Pathogenesis-Related PR-1 Proteins Are Antifungal

Isolation and Characterization of Three 14-Kilodalton Proteins of Tomato and of a Basic PR-1 of Tobacco with Inhibitory Activity against Phytophthora infestans

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Three distinct basic 14-kD proteins, P14a, P14b, and P14c, were isolated from tomato (Lycopersicon esculentum Mill. cv Baby) leaves infected with Phytophthora infestans. They exhibited antifungal activity against P. infestans both in vitro (inhibition of zoospore germination) and in vivo with a tomato leaf disc assay (decrease in infected leaf surface). Serological cross-reactions and amino acid sequence comparisons showed that the three proteins are members of the PR-1 group of pathogenesis-related (PR) proteins. P14a and P14b showed high similarity to a previously characterized P14, whereas P14c was found to be very similar to a putative basic-type PR-1 from tobacco predicted from isolated DNA clones. This protein, named PR-1g, was purified from virus-infected tobacco (Nicotiana tabacum Samsun NN) leaves and characterized by amino acid microsequencing, along with the well-known acidic tobacco PR-1a, PR-1b, and PR-1c. The various tomato and tobacco PR-1 proteins were compared for their biological activity and found to display differential fungicidal activity against P. infestans in both the in vitro and in vivo assays, the most efficient being the newly characterized tomato P14c and tobacco PR-1g.

In many plant species, response to infection by pathogenic bacteria, viruses, and fungi or to various abiotic stresses is accompanied by the synthesis of a variety of host proteins, several of which are termed PR proteins (Van Loon, 1985; Carr and Klessig, 1989; Bol et al., 1990; Linthorst, 1991; Stintzi et al., 1993). The first representatives of this type of proteins were detected by Van Loon and Van Kammen (1970) and by Gianinazzi et al. (1970) in leaves of tobacco (Nicotiana tabacum cv Samsun NN and Xanthi-nc, respectively) after tobacco mosaic virus infection. Since then, many proteins with similar properties have been isolated from tobacco and from other plant species as well, including both dicots and monocots (Carr and Klessig, 1989; Linthorst, 1991). These proteins have physicochemical properties that enable them to resist to acidic pH and proteolytic cleavage and thus survive in the harsh environments where they occur: the vacuolar compartment or cell wall or intercellular spaces. Based first on serological properties and later on sequence data, the major tobacco PR proteins have been classified into five major groups (Bol et al., 1990; Linthorst, 1991), which are also found in tomato.

These proteins have attracted considerable interest because of their possible causal role in resistance suggested by their high induction during induced local and systemic resistance. Two groups of tobacco PR proteins have known catalytic functions: PR-2 proteins have β-1,3-glucanase activity and PR-3 proteins have chitinase and lysozyme activity (Kauffmann et al., 1987; Legrand et al., 1987; Stintzi et al., 1993). Similarly, a number of tomato PR proteins were identified as chitinases and β-1,3-glucanases (Fischer et al., 1989; Joosten and De Wit, 1989). Chitinases and β-1,3-glucanases have been shown to have antifungal activity in vitro, particularly when assayed in combinations (Mauch et al., 1988; Sela-Buurlage et al., 1993). Recently, an antifungal activity has also been demonstrated for a tobacco PR protein of group 4 (Ponstein et al., 1994) and for thaumatin-like proteins of group 5 from maize (Vigers et al., 1991), barley (Heegaard et al., 1991), tobacco, and tomato (Woloshuk et al., 1991; Vigers et al., 1992).

Surprisingly, very little is known concerning the biological activity and function of the first discovered proteins, those of group PR-1. No resistance was found against viruses in transgenic tobacco plants expressing PR-1a and PR-1b (Cutt et al., 1989; Linthorst et al., 1989). Recently, however, it was reported (Alexander et al., 1993) that transgenic tobacco plants expressing high levels of protein PR-1a exhibited significantly reduced disease symptoms caused by infection with two oomycete pathogens, Peronospora tabacina and Phytophthora parasitica var nicotianae. This represented the first evidence, although indirect, of antifungal activity of a PR-1 protein.

In a preliminary short report we have described a direct fungicidal activity toward Phytophthora infestans of a PR-1 protein from tomato (Niderman et al., 1993). The protein assayed, P14, was found to be induced in response to infection by various pathogens (Camacho Henriquez and

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Abbreviation: PR, pathogenesis-related.
Sänger, 1982, 1984; De Wit and Van der Meer, 1986; Nas-suth and Sänger, 1986; Christ and Mösinger, 1989; Joosten and De Wit, 1989). It was purified and shown to be similar to the tobacco PR-1 proteins (Camacho Henríquez and Sänger, 1984; Lucas et al., 1985; Cornelissen et al., 1986). It was found later that the pattern of tomato PR proteins in the 15-kD range was more complicated than initially described, at least during the response to infection by *Cladosporium fulvum* (Joosten et al., 1990).

