The Plant Cell, Vol. 7, 573–588, May 1995 © 1995 American Society of Plant Physiologists

Small Cysteine-Rich Antifungal Proteins from Radish: Their Role in Host Defense

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Radish seeds have previously been shown to contain two homologous, 5-kD cysteine-rich proteins designated *Raphanus sativus*–antifungal protein 1 (Rs-AFP1) and Rs-AFP2, both of which exhibit potent antifungal activity in vitro. We now demonstrate that these proteins are located in the cell wall and occur predominantly in the outer cell layers lining different seed organs. Moreover, Rs-AFPs are preferentially released during seed germination after disruption of the seed coat. The amount of released proteins is sufficient to create a microenvironment around the seed in which fungal growth is suppressed. Both the cDNAs and the intron-containing genomic regions encoding the Rs-AFP preproteins were cloned. Transcripts (0.55 kb) hybridizing with an Rs-AFP1 cDNA–derived probe were present in near-mature and mature seeds. Such transcripts as well as the corresponding proteins were barely detectable in healthy uninfected leaves but accumulated systemically at high levels after localized fungal infection. The induced leaf proteins (designated Rs-AFP3 and Rs-AFP4) were purified and shown to be homologous to seed Rs-AFPs and to exert similar antifungal activity in vitro. A chimeric Rs-AFP2 gene under the control of the constitutive cauliflower mosaic virus 35S promoter conferred enhanced resistance to the foliar pathogen *Alternaria longipes* in transgenic tobacco. The term "plant defensins" is proposed to denote these defense-related proteins.

INTRODUCTION

In vegetative plant tissues, a series of dynamic defense mechanisms can be triggered upon wounding or perception of microorganisms. Newly formed carbohydrate material can be deposited in the cell wall in response to penetration attempts by fungal hyphae (Aist, 1976), and preexisting cell wall proteins can be oxidatively cross-linked upon wounding and elicitor treatment (Bradley et al., 1992). Both responses result in an induced fortification of the cell wall. Another strategy followed by plants to thwart invaders is based on the localized production of antimicrobial low molecular weight secondary metabolites known as phytoalexins (Van Etten et al., 1989; Maher et al., 1994). Furthermore, the synthesis of many presumed defense-related proteins is induced when plants are confronted with pathogens (Linthorst, 1991). Among these proteins are the pathogenesis-related (PR) proteins. Members of five different PR protein families have been shown to possess antifungal activity in vitro (Mauch et al., 1988; Woloshuk et al., 1991; Hejgaard et al., 1992; Niderman et al., 1993; Sela-Buurlage et al., 1993; Ponstein et al., 1994), and some PR proteins confer enhanced resistance to fungal diseases when expressed in transgenic plants (Broglie et al., 1991; but see Neuhaus et al., 1991; Alexander et al., 1993; Yoshikawa et al., 1993; Zhu et al., 1994). Many stress signals are able to induce the expression of defense-related proteins not only locally (e.g., in the vicinity of the infection site) but also in distant, nonstressed leaves. This phenomenon is known as systemic acquired resistance (Ross, 1961; Tuzun et al., 1989; Ward et al., 1991; Uknes et al., 1992). Candidate signal molecules involved in the immunization of a plant against subsequent infection are salicylic acid (Malamy et al., 1990; Métraux et al., 1990; Gaffney et al., 1993; Delaney et al., 1994) and methyl jasmonate (Farmer and Ryan, 1990; Farmer et al., 1992; Xu et al., 1994). Finally, some incompatible interactions between pathogens and plants can trigger localized necrosis of cells (Dixon and Lamb, 1990). This hypersensitive response can most probably be explained by "gene-for-gene" incompatibility (Ellingboe, 1981). A well-studied example of such a mechanism is the interaction between different tomato lines and different *Cladosporium fulvum* races. In the plant, *C. fulvum* produces race-specific elicitors (3-kD cysteine-rich peptides) that trigger the hypersensitive response only in tomato plants with the matching resistance gene (de Wit et

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al., 1992). The occurrence of the hypersensitive response is mediated by reactive oxygen species produced early in the plant–pathogen interaction (Levine et al., 1994).

Thus, plant defense mechanisms have been studied very intensively in vegetative tissues. However, little is known about the strategies used by seeds to survive and germinate in substrates densely populated with microorganisms. A particularly vulnerable stage occurs at early germination when the seed coat, which forms an effective physical barrier against microbes, is disrupted and the young seedling becomes exposed to the soil.

We have recently characterized a novel family of 5-kD cysteine-rich antifungal proteins (AFPs) from seeds of radish (Terras et al., 1992b) and four other crucifers, including Arabidopsis (Terras et al., 1993). Radish (*Raphanus sativus*) seeds contain almost equal amounts of two isoforms, Rs-AFP1 and Rs-AFP2, that exert antifungal activity against a broad spectrum of plant pathogenic filamentous fungi by causing hyperbranching and growth reduction of the hyphal tips. These proteins have little or no effect on bacteria and cultured human cells (Terras et al., 1992b). Using radish seed as a model system, we studied the release of Rs-AFPs during germination and the significance of this phenomenon with respect to seedling protection against fungal pathogens. In addition, the expression of Rs-AFPs or Rs-AFP–like proteins was examined in nonstressed and stressed leaves. To validate the presumed role of Rs-AFPs in host defense, Rs-AFP2 was constitutively expressed in transgenic tobacco plants that were subsequently analyzed for resistance to a foliar fungal pathogen.

RESULTS

Release of Rs-AFPs from Germinating Seeds

To study the release of antifungal compounds during germination of radish seeds, we developed a bioassay in which seeds were allowed to germinate on a medium supporting growth of a fungal colony. When the edges of the expanding colony approached the germinating seed, a growth inhibition halo appeared around the seed, as shown in Figure 1A. The growth inhibition effect could be mimicked by applying 1 μg of either purified Rs-AFP1 or Rs-AFP2 to a well in the agar medium (shown for Rs-AFP1 in Figure 1A). Addition of the endoprotease Pronase E to the medium resulted in the abolition of the inhibition zones caused by the germinating seed as well as by Rs-AFP1 (Figure 1B). Likewise, autoclaved seed or autoclaved Rs-AFP1 lost their inhibitory capacities (Figure 1C). When seed germination was prevented by addition of the plant hormone abscisic acid to the medium, no growth inhibition halo was observed around an intact seed. Under these conditions, however, seeds with a mechanically applied incision in their seed coat were still capable of releasing their antifungal components (Figure 1D). Thus, radish seed release a heat-sensitive proteinaceous antifungal compound only after disruption of their

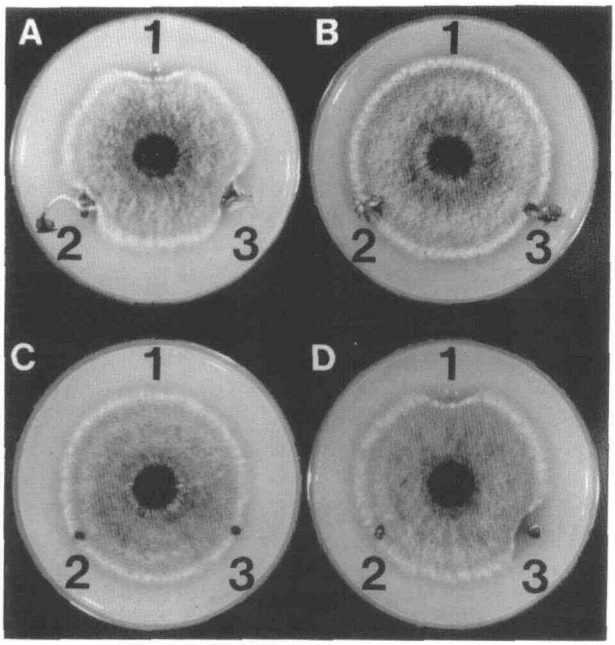

Figure 1. Release of Antifungal Compounds by Germinating Radish Seeds.

One microgram of purified Rs-AFP1 was applied at the positions indicated by the number 1. Radish seeds at the positions 2 had an intact seed coat, whereas seeds at positions 3 had an incised seed coat (along half of the seed periphery). The fungus *P. tritici-repentis* was used in this assay.

(A) Assay plates containing five cereal agar.

(B) Assay plates containing five cereal agar supplemented with 50 μg/mL Pronase E.

(C) Assay plates as given in **(A)**. The Rs-AFP1 solution and the seeds were autoclaved.

(D) Assay plates containing five cereal agar supplemented with 100 μM abscisic acid.

seed coat, either by germination or by mechanical incision. Of the previously purified radish seed proteins with antifungal properties, including Rs-AFPs, 2S albumins (Terras et al., 1992b), and a nonspecific lipid transfer protein (Terras et al., 1992a), only Rs-AFPs could restrict growth of fungal colonies in the agar diffusion bioassay at amounts <20 μg (data not shown). These results suggest that Rs-AFPs are the predominant proteinaceous antifungal compounds released from germinating radish seed. In the assay shown in Figure 1, the fungus *Pyrenophora tritici-repentis* was used because this fungus grows very evenly. Similar results (data not shown) were obtained when using other fungi, for example, *Fusarium culmorum* and *Pyricularia oryzae*.

