicated as X is again most likely a Cys residue as argued above. At the position of the question mark no amino acid identification could be made. A third amino acid sequence representing the C terminus of CBP20 was obtained as follows. CBP20 (4 nmol) was digested with trypsin, and the C-terminal peptide was purified from the digest via affinity chromatography on an immobilized anhydrotrypsin column (Yokosawa and Ishii, 1979; Ishii et al., 1983; Kumazaki et al., 1986). Sequencing of this peptide revealed the following amino acid sequence (CBP-PEP3): (Gly)-(His)-Leu-Ile-Val-Asn-Tyr-Glu-Phe-Val-Asn-Cys-Gly-Asp-Asn, which is presumably preceded by a Lys or an Arg residue.

To isolate clones corresponding to CBP20 a λZAP cDNA library of tobacco leaves infected with TMV was screened. A partial cDNA fragment was amplified from a total λZAP-cDNA library by using the oligonucleotides LS20 and LS46 in a PCR. The oligonucleotide LS20 was complementary to the pSK vector sequence of the λZAP arm at the 5′ end of the cDNA insert, and oligonucleotide LS46 was based on the amino acid sequence of fragment CBP-PEP2. The amplified DNA fragment (407 bp) was cloned as an *Eco*RI fragment into the *Eco*RI-linearized vector pBlueScript to yield clone pMOG684. The nucleotide sequence of the cloned *Eco*RI fragment confirmed that a partial CBP20 cDNA clone was isolated. To ensure the use of a CBP20-specific DNA probe, a 187-bp PCR fragment was amplified from plasmid pMOG684 using the oligonucleotides LS48 and LS49. This PCR fragment, encoding the N-terminal part of CBP20 up to the Gly-rich region present in CBP-PEP1, was subsequently used to screen a tobacco cDNA library.

The screening for CBP20-like cDNAs resulted in the isolation of several positive clones. Five clones containing 800 bp of cDNA inserts were sequenced. The derived sequences indicated that two types of cDNA clones had been isolated. The nucleotide sequence and the deduced amino acid sequence of the two different types of cDNA clones are shown in Figure 3. Clone *cbp*20–44 represents a nearly full-length cDNA. Because the deduced amino acid sequence exactly matched the determined sequences CBP-PEP1, CBP-PEP2, and CBP-PEP3 (Fig. 3), we concluded that *cbp*20–44 is a true CBP20 cDNA clone. Clone *cbp*20–52 encodes a protein containing two additional amino acid residues (Gly, Ala) in the Gly-rich region compared to CBP-PEP2 (Fig. 3).

Both cDNAs code at their 5′ end for a putative signal peptide that may be involved in transport of the protein across the membrane of the ER. The cleavage site would be between Ala[22] and Gln[23], analogous to the cleavage sites of other PR proteins, including β-1,3-glucanases (Shinshi et al., 1988; Linthorst et al., 1990a), PR-1 proteins (Cornelissen et al., 1987), chitinases (Linthorst et al., 1990b; Payne et al., 1990a), and class II PR-4 proteins (Linthorst et al., 1991). The resulting N-terminal Gln residue is readily modified, probably resulting in ineffective Edman degradation of the mature protein (see above). As mentioned above, CBP-PEP3 represents the C terminus of the mature CBP20 protein. However, as compared to this peptide the deduced amino acid sequence extends for another 11 residues. Apparently mature CBP20 is synthesized as a preproprotein from which the N-terminal

