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Proteolytic Processing of Class IV Chitinase in the Compatible Interaction of Bean Roots with *Fusarium solani*[1]

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Three chitinase isoenzymes, PvChiE, PvChiF, and PvChiG (molecular masses 29, 28, and 27 kD, respectively), were purified from bean (*Phaseolus vulgaris* L. cv Saxa) roots infected with the fungal pathogen *Fusarium solani* f. sp. *phaseoli*, and their amino acid sequence was partially determined. All sequences from all three isoenzymes exactly matched deduced amino acid sequences of the bean class IV chitinase PvChi4, formerly called PR4. The N terminus of PvChiF mapped to the hinge region, and the N terminus of PvChiG mapped to the catalytic domain of PvChi4. The N terminus of PvChiE was blocked. The appearance of PvChiE, PvChiF, and PvChiG correlated with an increase in protease activity in infected roots, and they could be generated in vitro by mixing extracts with high protease activity with extracts containing high amounts of PvChi4. Extracts from infected roots prepared in the presence of protease inhibitors also contained the processed forms of PvChi4, indicating that processing occurred in planta and not as an artifact of extraction. Processing of PvChi4 was not detected in incompatible interactions with a nonhost strain of *F. solani* and in symbiotic interactions with *Glomus mosseae*, and thus may be important only in compatible interactions with *F. solani*.

Plants respond to pathogen attack with an arsenal of defense responses, such as cell-wall reinforcement, production of antimicrobial compounds, and production of hydrolytic enzymes (Bowles, 1990). Chitinases (EC 3.2.1.14) are part of this response and have been studied in great detail (for reviews, see Collinge et al., 1993; Graham and Sticklen, 1994). Direct antifungal activity of chitinase was demonstrated in vitro (Schlumbaum et al., 1986), particularly in combination with β-1,3-glucanase (Mauch et al., 1988). In most plants studied, chitinases occur in several isoforms and, based on amino acid sequence information from purified proteins or cloned genes, they have been grouped into distinct classes (Meins et al., 1992, 1994). Class I chitinases are antifungal, vacuolar enzymes consisting of a Cys-rich domain, also called a hevein domain or a chitin-binding domain, a Pro-rich hinge or spacer region, and a catalytic domain. The Cys-rich domain is not necessary for antifungal or catalytic activity, but is essential for chitin binding, as shown for a tobacco class I chitinase (Iseli et al., 1993). Class IV chitinases are related in sequence but are secreted to the extracellular space (Collinge et al., 1993). Since not all chitinases have antifungal activity (Sela-Buurlage et al., 1993), specific chitinases might have different biological functions. Apart from a role in nonspecific defense, chitinases have also been implicated in plant growth and development (Neale et al., 1990; De Jong et al., 1993; Wemmer et al., 1994) and in the regulation of nodulation (Staehelin et al., 1994).

We are investigating the interactions between bean (*Phaseolus vulgaris*) roots and *Fusarium solani*, the causal agent of dry root rot. Bean plants are resistant to infection by the nonhost strain *F. solani* f. sp. *pisi*, a pathogen of peas, but highly susceptible to infection by the virulent strain, *F. solani* f. sp. *phaseoli* (Vögeli-Lange et al., 1995; note, we will call the nonhost strain "incompatible" and the virulent strain "compatible"). Previously, we presented a detailed microscopic analysis of bean roots inoculated with spores of compatible or incompatible strains of the fungus (Vögeli-Lange et al., 1995). We showed that spores of both *F. solani* f. sp. *pisi* and *F. solani* f. sp. *phaseoli* were able to bind to and germinate on the surface of bean roots, but only *F. solani* f. sp. *phaseoli* was able to penetrate into the root. Disease symptoms developed rapidly, indicating that the compatible fungus either avoided eliciting defense responses or, if defense responses were induced, they were probably ineffective. Here, we focus on chitinase expression in *Fusarium*-infected bean roots. Extracts of bean roots infected with a compatible strain of the fungus contained three new chitinase isoenzymes, designated PvChiE, PvChiF, and PvChiG, that occurred specifically in this type of interaction. N-terminal sequencing and sequencing of tryptic peptides revealed that these chitinases map to internal sequences of PR4 (Margis-Pinheiro et al., 1991), a bean class IV chitinase, which, according to recent recommendations concerning pathogenesis-related protein nomenclature, we propose to call PvChi4 (Meins et al., 1994; van Loon et al., 1994). The temporal pattern of appearance of PvChiE, PvChiF, and PvChiG correlated with an increase in protease activity in the roots. Furthermore, PvChiE, PvChiF, and PvChiG could be generated in vitro by mixing extracts with high protease activity with those containing high amounts of PvChi4. These results suggest that PvChiE, PvChiF, and PvChiG are proteolytic breakdown products of the extracellular, acidic bean class IV chitinase PvChi4. Proteolytic cleavage of PvChi4 occurred only in the compatible interaction of bean roots and

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Abbreviation: pCMB, *p*-chloromercuribenzoic acid.

F. solani, suggesting a biological function for processing of PvChi4 in this type of plant-fungus interaction.

MATERIALS AND METHODS

Plant and Fungal Material

Seeds of common bean (*Phaseolus vulgaris* L. cv Saxa) were surface-sterilized and grown in sterile vermiculite as described by Vögeli-Lange et al. (1995). The fungal strains *Fusarium solani* f. sp. *phaseoli* and *F. solani* f. sp. *pisi* were cultivated on V8 agar, and spores were collected as described by Vögeli-Lange et al. (1995).

