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RNase Activity Prevents the Growth of a Fungal Pathogen in Tobacco Leaves and Increases upon Induction of Systemic Acquired Resistance with Elicitin

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The hypersensitive response and systemic acquired resistance (SAR) can be induced in tobacco (*Nicotiana tabacum* L.) plants by cryptogein, an elicitin secreted by *Phytophthora cryptogea*. Stem application of cryptogein leads to the establishment of acquired resistance to subsequent leaf infection with *Phytophthora parasitica* var *nicotianae*, the agent of the tobacco black shank disease. We have studied early events that occur after the infection and show here that a tobacco gene encoding the extracellular S-like RNase NE is expressed in response to inoculation with the pathogenic fungus. Upon induction of SAR with cryptogein, the accumulation of NE transcripts coincided with a rapid induction of RNase activity and with the increase in the activity of at least two different extracellular RNases. Moreover, exogenous application of RNase activity in the extracellular space of leaves led to a reduction of the fungus development by up to 90%, independently of any cryptogein treatment and in the absence of apparent necrosis. These results indicate that the up-regulation of apoplastic RNase activity after inoculation could contribute to the control of fungal invasion in plants induced to SAR with cryptogein.

An outstanding opportunity to improve plant disease resistance is to understand the mechanisms involved in the induction of SAR. SAR is the ability of a whole plant to become resistant to a broad range of pathogens after a previous and local interaction with a pathogen. This nonspecific and long-lasting defense mechanism of plants is initiated after the induction of the HR, a local response involving cell death and resulting from the interaction with an avirulent pathogen. Now described in many plant species, SAR was first systematically analyzed in tobacco (*Nicotiana tabacum* L.). After inoculation with TMV, tobacco plants harboring the N-resistance gene develop a HR and acquire resistance against viral, bacterial, and fungal pathogens (Ross, 1961; Kuc, 1982). Many investigations have focused either on the identification of pathogen signals that trigger the HR (Ebel and Cosio, 1994) or on plant responses that govern SAR (Ryals et al., 1996).

Studies on the interactions of tobacco with the *Phytophthora* genus of fungi have led to the identification of elicitins, a family of fungal proteins able to induce the complete sequence of events involved in the induction of SAR (Ricci et al., 1993). For example, cryptogein is a 10-kD basic elicitin, secreted by *Phytophthora cryptogea*, a fungus nonpathogenic to tobacco, which induces a HR-like necrosis after application on tobacco (Bonnet et al., 1986; Billard et al., 1988). Physiological modifications associated with the HR, such as capsidiol and ethylene synthesis, H^+/K^+ exchange, and active oxygen species production are observed after cryptogein treatment of tobacco cell culture (Blein et al., 1991; Milat et al., 1991; Viard et al., 1994; Rustérucci et al., 1996). Moreover, tobacco plants treated by stem application with cryptogein become resistant to local inoculation with Ppn (Ricci et al., 1989), the agent of the black shank disease and the only pathogenic variety of *Phytophthora parasitica* known on tobacco. Resistance is not only restricted to the site of elicitin application, but is also observed after petiole inoculation at all levels of the stem (Bonnet et al., 1996). Therefore, elicitin-induced resistance appears to be systemic and also nonspecific, since under these conditions tobacco plants are protected against *Sclerotinia sclerotiorum*, a fungal pathogen unrelated to *Phytophthora* spp.

Cryptogein induces the expression of a set of PR protein genes (Keller et al., 1996a) previously described to be activated in tobacco plants in correlation with the onset of SAR (Métraux et al., 1991; Ward et al., 1991). PR genes are locally activated where the HR occurs in relation to the presence of cryptogein. A subset of these genes is also expressed in leaf tissues surrounding the necrotized areas, such as genes coding for PR-1a, PR-2, and PR-5 proteins. An important limitation to a better understanding of SAR is the ignorance of the biochemical activities of most PR proteins, with the exception of β-1,3-glucanases or chitinases, which can degrade the cell walls of fungal and bacterial pathogens (for review, see Ryals et al., 1994; Van Loon et al., 1994). Moreover, the molecular mechanisms involved in the regulation of the expression of PR protein genes are not well understood, and the transduction pathways leading to their activation in response to SAR inducers are poorly defined.