The aim of this study was to further characterize the fungicidal activity of the P14 protein (Niderman et al., 1993) and of other tomato and tobacco proteins related to the PR-1 family. Here we show that infection of tomato leaves with *P. infestans* induced the production of as many as three distinct P14 proteins, which were purified to homogeneity and demonstrated to be members of the PR-1 family by amino acid sequence comparisons. They exhibited differential antifungal activity in vitro and in vivo (leaf disc assay) toward *P. infestans*. The protein with the higher antifungal activity, P14c, showed only low sequence similarity (65%) to the initially described P14 (Lucas et al., 1985) but high similarity (94%) to the basic tobacco PR-1 predicted from a cDNA clone (Cornelissen et al., 1987) and from genomic DNA clones (Payne et al., 1989; Eyal et al., 1992). This putative basic PR protein, named PR-1g, was then isolated from tobacco leaves reacting hypersensitively to tobacco mosaic virus, along with the three well-known acidic proteins PR-1a, PR-1b, and PR-1c. Like the tomato P14 proteins, the tobacco PR-1 proteins exhibited different levels of antifungal activity in vitro and in vivo. The proteins with the highest level of activity against *P. infestans* were tomato P14c and tobacco PR-1g.

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**MATERIALS AND METHODS**

**Plant Material, Pathogens, and Inoculation Procedures**

Tomato (*Lycopersicon esculentum* Mill. cv Baby) and tobacco (*Nicotiana tabacum* cv Samsun NN) plants were grown in the greenhouse under a 16-h light/8-h dark photoperiod. Plants were used for experimentation when they had five or six fully expanded leaves (about 5 and 8 weeks after sowing for tomato and tobacco, respectively).

Tomato plants were inoculated with 15 mL per plant of sporangial suspension (4000 sporangia mL\(^{-1}\)) of *Phytophthora infestans* isolate S49 (Sandoz collection). Sporangia for inoculation were taken from infected potato tuber slices (*Solanum tuberosum* cv Bintje) kept at 15°C in the dark. Inoculated plants were placed in a moist chamber (15°C, darkness) for 20 h, put back into a growth chamber at 20°C for another day, and returned to the greenhouse until harvest.

Tobacco plants were infected with the U1 strain of tobacco mosaic virus by rubbing fully expanded leaves with a virus suspension (0.2 µg mL\(^{-1}\)) in water containing carborundum powder as abrasive. After inoculation, plants were incubated in a controlled growth chamber at 22°C for 7 d to allow the appearance and growth of necrotic lesions; the leaves were then harvested and frozen in liquid nitrogen and stored at −80°C.

**Preparation and Concentration of Protein Extracts**

In the case of tomato plants, the method was adapted from that of Christ and Mösinger (1989). Two hundred grams of fresh or liquid nitrogen-frozen leaves were homogenized in 500 mL of extraction buffer containing 5% acetic acid and 0.1% 2-mercaptoethanol at pH 2.8. The resulting mixture was centrifuged for 30 min at 35,000g at 4°C. The supernatant was decanted, adjusted to pH 5.5 with 5 N NaOH, and recentrifuged as above. Proteins were precipitated by adding solid ammonium sulfate to the supernatant to a final concentration of 70% saturation. After 1 h of continuous stirring at 4°C the precipitate was collected by centrifugation at 5,000g for 30 min. The pellet was resuspended in a minimal volume of 50 mM Tris-HCl, 500 mM NaCl, pH 7.5, buffer. The latter was subjected to dialysis against the same buffer.

In the case of tomato leaves, proteins were extracted at acidic pH as described previously (Heitz et al., 1991). The protein extract was centrifuged at 20,000g and the supernatant was desalted on a Sephadex G-25 (Pharmacia) column. This fraction was the starting material for purification to homogeneity of the tobacco PR-1 proteins.

**Purification of Tomato P14 Proteins**

The acidic homogenate was applied to a Sephadex G-25 column equilibrated with 50 mM 1,3-diaminopropane, pH 10, buffer. The void volume was then applied to a Mono Q column HR 10/10 (Pharmacia). Basic proteins were eluted directly through the column and concentrated with a Centricon PM-5 (Amicon Co, Beverly, MA). After a buffer exchange through a PD-10 column (Pharmacia) with a 50 mM acetate, pH 4.4, buffer, the eluate was loaded onto a Mono S column HR 10/10 (Pharmacia) and eluted with a NaCl gradient (0–1 M). The eluted fractions were UV monitored, analyzed for their protein content by SDS-PAGE and staining with Coomassie brilliant blue, and tested for chitinase, β-1,3-glucanase, and antifungal activity. The final purification step of the P14 proteins was gel filtration chromatography on a Superdex 75 HR 10/30 column (Pharmacia) equilibrated with 100 mM phosphate buffer, pH 6. The progress of protein purification during the overall procedure is described in detail in “Results.”