Plant Treatments

For fungal infections, roots of 10-d-old seedlings grown in vermiculite were briefly washed, dipped into a suspension of fungal spores (1×10^6 spores/mL), and then cultivated as described above. Roots of control seedlings were dipped in distilled water. Harvest was 1 to 4 d after treatment. For colonization of bean roots with the arbuscular mycorrhizal fungus *Glomus mosseae*, a previously described container system was used (Wyss et al., 1991). Briefly, 4-d-old bean seedlings, pregerminated on agar plates, were transferred to a 1:1 (v/v) sand:soil mixture with 10% (v/v) soil inoculum consisting of *G. mosseae*-infected *Physalis* roots. Plants were harvested 45 d after co-cultivation with the fungus. At harvest approximately 72% of the total root length was colonized by the fungus, as determined by the grid line-intersect method (Giovanetti and Mosse, 1980). Ethylene treatment was performed as described by Boller et al. (1983), and mercuric chloride treatment was performed either by spraying primary leaves with a 0.2% (w/v) solution of mercuric chloride (Margis-Pinheiro et al., 1993) or by dipping roots into 100 μM mercuric chloride. Seedlings were harvested 4 d after ethylene treatment or 2 d after mercuric chloride treatment. Where appropriate, seedlings were separated into leaves and roots and frozen until used at −20°C for protein extracts or at −70°C for RNA preparations.

Protein Extracts

Frozen roots were ground to a fine powder under liquid nitrogen and extracted with a mortar and pestle in 0.1 M sodium acetate, pH 5.0, and 2 mM DTT (1:2 tissue:buffer). Protein extracts for immunoblot analysis were prepared in 50 mM Tris-HCl, pH 8.0, containing 500 mM NaCl and 0.2% (v/v) Triton X-100 (Sigma). Homogenates were centrifuged for 10 min at 10,000*g*, and the supernatants were used as a source for enzyme assays or SDS-PAGE.

Analytical Procedures

The protein concentration in extracts and column fractions was measured according to the method of Bradford (1976) with BSA as a standard. Chitinase activity was measured radiochemically as previously described (Boller et al., 1983). For protease assays, extracts were incubated in a final volume of 1.0 mL in 0.1 M Tris-HCl, pH 8.9, containing either 0.15% (w/v) azocasein (Sigma) or 0.5% (w/v) azocoll (Sigma) as substrates. Incubation was for 2 h at 37°C. The reaction was stopped by adding 50% (w/v) TCA to achieve a final concentration of 10% (w/v). The mixture then was kept for 30 min at 4°C to precipitate TCA-insoluble proteins and centrifuged for 10 min at 10,000*g*. The absorption of TCA-soluble peptides in the supernatant was determined at A_{330} (azocasein) or A_{520} (azocoll). One unit is defined as an increase in A_{330} or A_{520} of 1 g^{-1} fresh weight h^{-1}. For inhibitor studies, extracts were preincubated with inhibitor or appropriate solvent for 1 h at 4°C. Inhibitors were dissolved in the following solvents: pCMB in 0.2 N NaOH, pepstatin A in ethanol, leupeptin in water, PMSF in isopropanol, and EDTA in water, with subsequent adjustment to pH 8.0 with NaOH. The effectiveness of the inhibitors was tested with collagenase (0.1 μg) for EDTA and papain (0.1 μg) for pCMB.

Purification of PvChiE, PvChiF, and PvChiG

PvChiE, PvChiF, and PvChiG, collectively called PvChiEFG, were purified from *Fusarium*-infected bean roots. Each purification step was monitored by measuring chitinase activity and by analyzing the migration position of chitinase isoenzymes on activity gels. Four hundred grams of *F. solani* f. sp. *phaseoli*-infected roots were harvested 4 d after inoculation with the fungus, and a crude extract was prepared as described above. Following a 90% ammonium sulfate precipitation, proteins were resuspended in 20 mM *N*-methyl piperazine, pH 4.5 (buffer A), containing 2 mM DTT, and dialyzed overnight against 10 mM buffer A and 2 mM DTT. The protein extract was subjected to anion-exchange chromatography using a Pharmacia XK16 column packed with DEAE-Trisacryl connected to an Econo-system (Bio-Rad) equilibrated with buffer A. Proteins were eluted by increasing the concentration of sodium chloride from 0 to 310 mM. Fractions containing PvChiEFG, according to activity gels, were pooled, dialyzed overnight against distilled water, lyophilized, and resuspended in 500 μL of buffer A. Proteins were applied to a Mono-Q anion-exchange column (Pharmacia) on a fast protein liquid chromatography system (Pharmacia) equilibrated with buffer A. The flow-through, containing PvChiEFG, was dialyzed against distilled water, lyophilized, and resuspended in 20 mM Bis-Tris, pH 6.2 (buffer B). The proteins were separated on a Mono-Q column (Pharmacia) equilibrated with buffer B using a linear gradient of sodium chloride from 0 to 400 mM. Active fractions containing PvChiEFG were dialyzed against distilled water and lyophilized. For gel filtration, lyophilized samples were resuspended in 100 mM Tris-HCl, pH 8.0, and 200 mM sodium chloride (buffer C) and loaded onto a fast protein liquid chromatography Superdex 75 h 10/30 column (Pharmacia) equilibrated with buffer C. Active fractions were pooled, dialyzed against distilled water, and stored at −20°C. The purity of this fraction was tested by nondenaturing SDS-PAGE and silver staining.

Electrophoretic Procedures and Immunoblot Analysis

SDS-PAGE was performed according to the method of Laemmli (1970). Prestained molecular weight markers were from Bio-Rad. Silver staining was carried out as described by Blum et al. (1987). For activity staining of chitinases, lyophilized extracts were resuspended in "nondenaturing" sample buffer containing 15% (w/v) Suc, 2.5% (w/v) SDS, and 0.01% (w/v) bromphenol blue in 125 mM Tris-HCl, pH 6.7, and subjected to electrophoresis in 10% SDS-polyacrylamide gels supplemented with 0.01% (w/v) glycol chitin essentially as described by Trudel and Asselin (1989). After electrophoresis, the gels were incubated at 37°C for 1 h in 0.1 M sodium acetate, pH 5.0, containing 1% (v/v) Triton X-100 (Sigma) and subsequently stained with calcofluor white. After the gels were destained in water, lytic zones were visible under UV light as nonfluorescent dark bands against a fluorescent background.