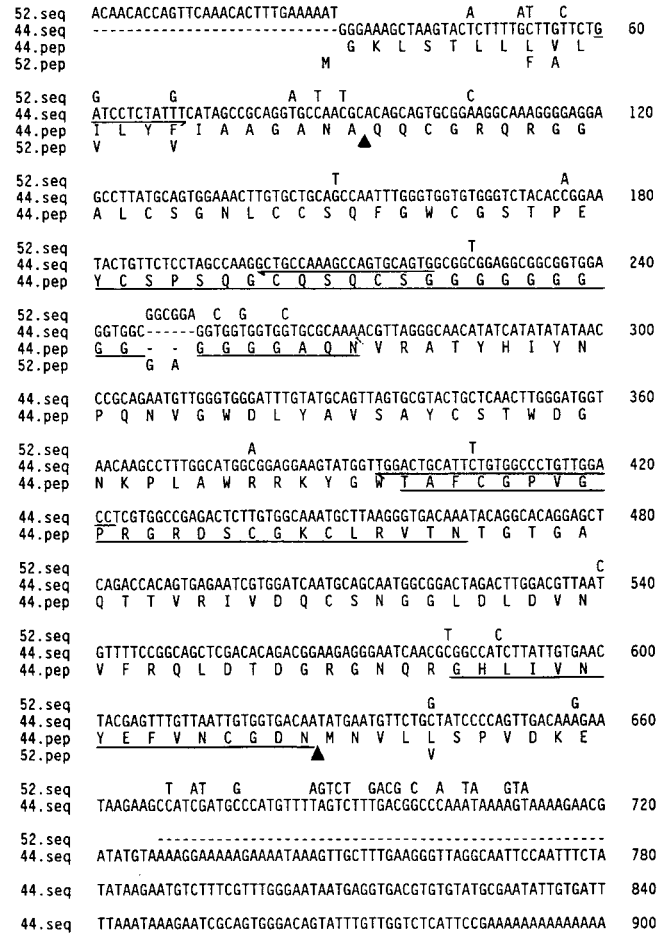

Figure 3. Sequence of *cbp*20 clones. Two *cbp*20 clones (designated *cbp*20–44 and *cbp*20–52) were isolated and sequenced (44.seq and 52.seq, respectively). The deduced amino acid is also given (44.pep and 52.pep, respectively). Nucleotides and deduced amino acid residues of *cbp*20–52 are given only when they differed from the *cbp*20–44 sequence. The peptide sequences obtained after V_8 protease treatment (CBP-PEP1), *N*-chlorosuccinimide/urea digestion (CBP-PEP2), and trypsin digestion (CBP-PEP3) are underlined. The putative N-terminal signal peptide cleavage site and the C-terminal cleavage site of the propeptide are indicated by arrowheads. The primers used in the PCR reactions are underlined.

signal peptide and the CTPP are cleaved off. The CTPP most likely functions as a vacuolar targeting signal, because the CTPPs of class I chitinases, class I β-1,3-glucanase, and AP24 were shown to be involved in vacuolar targeting (Neuhaus et al., 1991; Melchers et al., 1993).

CBP20 Is a Class I PR-4 Protein

Comparison of the deduced amino acid sequence of CBP20 with sequences stored in the SwissProt protein sequence data base revealed striking similarity with the primary structures of the putative wound-inducible proteins encoded by the *win1* and *win2* genes from potato (Stanford et al., 1989) and of prohevein from the rubber tree (Broekaert et al., 1990). Prohevein is the precursor of hevein, a small lectin found in

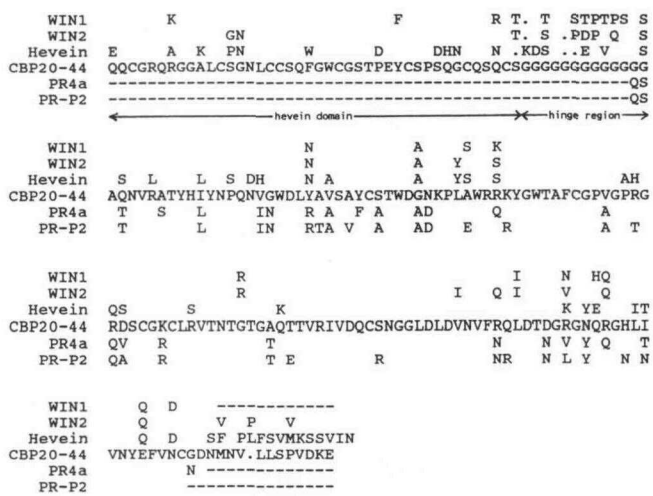

Figure 4. Sequence comparison of several PR-4 proteins. The sequence of proCBP20 is given and compared to several PR-4 proteins. Amino acid residues of tobacco PR-4a and tomato PR-P2 (Linthorst et al., 1991), prohevein (Broekaert et al., 1990), and the putative proWIN1 and proWIN2 proteins (Stanford et al., 1989) that are different from those in proCBP20 are indicated. Gaps introduced to optimize the alignment of the proteins are indicated as dots, whereas amino acids missing from the sequences are indicated by hyphens.

the latex of the rubber tree. A comparison of these four proteins is shown in Figure 4. In all cases the N-terminal signal peptide (not included in Fig. 4) is followed by a chitin-binding domain that is connected to a C-terminal domain by a hinge region. High identity of the CBP20 proprotein sequence was found with the putative WIN1 and WIN2 proproteins and prohevein (86.9, 87.5, and 70.5%, respectively). In contrast to the hinge regions of prohevein and the putative WIN proteins, the hinge region of CBP20 is extremely Gly rich. In clone *cbp*20–52, 14 of 15 amino acid residues are Gly, and in clone *cbp*20–44 all 13 residues are Gly (Fig. 4). In addition to the above similarities, the N-terminal sequence of CBP20 was found to be very similar to the chitin-binding domain of tobacco class I chitinases (Linthorst et al., 1990b; Shinshi et al., 1990) and stinging nettle lectin (Broekaert et al., 1989) (data not shown). In view of the chitin-binding capacity of CBP20, this similarity is not unexpected.