**Purification of Tobacco PR-1 Proteins**

The acidic tobacco PR-1a, PR-1b, and PR-1c were isolated using the procedure described previously (Jimet and Fritig, 1986). Protein PR-1g was predicted from sequence data (Cornelissen et al., 1987; Payne et al., 1989; Eyal et al., 1992) to be a basic protein. Therefore, we used a procedure similar to the one that allowed the isolation of other basic tobacco PR proteins (Geoffroy et al., 1990; Heitz et al., 1994b). The total protein fraction resulting from chromatography on Sephadex G-25 (Pharmacia) was loaded onto a cation-exchange column (S-Sepharose Fast Flow, Pharmacia, 2.2 × 8.5 cm) equilibrated with 20 mM sodium acetate, pH 5.2. The proteins were eluted with a linear gradient (0–0.4 M) of NaCl. Fractions were monitored by SDS-PAGE analysis followed by staining with Coomassie brilliant blue.
or by western blotting using a serum raised against tomato P14a. The fractions containing PR-lg were pooled and adjusted to 1.2 M ammonium sulfate with solid salt, and the solution was filtered through 0.22-μm filters (Millipore). After injection onto a Phenyl-Superose HR 5/5 column (Pharmacia), elution was performed with a decreasing gradient of salt concentration obtained by diluting a 1.2 M ammonium sulfate solution with water under the control of a programmer of a fast liquid chromatography apparatus (Pharmacia). Fractions containing PR-lg were pooled, concentrated to 200 μL on Centricon 10 concentrators (Amicon), filtered on a 0.22-μm filter, and injected onto a Superdex 75 HR 10/30 column (Pharmacia). Elution with 0.1 M sodium acetate, pH 5.2, containing 0.2 M NaCl, yielded the purified PR-lg.

**Protein Analysis and Quantitation**

For the tobacco proteins, quantitation was performed following the method of Bradford (1976), using BSA (Sigma) as standard. Electrophoresis was performed on slab gels according to Laemmli (1970) for SDS gels and according to Janet and Fritig (1986) for native basic gels. Gel fixation and staining were carried out as previously described (Legrand et al., 1987; Geoffroy et al., 1990).

For tomato proteins, quantitation was performed either by the method of Bradford (1976) or by the bicinchoninic acid assay, according to Smith et al. (1985), with BSA and IgG as standards. Native PAGE and SDS-PAGE were carried out on a Pharmacia PhastGel system. Samples were applied to 10 to 15% (w/v) acrylamide gradient PhastGels, and the appropriate buffer strips were used according to the supplier’s instructions. Prior to application on SDS-PAGE gels, the samples were boiled for 10 min in 10 mM Tris-HCl, pH 8, containing 1 mM EDTA, 1% SDS, 5% 2-mercaptoethanol. PhastGels were stained with Coomassie brilliant blue or silver stained according to the supplier’s instructions.

**Amino Acid Sequence Determinations from the Purified Proteins**

Each of the purified proteins (40 μg) was digested with 1 μg of trypsin (Stone et al., 1989). The peptide mixture was loaded on a C<sub>18</sub> reverse-phase column (Waters) equilibrated with 0.1% TFA. The elution was performed with a linear 0 to 70% gradient of acetonitrile in 0.1% aqueous TFA. Microsequencing of selected peptides was carried out by the Edman method using an Applied Biosystems Sequencer (model 473A).

**Production of Anti-P14a Serum**

Antibodies against P14a were raised in rabbits. The purified P14a was emulsified in Freund’s complete adjuvant for the first injection or in incomplete adjuvant for the subsequent three booster injections. Fifty micrograms of pure P14a antigen were used for the first injection and 20 μg were used for the others. Two weeks after the last booster immunization, the serum was collected. After clot removal, serum was clarified by centrifugation and stored in small batches at –80°C.

**Western Blotting**

Western blotting and subsequent incubation of the blots with antisera were performed according to the procedure of Towbin et al. (1979). Detection of the antigen-antibody complexes was carried out with the Bio-Rad immunoblot assay kit using goat anti-rabbit alkaline phosphatase.

**Enzymatic Assays**

Chitinase activity in chromographic fractions was measured according to a modification of the radiochemical method of Molano et al. (1977). The reaction mixture (250 μL) contained 100 mM sodium phosphate, pH 6.5, 100 μL of [<sup>3</sup>H]chitin (2.1 μCi mL<sup>–1</sup>) (a gift from Prof. T. Boller, University of Basel, Switzerland), and the appropriate protein solution. The rate of hydrolysis was determined using the method of Boller et al. (1983) and Métraux and Boller (1986).

For β-1,3-glucanase activity measurement, a modification of the colorimetric assay of Waffenschmidt and Jaenicke (1987) was used with laminarin as substrate. The reaction mixture (500 μL) contained 50 mM sodium acetate buffer, pH 5.0, 50 μL of protein solution, and 0.5 mg mL<sup>–1</sup> laminarin.