Proteins in polyacrylamide gels were electrotransferred to nitrocellulose membranes (Towbin et al., 1979) and blocked in buffer (20 mM Tris, pH 7.5, and 180 mM NaCl) containing 5% (w/v) nonfat dry milk. The blots then were incubated with antiserum directed against PvChi4 chitinase (kindly provided by Dr. G. Burkard, Strasbourg, France) in a dilution of 1:1500 overnight at room temperature. Goat anti-rabbit IgG horseradish peroxidase conjugate (Bio-Rad) was used as a secondary antibody, and serological reactions were detected by staining with hydrogen peroxide and 4-chloronaphthol.

Protein Sequencing

Purified chitinases were separated by nondenaturing electrophoresis as described above and transferred to Immobilon-P membranes (Millipore). Protein bands corresponding to PvChiE, PvChiF, and PvChiG were excised from the membrane and used for peptide sequencing. Tryptic peptides were generated as described by Fernandez et al. (1994), and N-terminal amino acid sequences and sequences of tryptic peptides were determined by automated Edman degradation using an Applied Biosystems 477A protein sequenator with on-line detection of phenylthiohydantoin-amino acids (Jenö et al., 1995).

IEF

Fifteen milliliters of protein extract containing 2% (v/v) ampholyte (Bio-Rad) with a pH range from 3.0 to 10.0 were separated in an 18-mL Rotofor cell (Bio-Rad) at 12 W of constant power for 3.5 h. Active fractions were pooled and refocused at 10 W of constant power for 1 h. pIs were determined by reading the pH values of active fractions.

Proteolytic Processing of PvChi4 in Vitro

Protein extracts containing high amounts of PvChi4 were prepared by homogenizing mercuric chloride-sprayed leaves in 0.1 M sodium acetate, pH 5.0 (de Tapia et al., 1986). After the sample was centrifuged at 10,000*g* for 10 min, the supernatant was adjusted to pH 8.0 by adding sodium hydroxide and used as a source of PvChi4. Protein extracts with high protease activity were prepared as described above from bean roots infected with the compatible pathogen, *F. solani* f. sp. *phaseoli*. For in vitro processing experiments, 30 μL of mercuric chloride-induced leaf extract were mixed with 3 μL of extract of *Fusarium*-induced roots and incubated for 45 min at 37°C. After incubation, samples were lyophilized and analyzed for the pattern of chitinase isoenzymes on enzyme activity gels. Either extract alone or a mixture of boiled extracts was used as a control.

Nucleic Acid Analyses

All manipulations were carried out according to standard procedures (Sambrook et al., 1989). RNA was extracted from frozen tissue by a hot phenol method. For RNA gel-blot analysis, total RNA was electrophoresed through formaldehyde gels and blotted onto nylon membranes (NY13N; Schleicher & Schuell). The blots were hybridized under low-stringency conditions, i.e. 50°C, in 6× SSC, 5 × Denhardt's solution, 0.5% (w/v) SDS, and 100 μg/mL carrier DNA with an α-^{32}P random primed labeled DNA probe of Pv*Chi*4 and washed three times for 20 min each at 50°C in 1× SSC and 0.1% (w/v) SDS. Hybridization signals were analyzed using a GS-250 molecular imager (Bio-Rad).

Sequencing of the Pv*Chi*4 cDNA clone (Margis-Pinheiro et al., 1991) was performed using a Sequencing Pro Kit (Toyobo, Osaka, Japan) following the manufacturer's instructions.

RESULTS

Induction of New Chitinase Isoenzymes in Bean Roots Infected with the Compatible Pathogen *F. solani* f. sp. *phaseoli*

Microscopic observations describing the interactions between bean roots and *F. solani* have been previously described (Vögeli-Lange et al., 1995). In this study, we measured chitinase activity in bean roots infected with *F. solani* and compared it with roots exposed to other biotic or abiotic treatments (Fig. 1A). Infection with the compatible pathogen *F. solani* f. sp. *phaseoli* caused an approximately 5-fold increase, and infection with the incompatible pathogen *F. solani* f. sp. *pisi* caused only an approximately 2-fold increase in chitinase activity. Roots heavily colonized by the arbuscular mycorrhizal fungus *G. mosseae* had the same activity as controls, whereas an ethylene treatment caused an approximately 14-fold induction of chitinase activity. A large part of the increase in total activity in infected roots and most of the increase in ethylene-treated roots were due to an increase in basic class I chitinase (data not shown). Enzyme activity gels were made with the various extracts, loading equal amounts of total activity. It is surprising that basic class I chitinase could not be seen on the gels, indicating that only select chitinases are visualized by the method used. On the other hand, a number of different chitinase isoenzymes were apparent on the activity gels: two constitutively expressed chitinase isoenzymes, PvChiA and PvChiB, were detected in all samples (Fig. 1B), and, more interestingly, five additional isoenzymes, PvChiC,