To verify and quantify the presumed Rs-AFP release from radish seeds, seeds with a mechanically applied incision in their seed coats were imbibed in water, after which the imbibition solution was analyzed by gel electrophoresis. Proteins released from a single imbibing seed were loaded on two

replica SDS–polyacrylamide gels. After a 30-min incubation, a clear band corresponding to the Rs-AFPs was detected on an immunoblot, as depicted in Figure 2B. After a 4-hr incubation, the amount of Rs-AFPs released in the imbibition medium exceeded 1 μg, the amount required to mimic the fungal growth inhibition halo formed around one germinating seed (see Figure 1). As shown on the Coomassie blue–stained gel (Figure 2A), other proteins were released as well, but Rs-AFPs were the most abundant proteins released in the imbibition medium. Neither Rs-AFPs nor any other protein could be detected in the imbibition medium of intact (nongerminated and nonincised) seed even after a 12-hr incubation period. Comparison of the total protein and immunoblot patterns of the imbibition medium from incised seed and of a crude seed extract further illustrated that Rs-AFPs were preferentially released. The amount of Rs-AFPs in a seed extract corresponding to one seed and in the imbibition medium of one incised seed (after a 4-hr incubation) was estimated to be 5 and 1.5 μg, respectively. For total protein, these values are 800 and 4.8 μg, respectively. Thus, ~30% of the total Rs-AFP content of a single incised seed was released into the imbibition medium after 4 hr. In contrast, the release of total protein from incised seed amounted only to 0.6% of the total soluble protein content of a crude seed extract.

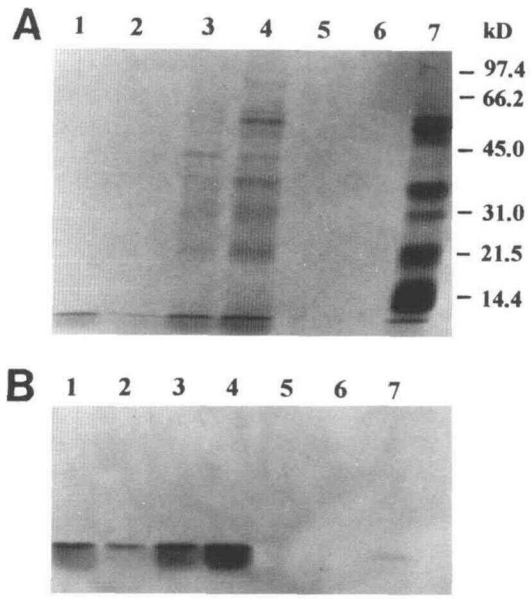

Figure 2. Electrophoretic Analysis of Proteins Released from Radish Seeds.

(A) Coomassie blue staining.
(B) Immunostaining.
Lanes 1 contain 1 μg of Rs-AFP1; lanes 2, 500 ng of Rs-AFP1; lanes 3, imbibition medium corresponding to one incised seed after 30 min; lanes 4, imbibition medium corresponding to one incised seed after 4 hr; lanes 5, imbibition medium corresponding to one intact seed after 4 hr; lanes 6, imbibition medium corresponding to one intact seed after 12 hr; lanes 7, crude seed extract corresponding to one-twentieth of a seed. Sizes of the molecular mass markers (not shown) are indicated at right in kilodaltons.

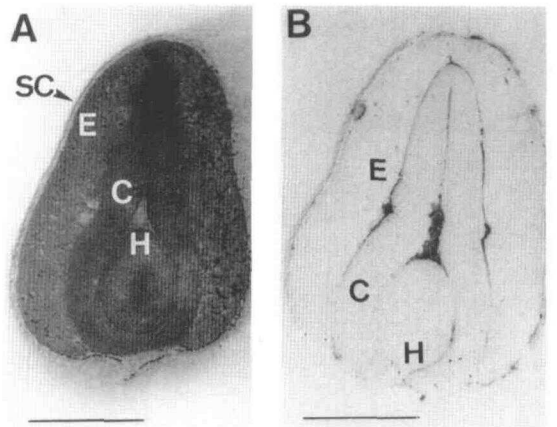

Figure 3. Tissue Print Immunolocalization of Seed Rs-AFPs.

(A) Section stained with amido black for total protein. The vascular bundle is visible in the center of the hypocotyl.
(B) Immune section.
Bars = 1 mm. C, cotyledon; E, endosperm; H, hypocotyl; SC, seed coat.

Preferential release of Rs-AFPs during seed imbibition suggests an extracellular location for these proteins. Tissue print immunolocalization revealed that Rs-AFPs are present at high levels in the outer cell layers of and in the spaces between the different seed organs, as illustrated in Figure 3. Closer examination by immunofluorescence microscopy of semithin seed sections confirmed that Rs-AFPs occur most abundantly in the outer cell wall layer lining the surface of cotyledons, hypocotyl, and endosperm, as depicted in Figures 4A to 4D. Rs-AFPs in the walls of inner cells of these organs were barely detectable by immunofluorescence microscopy. However, immunogold electron microscopic examination of ultrathin sections of mature radish seed revealed that Rs-AFPs specifically reside in the middle lamellae of cell walls throughout the different seed tissues (endosperm, see Figures 4E and 4F; cotyledons and hypocotyl, data not shown).

Rs-AFPs are thus extracellular seed proteins present at positions where the first contacts with invading fungi occur, namely, the outer surfaces of the different seed organs and the coatings of the intercellular spaces. This location is consistent with a role for Rs-AFPs in the protection of germinating seedlings against fungal infection.

cDNA Structure of Rs-AFPs

cDNA clones of Rs-AFPs were obtained by screening a radish seed cDNA library in λZAPII. Of 22 independent positive plaques, four contained an insert of ~400 bp, whereas the insert length of the remaining 18 phages varied from 250 to 300 bp. Nucleotide sequences were determined for the four 400-

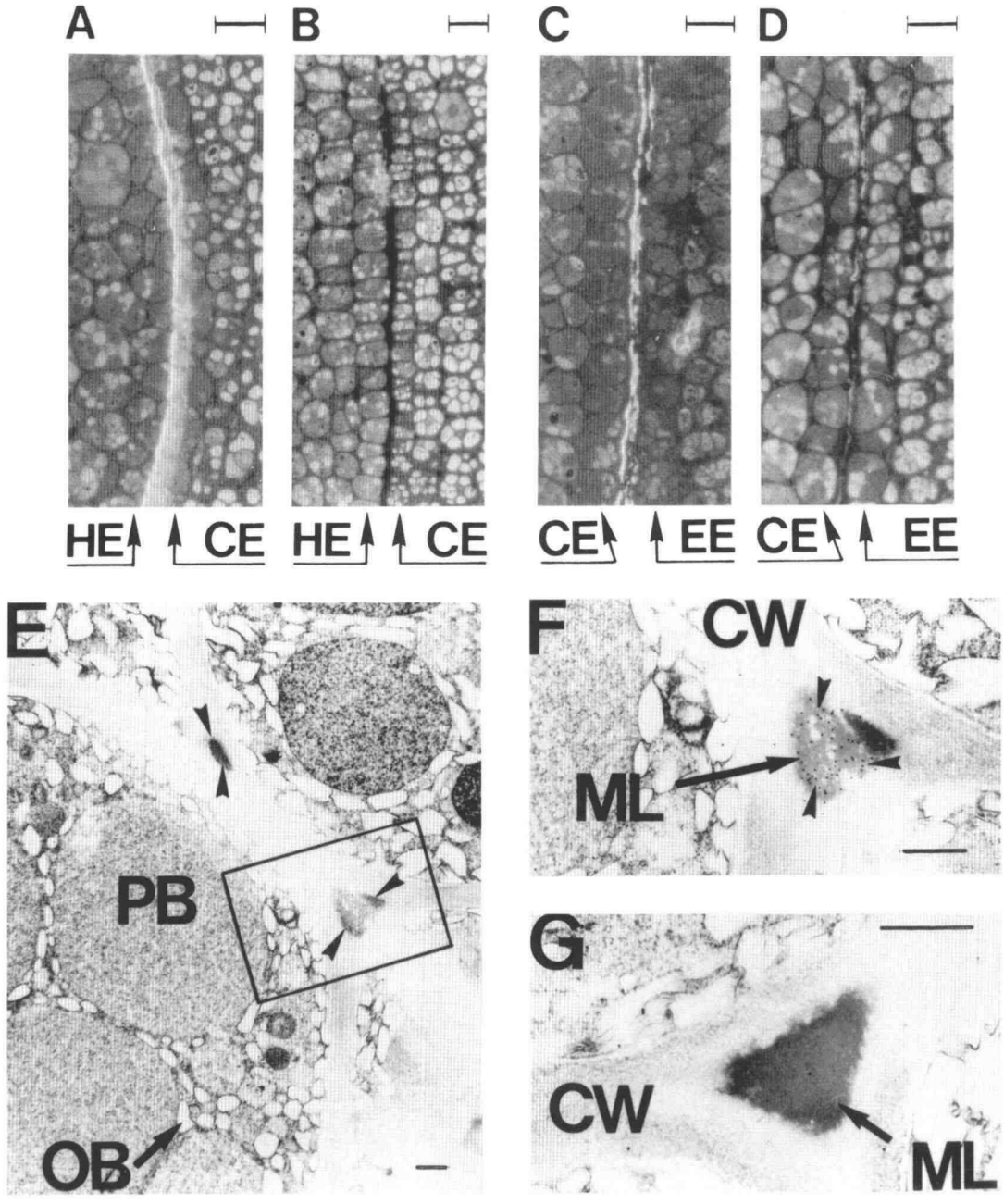

Figure 4. Immunolocalization of Seed Rs-AFPs in Seed Tissue Sections.