RNase activity was determined by measuring the amount of acid-soluble nucleotides produced by hydrolysis of yeast RNA, according to Sakakibara et al. (1992), with RNase A as positive control.

**In Vitro Antifungal Activity Test**

*P. infestans* isolate S49 (Sandoz collection) was grown on potato tuber slices at 15°C in the dark. Seven-day-old mycelium was harvested and sporangia were washed out from the mycelium with cold water. The washing fluid was then incubated at 15°C in the dark to allow zoospore release. The sporangia were separated from swimming zoospores by gentle centrifugation (20g). The protein solutions to be assayed for antifungal activity were mixed with 2 × 10<sup>4</sup> zoospores mL<sup>–1</sup> in a final volume of 20 μL on depression glass slides and incubated overnight at 15°C and 100% humidity in the dark. Germination inhibition was observed under a light microscope, and inhibitory activities of proteins were compared to control samples. Water and buffer were used as negative controls.

**In Vivo Leaf Disc Antifungal Activity Test**

Tissue culture multiwell plates (24 wells, Linbro, Flow Laboratories, Bioggio, Switzerland) were filled with 2 mL of 0.5% agar. Leaf discs (15 mm diameter) of tomato were cut and placed onto agar. Different concentrations of P14 proteins were sprayed onto the leaf discs (three or four replicates per concentration) using a Devilbiss spray gun (Servispray AG, Dülken, Switzerland) equipped with a 0.279-mm nozzle. The leaf discs were then inoculated by applying droplets of *P. infestans* (2 × 10<sup>4</sup> sporangia mL<sup>–1</sup>).
The multiwell plates were incubated for 5 d and the level of disease was visually rated as the infected leaf surface and was compared to that of buffer- or water-treated discs taken as controls. Inhibition was calculated as the reduction in percent of the infected leaf surface of the protein-treated discs versus the water-treated control discs.

RESULTS

Search for Tomato PR Proteins with Antifungal Activity against *P. infestans*

To obtain a strong induction of PR proteins, we inoculated tomato plants with sporangia of *P. infestans* (Christ and Mösinger, 1989). After incubation of the plants for 1 week to allow the formation of necrotic lesions, acidic homogenates were prepared from leaves of infected and control plants and proteins were analyzed by SDS-PAGE (Fig. 1). Homogenates from infected plants (lane I) showed the PR-protein pattern observed previously (Christ and Mösinger, 1989; Enkerli et al., 1993), with P14 as the most prominent band and the chitinase and β-1,3-glucanase bands in the 25- to 40-kD zone. Protein fraction I was further resolved by anion-exchange (Mono Q column) chromatography into two main fractions, one that contained the low-pI (i.e. acidic) proteins, named fraction A, and the second with high-pI proteins, named fraction B. SDS-PAGE showed that most of the PR proteins accumulated in fraction A (Fig. 1).

It was shown previously (Enkerli et al., 1993) that total PR-protein extracts from *P. infestans*-infected tomato plants have in vitro antifungal activity against *P. infestans*. We suspected that this activity was not solely due to proteins shown previously to be antifungal, such as β-1,3-glucanases and chitinases (Sela-Buurlage et al., 1993) or PR-5 proteins (Woloshuk et al., 1991). To confirm this, we monitored antifungal activity against *P. infestans* zoospores and sporangia as a guideline to detect new antifungal proteins.

Figure 1. SDS-PAGE patterns of PR proteins in extracts and chromatographic fractions obtained from tomato plants. Proteins were extracted from uninoculated control leaves (C) or from leaves 7 d after inoculation with *P. infestans* (I), separated on a 10 to 15% denaturing polyacrylamide gel, and stained with Coomassie brilliant blue. A and B are the acidic and basic protein fractions, respectively, obtained after chromatography of I extracts on a Mono Q column. Molecular mass markers are shown in kD in lanes M. Loaded samples were adjusted to constant amount (5 µg) of protein.

Figure 2. In vitro inhibitory activity toward *P. infestans*. Effects of total basic protein fraction B and total acidic protein fraction A (see Fig. 1) on release and germination of zoospores of *P. infestans*. Basic fraction B (50 µg ml⁻¹) (A), acidic fraction A (50 µg ml⁻¹) (B), or buffer (C) were added to 4 x 10⁴ sporangia ml⁻¹. Magnification: ×104.

The discharge of zoospores from sporangia was inhibited to a great extent by total PR proteins, and the germination of the zoospores was stopped or lysis was observed. This activity was not observed with a boiled homogenate or with a homogenate from noninduced tomato leaves (data not shown). The basic protein fraction B and the acidic protein fraction A were tested separately for their antifungal activity. Fraction A did not exhibit any inhibitory activity below a protein concentration of 100 µg ml⁻¹, in contrast to fraction B, which inhibited the germination of zoospores at concentrations below 50 µg ml⁻¹ (Fig. 2).