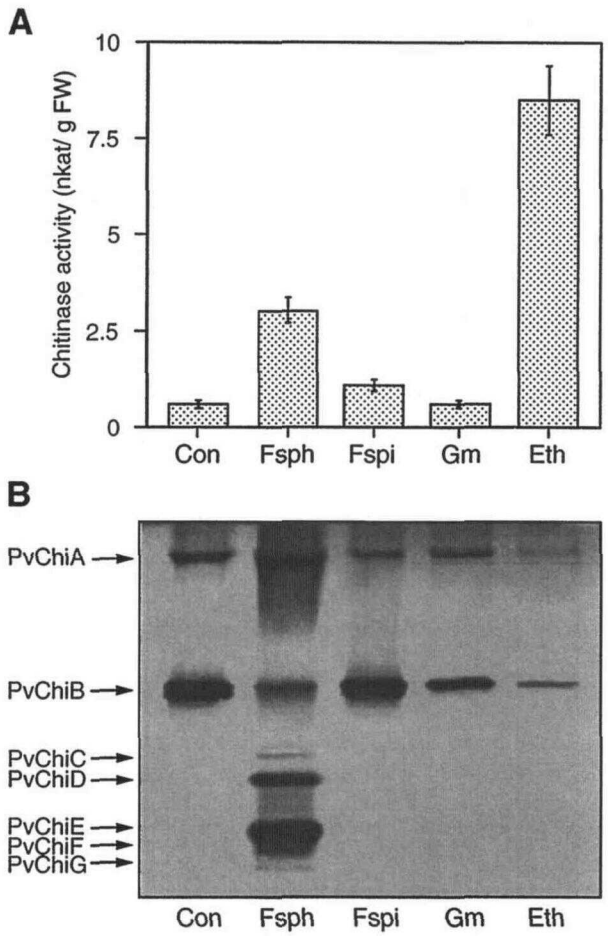

Figure 1. A, Comparison of total chitinase activity in extracts of bean roots exposed to different biotic or abiotic treatments. Data are means ± SE of 12 replicates from three independent experiments. B, Activity gel demonstrating isoforms of chitinase in protein extracts from bean roots. Ammonium sulfate-precipitated protein extracts were separated in glycol chitin-containing polyacrylamide gels and stained for chitinase activity with calcofluor white. Equal amounts of chitinase activity (7.5 pkat) were loaded per lane. Con, Control roots; Fsph, *F. solani* f. sp. *phaseoli*-infected roots; Fspi, *F. solani* f. sp. *pisi*-infected roots; Gm, roots colonized by the arbuscular mycorrhizal fungus *G. mosseae*; Eth, roots from ethylene-treated seedlings; FW, fresh weight.

PvChiD, PvChiE, PvChiF, and PvChiG, appeared after infection with the compatible pathogen. These proteins were absent from roots inoculated with spores of the incompatible fungus *F. solani* f. sp. *pisi*, from roots colonized by the arbuscular mycorrhizal fungus *G. mosseae*, and from roots treated with the stress hormone ethylene.

Two of the *F. solani* f. sp. *phaseoli*-induced chitinase isoenzymes, PvChiC and PvChiD, were also induced by the abiotic stress compound mercuric chloride. Therefore, PvChiE, PvChiF, and PvChiG, which appeared specifically in the compatible interaction between bean roots and *F. solani*, were chosen for further analysis. These isoenzymes migrated with R_f values of 0.81 (PvChiE), 0.85 (PvChiF), and 0.91 (PvChiG) in 10% polyacrylamide gels, with PvChiE producing the most prominent lytic zone in glycol chitin-containing polyacrylamide gels.

Purification and Properties of PvChiE, PvChiF, and PvChiG

To learn more about the nature of PvChiE, PvChiF, and PvChiG, collectively called PvChiEFG, the proteins were purified from *Fusarium*-infected bean roots using several steps of anion-exchange and gel-filtration chromatography (Table I). In step 2 (DEAE-Trisacryl, pH 4.5), isoenzymes PvChiEFG, PvChiB, PvChiC, and PvChiD were retained on the column, attesting to their acidic nature, whereas PvChiA and the dominant basic class I chitinase, providing most of the chitinase activity in infected roots, were in the flow-through. In step 3 (Mono-Q, pH 4.5), PvChiEFG could be separated from the more acidic isoforms PvChiB, PvChiC, and PvChiD, which were retained by the column. The flow-through of step 3, containing PvChiEFG, was reapplied to the same column, which was then equilibrated to pH 6.2 (step 4). The eluate of step 4, containing PvChiEFG, was then applied to a gel-filtration column, and in the final preparation all three proteins were eluted in a single peak. The purity of this fraction was tested by nondenaturing SDS-PAGE (Fig. 2). One lane was silver-stained for protein, and a replicate lane was stained for chitinase activity. PvChiE, PvChiF, and PvChiG could not be separated from each other by charge or according to size by the chromatographic methods used, suggesting that they have similar physicochemical properties. Silver staining, however, showed that the final preparation was free of contaminating proteins.

Table I. *Purification of the chitinase isoforms PvChiE, PvChiF, and PvChiG from F. solani f. sp. phaseoli-infected bean roots*

PvChiEFG were purified to homogeneity from 400 g of roots harvested 4 d after infection, as described in "Materials and Methods." The three isoforms were eluted in a single peak after the final gel-filtration step.

Purification Step	Total Chitinase Activity	Protein	Specific Activity[a]	Purification	Yield
	nkat	*mg*		*-fold*	*%*
Crude extract	1100	230	4.8	1	100
Protein extract[b]	676	172	3.9	1	61.5
DEAE, pH 4.5 eluate	39.8	2.8	14.4	3	3.6
Mono-Q, pH 4.5 flow-through	19.9	1.4	14.2	3	1.8
Mono-Q, pH 6.2 eluate	19.1	0.09	212.2	44	1.8
Gel filtration	8.3	0.03	276.7	58	0.8

[a] Specific chitinase activity = nkat mg^{-1} protein. [b] Ammonium sulfate-precipitated crude extract.

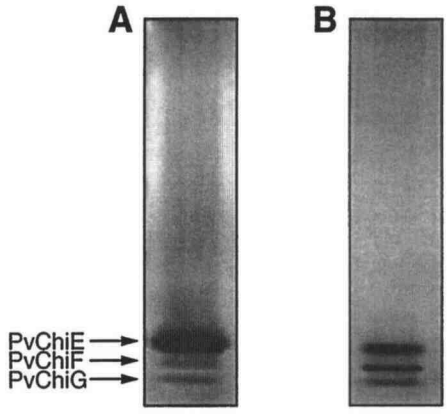

Figure 2. Analysis of chitinase isoforms PvChiE, PvChiF, and PvChiG induced by *F. solani* f. sp. *phaseoli*. Purified proteins were resuspended in nondenaturing sample buffer and separated in glycol chitin-containing polyacrylamide gels. A, Chitinase enzyme activity staining. B, Silver staining.

The pI of PvChiEFG was at approximately pH 6.0 as determined by IEF, and gel-filtration analysis revealed a molecular mass of about 24 kD (data not shown). PvChiEFG could be separated by denaturing SDS-PAGE to yield 29, 28, and 27 kD for PvChiE, PvChiF, and PvChiG, respectively (data not shown). The pH optimum of PvChiEFG for hydrolysis of [^{3}H]chitin was pH 5.0, and the temperature optimum was 46°C (data not shown).