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Abbreviations: HR, hypersensitive response; ICF, intercellular fluid; Ppn, *Phytophthora parasitica* var *nicotianae*; PR, pathogenesis related; SAR, systemic acquired resistance; TMV, tobacco mosaic virus.

Salicylic acid and 2,6-dichloroisonicotinic acid are synthetic compounds that also induce acquired resistance and PR protein gene expression. The requirement of endogenous salicylic acid in SAR has been demonstrated by the use of transgenic tobacco expressing the bacterial *nahG* gene encoding a salicylate hydroxylase, which converts salicylic acid to catechol (Gaffney et al., 1993). After TMV inoculation, these plants do not accumulate salicylic acid or develop acquired resistance to subsequent inoculations. Upstream of salicylic acid induction, GTP-binding proteins could be part of the transduction pathway leading to SAR, as was suggested by the induction of PR gene expression and the increase of resistance to *Pseudomonas tabaci* in transgenic tobacco expressing the A1 subunit of the cholera toxin (Beffa et al., 1995).

In plants functional roles of extracellular RNases have been suggested in nutrient remobilization and shown in gametophytic self-incompatibility (for review, see Green, 1994; Dodds et al., 1996a). The expression of S-RNase genes in the pistils of self-incompatible flowers is required for the rejection of the pollen bearing the same S-allele (McClure et al., 1989; Lee et al., 1994; Murtlett et al., 1994). On the other hand, the induction of S-like RNase genes has been described in response to phosphate limitation in *Arabidopsis thaliana*, tomato, and *Nicotiana alata* (Bariola et al., 1994; Köck et al., 1995; Dodds et al., 1996b). Based on the correlation between the high level of RNase activity in the pistil and the low susceptibility of this tissue to pathogens, it has been proposed that RNases could participate in the defense mechanisms of plants (Lee et al., 1992). An increase in extracellular RNase activity in rust-infected wheat leaves has been observed compared with control leaves (Barna et al., 1989). Lusso and Kuc (1995) have reported the increase of RNase activity in tobacco plants induced to SAR with TMV after challenge inoculation with viral and fungal pathogens. This work suggests a role for this enzymatic activity in SAR and supports the hypothesis that a significant and dynamic step in the expression of the SAR phenotype takes place only after subsequent inoculation with the pathogen.

In this paper we present data that suggest the involvement of extracellular RNases in acquired resistance in tobacco. We show that a gene encoding an S-like extracellular RNase, NE, is expressed transiently in tobacco leaves in response to Ppn inoculation. Its expression coincides with a rapid increase of RNase activity in tobacco induced to SAR with cryptogein. Under these conditions two extracellular RNase activities with approximate sizes of 22 and 18 kD were stimulated. Our data also demonstrate that the presence of RNase activity in the extracellular space is sufficient to prevent invasion of tobacco leaf by Ppn. The latter observation was made independently of any elicitin treatment and in the absence of any apparent necrosis. According to these results, we propose that an activation of extracellular RNase activity specifically upon challenge infection could be an important requirement for the establishment of acquired resistance in tobacco.

MATERIALS AND METHODS

Plant and Fungal Material

Experiments were performed with tobacco (*Nicotiana tabacum* L. cv Xanthi nc.) plants or with the transgenic line NahG-8 expressing the *nahG* gene (Gaffney et al., 1993), which was generously provided by J. Ryals (CIBA-GEIGY Corp., Research Triangle Park, NC). Plants were grown in a growth chamber at 24°C with a 16-h photoperiod at a light intensity of 100 μE m^{-2} s^{-1}. Ppn isolate 329 (Lacourt et al., 1994) was from the *Phytophthora* collection of the Institute National de la Recherche Agronomique (Antibes, France) and was cultivated on malt-agar at 24°C in the dark. For the production of zoospores, Ppn mycelium was cultivated for 1 week in V8 liquid medium (Ribeiro, 1978) at 24°C under continuous light conditions, macerated, and subsequently incubated for 4 d on water containing 2% agar. The zoospores were released after a heat-shock procedure consisting of two incubations, the first at 4°C for 1 h and the second at 37°C for 30 min, after the addition of 10 mL of water. A suspension of zoospores in water at 1 zoospore per microliter was prepared just before the inoculation procedure.