Tissue sections in **(A)** to **(D)** are immunofluorescence micrographs; sections in **(E)** to **(G)** are immunogold micrographs.
(A) Immune section with a border between hypocotyl and cotyledon.
(B) Preimmune section as shown in **(A)**.
(C) Immune section with a border between cotyledon and endosperm.
(D) Preimmune section as shown in **(C)**.
(E) Immune section of endosperm tissue.
(F) Enlargement of the zone in **(E)** indicated with a rectangle.
(G) Preimmune section of endosperm tissue.
CE, cotyledon epidermis; CW, cell wall; EE, endosperm epidermis; HE, hypocotyl epidermis; ML, middle lamella; OB, oil body; PB, protein body.
Bars in **(A)** to **(D)** = 20 μm. Bars in **(E)** to **(G)** = 0.5 μm. Arrowheads in **(E)** and **(F)** show the presence of gold particles.

bp inserts and for six of the other inserts. All of the 400-bp cDNAs corresponded to the full-length Rs-AFP1 transcripts, which contained open reading frames of 240 bp. The nucleotide sequence and deduced amino acid sequence for one of these clones, pFRG1, are depicted in Figure 5A. From a comparison of the experimentally determined N-terminal sequence of Rs-AFP1 (Terras et al., 1992b) with the deduced amino acid sequence of pFRG1, we concluded that the mature protein (51 amino acids) is preceded by a 29–amino acid peptide showing all the characteristics of a signal peptide (von Heijne, 1986). The occurrence of a signal peptide is in agreement with the extracellular location of the mature protein. The calculated molecular mass of the predicted mature protein (5685 D) corresponds well with the mass estimated by SDS-PAGE (5 kD; Terras et al., 1992b). Five of the smaller sequenced inserts were 5′ truncated cDNAs corresponding to Rs-AFP1, whereas the remaining characterized positive clone contained a 5′ truncated cDNA corresponding to Rs-AFP2. The 5′ end of the Rs-AFP2 cDNA was rescued by ligation of an oligonucleotide to the 3′ end of single-stranded cDNAs followed by polymerase chain reaction (PCR) amplification. The nucleotide sequence of the full-length Rs-AFP2 cDNA and the deduced amino acid sequence are given in Figure 5B. Again, the mature protein (51 amino acids) is preceded by an N-terminal prepeptide of 29 amino acids. PCR amplification of the genomic fragments encompassing the Rs-AFP1 and Rs-AFP2 coding region yielded bands larger in size than the products obtained by amplification of the corresponding cDNA regions, indicating the presence of introns. Therefore, the nucleotide sequences of both genomic fragments were determined. The intron in the Rs-AFP1 coding sequence is 112 bp in length, whereas the Rs-AFP2 intron spans 98 bp. Both introns occur at the same position in the open reading frames, namely, between the codons for amino acids −9 and −8 of the N-terminal prepeptide.

From the nucleotide sequence data, the complete amino acid sequences of Rs-AFP1 and Rs-AFP2 could be deduced (Figure 5C). It thus appears that Rs-AFP1 and Rs-AFP2 are nearly identical peptides differing only at residues 5 and 27. The open reading frame encompassing the Rs-AFP2 preprotein is 91% identical to the Rs-AFP1 coding sequence.

Expression of Rs-AFPs during Seed Development and in Biologically Stressed Leaves

The tissue-specific expression patterns of the Rs-AFPs as well as the temporal expression pattern of the Rs-AFPs during seed development were determined by RNA gel blot analysis using an Rs-AFP1 cDNA–derived RNA probe. In seeds, Rs-AFP mRNAs start to accumulate at the onset of desiccation (45 days postanthesis) and are still present in mature, dry seed, as shown in Figure 6. Therefore, Rs-AFPs are expressed only during the final stage of seed development when the seeds are prepared to detach from the mother plant. Cross-hybridizing transcripts of the same length (0.55 kb) were also present at

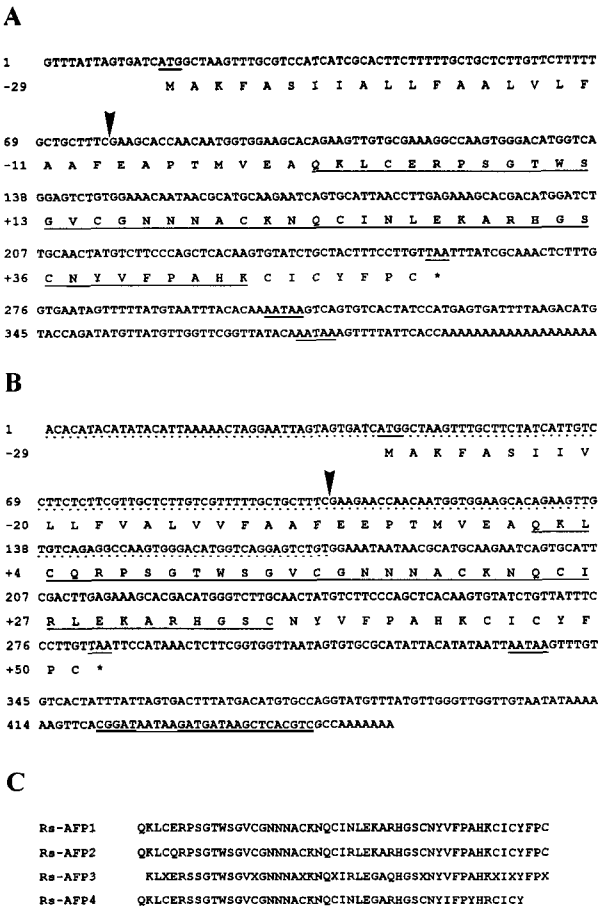

Figure 5. Nucleotide Sequence of cDNAs and Genomic Regions and Amino Acid Sequences of Rs-AFPs.

(A) Nucleotide sequence and deduced amino acid sequence of the full-length Rs-AFP1 cDNA insert of pBluescript SK− phagemid pFRG1.
(B) Nucleotide sequence and deduced amino acid sequence of the full-length Rs-AFP2 cDNA. The depicted nucleotide sequence was obtained by combining a 5′ truncated cDNA sequence isolated from the seed cDNA library and the 5′ end rescued by anchor PCR (underscored with dotted line) with an antisense primer specific to a part of the 3′ noncoding region (double underlined).
(C) Complete amino acid sequences of mature seed Rs-AFP1 and Rs-AFP2 deduced from **(A)** and **(B)** and N-terminal sequence data of the Rs-AFPs (Terras et al., 1992b). N-terminal amino acid sequences of leaf Rs-AFP3 and Rs-AFP4 were determined in this study.
Start and stop codons, experimentally determined N-terminal amino acid regions, and putative polyadenylation signals are underlined in **(A)** and **(B)**. The cysteines of Rs-AFP3 were not derivatized before automated Edman degradation; therefore, the putative cysteine residues of Rs-AFP3 are indicated by X's in **(C)**. The positions of the intron sequences are indicated by arrowheads in **(A)** and **(B)**. Asterisks in **(A)** and **(B)** indicate stop codons. Nucleotide sequences were submitted to GenBank and have accession numbers U18557 (Rs-AFP1) and U18556 (Rs-AFP2).

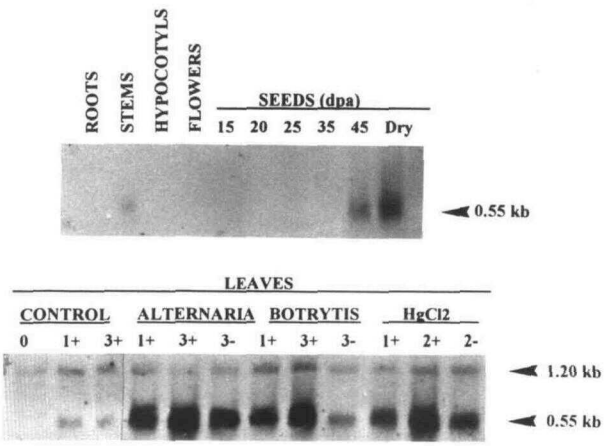

Figure 6. RNA Gel Blot Analysis of Rs-AFP Expression in Radish.

The analyzed samples represent 15 μg of total RNA extracted from the indicated tissues. The designation hypocotyls refers to the succulent storage organs of the radish plant. dpa, days postanthesis; 0, total RNA isolated from leaves collected just after inoculation with 50 5-μL drops of H_2O (CONTROL), 50 5-μL drops of *A. brassicola* spores (at 5×10^5 spores per mL), 50 5-μL drops of *Botrytis cinerea* spores (at 5×10^5 spores per mL), or 50 5-μL drops of 0.2% (w/v) mercuric chloride; 1+, 2+, and 3+ represent total RNA isolated from leaves collected 24, 48, and 72 hr, respectively, after inoculation; 2− and 3− represent total RNA isolated from noninoculated leaves from the same plants as given above after 48 and 72 hr, respectively.

low levels in leaves and stems, but not in roots, the hypocotyl-derived succulent storage organ (all harvested from 5-week-old plants), or flowers (harvested from adult radish plants). In leaves, a second hybridizing transcript of 1.2 kb was observed. This transcript was also apparent in stems and flowers after overnight exposure (data not shown). This mRNA species has not yet been identified.

Because Rs-AFP–like proteins seem to be at least weakly expressed in leaves, we were interested in whether their expression was affected by stress. Therefore, different stress factors were applied to leaves of 5-week-old plants, after which treated and untreated leaves from the same plants were collected separately for isolation of total RNA. RNA gel blot analysis (Figure 6) revealed that induction above the low constitutive expression level of leaf Rs-AFPs was triggered by fungal infection and by treatment with mercuric chloride. This induction was not restricted to stressed leaves because untreated leaves showed an almost identical response, implying the transduction of a stress signal through the plant. Moreover, the systemic accumulation of the 0.55-kb transcript was accompanied by the accumulation of 5-kD proteins that were recognized by antibodies raised against Rs-AFP1, as shown by the immunoblot analysis in Figure 7.