PvChiE, PvChiF, and PvChiG Are Related to PvChi4, a Bean Class IV Chitinase

Automated Edman degradation was performed to obtain N-terminal amino acid sequences of the three *Fusarium*-induced chitinases. The N terminus of PvChiE was blocked. However, unexpectedly, the N termini of both PvChiF and PvChiG could be aligned to internal sequences of the deduced amino acid sequence of Pv*Chi*4, a bean class IV chitinase (Margis-Pinheiro et al., 1991) (Fig. 3A). The N terminus of PvChiF mapped to the start of the hinge region, and the N terminus of PvChiG mapped to the transition between the hinge region and the catalytic domain of PvChi4. Initial discrepancies in the alignment of peptide sequences and deduced amino acid sequences of Pv*Chi*4 were eliminated after the Pv*Chi*4 cDNA clone was resequenced. An updated version of the Pv*Chi*4 cDNA sequence has been deposited in the database. All three prolyl residues in the N terminus of PvChiF were partially hydroxylated. The occurrence of Hyp in the Pro-rich hinge region was previously demonstrated for a tobacco class I (Sticher et al., 1993) and a sugar beet class IV (Nielsen et al., 1994) endochitinase. The function of prolyl hydroxylation in chitinases is unknown. Amino acid sequences of tryptic peptides obtained from PvChiE and PvChiF were identical with deduced amino sequences of Pv*Chi*4. In this case, peptide sequences could be aligned to sequences in the catalytic domain of PvChi4 (Fig. 3B). These data suggest that PvChiE, PvChiF, and PvChiG either are proteolytic breakdown products of the extracellular acidic bean class IV chitinase PvChi4 or are derived from Pv*Chi*4-related transcripts lacking the chitin-binding domain. Thus, we predicted that full-length transcripts encoding PvChi4 and/or shorter versions accumulated in infected roots.

PvChiEFG Are Posttranslational Processing Products of PvChi4

To determine which form(s) of Pv*Chi*4 transcripts accumulated in infected roots, RNA gel blots were performed under low-stringency conditions (Fig. 4A). In *Fusarium*-infected roots, we detected only one Pv*Chi*4 transcript, which was identical in size with the one induced by mercuric chloride, a known inducer of PvChi4 (de Tapia et al., 1986; Margis-Pinheiro et al., 1991). Similar results were obtained when RNA gel blots were processed under high-stringency conditions (data not shown). Thus, it appears that only full-length transcripts of Pv*Chi*4 were induced, and it therefore is unlikely that PvChiE, PvChiF, and PvChiG are products of Pv*Chi*4-related transcripts lacking the chitin-binding domain. Conversely, several proteins cross-reacting with the PvChi4 antibody were detected in infected roots (Fig. 4, B and C). Based on comparison with extracts from mercuric chloride-treated leaves, which served as a positive control, the largest of these proteins (apparent molecular mass 33.5 kD) corresponded to the authentic PvChi4 protein. The other three proteins cross-reacting with the PvChi4 antibody, migrating with appar-

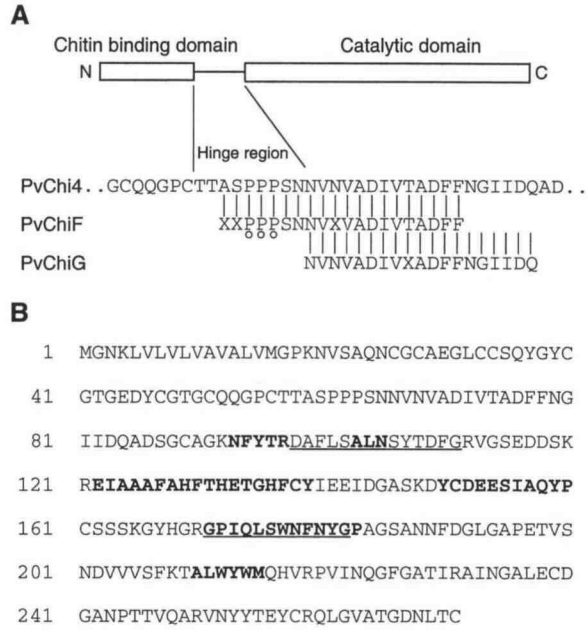

Figure 3. Comparison of the deduced amino acid sequence of Pv*Chi*4 (Margis-Pinheiro et al., 1991) to N-terminal and internal amino acid sequences of PvChiE, PvChiF, and PvChiG determined by Edman degradation. A, Alignment of N-terminal amino acid sequences of PvChiF and PvChiG to PvChi4. X, Unidentified residues; o, partially hydroxylated prolyl residues. B, Alignment of tryptic peptides of PvChiE (bold) and PvChiF (underlined) to the deduced amino acid sequence of Pv*Chi*4. The deduced amino acid sequence shown is the one obtained after resequencing the Pv*Chi*4 cDNA clone.