For a further identification of the basic antifungal proteins, fraction B was chromatographed on a cation-exchange column (Mono S, Pharmacia) and 19 fractions (Fig. 3A) were collected for further analysis. Every fraction
Pathogenesis-Related PR-1 Proteins Are Antifungal

was subjected to the zoospore germination test to detect antifungal activity (Fig. 3B) but was also assayed for chitinase and β-1,3-glucanase activity (data not shown). The hydrolytic activities and the SDS-PAGE patterns (Fig. 3C) of the fractions showing zoospore inhibition indicated that the strongest antifungal activity was associated with a glucanase (fraction 19), a PR-5 (fraction 17), and a chitinase (fraction 12). But there was also a clear antifungal activity in fractions 4, 11, and 13, all of which contained a major protein band in the range of 14 kD (Fig. 3C). A P14 protein had been identified previously (Camacho Henriquez and Sänger, 1982, 1984; Enkerli et al., 1993) as the most prominent band in extracts of infected tomato leaves and shown to belong to the PR-1 family.

For this group of proteins, no direct antifungal activity was known, and, therefore, we focused on these fractions and characterized further their protein contents.

Characterization of Two New PR-1 Proteins Induced in Infected Tomato Leaves

Fractions 4, 11, and 13 were concentrated and subjected to gel filtration. This chromatographic step led to three different 14-kD proteins of high purity, as evidenced by SDS-PAGE (data not shown) and by low-pH native PAGE (Fig. 4A). We named the major and already known P14 P14a, and the two new proteins from fractions 4 and 11 P14b and P14c, respectively. Analysis by native PAGE at low pH of the purified P14 proteins revealed that P14c migrated with the highest mobility, followed by P14a and P14b (Fig. 4A). The yields of the three P14 proteins from 500 g of leaves (and their concentrations) were 2 mg (4 μg g⁻¹ fresh weight), 300 μg (0.6 μg g⁻¹ fresh weight), and 500 μg (1 μg g⁻¹ fresh weight) for P14a, P14b, and P14c, respectively.

Each P14 was subjected to a set of enzymatic assays (chitinase, β-1,3-glucanase, and RNase). None of these en...
zymotic activities could be detected in any of the tests (data not shown), extending the negative results in a similar search for enzyme activities for the tobacco PR-1 proteins (Van Loon, 1985). Western blots performed on the three pure proteins after migration upon native PAGE were probed with antiserum raised against P14a. They revealed the expected strong homologous reaction with P14a but also strong and specific heterologous reactions with the two novel P14 proteins (Fig. 4B), suggesting that they were likely to be new members of the tomato PR-1 family.

This was confirmed by partial microsequencing of the three purified proteins P14a, P14b, and P14c and comparison with the amino acid sequence deduced from the nucleotideic sequence of a tomato cDNA clone corresponding to protein P14 (Tornero et al., 1993). Figure 5 shows that there is 100% identity between our partially sequenced P14a (48 amino acids out of a total of 138 have been determined and aligned) and the sequence named Tom P14a (Camacho et al., 1986) and published by Tornero et al. (1993). The P14b and P14c proteins show 95 and 65% identity, respectively, to the tomato PR-1a. Thus, P14b and P14c are indeed two newly characterized tomato proteins of the PR-1 family. P14b being very similar to the well-known P14 (Camacho and Sänger, 1982, 1984), henceforth referred to as P14a. Further sequence comparisons (Fig. 5) between cloned PR-1 sequences of tobacco and the partial amino acid sequences of the purified tomato proteins indicated, surprisingly, 94% identity between tomato P14c (107 amino acids determined and aligned) and the sequence deduced from clone G (Cornelissen et al., 1987) corresponding to a putative basic tobacco PR-1, a basic counterpart to the well-known acidic PR-1a, PR-1b, and PR-1c. Since tomato P14c exhibited the strongest antifungal activity against P. infestans (see Fig. 3B, fraction 11), we decided to isolate the tobacco PR-1 proteins, in particular the basic member corresponding to clone G, to investigate their antifungal activities and compare them to those of the tomato PR-1 members.

Isolation of the Tobacco PR-1 Proteins and Characterization of the Predicted Basic PR-1g

The isolation and sequence of a tobacco basic-type cDNA (Cornelissen et al., 1987) and of basic-type PR-1 genomic clones (Payne et al., 1989; Eyal et al., 1992) have been reported. However, there is no report on the isolation of the corresponding gene product from tobacco. Expression studies have shown accumulation of transcripts of basic PR-1 in roots and in leaves of axenically cultured plants (Memelink et al., 1987, 1990) and in ethephon-treated and virus-infected leaves (Brederode et al., 1991; Ward et al., 1991; Eyal et al., 1992). We used the latter biological system, leaves reacting hypersensitively to tobacco mosaic virus, to isolate the highly induced and well-known acidic proteins PR-1a, PR-1b, and PR-1c and to tentatively characterize their basic counterpart. This protein was named PR-1g, according to the most recent recommendations for naming plant PR proteins (Van Loon, 1990; Van Loon et al., 1994).