In an effort to isolate the induced Rs-AFP–like proteins from infected leaves, the total basic protein fractions from healthy and *Alternaria brassicola*–infected leaves were separated on a reverse-phase chromatography column. When comparing the chromatographic profiles of both samples (depicted in Figures 8A and 8B, respectively), two peaks become prominent in *A. brassicola*–infected leaves that are virtually absent in healthy leaves. Both peaks coeluted with antifungal activity and contained peptides of 5 kD (inset of Figure 8B), which are called Rs-AFP3 and Rs-AFP4 for the peptides in the earliest and latest eluting peaks, respectively. Both Rs-AFP3 and Rs-AFP4 cross-reacted with an antiserum raised against Rs-AFP1 when assessed by immunoblotting of SDS-PAGE gels (data not shown). The concentrations of Rs-AFP3 and Rs-AFP4 required to obtain 50% inhibition of fungal growth were similar to those of the seed Rs-AFPs (Table 1). Moreover, fungi treated with Rs-AFP3 or Rs-AFP4 displayed a hyperbranched morphology similar to that of fungi treated with seed Rs-AFPs (data not shown). The final proof that leaf Rs-AFPs are homologous to seed Rs-AFPs was provided by the amino acid sequences. The complete amino acid sequence consisting of 51 residues was determined for Rs-AFP3, whereas the putative last three amino acids were missing for Rs-AFP4. Both sequences are shown in Figure 5C and can be compared with the amino acid sequences of seed Rs-AFPs. The overall amino acid sequence homology of leaf Rs-AFPs with seed Rs-AFPs is ~90%. Rs-AFP4, but not Rs-AFP3, had to be digested with the enzyme pyroglutamate aminopeptidase to obtain amino acid sequence signals. Similar to the seed Rs-AFP isoforms (Terras et al., 1992b), Rs-AFP4 thus has a cyclized glutamine as the N-terminal amino acid.

Enhanced Disease Tolerance of Transgenic Tobacco Expressing Rs-AFP2

The characteristics of the Rs-AFPs emerging from this and previous work (Terras et al., 1992b) prompted us to analyze expression of these proteins in a heterologous tobacco system to assess the disease resistance of such transgenic plants.

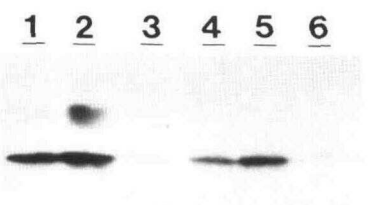

Figure 7. Induction of Rs-AFP–Like Proteins in Infected Radish Leaves.

Four-microgram amounts of leaf protein after partial purification (see Methods) were electrophoresed on an SDS–polyacrylamide gel and subjected to immunoblot analysis using anti–Rs-AFP1 antibodies. Lane 1, 100 ng of Rs-AFP1; lane 2, 200 ng of Rs-AFP1; lane 3, uninfected leaves; lane 4, uninfected leaves of radish plants infected with *A. brassicola* (see legend to Figure 6) collected 72 hr after inoculation; lane 5, infected leaves of the same plants as used for lane 4; lane 6, leaves inoculated with H_2O collected 72 hr after inoculation. Samples were prepared as described for tobacco (see Methods).

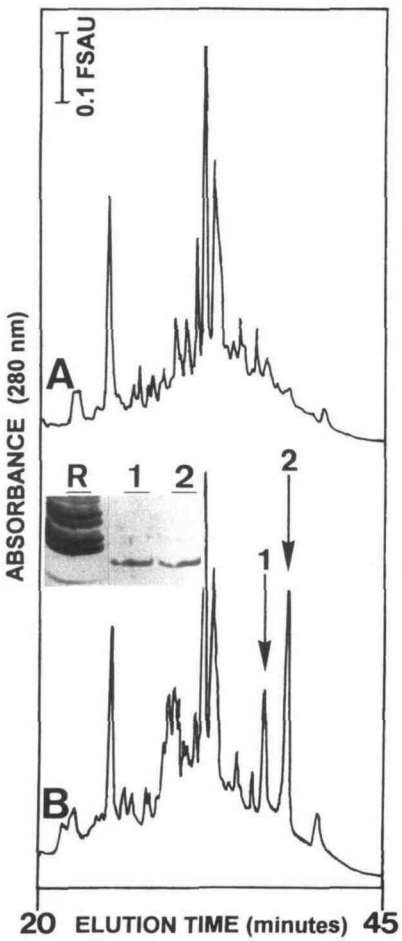

Figure 8. Purification of Rs-AFP–Like Proteins from Infected Radish Leaves.

Separation of the basic protein fraction of radish leaves on a reverse-phase chromatography column (see Methods).
(A) Basic protein fraction of 20 g of healthy leaves.
(B) Basic protein fraction of 20 g of *A. brassicola*–infected leaves. The inset shows a gel blot of proteins contained in peak 1 (lane 1) and peak 2 (lane 2) after SDS-PAGE. Lane R contains the molecular mass markers: myoglobin (17 kD), myoglobins I and II (14.4 kD), myoglobin I (8 kD), myoglobin II (6 kD), and myoglobin III (2.5 kD).
FSAU, full-scale absorption units.

Tobacco leaf discs were transformed by cocultivation with a disarmed Agrobacterium strain harboring the plant transformation vector pFRG8 (containing the chimeric gene for constitutive expression of Rs-AFP2; schematically shown in Figure 9A). Forty T_0 kanamycin-resistant plants were selected based on PCR analysis of their genome for the presence of the introduced gene as well as on Rs-AFP2 expression as assessed by immunoblotting. After self-pollination, segregation analysis of the progeny was performed by a germination assay on kanamycin-containing medium. Five T_1 lines segregating 3:1 (resistant/susceptible to kanamycin) were retained and self-pollinated, and the progeny were screened for homozygous, hemizygous, and azygous genotypes. Expression of Rs-AFP2 in the homozygous T_2 lines was again checked by immunoblot analysis of crude leaf protein fractions (Figure 9B). Based on immunoblot analysis of two independent leaf protein samples per plant from at least two siblings per T_2 line, we concluded that Rs-AFP2 was expressed at levels ranging from 0.6 to 2.4 μg per mg of total leaf protein (3 to 12 μg of Rs-AFP2 per g of fresh leaf tissue) in the five independent homozygous T_2 lines, with line 6014 displaying the highest expression level (Table 2).

Crude leaf protein fractions were also tested for in vitro antifungal activity against the tobacco foliar pathogen *A. longipes* using a quantitative microplate assay (Broekaert et al., 1990). The antifungal activity of the crude leaf protein fractions prepared from the transgenic homozygous T_2 line expressing Rs-AFP2 at the highest levels (line 6014) was more than 10-fold higher than that of extracts from untransformed or T_2 azygous (null line) plants. The other homozygous T_2 lines displayed only a two- to fourfold higher antifungal activity relative to the controls (Table 2). Under the given assay conditions, purified seed Rs-AFP2 inhibited growth of *A. longipes* by 50% at a concentration of 1.5 μg/mL. The *A. longipes* microcultures treated with crude leaf protein fractions from transgenic line 6014 clearly displayed the hyperbranched morphology typically caused by Rs-AFPs (Terras et al., 1992b; this study, Figure 9C). Thus, Rs-AFP2 can be functionally expressed in a heterologous tobacco system.

The transgenic T_2 lines expressing Rs-AFP2 were analyzed for disease resistance to *A. longipes*. The area of the lesions formed on leaves of the homozygous T_2 line 6014 was on average eight- and sevenfold lower than that of lesions formed on an azygous T_2 line derived from the same primary transformant and on an untransformed plant, respectively, as depicted in Figure 10. No lesions were visible on 64% of the infection spots of the homozygous line, versus 31 and 34% for the azygous and untransformed lines, respectively. Similar results were obtained in three additional independent tests. On the other hand, none of the other homozygous transgenic lines were more resistant than the control lines (data not shown), indicating that Rs-AFP2 expression in transgenic

Table 1. In Vitro Antifungal Activity of Radish AFPs in a Synthetic Low Ionic Strength Medium

Fungus	Rs-AFP1[a]	Rs-AFP2[a]	Rs-AFP3	Rs-AFP4
A. brassicola	15	2	2	5
Botrytis cinerea	8	2	2	9
F. culmorum	5	2	2	11

The given quantitative measure for in vitro antifungal activity is the protein concentration (micrograms per milliliter) required to obtain 50% growth inhibition and is derived from dose–response curves (Broekaert et al., 1990).
[a] In vitro antifungal activities as previously determined (Terras et al., 1992b).

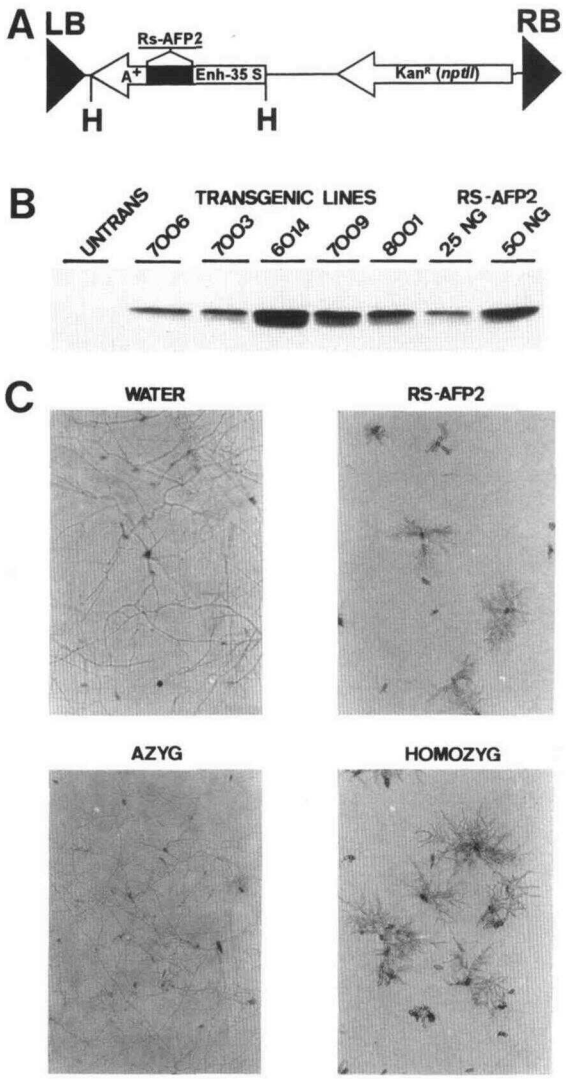

Figure 9. Analysis of Transgenic Tobacco.