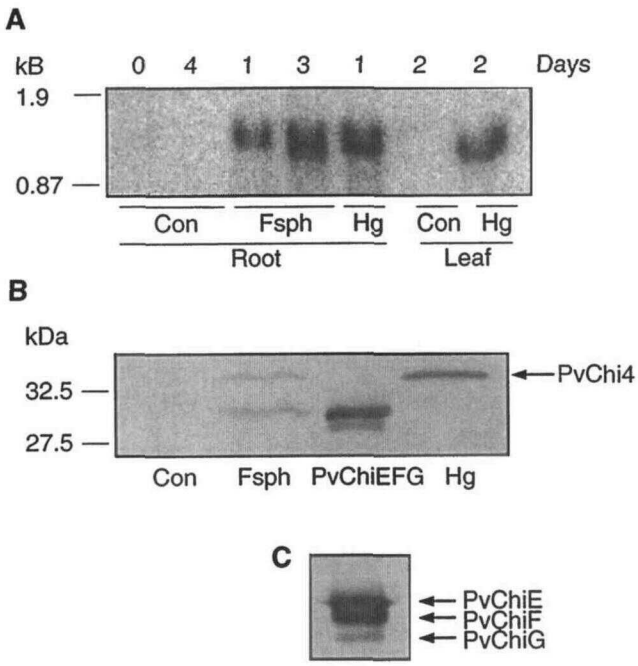

Figure 4. Accumulation of PvChi4 and its transcripts compared with the accumulation PvChiEFG. A, RNA gel-blot analysis. Total RNA (20 μg) isolated from control (Con) samples or from samples treated with *F. solani* f. sp. *phaseoli* (Fsph) or mercuric chloride (Hg) was separated on a denaturing agarose gel and blotted onto a nylon membrane. The blot was hybridized with a ^{32}P-labeled *PvChi4* cDNA probe under low-stringency conditions, i.e. 50°C, and washed at the same temperature three times in 1× SSC and 0.1% (w/v) SDS. B, Immunoblot analysis. Crude extracts from untreated (Con) or *F. solani* f. sp. *phaseoli*-infected (Fsph) roots or from mercuric chloride-treated (Hg) leaves were compared with purified PvChiEFG on immunoblots probed with antiserum against PvChi4. In C, higher amounts of PvChiEFG were loaded.

ent molecular masses of 29, 28, and 27 kD, corresponded to purified PvChiE, PvChiF, and PvChiG. These results indicate that PvChi4 accumulates in response to infection and mercuric chloride and that PvChiEFG are products of post-translational processing events of PvChi4 that are formed only in infected roots and not in those treated with mercuric chloride.

We examined the appearance of PvChi4 and its processing products in a time-course experiment (Fig. 5A). A strong signal for PvChi4 was apparent after 1 d of infection, and the proteolytic processing product PvChiE became visible 1 d later. This result correlated with the appearance of PvChiEFG on chitinase enzyme activity gels (Fig. 5B). On these activity gels, PvChi4 was visible as a faint band just below PvChiB. The identity of this band as PvChi4 was established by immunoblot analysis of such gels (data not shown). Protease activity, measured with the substrate azocoll, accumulated in bean roots infected with the compatible but not the incompatible fungal pathogen (Fig. 5C). Negligible amounts of protease activity accumulated in mercuric chloride-treated roots. Similar results were obtained when azocasein was used as a substrate (data not shown). The increase in protease activity correlated with the appearance of PvChiEFG on chitinase enzyme activity gels (Fig. 5B) and on immunoblots probed with antibodies directed against PvChi4 (Fig. 5A), suggesting that PvChiEFG are proteolytic processing products of PvChi4. To directly test this hypothesis, extracts containing high amounts of PvChi4 and a dilute extract of *Fusarium*-infected roots containing high protease activity were mixed and, after incubation for 45 min at 37°C, analyzed on chitinase enzyme

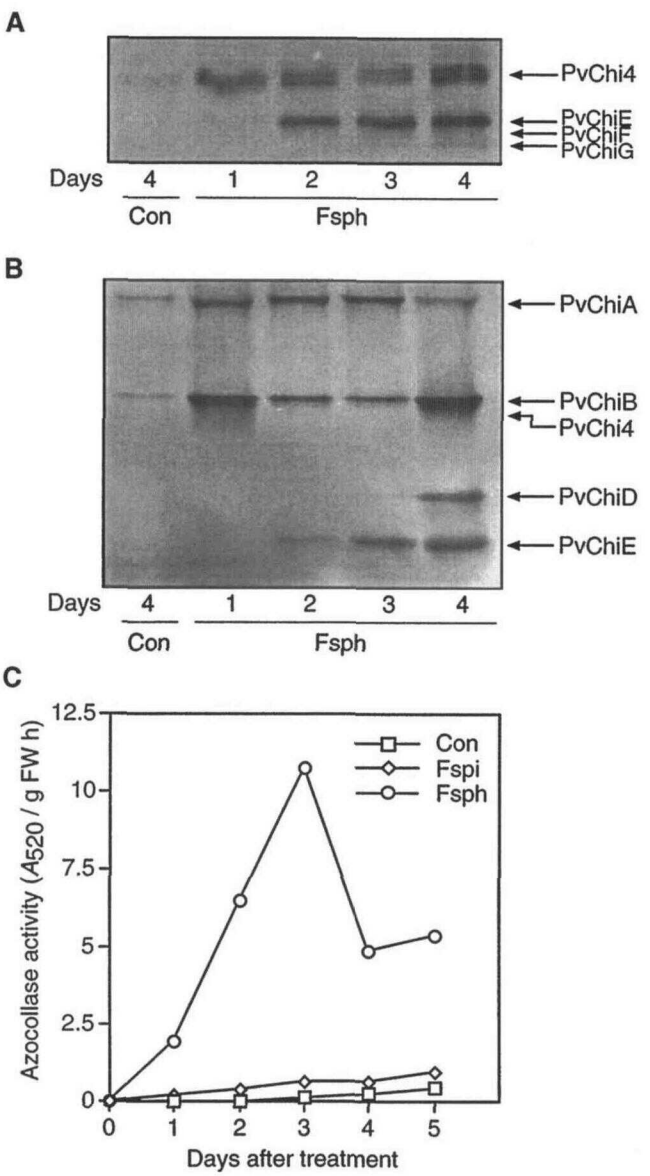

Figure 5. Correlation between protease activity and appearance of PvChiE in *F. solani* f. sp. *phaseoli*-infected bean roots. Detection of chitinases was accomplished by immunoblot analysis using antiserum against PvChi4 (A) or in enzyme activity gels (B). A time course of protease activity was measured with the substrate azocoll (C). Occasionally, a double band was observed for PvChi4 on immunoblots. Samples were compared on a fresh weight (FW) basis. Con, Control; Fsph, *F. solani* f. sp. *phaseoli*-infected roots; Fspi, *F. solani* f. sp. *pisi*-infected roots.