The three acidic PR-1 proteins were purified from virus-infected Samsun-NN leaves by a procedure already described (Jamet and Fritig, 1986) and appeared homogeneous upon SDS-PAGE (Fig. 6B). Concerning the search for the basic counterpart, our previous attempts using antibodies raised against PR-1a or PR-1b had not revealed any band in the basic protein fraction, either because the amounts of the putative protein were too low or because there was no serological cross-reactivity between these proteins, which show only 65% sequence similarity. Since the anti-tomato P14a serum readily recognized the tomato P14c protein (Fig. 4B), whose amino acid sequence showed 94% identity with the predicted sequence (clone G) of tobacco PR-1g, we used this serum as a guideline for the purification of P14c. After removal of the acidic proteins on an anion-exchange column, the basic proteins were chromatographed on a cation-exchange column (S-Sepharose, Pharmacia) and the resulting fractions were analyzed for their protein content by SDS-PAGE followed by immunodetection with either anti-tobacco PR-1b or anti-tomato P14a serum (Fig. 6A). The latter but not the former serum revealed a protein of the expected size (Fig. 6A, lanes 5). The fractions containing this protein were pooled to perform two further purification steps, hydrophobic interaction chromatography followed by size exclusion chromatography, which led to the isolation of a protein that appeared homogeneous upon SDS-PAGE (Fig. 6B, lane 1g). Immunoblots again revealed a clear reaction of the isolated protein with anti-tomato P14a but not with anti-tobacco PR-1b serum (Fig. 6A).

Figure 5. Comparison of the amino acid sequences of tomato and tobacco PR-1 proteins. Tomato (Tom) PR-1a, clone G, and tobacco (Tob) PR-1a are amino acid sequences deduced from published cDNA sequences (Cornelissen et al., 1987; Tornero et al., 1993). Tom P14a, Tom P14b, Tom P14c, and Tob PR-1g are partial amino acid sequences determined by microsequencing of the purified proteins. Gaps, represented by dashes (-), have been included in the sequences to maximize similarities. Residues identical to the tomato PR-1a sequence are in uppercase letters, and those differing from PR-1a are in lowercase letters. Residues conserved in clone G, Tob-1g, and/or Tom 14c are boxed. Residues not identified are designated by X.
larly, in contrast to most basic tobacco PR proteins class II chitinases, known to be extracellular. This sug-

gested that PR-lg is at least partially located extracellu-
lar washing fluids. Protein contents of such fluids ob-
ained from infected leaves bearing necrotic lesions must be analyzed carefully, since extensive cell damage caused by necrotization might be responsible for the presence of intracellular proteins as contaminants in the fluids. The analytical procedure should include proteins of known and intracellular fluids of high quality, as evidenced by the complete absence of the basic class I chitinases, known to be vacuolar, and the presence of high amounts of the acidic class II chitinases, known to be extracellular. This sug-
gested that PR-lg is at least partially located extracel-
larly, in contrast to most basic tobacco PR proteins (Linthorst, 1991).

The identification of this protein as PR-1g was confirmed by partial amino acid sequencing (59 amino acids out of 179 were determined and aligned). Figure 5 shows that there is 97% sequence identity between our partially sequenced PR-1g and the sequence deduced from clone G (Cornelissen et al., 1987). There is also (data not shown) 97% se-
quence identity to the genomic clone obtained by Eyal et al. (1992) and 94% to that of Payne et al. (1989).

The acidic tobacco PR-1 proteins are known to be extra-
cellular (Linthorst, 1991) and easily detectable in intercel-
lar washing fluids. Protein contents of such fluids ob-
tained from infected leaves bearing necrotic lesions must be analyzed carefully, since extensive cell damage caused by necrotization might be responsible for the presence of intracellular proteins as contaminants in the fluids. The analytical procedure should include proteins of known and clear-cut localization, as already shown for tobacco leaves (Heitz et al., 1991). Preliminary results (data not shown) indicated the presence of significant amounts of PR-1g in an intercellular fluid of high quality, as evidenced by the complete absence of the basic class I chitinases, known to be vacuolar, and the presence of high amounts of the acidic class II chitinases, known to be extracellular. This sug-
gested that PR-lg is at least partially located extracel-
larly, in contrast to most basic tobacco PR proteins (Linthorst, 1991).

Figure 6. Immunodetection after SDS-PAGE of tobacco basic PR-1g and SDS-PAGE of purified tobacco PR-1 proteins. A, Western blots with either anti-tobacco PR-1b or anti-tomato P14a serum; lanes S were loaded with an aliquot (1 μg of protein) of a S-Sepharose chromatographic fraction enriched with PR-1g; lanes lg were loaded with 1 μg of purified PR-1g. B, Lanes were loaded with 1 μg of purified proteins PR-1a, PR-1b, PR-1c, and PR-1g and the proteins were revealed by staining with Coomassie brilliant blue.