(A) Diagram of the T-DNA region of pFRG8. LB, left border; RB, right border; H, HindIII site; A^+, polyadenylation signal of the CaMV 35S promoter; Rs-AFP2, coding region of Rs-AFP2; Enh-35 S, enhanced CaMV 35S promoter (Kay et al., 1987); Kan^R (*nptII*), chimeric gene conferring kanamycin resistance with a nopaline synthase promoter and terminator and the neomycin phosphotransferase II coding region.
(B) Expression of Rs-AFP2 in homozygous T_2 lines visualized by immunoblotting. UNTRANS, untransformed.
(C) In vitro antifungal activity of water against *A. longipes* (negative control), purified Rs-AFP2 at 3 μg/mL (positive control), and partially purified leaf extracts of azygous (AZYG) and homozygous (HOMOZYG) T_2 6014 lines derived from the same primary transformant. The dilutions of the extracts are equivalent to the protein fraction derived from 0.15 g of leaves (fresh weight) per mL of culture medium. Micrographs were taken after 24 hr of incubation at 22°C.

plants must reach a threshold level before displaying an increased disease tolerance phenotype.

DISCUSSION

The data presented in this paper strongly suggest that the antifungal Rs-AFPs are an important component of the defense system of radish seeds. The extracellular localization of the Rs-AFPs in seed and the preferential release of these proteins in the imbibition medium during germination can contribute to the control of seed- and soil-borne fungal diseases, thus enhancing the chances of seedling survival and reproduction. Many seed- and soil-borne fungal diseases causing important losses in yield and seedling stand are effectively controlled by treating seeds with chemical fungicides (Bateman, 1977; Koch and Leadbeater, 1992), which is a common practice in modern agriculture. Hence, Rs-AFPs may be considered a biological equivalent of seed-applied chemical fungicides. Although Swegle et al. (1992) have previously shown that antifungal chitinases are released from barley seeds during imbibition, it was not demonstrated that the released amounts are physiologically meaningful for protection of the seed.

Surprisingly, the expression of Rs-AFP–like proteins in radish is not restricted to seeds but also occurs in leaf tissues. The Rs-AFP counterparts in leaves are strongly and systemically induced upon fungal infection and can therefore be classified as a new type of PR protein. We isolated two Rs-AFPs from *A. brassicola*–infected radish leaves: they are ~90% homologous to the previously purified seed isoforms (Terras et al., 1992b) and exert comparable in vitro antifungal activity. These results also indicate that the pathogen-induced leaf Rs-AFPs are not identical to seed Rs-AFPs, which implies that

Table 2. Rs-AFP2 Expression Levels and in Vitro Antifungal Activity of Leaf Protein Extracts from Transgenic Tobacco Lines

Plant Line	Rs-AFP2 Content[a] (μg/mL Total Leaf Protein)	In Vitro Antifungal[b] Activity (Units/mL)
Untransformed	<0.2	30
Azygous line 6014	<0.2	30
Homozygous line 6014	2.4	320
Homozygous line 7003	0.6	60
Homozygous line 7006	0.6	60
Homozygous line 7009	1.3	120
Homozygous line 8001	1.1	120

[a] Rs-AFP2 levels were determined by densitometric scanning of immunoblot luminograms. Total protein levels were determined by the Coomassie blue dye binding method (Bradford, 1976).
[b] In vitro antifungal activity was measured on partially purified leaf extracts using *A. longipes* as a test fungus (see Methods for additional experimental details).

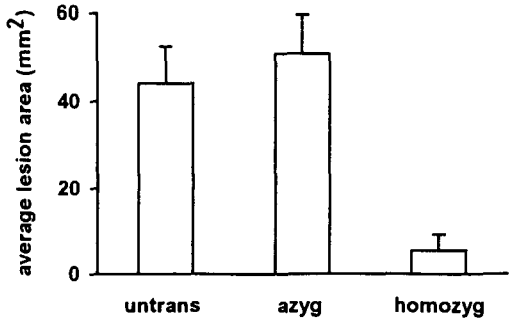

Figure 10. Transgenic Lines Analyzed for Disease Resistance.

Shown is a quantitative analysis of the tolerance to *A. longipes* infection of the Rs-AFP2–expressing tobacco line 6014 (homozyg) relative to azygous T_2 plants derived from the same primary transformant (azyg) and untransformed control (untrans) plants. The average lesion area was determined from a total of 64 spot infections (four spots per leaf, two leaves per plant, and eight plants per line) 7 days after inoculation with 5-μL drops containing 1000 spores per mL in 50 mM glucose. Lines on top of bars represent standard errors.

they are products of different genes. This finding is consistent with the existence of eight to 10 genes that hybridize with an Rs-AFP1 cDNA probe, as shown by DNA gel blot analysis (F.R.G. Terras, unpublished results). Of interest in this context is the observation of Chiang and Hadwiger (1991) that pea pods challenged with *F. solani* accumulate mRNAs transcribed from two genes encoding 5-kD proteins whose deduced amino acid sequences show 45 and 51% identity with Rs-AFP1. The onset of the accumulation of these pea transcripts coincides with the initial suppression of the growth of the pathogen (Chiang and Hadwiger, 1991). Thus, Rs-AFP–like proteins could, together with other pathogenesis-induced antifungal proteins such as chitinases and glucanases, contribute to the control of fungal diseases in vegetative tissues. Although we were unable to demonstrate expression of Rs-AFP–like genes in flowers, expression of genes encoding Rs-AFP–like proteins has been reported to occur in tobacco flowers (Gu et al., 1992), potato flowers (Moreno et al., 1994), and *Petunia inflata* pistils (Karunanandaa et al., 1994).

Additional evidence for a role of Rs-AFPs in plant defense came from the analysis of the disease tolerance of transgenic tobacco plants constitutively expressing Rs-AFP2. The leaves of such Rs-AFP2–expressing plants displayed a significantly decreased susceptibility to *A. longipes* infection relative to untransformed control plants. The difference in disease tolerance between T_2 line 6014 and the other (lower expressing) transgenic lines indicates that Rs-AFP2 must be expressed above a certain threshold level before a disease tolerance phenotype can be observed. This is consistent with the fact that purified seed Rs-AFP2 also has a sharp dose-dependent growth inhibition effect on fungi in in vitro assays. Moreover, we also observed disease resistance to *A. longipes* when assaying high-expressing multilocus integrant T_1 lines in preliminary tests (data not shown). We have also verified that the enhanced tolerance of Rs-AFP2–expressing tobacco line 6014 could be caused by enhanced expression of defense-related proteins. However, the activity of chitinases, glucanases, and peroxidases in line 6014 measured both before and after infection with *A. longipes* was not significantly different from that in similarly treated untransformed plants (I. Penninckx and W.F. Broekaert, unpublished results).

From the *A. brassicola*–infected radish leaves, we were able to purify ~2 μg of Rs-AFPs (Rs-AFP3 and Rs-AFP4 together) per g of fresh leaf tissue. Taking into account the results obtained with the transgenic tobacco plants (increased disease tolerance at Rs-AFP2 levels of 12 μg per g of fresh leaf tissue), this would mean that the accumulated amounts of Rs-AFPs in infected radish leaves are not sufficient to stop the pathogen from growing within the plant. However, preliminary results obtained from tissue printing diseased leaves showed that the amount of Rs-AFPs accumulating locally around infection sites is higher than that in the remaining part of the leaf (F.R.G. Terras, unpublished results).

Moreover, spray application of Rs-AFP2 on the surface of sugar beet leaves controls disease development by *Cercospora beticola* as efficiently as the chemical fungicide hexaconazole, when compared on a molar base (De Bolle et al., 1993), and chemical compounds that, like Rs-AFPs, cause increased hyphal branching in vitro are used to control foliar diseases in crops (Robson et al., 1989; Wiebe et al., 1990).

A closer examination of the arrangement of the cysteine and glycine residues in the Rs-AFP amino acid sequences reveals the putative existence of a "cysteine-stabilized α-helix" motif characterized by the occurrence of the sequences CXXXC, GXC, and CXC (where X stands for any amino acid). This motif was originally reported to occur in endothelin (Kobayashi et al., 1991; Tamaoki et al., 1991), a mammalian peptide with vasoconstricting activity (Yanagisawa et al., 1988), and later shown to dictate the three-dimensional structure of insect defensin A (Bonmatin et al., 1992). Insect defensin A is a 40–amino acid cysteine-rich peptide with antibacterial properties. It is induced in the hemolymph of the fleshfly (*Phormia terranovae*) after challenge with bacteria (Lambert et al., 1989). The γ-thionins isolated from cereal seeds (Colilla et al., 1990; Mendez et al., 1990) have the same arrangement of their cysteine residues as the Rs-AFPs (and thus are more homologous to the Rs-AFPs than to the α- or β-thionins; Terras et al., 1992b; Bohlmann, 1994) and display a three-dimensional fold highly similar to that of insect defensins (Bruix et al., 1993). The preliminary nuclear magnetic resonance–based three-dimensional structure of Rs-AFP1 (F. Fant, L. Santos, W. Vranken, K. Boulez, J.C. Martins, and F.A.M. Borremans, personal communication) fully supports the structural homology of this protein with γ-thionins and insect defensins. Recently, a cysteine-rich peptide with sequence homology to the Rs-AFPs (18 of 44 residues identical after alignment to the Rs-AFP1 sequence, including the cysteine-stabilized α-helical motif) was isolated from the hemolymph of bacterially infected fruit flies. Like the Rs-AFPs, this peptide is not active against bacteria but displays strong antifungal activity. This 44-residue peptide starts to accumu-

late in the insect hemolymph as early as 1 hr after bacterial challenge and is the major immune-induced peptide produced by fruit flies (Fehlbaum et al., 1994). Hence, it appears that plants and animals have developed similar weapons to combat microbial infection.