A strong identity in primary structure was also observed between the C-terminal domain of the CBP20 proprotein and the class II PR 4 proteins PR-4a and PR-4b (78.7 and 78.7%, respectively; Fig. 4) from tobacco and PR-P2 (75%) from tomato (Linthorst et al., 1991). This observation prompted us to investigate the immunological relationship between the class II PR-4 proteins and CBP20. To this end the pool of chitin-binding proteins (Fig. 1, lane C) and class II PR-4 proteins from tobacco were incubated with antiserum raised against PR-P2 from tomato. The antiserum recognized the purified class II PR-4 proteins as well as CBP20 (Fig. 5, lanes A and C, respectively). No cross-reactions were observed with the class I chitinases that were present in the pool of chitin-binding proteins (Fig. 5, lane B). Similar results were

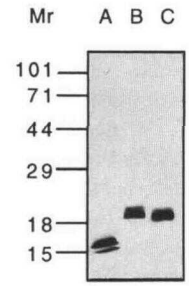

Figure 5. Immunological identification of CBP20. CBP20-containing fractions were used to screen for immunological cross-reactivity. Samples of purified PR-4a and PR-4b, the chitin eluate, and purified CBP20 were separated on 12.5% SDS-polyacrylamide gels (lanes A, B, and C, respectively) and electroblotted to nitrocellulose membranes. Immunodetection was performed with the antiserum to the PR-4 protein of tomato (PR-P2). Lane Mr shows prestained markers. The corresponding molecular masses in kD are as indicated.

obtained with antiserum specific for the tobacco class II PR-4 proteins (data not shown).

The serological relation between CBP20 and the class II PR-4 proteins made it possible to study the cellular localization of CBP20 in TMV-infected tobacco plants. Primary infected and healthy tobacco leaves were used for the isolation of EFs. Leaves from which the EFs had been removed and freshly harvested leaves were used to prepare extracts further referred to as "−EF" and "T" (total), respectively. Immunoblots of these protein samples showed a clear induction of CBP20 as a result of TMV infection (compare lanes A and D in Fig. 6). Furthermore, it was shown that CBP20 was present in T and −EF extracts prepared from TMV-inoculated leaves (Fig. 6, lanes D and E) and not in the EFs isolated from these leaves (Fig. 6, lane F). This indicates that CBP20 is intracellularly located. A protein of 14 to 15 kD present in the protein samples T and EF (lanes D and F) cross-reacted with the antiserum as well. This protein band was induced by TMV

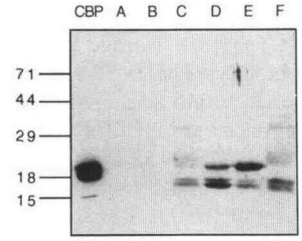

Figure 6. Cellular localization of CBP20. The antiserum to the tomato PR-4 protein PR-P2 was used to determine the cellular localization of CBP20. Uninfected (lanes A, B, and C) and TMV-infected (lanes D, E, and F) tobacco plants were sampled for total soluble leaf protein (lanes A and D), the extracellular washing fluid (lanes C and F), and soluble proteins remaining after the removal of the EF fraction (lanes B and E). Samples of 5 μg of protein were separated by 12.5% SDS-PAGE and electroblotted to a PVDF membrane. Immunodetection was performed with the antiserum to tomato PR-P2 (diluted 1:1000). A purified sample of CBP20 served as reference as well as a prestained molecular mass standard. The corresponding molecular masses in kD are as indicated.

(compare Fig. 6, lanes A and D) and represents the class II (acidic) PR-4 proteins from tobacco, which are known to be located extracellularly (Linthorst et al., 1991).

The identity in primary structure of CBP20 and the class II PR-4 proteins from tobacco, as well as the serological relationship between these proteins, and the intracellular localization of CBP20 led us to the conclusion that CBP20 is a class I PR-4 protein.

CBP20 Is Encoded by a Small Gene Family

The cDNA insert of the *cbp*20–44 clone and a class II PR-4 clone (Linthorst et al., 1991) were hybridized to blots containing tobacco DNA digested with four different restriction enzymes. The Southern blot hybridized with the CBP20 cDNA showed a maximum of two hybridizing fragments in each lane (Fig. 7, right). This suggests that at least two copies of the CBP20 gene are present in tobacco. The Southern blot hybridized with the class II PR-4 clone indicates the presence of two to four class II PR-4 genes (Fig. 7, left), which is in agreement with a previous report by Linthorst et al. (1991). Because distinct DNA fragments hybridized with the probes, it was concluded that CBP20 and class II PR-4 genes were specifically detected and that no strong cross-hybridization occurred.

Accumulation of CBP20 mRNA upon TMV Infection, Wounding, and Other Forms of Stress

The induction of CBP20 by TMV and the high similarity of the polypeptide with the putative wound-inducible proteins WIN1 and WIN2 led us to investigate whether the expression of CBP20 mRNA is responsive to stress. Samsun NN tobacco plants were subjected to different stress conditions, and leaf samples were taken 3 d after inoculation with

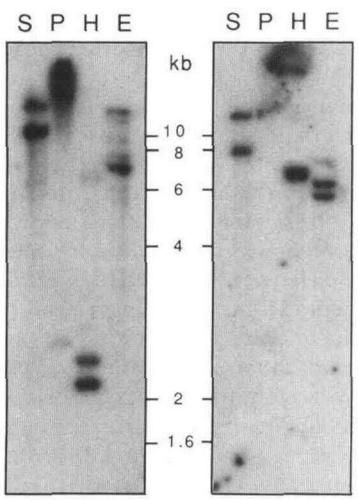

Figure 7. Determination of the size of the PR-4 gene family. Tobacco DNA was digested with four restriction enzymes: *Sst*I (S), *Pst*I (P), *Hin*dIII (H), and *Eco*RI (E). The resulting fragments were separated on a 1% agarose gel and blotted to Hybond N$^+$. Fragments were hybridized with either a ^{32}P-labeled cDNA of clone PR-4a (left) or *cbp*20–44 (right). Molecular size standards are as indicated.