Cryptogein Treatments and Challenge Inoculations

All experiments were performed on 7- or 8-week-old tobacco plants. For elicitin application, four plants were decapitated and stem treated with 20 μL of water or an aqueous solution of cryptogein (5 μM), which was purified as previously described by Bonnet et al. (1996). For SAR-induction assays on leaves, Ppn was inoculated 48 h after the elicitin treatment by infiltrating a 100-μL suspension containing 100 zoospores into the parenchymatic tissue of nonnecrotic leaf areas, which represents a weak inoculum. In each experiment two infiltrations of zoospore suspension per leaf were performed on the second to the sixth leaf below the spot of elicitin application. To evaluate the level of protection, the invaded areas of each leaf were measured at different times after inoculation and compared with those of water-treated and inoculated control plants.

PCR Amplification of RNA and Cloning of RNase Probes

Reverse transcription of mRNA and PCR amplification were carried out as described (Kawasaki, 1990). Polyadenylated RNA from Ppn-inoculated leaves of tobacco plants treated with water or cryptogein was purified by using the Quickprep Micro mRNA Purification Kit (Pharmacia). First-strand cDNA was obtained by using a reverse-transcription system (Promega). Degenerate oligonucleotides used as PCR primers were designed from the coding sequences of S-like RNases: LE of tomato (*Lycopersicon esculentum* L.) (Köck et al., 1994), NE of *Nicotiana alata* (Dodds et al., 1996b), and RNS1 and RNS2 of *Arabidopsis thaliana* (Bariola et al., 1994). The sense primer was derived from the consensus sequence QWPGSYCD (Green, 1994) and corresponds to the oligonucleotide 5′-CAGTGGCCAG-GATCXTAYTGY-3′. The antisense primer was derived

from the consensus sequence HEWEKHGTC and corresponds to the oligonucleotide 5′-ACAAGTGCCATGYTTYTCCCA-3′. The PCR products were separated by agarose-gel electrophoresis, cloned in the pGEM-T plasmid (Promega), and sequenced by the dideoxynucleotide method using the Sequenase system (United States Biochemical). The complete coding sequence of *N. tabacum* NE cDNA was also obtained by reverse transcription-PCR with primers derived from the 5′ and 3′ ends from *N. alata* NE cDNA. The sense primer corresponds to the oligonucleotide 5′-AAAAGCTTCATTTTTATAAGAA-3′ (nucleotides 1–22). The antisense primer corresponds to the oligonucleotide 5′-GGCATAGCCTCTTACAATGATT-3′ (nucleotides 794–814).

Northern-Blot Hybridizations

Total RNA was purified as described by Logemann et al. (1987). For each point, RNA was purified from 10 inoculated areas on five leaves taken from different plants and at all levels on the stem. RNA (5 μg) was subjected to electrophoresis in a 1.4% agarose-formaldehyde gel, transferred, and cross-linked onto Hybond N^+ filters (Amersham). Hybridizations were performed under stringent conditions according to the manufacturer's instructions with the following random-primed probes: the 1-kb *Eco*RI fragment corresponding to PR-1a cDNA (Ward et al., 1991), and the 0.25-kb *Sac*II-*Nde*I fragment corresponding to one of our PCR products and homologous to the coding sequence of the S-like RNase NE gene (Dodds et al., 1996b) (nucleotides 140–406).