Differential Antifungal Activities against *P. infestans* in Vitro of the Various Tomato and Tobacco PR-1 Proteins

The various purified tobacco and tomato proteins were assayed for their antifungal activity against *P. infestans*. Most of the antifungal assays were performed in vitro and consisted of measuring the degree of inhibition of zoospore germination. Tomato P14c, along with tobacco PR-1g, turned out to be the most effective in this antifungal assay. Table I indicates the minimum concentration of the various tomato and tobacco PR-1 proteins needed to prevent zoospore germination, and very clearly shows differential biological activities of the different PR-1 group proteins. This is also demonstrated by the dose-response curves of this assay for the different proteins, as illustrated in Figure 7. Tomato P14c and tobacco PR-1g, which show considerable sequence similarity, also exhibited similar antifungal activities at all concentrations assayed, so that the correspond-
ing dose-response curves were almost superimposed. They were significantly more inhibitory than the other two basic proteins, P14a and P14b. The acidic tobacco PR-1 proteins were only slightly inhibitory (Table I).

### Table I. Differential inhibitory activities of tomato and tobacco PR-1 proteins on *P. infestans* zoospore germination

<table>
<thead>
<tr>
<th>Protein Assayed</th>
<th>Concentration of Protein (μg ml⁻¹)</th>
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<tr>
<td>Tobacco PR-1c</td>
<td>&gt;200</td>
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<tr>
<td>Tobacco PR-1a</td>
<td>200</td>
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<tr>
<td>Tobacco PR-1b</td>
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<td>Tobacco PR-1g</td>
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<td>Tomato P14c</td>
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*Minimal concentration of the various proteins required to inhibit the germination of zoospores by more than 90%.*
DISCUSSION

In tomato, one of the most predominant and best-characterized tomato PR proteins is P14 (Camacho Henriquez and Sänger, 1982, 1984; Granell et al., 1987), a basic protein that shows homology to the tobacco PR-1 protein family (Lucas et al., 1985; Cornelissen et al., 1986). It was shown later that after infection with virulent or avirulent races of Cladosporium fulvum the pattern of induction of tomato basic 14- to 15-kD proteins was more complicated, and three distinct proteins named P2, P4, and P6 were purified (Joosten et al., 1990). The authors obtained evidence that P2 was related to the PR-4 family of tobacco PR proteins, whereas P4 and P6 were related to the PR-1 family. Electrophoretic migrations under native and denaturing conditions suggested that P6, the most abundant of the basic 15-kD tomato proteins, corresponded to the well-known PI 4, whereas P4 was a newly characterized tomato PR-1 protein.

In P. infestans-infected tomato leaves we found not only two, but three, distinct members of the PR-1 family. Comparison of the electrophoretic patterns under native conditions described in the present study (Fig. 4B) to those reported by Joosten et al. (1990) suggests that (a) our major protein, P14a, corresponds to P6 and is indeed the most prominent of the 15-kD proteins; (b) our P14b corresponds to P4; and (c) our P14c was not detected by Joosten et al. (1990), since it migrates only slightly faster than P14a on native acidic gels and is separated from P14a upon cation-exchange chromatography (see Fig. 3A, fractions 11 and 13) only with the use of a highly resolving NaCl gradient. We were able to purify P14a, P14b, and P14c to homogeneity and to identify them as members of the PR-1 family by comparison of their amino acid sequences (Fig. 5) to that of P14 (Lucas et al., 1985) or to that deduced from a P14 cDNA clone (Tornero et al., 1993). P14a was found to be identical and P14b was found to be very similar to the latter, whereas P14c showed only 65% similarity to P14 but very high (94%) similarity to a putative basic tobacco PR-1 protein.

Since the fractionation of the tomato PR-1 proteins was also initially monitored (Fig. 3B) by an in vitro antifungal activity test against P. infestans (Fig. 2), and since P14c appeared to be the most active, we were prompted to extend the search for a direct biological activity of the well-known tobacco PR-1 proteins and to try to isolate the predicted basic tobacco PR-1 analog named PR-1g (Van Loon, 1990; Van Loon et al., 1994). Although transcripts for basic-type tobacco PR-1 genes had been detected under various induction conditions, a polypeptide product for basic-type tobacco PR-1 genes had still to be isolated. There is only one report of detection of polypeptide products for basic PR-1 genes (Eyal et al., 1993), using antibodies raised against a fusion protein obtained by expressing a basic PR-1 sequence in bacteria. These antibodies revealed two polypeptides of 16 and 20 kD produced in ethylene-treated leaves (Eyal et al., 1993). In the present study, an antitomato P14a serum detected one band in the basic protein fraction from virus-infected tobacco leaves (Fig. 6A, lane S), and this protein was shown to be PR-1g with the expected amino acid sequence corresponding to a cDNA and two
Pathogenesis-Related PR-1 Proteins Are Antifungal