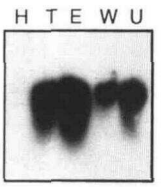

Figure 8. Induction pattern of CBP20 mRNA. mRNA was isolated from healthy tobacco plants (H) and tobacco plants stressed by inoculation with TMV (T), ethephon treatment (E), wounding (W), or UV light treatment (U). The amount of CBP20-specific mRNA was determined by hybridizing with a ^{32}P-labeled probe.

TMV, 2 d after wounding, 1 d after ethephon treatment, and 1 d after UV light irradiation. At these times, maximal expression of the class I PR-2 and PR-3 genes was reported by Brederode et al. (1991). The leaf samples were extracted and analyzed on a northern blot. Figure 8 shows that the expression of *cbp*20 in nonstressed tobacco leaves is not detectable. TMV infection of tobacco leaves induced the expression of *cbp*20 gene to high levels (Fig. 8, lane T). Treatment with ethephon resulted in an even higher level of CBP20 mRNA (Fig. 8, lane E). Moderate induction of CBP20 expression was found after wounding the leaves or UV light treatment (Fig. 8, lanes W and U, respectively). Thus, the stress induction pattern of *cbp*20 matches the stress induction pattern of other class I PR proteins (Brederode et al., 1991).

DISCUSSION

Characterization of CBP20

In this paper we describe the purification and characterization of a novel stress-inducible, antifungal protein from TMV-infected tobacco leaves. Its native and denatured molecular masses, 8 and 20 kD, respectively, indicate that the protein is probably purified as a monomer. Because the 20-kD protein binds to chitin, it is referred to as chitin-binding protein (CBP20). Low levels of chitinase and lysozyme activities are associated with the protein. Furthermore, the protein inhibits the growth of various fungi tested, especially in the presence of a tobacco class I chitinase or a tobacco class I β-1,3-glucanase.

Based on partial protein sequences obtained from the purified protein (CBP-PEP1 and CBP-PEP2), DNA primers were designed to isolate cDNA clones corresponding to CBP20. Two types of clones were isolated. The deduced primary structure of the protein encoded by one type (*cbp*20–44) matches exactly the three peptide sequences that were determined for CBP20.

The other type encodes a protein that differs from *cbp*20–44 by five conservative amino acid changes and a small insertion of two amino acids. Four of the five amino acid changes are within the N-terminal signal peptide and, consequently, are absent in the mature protein. The insertion is in the hinge region of the protein, which consists of 13 Gly residues. The finding of two types of cDNA clones is in agreement with the observation that CBP20 is encoded by a small family of at least two genes.

CBP20 is both structurally and immunologically related to

the class II PR proteins PR-4a and PR-4b of tobacco and PR-P2 of tomato. From these observations and its intracellular localization we conclude that CBP20 is a class I PR-4 protein. It is interesting that the relationship between the class I and class II PR-4 proteins is analogous to the relationship between the class I and class II PR-3 proteins (Linthorst et al., 1990b; Shinshi et al., 1990): the class I proteins of both the PR-3 and PR-4 group consist of an N-terminal chitin-binding domain linked to a C-terminal domain by a hinge region, whereas the class II proteins of both groups consist of the C-terminal domain only. In tobacco both class I and class II PR proteins are induced under various stress conditions. However, their induction patterns are not identical (Brederode et al., 1991). A striking difference is the reaction upon wounding. Whereas the class I proteins are strongly induced upon wounding, the class II proteins are not. CBP20 is induced upon wounding and otherwise the induction pattern of this protein also resembles that of the class I PR proteins. This further substantiates the conclusion that CBP20 is a class I PR protein.

Comparison of CBP20 to Other Chitin-Binding Proteins

The chitin-binding domain of CBP20 shows extensive identity to the chitin-binding domains of other proteins like the class I chitinases (Linthorst et al., 1990b), hevein (Broekaert et al., 1990), stinging nettle lectin (Lerner and Raikhel, 1992), and the putative WIN proteins from potato (Stanford et al., 1989). Except for the class I chitinases and stinging nettle lectin, identity is not restricted to the chitin-binding domain: the C-terminal domain of CBP20 is also homologous to the deduced C-terminal domain of prohevein (Broekaert et al., 1990) and the putative WIN proteins (Fig. 4).