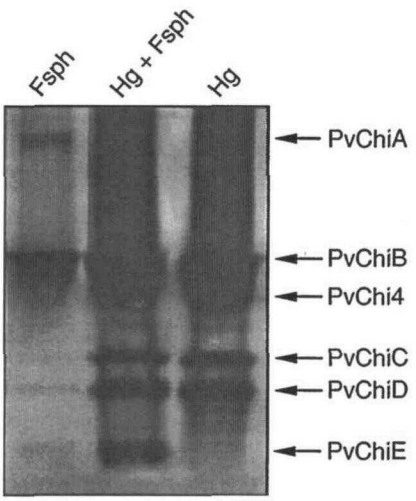

Figure 6. In vitro generation of PvChiE. Extracts from mercuric chloride-treated bean leaves (Hg) and a diluted sample from *F. solani* f. sp. *phaseoli*-infected bean roots (Fsph) were incubated either alone or in combination (Hg + Fsph) at 37°C for 45 min. Subsequently, the samples were separated in a glycol chitin-containing polyacrylamide gel and stained for chitinase activity.

activity gels (Fig. 6). Very little or no PvChiEFG was detected in either extract incubated alone; however, a strong band migrating with an R_f value corresponding to PvChiE was detected after the two extracts were mixed.

In other experiments, PvChiF and PvChiG were also detected. PvChiEFG could not be generated by mixing extracts containing high amounts of PvChi4 with known proteases such as collagenase or papain (data not shown). After treatment with papain, PvChi4 was completely degraded, whereas collagenase treatment had no effect. Protease inhibitor studies (Table II) showed that in *Fusarium*-infected bean roots protease activity was strongly inhibited by EDTA, an inhibitor of metalloproteases, and to a lesser extent by pCMB, which inhibits Cys proteases. Inhibitors of Ser or aspartyl proteases had no significant effect on protease activity in extracts of *Fusarium*-infected bean roots. Protease activity was maximal when measured at an alkaline pH (data not shown). The origin of the PvChi4-processing protease is unknown, since this activity was absent from control roots and from fungal hyphae grown in vitro (data not shown).

Table II. *Effects of inhibitors on protease activity*

Protease activity was determined in *F. solani* f. sp. *phaseoli*-infected bean roots in either the presence or absence of protease inhibitors with azocasein as the substrate as described in "Materials and Methods." Activities are expressed as percentages of the corresponding solvent controls.

Inhibitor	Concentration	Remaining Activity
	mM	%
EDTA	1	14
pCMB	2	71
PMSF	2	90
Pepstatin A	0.03	97
Leupeptin	0.04	100

Proteolytic Processing of PvChi4 Occurs in Vivo and Is Not an Artifact of Extraction

To distinguish whether processing of PvChi4 occurs in vivo or whether it is an artifact of extraction, protein extracts were prepared from *Fusarium*-infected roots in the absence and presence of 2 mM EDTA, an inhibitor of protease activity in *Fusarium*-infected bean roots (Table II). Immunoblot analysis was used for detection of PvChi4 and its breakdown products. As shown in Figure 7A, PvChiE, the major proteolytic breakdown product of PvChi4, was present in similar amounts in extracts prepared either with or without the protease inhibitor EDTA. Similarly, PvChiE also was detected when samples were extracted directly into SDS-PAGE sample buffer (data not shown). No breakdown products were observed in either leaves or roots treated with mercuric chloride. These results indicate that proteolytic processing of PvChi4 occurs in vivo and is not an artifact of extraction. As a control, immunoblots were probed for expression of the vacuolar class I chitinase, which is related in sequence to the extracellular class IV chitinase. Like the class IV chitinase, we also detected a smaller molecular weight band, PvChiH, cross-reacting with the class I chitinase antibody, which was present in bean roots infected with the compatible but not the incompatible fungal pathogen (Fig. 7B). Affinity chromatography on colloidal chitin suggested that PvChiH is a breakdown product of class I chitinase, which lacks the chitin-binding domain (data not shown). However, in this case, PvChiH appeared only when extracts were prepared in the absence of EDTA, the protease inhibitor. Taken together these results suggest that class I chitinase and the processing protease only have contact during tissue disintegration, but

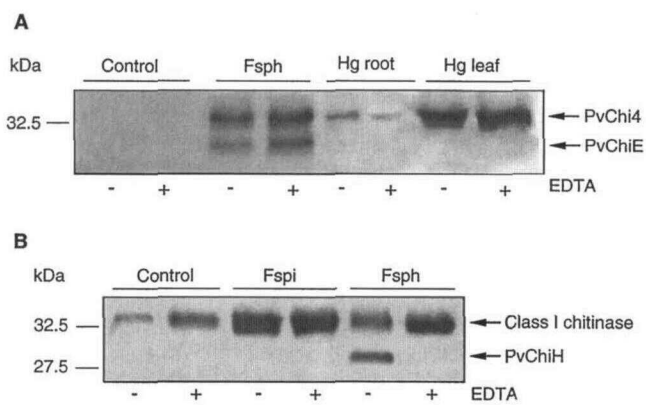

Figure 7. Immunoblot analysis demonstrating the effect of EDTA on the processing of class IV and class I chitinase. Extracts from roots treated with *F. solani* f. sp. *phaseoli* (Fsph) or *F. solani* f. sp. *pisi* (Fspi) or from mercuric chloride-treated (Hg) roots and leaves were extracted in either the absence (−) or presence (+) of 2 mM EDTA. Equal amounts of total protein (10 μg) were loaded per lane. In A, the immunoblot was probed with antiserum directed against PvChi4, and in B, the immunoblot was probed with antiserum directed against class I chitinase.

that class IV chitinase and the processing protease are co-localized in the same cellular compartment. Our results also suggest that class I and class IV chitinase are processed by the same enzyme.