Based on the well-documented antifungal activities of the Rs-AFPs (Terras et al., 1992b; this study), their presumed role in host defense, as well as their structural and functional homologies with pathogen-inducible insect defensins, we propose the term "plant defensins" to describe the Rs-AFPs and their homologs in plants.

METHODS

Biological Material

Radish (*Raphanus sativus* cv Ronde Rode Kleine Witpunt) seeds were obtained from AVEVE (Leuven, Belgium). Growth of fungi and harvesting and storage of fungal spores were done as described previously (Broekaert et al., 1990). The following fungal strains were used: *Alternaria brassicola* (MUCL 20297; Mycothèque Université Catholique de Louvain, Louvain-la-Neuve, Belgium), *A. longipes* (CBS 620.83; Centraalbureau voor Schimmelcultures, Baarn, The Netherlands), *Fusarium culmorum* (IMI 180420; International Mycological Institute, Kew, UK), and *Pyrenophora tritici-repentis* (MUCL 30217).

Agar Diffusion Bioassay for Antifungal Activity

Radish seeds were surface sterilized by the following procedure: 1 min in 70% (v/v) ethanol, 10 min in a sixfold diluted commercial hypochlorite solution containing 0.1% (w/v) SDS, and five washes of 5 min in sterile water. The fungus *P. tritici-repentis* was grown on five cereal agar (five cereal baby food instant flakes, 20 g/L, and agar, 8 g/L; Broekaert et al., 1990). With a sterile cork borer, a mycelial plug was removed from a plate colony and placed mycelium-side down in the center of a 9-cm Petri dish containing five cereal agar. Surface-sterilized seeds were buried in the agar 3 cm from the center. Purified proteins were applied in a total volume of 5 μL in a well (3-mm diameter and 3-mm depth) punched in the medium 3 cm from the center. The seeds were positioned in the agar simultaneously with the mycelial plug; purified proteins were applied 24 hr after inoculation of the plates with the mycelial plug. The assay plates were incubated for a total of 5 days.

Imbibition of Radish Seed

Radish seeds were soaked in running tap water (RTW) for 30 min. With a scalpel, an incision was made in the seed coat (along half of the seed periphery), and the incised seeds were incubated (in a 1.5-mL Eppendorf tube; five seeds in 250 μL of RTW) for an additional 30 min or 4 hr at room temperature. Intact seeds were incubated (five seeds in 250 μL of RTW) for 4 or 12 hr. A small hole was then punched in the bottom of the Eppendorf tube, and the imbibition medium was separated from the seeds by centrifugation (5 min at 3000 rpm). The fluid was collected in a second Eppendorf tube. Particulate material in the imbibition medium was removed by centrifugation (5 min at 11,000 rpm). A crude seed extract was obtained by grinding 50 seeds in a mortar containing 2 mL of RTW. The suspension was then allowed to stand for 3 hr at 4°C, followed by removal of particulate material by centrifugation (5 min at 11,000 rpm).

Estimation of radish antifungal proteins in the seed imbibition solution or seed extract was done by densitometry scanning of an x-ray film obtained after chemiluminescent detection of Rs-AFPs on an immunoblot (see the following section) and standardized to a twofold dilution series of purified Rs-AFP1. Determination of total protein was performed by the Bradford protein assay (Bradford, 1976) using BSA as a standard.

Protein Gel Electrophoresis and Immunoblotting

Samples were analyzed by SDS-PAGE (separating gel: 15% total acrylamide, 0.5% bis-acrylamide; stacking gel: 5% total acrylamide, 2% bis-acrylamide) on two replicate gels. One gel was stained with Coomassie Brilliant Blue R 250; proteins separated on the other were electroblotted (semidry, 1.5 hr in 48 mM glycine, 39 mM Tris, 20% [v/v] methanol at 1 mA/cm^2) onto nitrocellulose (0.2-μm pore size; Hoefer Scientific Instruments, San Francisco, CA). Immunoblots were processed as described (Boehringer Mannheim Colloquium No. 2/1992), and detection was performed using the enhanced chemiluminescence method (Amersham). Anti-Rs-AFP1 IgGs were obtained by treating antiserum from immunized rabbits with caprylic acid followed by $(NH_4)_2SO_4$ precipitation according to Harlow and Lane (1988). The IgG precipitate was then redissolved in PBS (the initial serum volume) and dialyzed against PBS. The IgG fraction was passed over an affinity matrix consisting of cyanogen bromide–activated Sepharose 6MB (Pharmacia) to which total radish seed proteins were coupled (specific anti-Rs-AFP antibodies do not bind to purified Rs-AFP1 coupled to this matrix; F.R.G. Terras, unpublished results). The partially purified primary antibodies were diluted 1:5000 in the immunoblotting experiments.

Immunolocalization

For tissue print immunolocalization, mature radish seed were cut in halves. With each half, a print was made on nitrocellulose (0.22-μm pore size) prewetted in Tris-buffered saline (100 mM Tris, 150 mM NaCl, pH 7.5). Prints were stained for total protein with 0.1% (w/v) amido black in 25% (v/v) isopropanol, 10% (v/v) acetic acid for 1 min and destained by three 10-min washes in 25% (v/v) isopropanol, 10% (v/v) acetic acid. Prints for immunolocalization of Rs-AFPs were processed as described above, except that the secondary antibodies were conjugated to alkaline phosphatase and the detection was performed with 5-bromo-4-chloro-3-indolyl phosphate and nitro blue tetrazolium.

Whole radish seed were fixed and subsequently embedded in London Resin White resin (Polysciences, Warrington, PA) according to De Clercq et al. (1990). Ultrathin (60 nm) sections were made with an Ultracut S microtome (Reichert-Jung, Vienna, Austria) equipped with a diamond knife and collected on nickel grids precoated with Formvar (Polyscience, Niles, IL). Semithin sections (1 μm) were produced with a glass knife and collected on glass slides pretreated with 4% (w/v) gelatin and 0.4% (w/v) $KCr(SO_4)_2$. Ultrathin sections were further processed as follows: 30 min in 5% (w/v) $NaIO_4$; three times for 2 min in H_2O; 10 min in 0.1 N HCl; three times for 2 min in H_2O; two times for 30 min in PBS, 0.1% (v/v) Tween 20, 2% (w/v) BSA, pH 7.2; 1.5 hr with partially purified anti-Rs-AFP1 antibodies or preimmune serum diluted 50-fold in PBS, 0.1% (v/v) Tween 20, 2% (w/v) BSA, pH 7.2;

four times for 5 min in PBS, 0.5% (v/v) Tween 20, pH 7.2; two times for 15 min in PBS, 0.1% (v/v) Tween 20, 2% (w/v) BSA, pH 7.2; 1 hr with protein A–gold (15-nm particles; EY Lab Inc., San Mateo, CA) conjugate diluted 50-fold in PBS, 0.1% (v/v) Tween 20, 1% (w/v) BSA, pH 7.2; three times for 6 min in PBS, 0.5% (v/v) Tween 20, pH 7.2; three times for 6 min in H_2O; 1 hr in 2% (w/v) uranyl acetate in H_2O; and 4 min in $Pb(OH)_2$ chelated with citrate (Reynold's solution). Electron microscopy was performed with a JEOL 100CXII transmission microscope (JEOL, Tokyo, Japan).

Semithin sections were incubated as follows: 20 min in 0.5% (v/v) Triton X-100; three times for 10 min in PBS, 10 mM glycine; 15 min in undiluted goat serum; 12 hr at 4°C with partially purified anti–Rs-AFP1 antibodies or preimmune serum diluted 50-fold in 10% (v/v) goat serum, 90% (v/v) PBS, 0.02% (v/v) Triton X-100; three times for 10 min in PBS, 10 mM glycine; 15 min in undiluted goat serum; 1 hr in rhodamine-conjugated goat anti–rabbit IgGs (Pierce, Rockford, IL) diluted 50-fold in 10% (v/v) goat serum, 90% (v/v) PBS, 0.02% (v/v) Triton X-100; and an additional three times for 10 min in PBS. For immunofluorescence, sections were viewed with epifluorescence optics with a 450- to 495-nm filter (Zeiss Axiophot microscope, Oberkochen, Germany).

Molecular Cloning of Rs-AFP cDNAs

Seeds at six different developmental stages were collected from outdoor-grown radish plants, frozen in liquid nitrogen, and stored at −80°C. After pulverization, total RNA was extracted from 15 g of a mixture of the six different developmental stages, using the method of de Vries et al. (1988), with the exception that 6 mL of a 1:2 phenol–RNA extraction buffer mixture and 2 mL of chloroform were used per g of tissue. Poly(A)$^+$ mRNA was purified by affinity chromatography on oligo(dT)–cellulose as described by Sambrook et al. (1989), yielding ~10 μg of poly(A)$^+$ RNA per g of tissue. Double-stranded cDNAs were prepared from 1.5 μg of poly(A)$^+$ RNA according to Gubler and Hoffman (1983) and ligated to EcoRI-NotI adapters (cDNA synthesis kit, Pharmacia). The cDNAs were cloned into the λZAPII phage vector (Stratagene) according to the manufacturer's instructions. A DNA probe for screening the cDNA library was produced by polymerase chain reaction (PCR) as follows. Two degenerate oligonucleotides were synthesized: OWB15 (5′-AAAGAATTCAARYTNTGYSARMGNCC-3′; EcoRI recognition sequence underlined) and OWB17 (5′-AAAGAATTC-RTGNGCNGGRAANACRTARTTRC-3′; EcoRI recognition sequence underlined). OWB15 corresponds to amino acids 2 to 7 of Rs-AFP1 and has a sense orientation, whereas OWB17 corresponds to amino acids 36 to 43 of Rs-AFP1 and has an antisense orientation. PCR was performed with the *Taq* polymerase under standard conditions (Sambrook et al., 1989) using OWB15 and OWB17 as primers and 25 ng of cDNA as target DNA. The amplification program included an initial step at 94°C for 5 min, 30 cycles of 1 min at 94°C, 2 min at 45°C, 3 min at 72°C, and a final step at 72°C for 10 min. The 144-bp PCR amplification product was purified on a 3% (w/v) agarose (NuSieve; FMC, Rockland, ME) gel and partially reamplified with the degenerate sense oligonucleotide OWB16 (5′-AAAGAATTCGGNACNTGGWSNGGN-GTNTG-3′; EcoRI recognition sequence underlined) and OWB17. OWB16 corresponds to amino acids 9 to 15 of Rs-AFP1. The resulting 123-bp PCR amplification product was again purified on a 3% (w/v) agarose (NuSieve; FMC) gel and reamplified by PCR under the same conditions, except that the reaction mixture contained 130 μM deoxythimidine triphosphate (dTTP) and 70 μM digoxigenin-11-dUTP rather than 200 μM dTTP. The digoxigenin-labeled PCR product was purified on a 3% (w/v) NuSieve agarose gel and stored as an agarose slice at −20°C.