Tomato leaves infected by *C. fulvum* synthesize a basic chitin-binding, intracellularly located 20-kD protein cross-reacting with the antiserum to PR-P2 (data not shown). In contrast to the monomeric nature of CBP20, the native tomato protein appears to be a dimer. We anticipate that this protein is homologous to CBP20. Tamarillo fruits (*Cynhomandra betacea*, also a solanaceous species) has been shown to contain 25-kD chitin-binding lectins (Xu et al., 1992). Because the amino acid composition of these proteins and CBP20 is comparable, it might turn out that these lectins are also representatives of the class I PR-4 group of proteins.

Targeting and Processing of Class I PR-4 Proteins

Immunological studies indicate that CBP20 is localized intracellularly. Like the tobacco class I PR-2 (Van den Bulcke et al., 1989) and PR-5 (Singh et al., 1987) proteins and the bean class I PR-3 (Boller and Vögeli, 1984) protein, CBP20 is likely to be localized in the vacuole. Transport of the immature CBP20 across the membrane of the ER is facilitated by the N-terminal signal peptide. Unique to the class I PR proteins is the presence of a CTPP that is involved in intracellular (vacuolar) targeting of these proteins and that is cleaved off during maturation (Shinshi et al., 1988; Neuhaus et al., 1991; Melchers et al., 1993). We showed that the CTPP was removed during maturation of the protein in the case of CBP20 as well. From the sequence comparison of CBP20 and prohevein one would expect that hevein is located intracellularly and processed in a manner comparable to CBP20. However, apart from the expected 20-kD protein, prohevein has been shown to be processed into a 5-kD N-terminal chitin-binding domain (hevein) and a 14-kD C-terminal domain (Lee et al., 1991). All three proteins appear to be localized intracellularly. Although we never obtained any indication of this kind of processing taking place in tobacco, we cannot exclude the possibility that proCBP20 is also processed in a way similar to prohevein. About the nature of the mature WIN1 and WIN2 proteins from potato one can only speculate, because these proteins have not been described in the literature. The putative WIN2 protein presumably is localized intracellularly because of the presence of a CTPP (Stanford et al., 1989). Mature proteins arising from proWIN2 may be either the full-length 20-kD protein or the 5- and 14-kD N- and C-terminal domains of the protein. Because the putative WIN1 protein seems to lack a CTPP, it is likely that this protein is extracellularly localized. It thereby resembles the class IV chitinases consisting of an N-terminal signal peptide and a hevein domain connected to a C-terminal domain by a hinge region (Collinge et al., 1993).

Antifungal Activity of Chitin-Binding Proteins

The association of antifungal activity with several chitin-binding proteins led us to look into the antifungal effect of CBP20. The chitin-containing fungi *T. viride*, *F. solani*, and *A. radicina* were used to study the effect of CBP20. *T. viride* appeared to be the most sensitive fungus. Low amounts of CBP20 (6.7 $\mu g\ mL^{-1}$ = 1 μg/well) resulted in lysis and total growth inhibition of this fungus. This amount of CBP20 is in the same range as that of PR-4 type proteins from barley grain needed for growth inhibition of *Trichoderma harzianum* (Hejgaard et al., 1992). These authors stated that the basic PR-4 type proteins used in their studies lack a chitin-binding domain yet bind to chitin. If so, they should be regarded as class II type PR-4 proteins. It is interesting that we found that the class II PR-4 type proteins from tobacco lack antifungal activity (data not shown). The amount of CBP20 needed for growth inhibition of *T. viride* is lower than the amount of hevein and stinging nettle lectin (both about 45 $\mu g\ mL^{-1}$) needed for 50% growth reduction of *Trichoderma hamatum* (Van Parijs et al., 1991) and *T. viride*, respectively (Broekaert et al., 1989). However, differences in the extraction and/or assay procedures may account for these effects. To account for the difference in extraction, CBP20 was isolated in a buffer in which β-mercaptoethanol had been replaced by 10 mM thiourea (Van Parijs et al., 1991; Broekaert et al., 1992). Further purification of the protein was performed, and antifungal assays were run with *F. solani* as the test fungus. It appeared that CBP20 obtained in this way had a 2- to 5-fold stronger antifungal effect than CBP20 obtained in the usual way. Apparently, CBP20 is partly inactivated by the addition of β-mercaptoethanol.

The germ tubes of *F. solani* were less sensitive to CBP20 as compared to the germ tubes of *T. viride*. However, growth inhibition was clearly observed in the presence of 30 $\mu g\ mL^{-1}$ (= 4.5 μg/well) of CBP20. Again, this seems to be lower than the amount of hevein needed (600–1250 $\mu g\ mL^{-1}$) for 50%

growth inhibition of two *Fusarium* species (Van Parijs et al., 1991). Using *F. solani* as the test fungus we showed that CBP20 interacts synergistically with both a class I β-1,3-glucanase and a class I chitinase of tobacco. Binding of CBP20 to the chitin matrix of the cell wall apparently renders the fungus more sensitive to enzymes capable of hydrolyzing cell wall components.