RNase Activity Assays

Inoculated tissues (1 g) were disrupted under liquid nitrogen and suspended in 1 mL of 50 mM Tris-HCl (pH 7.0) buffer. After centrifugation at 10,000*g* for 10 min at 4°C, the supernatant fraction was used for the RNase enzymatic assays after determination of the protein concentration, according to Bradford (1976). Protein extracts (5 μg) were incubated for 2 h at 37°C in 250 μL of 50 mM Tris-HCl (pH 7.0) buffer containing BSA (0.01%) and RNA from yeast (400 μg/mL). After incubation, the remaining RNA was precipitated with ethanol in the presence of 2.5 M ammonium acetate and resuspended in 500 μL of water. The optical density was measured at 260 and 280 nm (the ratio ranged from 1.7 to 2). Total RNase activity was determined as the decrease of absorption at 260 nm relative to the control without protein extract. Its activity was standardized for 1 mg of protein.

Electrophoresis and RNase Activity Staining

The detection of RNase activity in gels was performed as previously described by Yen and Green (1991) with intercellular fluid prepared in 50 mM Tris-HCl buffer (pH 7.0), as described by Hammond-Kosak (1992). These extracts were devoid of cytoplasmic contaminants, as attested by the absence of Glc-6-P dehydrogenase activity (data not shown). Protein extracts (2 μg) were subjected to SDS-PAGE gels that contained *Torulopsis utilis* RNA (2.5 mg/mL). After electrophoresis, the gels were washed twice for 10 min with 25% (v/v) isopropanol in 10 mM Tris-HCl buffer (pH 7.0) and twice for 10 min with the buffer alone. The gels were then incubated for 50 min at 50°C in 100 mM Tris-HCl buffer (pH 7.0) and washed for 10 min with 10 mM Tris-HCl buffer (pH 7.0). The activity of the renatured RNases was observed by staining the gels for RNA (negatively stained for RNase activity) with 0.2% toluidine blue in 10 mM Tris-HCl buffer (pH 7.0) for 10 min. After extensive washing in 10 mM Tris-HCl buffer (pH 7.0) and in 10% (v/v) glycerol-10 mM Tris-HCl buffer (pH 7.0), the gels were photographed.

Assays for Protection with RNase A

In these experiments the elicitin treatment was omitted and the mode of inoculation used was the same as described above. The zoospores were in suspension in water at the same concentration (1 zoospore per μL) in the presence of RNase A from bovine pancreas (10 μg/mL, 1 Kunitz unit/mL; Sigma type III-A), RNase A from bovine pancreas oxidized with performic acid and devoid of RNase activity (10 μg/mL, Sigma), myoglobin from horse heart (10 μg/mL, Sigma), or DNase I from bovine pancreas (10 μg/mL, 20 Kunitz units/mL). For each experiment, 12 leaves from four plants were inoculated twice: the right part of each leaf was inoculated with zoospores in the presence of RNase A; the left part was inoculated with control zoospore suspensions (water, myoglobin, DNase 1, and inactive RNase A). The areas invaded by the fungus were measured each day to determine the kinetics of leaf invasion. The effect of these proteins on the in vitro growth of Ppn was tested by seeding 100 zoospores in water supplemented with the different proteins at 10 μg/mL or on malt-agar plates in the same conditions. Trypan blue staining was performed as previously described by Keogh et al. (1980) and Delaney et al. (1994): leaves were boiled in ethanol with 0.1% lactophenol-trypan blue for 40 min before being washed in ethanol, then in water, and finally decolorized in chloral hydrate for 24 h.

TMV inoculations were performed by mechanical injury of the upper epidermis of cv Xanthi nc. leaves. For each experiment, 12 leaves from three plants were inoculated twice: the right part of each leaf was inoculated with a suspension of TMV particles in 50 mM Tris-HCl (pH 7.4) and in the presence of RNase A (10 μg/mL); the left part was inoculated with the same viral suspension in 50 mM Tris-HCl (pH 7.4) without RNase A, or supplemented with inactive RNase A (10 μg/mL).

RESULTS

Tight Control of the Systemic Induction of PR-1a Transcript Accumulation in Response to Ppn Challenge Leaf Inoculation on Cryptogein-Treated Plants

Tobacco plants were treated with cryptogein by stem application. Under these conditions cryptogein is rapidly translocated in the plant and induces a HR-like necrosis.