Approximately 10,000 plaque-forming units of the λZAPII cDNA library were screened with the digoxigenin-labeled PCR product by in situ plaque hybridization using nylon membranes (Hybond-N; Amersham). Membranes were air dried, and DNA was cross-linked to the membranes under UV light. Prehybridization was performed for 4 hr at 64°C in 5 × SSC (1 × SSC is 0.15 M NaCl, 0.015 M sodium citrate), 1% (w/v) blocking reagent (Boehringer Mannheim), 0.1% (w/v) *N*-lauroylsarcosine, 0.02% (w/v) SDS. The agarose slice containing the digoxigenin-labeled DNA probe was molten, and the appropriate amount was diluted to 0.5 mL with prehybridization buffer. After heat denaturing (boiling for 10 min), the probe was added at a final concentration of 10 ng/mL to fresh prehybridization buffer. Hybridization was performed for 16 hr at 64°C. Excess probe was removed by washing two times for 5 min in 2 × SSC, 0.1% (w/v) SDS at room temperature. Stringency washes (two times for 15 min) were performed in 0.1 × SSC, 0.1% (w/v) SDS at 64°C. Detection of the bound probe was done using anti-digoxigenin antibodies linked to alkaline phosphatase (Boehringer Mannheim) and 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium as color reagent according to the manufacturer's instructions. Positive plaques were purified by two additional screening rounds with the same probe under the same conditions. Inserts from purified plaques were excised in vivo and rescued as pBluescript SK– phagemids with the aid of the helper phage R408 (Stratagene), according to the supplier's instructions. The inserts from the positive clones were excised by EcoRI digestion, and their sizes were determined by agarose gel electrophoresis.

Nucleotide sequence determinations were performed with an automatic sequencer (A.L.F; Pharmacia) based on the dideoxynucleotide chain termination method (Sanger et al., 1977).

Cloning of the Full-Length Rs-AFP2 cDNA

To obtain a full-length Rs-AFP2 cDNA clone, 10-μg amounts of total RNA isolated from mature radish seed were reverse transcribed with the avian myeloblastosis virus reverse transcriptase (Boehringer Mannheim) for 30 min at 52°C. The reverse transcription reactions were primed by a homopolymeric deoxythymidine oligonucleotide (20-mer). Excess reagents were removed by passing the reaction mixture over a Chroma Spin+TE100 column (Clontech, Palo Alto, CA) equilibrated with 10 mM Tris, 1 mM EDTA, 300 mM NaCl, 0.05% (w/v) SDS, pH 8 (Delort et al., 1989). RNA was degraded by alkaline hydrolysis in 0.2 N NaOH for 30 min at 50°C. After neutralization, the single-stranded cDNAs were precipitated with LiCl and ethanol. The anchor oligonucleotide OWB116 (5′-PO_4^{3-}-AGAATTCGCATTGCATCGGATCCATGA-TCGAT-NH_2-3′; EcoRI recognition sequence underlined and BamHI recognition sequence double underlined) was subsequently ligated to one-fourth of the single-stranded cDNAs with the T4 RNA ligase (New England Biolabs, Beverly, MA) for 24 hr at 22°C as described by Dumas et al. (1991). One-tenth of the anchor-ligated single-stranded cDNAs were used to perform PCR with the oligonucleotides OWB117 (5′-ATCGATCATGGATCCGATGCAATGC-3′; BamHI recognition sequence underlined; OWB117 is the inverse complement of a part of OWB116) and OWB23 (5′-ATAGAATTCGACGTGAGCTTATCATCTTATT-ATCCG-3′; EcoRI recognition sequence underlined; OWB23 is the inverse complement of nucleotides 421 to 447 of the Rs-AFP2 cDNA; see Figure 5B) as amplimers. The nucleotide sequence of several cloned PCR products was determined to rule out possible PCR errors.

Cloning of the Genomic Regions Encoding Rs-AFP1 and Rs-AFP2

Radish genomic DNA for use in PCRs was isolated by grinding 0.5 cm^2 of radish leaf tissue in an Eppendorf tube containing 400 μL of extraction buffer (200 mM Tris, 25 mM EDTA, 250 mM NaCl, 0.5% [w/v] SDS, 10 μM β-mercaptoethanol, pH 8). After centrifugation (5 min at 11,000 rpm), 300 μL of the supernatant was transferred to a new Eppendorf tube, and an equal volume of isopropanol was added to precipitate the DNA. The precipitated DNA was collected by centrifugation (5 min at 11,000 rpm), dried, and redissolved in 100 μL of 10 mM Tris, 1 mM EDTA, pH 8. Five microliters of this DNA solution was used as a template in the PCR amplification (in a total volume of 20 μL). The specific primers OWB201 (5′-TAATGAATTCGTTTTATTAGTGATCATGGC-3′, corresponding to nucleotides 1 to 20 of pFRG1; EcoRI recognition sequence underlined) and OWB202 (5′-TAATGGATCCTAAAACTTTATTTGTATAACCG-3′, the inverse complement of nucleotides 368 to 289 of pFRG1; BamHI recognition sequence underlined) were used to amplify the Rs-AFP1–encoding genomic region. The specific primers OWB232 (5′-AAGCTGTCGACCACATACATATACATTAAAAACTAGG-3′, corresponding to nucleotides 2 to 27 of the full-length Rs-AFP2 cDNA; SalI recognition site underlined) and OWB23 (see previous section) were used to amplify the Rs-AFP2–encoding genomic region. PCR products were digested with the appropriate restriction enzymes and cloned into pEMBL18+. Nucleotide sequences were determined as described above.

RNA Gel Blotting

Total RNA was isolated according to Logemann et al. (1987). Samples of 15 μg were glyoxal denaturated (Thomas, 1983) and separated in a 1.4% (w/v) agarose gel (in 10 mM sodium phosphate, pH 7). RNA was blotted onto a positively charged nylon membrane (Boehringer Mannheim) via capillary transfer in 20 × SSC, cross-linked by UV illumination (5 min; both sides of the blot), and deglyoxalated by incubating the blot for 10 min in 20 mM Tris, pH 10, at 90°C followed by immediate transfer to 20 mM Tris, pH 8, at room temperature. Prehybridization with 50% (v/v) formamide, 5 × SSC, 2% (w/v) blocking reagent (Boehringer Mannheim), 0.1% (w/v) *N*-lauroylsarcosine, 0.02% (w/v) SDS was performed at 68°C for 6 hr. A digoxigenin-labeled antisense RNA probe (Dig RNA labeling kit; Boehringer Mannheim) was made by run-off transcription (with T7 RNA polymerase) from the BamHI-linearized plasmid pFRG1. Hybridization with this probe included at 100 ng/mL in prehybridization buffer was performed overnight at 68°C in a rolling bottle oven. Excess probe was removed by two 5-min washes with 2 × SSC, 0.1% (w/v) SDS at room temperature. Stringency washes (twice for 20 min) were performed at 68°C with 0.1 × SSC, 0.1% (w/v) SDS. Bound probe was visualized following the immunochemiluminescence detection protocol of Boehringer Mannheim. X-ray films were exposed for 3 hr.

Purification of Antifungal Proteins from *A. brassicola*–Infected Radish Leaves

Leaves of 4-week-old radish plants were inoculated with 50 5-μL drops of H_2O (control) or *A. brassicola* spore suspension (at 5×10^5 spores per mL in H_2O) and collected after 3 days of incubation at 100% relative humidity. The remaining liquid of the drops was taken up with absorbing paper, and the fresh weight of the leaf samples was determined. After grinding the leaf material in liquid nitrogen, two volumes (twice the fresh weight of the leaves) of extraction buffer (50 mM Tris, pH 7.5, 1 mM phenylmethylsulfonyl fluoride, 2 mM thiourea, 1.5% [w/v] polyvinylpolypyrrolidone) were added. This suspension was continuously stirred at room temperature for 2 hr. The particulate material was removed by centrifugation (30 min at 7000*g*), and the supernatant was subsequently filtered through Whatman No. 1 filter paper and through 0.45- and 0.22-μm filters (Millipore Corp., Milford, MA). The filtrate was loaded on a Q-Sepharose Fast Flow anion exchange column (5 × 5 cm; Pharmacia, Uppsala, Sweden) equilibrated with 50 mM Tris, pH 7.5. The flow-through fraction was dialyzed overnight against distilled water in a dialysis bag with a molecular mass cut-off of 1000 D (Spectra/Por 6; Alltech, Deerfield, IL) and brought to 50 mM Mes (2-(*N*-morpholino)ethanesulfonic acid, pH 5.5). The dialyzed basic protein fraction at pH 5.5 was then loaded on an S-Sepharose high-performance cation exchange column (10 × 1.6 cm; Pharmacia) equilibrated with 50 mM Mes, pH 5.5. Bound proteins were eluted with 0.5 M NaCl, 50 mM Mes, pH 5.5. This fraction was finally separated on a reverse-phase chromatography column (C_2/C_{18} 5-μm porous silica, 25 × 0.4 cm; Pharmacia) equilibrated with 0.1% (v/v) trifluoroacetic acid (TFA). The following gradient (at a flow rate of 1 mL/min) was applied: 0 to 2 min, 0.1% (v/v) TFA; 2 to 52 min, 0 to 50% (v/v) acetonitrile in 0.1% (v/v) TFA. The eluate was monitored at 280 nm for detection of proteins. Peak fractions were collected manually, vacuum dried, and redissolved in Milli Q-water (Millipore, Bedford, MA). Protein determination, SDS-PAGE analysis on precast PhastGel High Density (Pharmacia), amino acid sequence determination, and in vitro antifungal activity determination were performed as previously described (Terras et al., 1992b). Rs-AFP3 was not derivatized before automated Edman degradation. Cysteines in Rs-AFP4 were reduced, thiol groups were S-carboxyamidomethylated, the derivatized Rs-AFP4 was repurified by reverse-phase chromatography, and the N terminus was deblocked by incubation with pyroglutamate aminopeptidase (Boehringer Mannheim) before automated Edman degradation as described by Terras et al. (1992b).