Because chitin-containing fungi are very sensitive to chitin-binding chitinases (Sela-Buurlage et al., 1993), it was essential to achieve complete separation of the class I chitinases and CBP20 before performing antifungal assays. This was achieved by repeated gel filtration. Nevertheless, control experiments were incorporated to rule out the possibility that traces of class I chitinases were responsible for the observed antifungal effects. This appeared not to be the case (data not shown). The most direct proof that CBP20 itself exhibits antifungal activity rather than contaminating class I chitinases is the synergistic activity of these two chitin-binding proteins on *F. solani*. If the chitinase activity in the CBP20 preparation resulted from contaminating chitinases, and CBP20 itself were not antifungal at all, then no synergistic effect against *F. solani* would be expected when 1 μg of CBP20 was mixed with 0.5 μg of class I chitinase (Sela-Buurlage et al., 1993).

Biological Role of CBP20

From the results presented in this paper it is clear that CBP20 is induced after several forms of stress regimes, including pathogen attack and wounding, and exhibits antifungal activity. Potato is known to react to wounding by inducing the expression of two wound-inducible genes (Stanford et al., 1989) that are both highly homologous to CBP20. It would be interesting to know whether these genes are also induced by pathogen attack. If we assume that this is the case, it seems likely that CBP20 and related proteins play (among others) a role in plant defense. Because chitin is present in many fungal cell walls and absent from plant cell walls, chitinases and chitin-binding proteins are excellent and specific defense barriers for plants. The importance of chitinases is probably well illustrated by the diversity of chitinases that are induced upon pathogen attack (Legrand et al., 1987; Lawton et al., 1992): chitin-binding proteins with low and high levels of chitinase activity and several chitinases that are not able to bind to chitin yet hydrolyze (partly solubilized) chitin. By producing so many apparently harmless plant defense enzymes, a large spectrum of substrate molecules (present in the fungal cell wall) may be hydrolyzed into a spectrum of reaction products that, in turn, may function as aspecific elicitors in the plant defense reaction.

ACKNOWLEDGMENTS

We thank Marion Apotheker-de Groot for technical assistance during DNA sequencing, Ruud van den Broek and Ilma Dekker for the continuous supply of TMV-infected tobacco leaves, Dr. Matthieu Joosten for the kind gift of PR-P2 antiserum, Dr. Huub Linthorst for the generous gift of the tobacco cDNA library, and Dr. Wicher Weijer for the determination of the amino acid sequences.

Received June 11, 1993; accepted September 2, 1993.

LITERATURE CITED

Bol JF, Linthorst HJM, Cornelissen BJC (1990) Plant pathogenesis-related proteins induced by virus infection. Annu Rev Phytopathol **28:** 113–138

Boller T, Vögeli U (1984) Vacuolar localization of ethylene-induced chitinase in bean leaves. Plant Physiol **74:** 442–444

Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of proteins utilizing the principle of protein-dye binding. Anal Biochem **72:** 248–254

Brederode FT, Linthorst HJM, Bol JF (1991) Differential induction of acquired resistance and PR gene expression in tobacco by virus infection, ethephon treatment, UV light and wounding. Plant Mol Biol **17:** 1117–1125

Broekaert W, Lee H, Kush A, Chua NH, Raikhel N (1990) Wound-induced accumulation of mRNA containing a hevein sequence in laticifers of rubber tree (*Hevea brasiliensis*). Proc Natl Acad Sci USA **87:** 7633–7637

Broekaert WF, Mariën W, Terras FRG, de Bolle MFC, Proost P, Van Damme J, Dillen L, Claeys M, Rees SB, Vanderleyden J, Cammue BPA (1992) Antimicrobial peptides from *Amaranthus caudatus* seeds with sequence homology to the cysteine/glycine-rich domain of chitin-binding proteins. Biochemistry **31:** 4308–4314

Broekaert WF, Van Parijs J, Allen AK, Peumans WJ (1988) Comparison of some molecular, enzymatic and antifungal properties of chitinases from thorn-apple, tobacco and wheat. Physiol Mol Plant Pathol **33:** 319–331

Broekaert WF, Van Parijs J, Leyns F, Joos H, Peumans WJ (1989) A chitin-binding lectin from stinging nettle rhizomes with antifungal properties. Science **245:** 1100–1102

Chen EY, Seeburg PH (1985) Supercoil sequencing: a fast and simple method for sequencing plasmid DNA. DNA **4:** 165–170

Chrispeels MJ, Raikhel NV (1991) Lectins, lectin genes and their role in plant defense. Plant Cell **3:** 1–9

Cleveland DW, Fischer SG, Kirschner MW, Laemmli UK (1977) Peptide mapping by limited proteolysis in sodium dodecyl sulfate and analysis by gel electrophoresis. J Biol Chem **252:** 1102–1106

Collinge DB, Kragh KM, Mikkelsen JD, Nielsen KK, Rasmussen U, Vad K (1993) Plant chitinases. Plant J **3:** 31–40

Cornelissen BJC, Horowitz J, Van Kan JAL, Goldberg RB, Bol JF (1987) Structure of tobacco genes encoding pathogenesis-related proteins from the PR-1 group. Nucleic Acids Res **15:** 6799–6811