DISCUSSION

Fusarium-Infected Bean Roots Contain Proteolytically Processed Forms of PvChi4

Three new chitinase isoenzymes were detected specifically in bean roots infected with the compatible fungal pathogen *F. solani* f. sp. *phaseoli*. These isoenzymes were absent from roots inoculated with an incompatible strain of the fungus, from roots heavily colonized by the arbuscular mycorrhizal fungus *G. mosseae*, and from roots treated with abiotic elicitors. We provide evidence that these three chitinase isoenzymes are proteolytic processing products of the extracellular, acidic bean class IV chitinase PvChi4: (a) N-terminal amino acid sequences and sequences of tryptic peptides of PvChiE, PvChiF, and PvChiG mapped to internal regions of the deduced amino acid sequence of Pv*Chi*4; (b) PvChi4, purified PvChiE, PvChiF, and PvChiG cross-reacted with antibodies directed against PvChi4, but there was only one Pv*Chi*4 transcript; (c) protease activity increased in bean roots treated with the compatible but not the incompatible fungal pathogen, and correlated in time with the appearance of PvChiEFG; and (d) PvChiEFG could be produced in vitro by mixing extracts with high protease activity with extracts containing high amounts of PvChi4. PvChiEFG could also be detected when roots were extracted in the presence of protease inhibitors (Fig. 7A), or when roots were extracted directly into SDS-PAGE sample buffer (data not shown). These data indicate that PvChiEFG are generated in planta and not during or after extraction.

Protein-sequencing data in conjunction with size determination analysis by SDS-PAGE and gel filtration suggest that PvChiE, PvChiF, and PvChiG are sequential processing products of PvChi4, that lack the chitin-binding domain. The N terminus of PvChiF mapped to the hinge region and the N terminus of PvChiG mapped to the beginning of the catalytic domain of the deduced amino acid sequence of Pv*Chi*4. Even though the N terminus of PvChiE, the largest of the three chitinase isoenzymes, was blocked, mixing experiments (Fig. 6) strongly suggest that PvChiE is also a proteolytic breakdown product of PvChi4 and not an unmodified form of the enzyme lacking prolyl hydroxylation (Fig. 3). Furthermore, despite the fact that the chitin-binding domain of PvChi4 contains potential trypsin cleavage sites, none of the tryptic peptides obtained from PvChiE mapped to this region of the protein (Fig. 3). Two additional acidic chitinase isoenzymes, PvChiC and PvChiD, were detected in extracts of bean roots infected with the compatible fungal pathogen (Fig. 1B). On chitinase enzyme activity gels, these isoforms migrated to positions between PvChi4 and PvChiEFG (Fig. 5B). Although we cannot completely rule out the possibility that PvChiC and PvChiD are intermediate processing products of PvChi4 leading to the accumulation of PvChiEFG, we think it is unlikely that they are precursors to PvChiEFG. $HgCl_2$-treated leaves accumulated large amounts of PvChiC and PvChiD, and yet PvChiEFG were not detected in such leaves (Fig. 6).

Processing of PvChi4—Activation or Inactivation of the Plant Defense?

In compatible plant microbe interactions, the microorganism can live and replicate within the host tissue. Thus, adaptation to the host's defense responses must have occurred. Therefore, proteolytic processing of PvChi4 might be part of the pathogen's strategy to inactivate the plant's defense response. Alternatively, processing of PvChi4 could diversify the plant's defense response by generating multiple chitinase isoforms with perhaps different substrate specificities. Chitinase enzyme activity tests with purified enzymes showed that the processed forms of PvChi4, PvChiE, PvChiF, and PvChiG are active chitinases. Removal of the chitin-binding domain may result in enzymes with altered substrate-binding properties or altered substrate specificity (Iseli et al., 1993). It cannot be excluded, however, that the processed forms of PvChi4 are more accessible to degradation. An exochitinase from *Streptomyces olivaceovirides* exists in an unprocessed form and in a proteolytically processed form lacking the chitin-binding domain (Blaak and Schrempf, 1995). The processed enzyme was shown to hydrolyze crystalline α-chitin (as it occurs in fungi) less efficiently than the unprocessed one (Blaak and Schrempf, 1995). Colloidal chitin and chitooligomers, on the other hand, were degraded equally well by the two forms of the enzyme. Similar results were obtained for a chitinase from *Bacillus circulans* (Watanabe et al., 1990) and a cellulase from *Streptomyces reticuli* (Schlochtermeier et al., 1995), neither of which bound to their respective substrates when the binding domains had been removed.

Previously described pathogenesis-related protein-processing proteases were of plant origin and were expressed constitutively (Rodrigo et al., 1989, 1991). Conversely, several inducible microbial proteases have been implicated in the establishment of antagonistic and mutually beneficial symbioses (Ball et al., 1991; Goetting-Minesky and Mullin, 1994; Lindstrom and Belanger, 1994). The PvChi4-processing protease was absent from control roots and from fungal hyphae grown in vitro (data not shown). Thus, this activity is induced specifically in the compatible interaction between bean roots and the root rot pathogen. The protease was most active at alkaline pH, and activity was strongly inhibited by EDTA, an inhibitor of metalloproteases. Proteases with similar properties have previously been detected in the cell walls of common bean (Van der Wilden et al., 1983), in soybean leaves (Ragster and Chrispeels, 1979), and also in *Fusarium* (Morita et al., 1994). Future studies will show whether the PvChi4-processing protease is of plant or fungal origin.

The processing of chitinase in *Fusarium*-infected bean roots was not only demonstrated for the extracellular class IV chitinase PvChi4, but also for the vacuolar class I enzyme (Fig. 7). In this case, however, removal of the chitin-binding domain occurred during tissue homogenization and not in vivo, as in the case of PvChi4. Since protease activity was detected in the intercellular washing fluid

from *Fusarium*-infected bean roots (data not shown), PvChi4 and protease might be localized in the same cellular compartment. The vacuolar class I chitinase, on the other hand, may only contact the enzyme during homogenization and perhaps upon fungal penetration of host cells. No processing products were detected on immunoblots developed with class I β-1,3-glucanase antibodies (data not shown), indicating that pathogenesis-related protein processing is specific.

Posttranslational removal of the chitin-binding domain has been demonstrated for hevein, a lectin from the latex of the rubber tree (Lee et al., 1991), and suggested for stinging nettle lectin, a protein composed of two copies of the chitin-binding domain fused to a chitinase-like catalytic domain (Lerner and Raikhel, 1992), but the corresponding proteases are not known. Future studies will show whether the PvChi4-processing protease may be such a candidate. Furthermore, it will be of importance to determine whether processing of PvChi4 is of biological importance for the bean-*F. solani* interaction.

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