Site-Directed Mutagenesis

For construction of a plant transformation vector, we have chosen the Rs-AFP2 coding sequence because Rs-AFP2 has a higher antifungal activity than Rs-AFP1 (Terras et al., 1992b). Because a full-length cDNA clone for Rs-AFP2 was not available at the onset of the plant transformation experiments, we decided to convert the Rs-AFP1 coding sequence to an Rs-AFP2 coding sequence, using site-directed mutagenesis performed essentially as described by Merino et al. (1992). In a first step, PCR (30 cycles; 1 min at 94°C, 2 min at 45°C, 3 min at 72°C) was performed on KpnI-linearized pFRG1 (100 ng) with the first antisense mismatch primer OWB28 (5′-CTTGGCCTTTGGCACAACTTC-3′; converting Glu-5 to Gln-5) and the M13 reverse primer extended with a 5′ tag, OWB35 (5′-GGAATAGCCGATCGAGATCTAGGAAACAGCTATGACCATG-3′). The resulting product was extended (five cycles; 1 min at 94°C, 1 min at 50°C, 1 min at 72°C) in the presence of pFRG1, followed by the addition of the second antisense mismatch primer, OWB29 (5′-GCTTTCTCAAGTCTAATGCAC-3′; converting Asn-27 to Arg-27), and an oligonucleotide identical to the 5′ tag of the M13 reverse primer OWB36 (5′-GGAATAGCCGATCGAGATCTAGGA-3′). Standard amplification was used (see first step). The same procedure was repeated once more, except that the antisense primer OWB30 (5′-AACTCGAGCTGCAGTGTCGACCTATTAACAAGGAAAGTAGC-3′) was used. It is complementary to the 3′ end of the coding sequence

of the Rs-AFP1 cDNA. This latter primer introduced a second stop codon and the SalI, PstI, and XhoI restriction sites behind the natural stop codon of the Rs-AFP1 cDNA. Due to its higher fidelity, *Pfu* polymerase (Stratagene) was used instead of *Taq* polymerase. The final product was digested with BamHI and SalI and cloned in the corresponding sites of pEMBL18+ (yielding pFRG4) and checked by nucleotide sequence determination.

Construction of the Plant Transformation Vector pFRG8 and Transformation of Tobacco

The Rs-AFP2 coding sequence was cloned as a 5′ BamHI–SalI 3′ fragment (isolated from pFRG4) between the enhanced cauliflower mosaic virus (CaMV) 35S promoter and the CaMV 35S terminator regions of pFAJ3002, yielding pFRG7. pFAJ3002 is a derivative of pFF19 (Timmermans et al., 1991) in which the unique EcoRI site of pFF19 is replaced by a HindIII site. The resulting chimeric gene has the following leader sequence (from the CaMV 35S promotor transcription start site to the Rs-AFP2 start codon, with sequences derived from the Rs-AFP1 cDNA 5′ leader underlined): ACACGCTGAAATCACCAGTCTCTCTCTACAAATCTATCTCTCTCGATCGCGAGCTCGGTACCCGGGGATCCCCCCGGGCTGCAGGAATTCGCGGCCGCGTTTTATTAGTGATC. The chimeric Rs-AFP2 expression cassette (HindIII fragment of pFRG7) was subsequently cloned in the HindIII site of the T-DNA region of the binary pBin19Ri plant transformation vector, yielding pFRG8. pBin19Ri is identical to pBin19 (Bevan, 1984), except that the orientation of the multiple cloning site is reversed and the original chimeric neomycin phosphotransferase II (*nptII*) gene between the T-DNA borders is replaced by the chimeric *nptII* gene described in An et al. (1984). pFRG8 was introduced into *Agrobacterium tumefaciens* LBA4404 (Hoekema et al., 1983) by electroporation. The resulting Agrobacterium strain was used for transformation of tobacco (cv Samsun NN) leaf explants by cocultivation, and transgenic plants were regenerated under kanamycin selection (100 mg/L) as described by Horsch et al. (1985).

Analyses on Transgenic Tobacco

Leaf extracts for immunoblot analysis were prepared by extracting 1 g of fresh leaf tissue with 1 mL of seed extraction buffer (Terras et al., 1992b) for 1 hr at room temperature, followed by heating of the suspension at 80°C for 10 min. Particulate material was subsequently removed by centrifugation, and samples were analyzed by immunoblotting as described above, except that 5% (w/v) nonfat dried milk in Tris-buffered saline, 0.1% (v/v) Tween 20 was used as blocking reagent (rather than 2% [w/v] blocking reagent [Boehringer Mannheim] in Tris-buffered saline, 0.1% [v/v] Tween 20) and incubations with antibodies and washing steps were performed in Tris-buffered saline, 0.1% (v/v) Tween 20 (rather than Tris-buffered saline containing 0.1% [v/v] Tween 20 and 2% [w/v] blocking reagent [Boehringer Mannheim]).

Antifungal activity of crude protein fractions isolated from leaves was determined as follows. Five-gram amounts (fresh weight) of leaves were homogenized in 10 mL of seed extraction buffer as previously described. The suspension was heated at 80°C for 5 min and clarified by centrifugation. Solid $(NH_4)_2SO_4$ was added to the supernatant to 75% relative saturation. Proteins precipitated after standing for 1 hr at room temperature were collected by centrifugation (30 min at 7000*g*). The pellet was resuspended in 2 mL of sterile distilled water, and the solution was subsequently desalted by passage over a disposable reverse-phase column (C_8 silica, 1-mL bed volume; SEP-PAK; Waters, Milford, MA). The column was rinsed with 5% (v/v) acetonitrile, 0.1% (v/v) TFA, and proteins were eluted with 50% (v/v) acetonitrile, 0.1% (v/v) TFA. The eluted protein fraction was freeze dried, taken up in 200 μL of sterile distilled water, filter sterilized (0.22 μm), and tested in a microplate antifungal activity assay (Broekaert et al., 1990). *A. longipes* was used as a test fungus in a growth medium consisting of 16-fold diluted potato dextrose broth (Difco, Detroit, MI). In this medium, purified Rs-AFP2 inhibits *A. longipes* by 50% at 1.5 μg/mL. The antifungal activity of a sample is given in units per milliliter and is defined as the total volume of the assay mixture divided by the volume of the sample in the assay mixture that gives 50% growth inhibition (i.e., the dilution factor for obtaining 50% growth inhibition).

Germination assays for checking the segregation of the chimeric *nptII* gene in transgenic tobacco were performed on half-strength Murashige and Skoog medium (Murashige and Skoog, 1962) containing 100 mg/L kanamycin and 3% (w/v) sucrose as described by Deblaere et al. (1987).

To assay the tolerance to infection of tobacco leaves by *A. longipes*, tobacco seeds were surface sterilized (see agar diffusion bioassay), sown on solid half-strength Murashige and Skoog medium containing 2% (w/v) sucrose in sterile plastic containers (10-cm diameter, 6-cm height), and grown at 24°C under a 16-hr-light/8-hr-dark regime. Plants were subcultured by placing the part with the two top leaves in fresh medium. Disease tests were performed on plants 3 weeks after subculturing by applying 5-μL drops of an *A. longipes* spore suspension (1000 spores per mL) in 50 mM glucose at the adaxial side of the leaves. Inoculated plants were further incubated, and disease symptoms were scored 7 days after infection by measuring the size of the lesions visible at the abaxial side of the leaves.

ACKNOWLEDGMENTS

The authors are indebted to Inge Goderis for determining the nucleotide sequences and for performing part of the cloning work. The authors also thank Cliff Hart (ZENECA Agrochemicals, Bracknell, UK) for making the photograph shown on the front cover. This work was supported in part by the European Collaborative Linkage of Agriculture and Industry through Research (ECLAIR) Programme (AGRE-0005) of the Commission of the European Union. F.R.G.T. is the recipient of a predoctoral fellowship from the Belgian Instituut ter Aanmoediging van het Wetenschappelijk Onderzoek in Nijverheid en Landbouw and acknowledges a grant from the Belgian Nationaal Fonds voor Wetenschappelijk Onderzoek to stay as a visiting researcher at the Michigan State University Department of Energy Plant Research Laboratory. N.V.R. acknowledges the receipt of National Science Foundation Grant No. DCB-9002652. W.F.B. is a Research Associate of the Belgian Nationaal Fonds voor Wetenschappelijk Onderzoek.

Received February 15, 1995; accepted March 28, 1995.

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