De Wit PJGM, Spikman G (1982) Evidence for the occurrence of race and cultivar-specific elicitors of necrosis in intracellular fluids of compatible interactions of *Cladosporium fulvum* and tomato. Physiol Plant Pathol **21:** 1–11

Hejgaard J, Jacobsen S, Bjorn SE, Kragh KM (1992) Antifungal activity of chitin-binding PR-4 type proteins from barley grain and stressed leaf. FEBS Lett **307:** 389–392

Ishii SI, Yokosawa H, Kumazaki T, Nakamura I (1983) Immobilized anhydrotrypsin as a specific affinity adsorbent for tryptic peptides. Methods Enzymol **91:** 378–383

Joosten MHAJ, Bergmans CJB, Meulenhoff EJS, Cornelissen BJC, De Wit PJGM (1990) Purification and serological characterization of three basic 15-kilodalton pathogenesis-related proteins from tomato. Plant Physiol **94:** 585–591

Kauffmann S, Legrand M, Geoffrey P, Fritig B (1987) Biological function of 'pathogenesis-related' proteins: four PR proteins of tobacco have 1,3-β-glucanase activity. EMBO J **6:** 3209–3212

Kumazaki T, Nakako T, Ariska F, Ishii SI (1986) A novel method for selective isolation of C-terminal peptides from tryptic digests of proteins by immobilized anhydrotrypsin: application to structural analyses of the tail sheath and tube proteins from bacteriophage T4. Proteins Structure Function Genet **1:** 100–107

Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of the bacteriophage T4. Nature **227:** 680–685

Lawton K, Ward E, Payne G, Moyer M, Ryals J (1992) Acidic and basic class III chitinase mRNA accumulation in response to TMV infection of tobacco. Plant Mol Biol **19:** 735–743

Lee H, Broekaert WF, Raikhel NV (1991) Co- and post-translational processing of the hevein preproprotein of latex of the rubber tree (*Hevea brasiliensis*). J Biol Chem **266:** 15944–15948

Legrand M, Kauffmann S, Geoffroy P, Fritig B (1987) Biological function of pathogenesis-related proteins: four tobacco pathogenesis-related proteins are chitinases. Proc Natl Acad Sci USA **84:** 6750–6754

Lerner DR, Raikhel NV (1992) The gene for stinging nettle lectin (*Urtica dioica* agglutinin) encodes both a lectin and a chitinase. J Biol Chem **267:** 11085–11091

Linthorst HJM (1991) Pathogenesis-related proteins of plants. Crit Rev Plant Sci **10:** 123–150

Linthorst HJM, Danhash N, Brederode FT, Van Kan JAL, De Wit PJGM, Bol JF (1991) Tobacco and tomato PR proteins homologous to *win* and pro-hevein lack the "hevein" domain. Mol Plant-Microbe Interact **4:** 586–592

Linthorst HJM, Melchers LS, Mayer A, Van Roekel JSC, Cornelissen BJC, Bol JF (1990a) Analysis of gene families encoding acidic and basic β-1,3-glucanases of tobacco. Proc Natl Acad Sci USA **87:** 8756–8760

Linthorst HJM, Van Loon LC, Van Rossum CMA, Mayer A, Bol JF, Van Roekel JSC, Meulenhoff EJS, Cornelissen BJC (1990b) Analysis of acidic and basic chitinases from tobacco and petunia and their constitutive expression in transgenic tobacco. Mol Plant-Microbe Interact **3:** 252–258

Lischwe MA, Ochs D (1982) A new method for partial peptide mapping using N-chlorosuccinimide/urea and peptide silver staining in sodium dodecyl sulfate-polyacrylamide gels. Anal Biochem **127:** 453–457

Maniatis T, Fritsch EF, Sambrook J (1982) A Laboratory Manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY

Matsudaira P (1987) Sequence from picomole quantities of proteins electroblotted onto polyvinylidene difluoride membranes. J Biol Chem **262:** 10035–10038

Mauch F, Mauch-Mani B, Boller T (1988) Antifungal hydrolases in pea tissue II. Inhibition of fungal growth by combinations of chitinase and β-1,3-glucanase in bean leaves. Plant Physiol **88:** 936–942

Melchers LS, Sela-Buurlage MB, Vloemans SA, Woloshuk CP, Van Roekel JSC, Pen J, Van den Elzen PJM, Cornelissen BJC (1993) Extracellular targeting of the vacuolar tobacco proteins AP24, chitinase and β-1,3-glucanase in transgenic plants. Plant Mol Biol **21:** 583–593

Molano J, Duran A, Cabib E (1977) A rapid and sensitive assay for chitinase using tritiated chitin. Anal Biochem **83:** 648–656

Moos M, Nguyen NY, Liu TY (1988) Reproducible high yield sequencing of proteins electrophoretically separated and transferred to an inert support. J Biol Chem **263:** 6005–6008