Table II. In vivo inhibitory activity of tomato and tobacco PR-1 proteins on the growth of P. infestans on tomato leaf discs

<table>
<thead>
<tr>
<th>Protein Assayed</th>
<th>Percent Inhibition with Protein Amounts of</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.5 μg</td>
</tr>
<tr>
<td>Tomato</td>
<td></td>
</tr>
<tr>
<td>P14a</td>
<td>45</td>
</tr>
<tr>
<td>P14b</td>
<td>40</td>
</tr>
<tr>
<td>P14c</td>
<td>80</td>
</tr>
<tr>
<td>Tobacco</td>
<td></td>
</tr>
<tr>
<td>PR-1a</td>
<td>10</td>
</tr>
<tr>
<td>PR-1b</td>
<td>20</td>
</tr>
<tr>
<td>PR-1c</td>
<td>20</td>
</tr>
<tr>
<td>PR-1g</td>
<td>10</td>
</tr>
</tbody>
</table>

genomic clones already described (Cornelissen et al., 1987; Payne et al., 1989; Eyal et al., 1992).

Preliminary data (not shown) suggest that at least a proportion of PR-1g is targeted to the extracellular compartment. However, when leaves had been infiltrated for the production of the intercellular washing fluid, the extracts obtained from such leaves still contained PR-1g in higher relative amounts than other strictly extracellular PR proteins. These results suggest a dual localization of PR-1g. In the present study the localization of tomato PR-1 proteins was not investigated, but the P4 (i.e. P14b) and P6 (i.e. P14a) proteins have previously been found in apoplastic fluids from C. fulvum-infected tomato leaves, indicating an extracellular localization (Joosten and De Wit, 1989). There is also a report on a dual localization in the vacuole and in the intercellular spaces of the P1 (p14) tomato protein induced by infection with citrus exocitosis viroid (Vera et al., 1989). It is not clear from this immunochromatography whether the antibodies used have recognized one or more of the different tomato P14s. The targeting and localization of the basic PR-1 protein obviously have to be examined further in both tomato and tobacco and are of importance, especially in view of the direct antimicrobial activity demonstrated for these proteins in the present report.

PR-1 proteins have long been suspected to possess antiviral activity. However, transgenic plants expressing PR-1 cDNA showed no significant resistance against viruses such as tobacco mosaic virus, alfalfa mosaic virus, and potato virus Y (Cutt et al., 1989; Linthorst et al., 1989; Alexander et al., 1993). On the other hand, transgenic tobaccos expressing high levels of PR-1a showed no resistance to Pseudomonas syringae pv tabaci or to Cercospora nicotianae, but showed an increased tolerance, estimated from visual ranking of disease symptoms, to infection with Phytophthora parasitica var nicotianae (black shank disease) and with Peronospora tabacina (blue mold disease) (Alexander et al., 1993). These results provided indirect evidence for antifungal activity of a PR-1 protein. However, the authors could not exclude the possibility that high in vivo constitutive levels of PR-1a allowed the plant to activate additional defense responses that limited the spread of the disease.

In the present study the direct fungicidal activity against P. infestans of both tomato and tobacco PR-1 proteins was demonstrated both with an in vitro test measuring inhibition of zoospore germination (Fig. 2) and with an in vivo leaf disc test in which the reduction of infected leaf surface was scored (Fig. 8). The results obtained with the two methods were consistent and indicated clear differential activities of the various PR-1 proteins and a dose dependency (Figs. 7 and 8; Tables I and II). The concentrations needed to completely inhibit the germination of zoospores ranged between 10 and 100 μg mL⁻¹ for the most active proteins. These concentrations are much higher than the mean concentrations calculated, for instance, for the purified tomato P14 proteins (0.6–4 μg g⁻¹ fresh weight), assuming that 1 g of fresh weight contains about 1 mL of liquid. However, a higher proportion of the induced PR-1 proteins is known to accumulate locally around the infection site in zones that represent only a small percentage (<10%) of the leaf surface (Antoniw and White, 1986; Heitz et al., 1994a). Thus, the concentrations in vivo are at least 10-fold higher than the mean values for the intracellular proteins and are even much higher for the extracellular proteins present in the small intercellular fluid volume. Hence, the concentrations that are likely to occur in vivo, especially in the intercellular spaces, are in the range of those that were shown to be effective in this report.

In our study we found that tobacco PR-1a was by far not the most efficient antifungal protein against P. infestans. The two newly characterized PR-1 proteins, tomato P14c and tobacco PR-1g, exhibited the highest fungicidal activity, and their comparable efficiency was consistent with their structural similarity. It should be interesting to express these proteins constitutively in transgenic plants, alone or in combination with another PR protein of either the PR-2, PR-3, or PR-5 family, and search for increased tolerance against a variety of fungi.

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