Neuhaus J-M, Sticher L, Meins F, Boller T (1991) A short C-terminal sequence is necessary and sufficient for the targeting of chitinases to the plant vacuole. Proc Natl Acad Sci USA **88:** 10362–10366

Neuhoff V, Arold N, Taube D, Ehrhardt W (1988) Improved staining of proteins in polyacrylamide gels including isoelectric focusing gels with clear background at nanogram sensitivity using Coomassie brilliant blue G-250 and R-250. Electrophoresis **9:** 255–262

Payne G, Ahl P, Moyer M, Harper A, Bech J, Meins F, Ryals J (1990a) Isolation of complementary DNA clones encoding pathogenesis-related proteins P and Q, two acidic chitinases from tobacco. Proc Natl Acad Sci USA **87:** 98–102

Payne G, Ward E, Gaffney T, Ahl Goy P, Moyer M, Harper A, Meins F, Ryals J (1990b) Evidence for a third structural class of β-1,3-glucanase in tobacco. Plant Mol Biol **15:** 797–808

Raikhel NV, Wilkins TA (1987) Isolation and characterization of a cDNA clone encoding wheat germ agglutinin. Proc Natl Acad Sci USA **84:** 6745–6749

Schlumbaum A, Mauch F, Vögeli U, Boller T (1986) Plant chitinases are potent inhibitors of fungal growth. Nature **324:** 365–367

Sela-Buurlage MB, Ponstein AS, Vloemans SA, Melchers LS, Van den Elzen PJM, Cornelissen BJC (1993) Only specific tobacco chitinases and β-1,3-glucanases exhibit antifungal activity. Plant Physiol **101:** 857–863

Selsted ME, Martinez RJ (1980) A simple and ultrasensitive assay for the quantitative determination of lysozyme in the picogram range. Anal Biochem **109:** 67–70

Sequeira L (1983) Mechanisms of induced resistance in plants. Annu Rev Microbiol **37:** 51–97

Shinshi H, Neuhaus J-M, Ryals J, Meins F (1990) Structure of a tobacco endochitinase gene: evidence that different chitinase genes can arise by transposition of sequences encoding a cystein-rich domain. Plant Mol Biol **14:** 357–368

Shinshi H, Wenzler H, Neuhaus J-M, Felix G, Hofsteenge J, Meins F (1988) Evidence for N- and C-terminal processing of a plant defense-related enzyme: primary structure of tobacco prepro-β-1,3-glucanase. Proc Natl Acad Sci USA **85:** 5541–5545

Singh NK, Bracker CA, Hasegawa PM, Handa AK, Buckel S, Hermodson MA, Pfankoch E, Regnier FE, Bressan RA (1987) Characterization of osmotin: a thaumatin-like protein associated with osmotic adaptation in plant cells. Plant Physiol **85:** 529–536

Stanford A, Bevan M, Northcote D (1989) Differential expression within a family of novel wound-induced genes in potato. Mol Gen Genet **215:** 200–208

Van den Bulcke M, Bauw G, Castresana C, Van Montagu M, Vandekerckhove J (1989) Characterization of vacuolar and extracellular β(1,3)-glucanases of tobacco: evidence for a strictly compartmentalized plant defense system. Proc Natl Acad Sci USA **86:** 2673–2677

Van Loon LC (1990) The nomenclature of pathogenesis-related proteins. Physiol Mol Plant Pathol **37:** 229–230

Van Parijs J, Broekaert WF, Goldstein IJ, Peumans WJ (1991) Hevein: an antifungal protein from rubber-tree (*Hevea brasiliensis*) latex. Planta **183:** 258–264

Verwoerd TC, Dekker BMM, Hoekema A (1989) A small-scale procedure for the rapid isolation of plant RNAs. Nucleic Acids Res **17:** 2362

Vogelsang R, Barz W (1993) Purification, characterization and differential hormonal regulation of a β-1,3-glucanase and two chitinases from chickpea (*Cicer arietinum* L.). Planta **189:** 60–69

Ward ER, Uknes SC, Williams SC, Dincher SS, Wiederhold DL, Alexander DC, Ahl Goy P, Metraux J, Ryals JA (1991) Coordinate gene activity in response to agents that induce systemic acquired resistance. Plant Cell **3:** 1085–1094

Wirth SJ, Wolf GA (1990) Dye-labelled substrates for the assay and detection of chitinase and lysozyme activity. J Microbiol Methods **12:** 197–205

Woloshuk CP, Meulenhoff JS, Sela-Buurlage M, Van den Elzen PJM, Cornelissen BJC (1991) Pathogen-induced proteins with inhibitory activity toward *Phytophthora infestans*. Plant Cell **3:** 619–628

Yokosawa H, Ishii SI (1979) Immobilized anhydrotrypsin as a biospecific adsorbent for the peptides produced by trypsin-like proteases. Anal Biochem **98:** 198–203

Xu C, Moore CH, Fountain DW, Yu PL (1992) Purification and characterization of a new lectin from tamarillo fruit (*Cynhomandra betacea*). Plant Sci **81:** 183–189