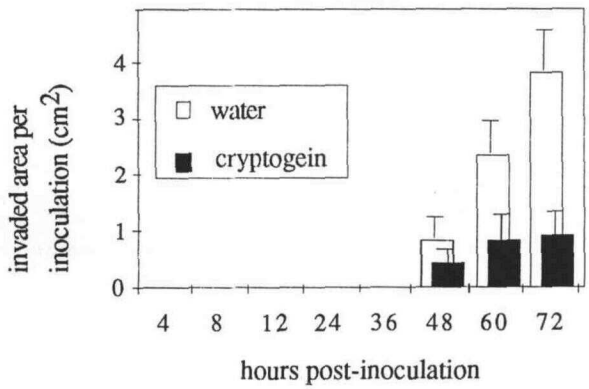

Figure 1. Effect of stem application of cryptogein on Ppn invasion after leaf inoculation. cv Xanthi nc. plants were stem treated with water or cryptogein (100 pmol). Two days later, Ppn zoospores were infiltrated in two distinct places on each of the second to the sixth leaf below the spot of elicitin application. The kinetics of leaf invasion were determined by measuring the invaded areas at the indicated time points. Each bar represents the means ± SD of four replicates from three different experiments. A replicate corresponds to eight inoculated areas on four leaves of one plant.

The distribution of the elicitin in the leaves is irregular: [^{125}I]cryptogein is detected only in areas that turn necrotic (Keller et al., 1996a). In these necrotic patches SAR genes are locally expressed. Only a subset of SAR genes, including PR-1a, is expressed systemically in nonnecrotic leaf areas in which cryptogein cannot not be detected. After the development of this HR-like necrosis, elicitin treatment also induces SAR against Ppn in the nonnecrotic leaves and parts of leaves devoid of elicitin (P. Bonnet, H. Keller, E. Galiana, J.P. Blein, and P. Ricci, unpublished results). Two days after the addition of cryptogein, a suspension of 100 zoospores of Ppn was infiltrated into the nonnecrotic parenchymatic tissue from the second to the sixth leaf below the site of elicitin application. Figure 1 shows the kinetics of disease symptom expansion: symptoms appeared at the site of infiltration 48 h after inoculation. In control plants treated with water, areas exhibiting disease symptoms expanded rapidly, with an average increase of 3 cm^2 in 24 h. In cryptogein-treated plants the growth was significantly reduced by at least 70%, the invasion rate not exceeding 0.5 cm^2 per day.

Gel-blot hybridization analysis with RNA from nonnecrotic leaf areas (devoid of cryptogein) 2 d after elicitin treatment revealed the systemic PR-1a transcript accumulation previously described by Keller et al. (1996a) (Fig. 2, lane BI). Challenge inoculation of these systemic tissues with Ppn led to the rapid, transient suppression of PR-1a transcript accumulation (Fig. 2A). After this short period of mRNA suppression, 12 h after inoculation a continuously increasing high level of PR-1a gene expression became evident in the systemic tissues of cryptogein-treated plants. Thus, the establishment of SAR in leaves of cryptogein-treated plants appears to be correlated with a three-step regulation of PR-1a transcript accumulation: (a) mRNA accumulation in response to the induction of SAR with cryptogein, (b) transient suppression immediately after challenge inoculation, and (c) reinduction of mRNA accumulation during the expression of the SAR phenotype. Inoculation of water-treated control plants led to detectable transcript accumulation only 72 h after Ppn infiltration. This three-step process in the regulation of PR-1a transcript accumulation upon induction of SAR and after inoculation with Ppn appears to be specific, because it was not observed for basic PR-1 and PR-2 mRNAs (data not shown).

Induction of RNase NE Gene Expression in Response to Ppn Inoculation

To investigate a putative involvement of plant RNases in SAR, we first looked for the expression of genes encoding RNases after infiltration of Ppn zoospores. We used reverse transcription-PCR to amplify cDNAs with degenerated primers corresponding to consensus sequences of S-like RNase genes of tomato, Arabidopsis, and *N. alata*. The PCR products obtained from mRNA of cryptogein-treated to-

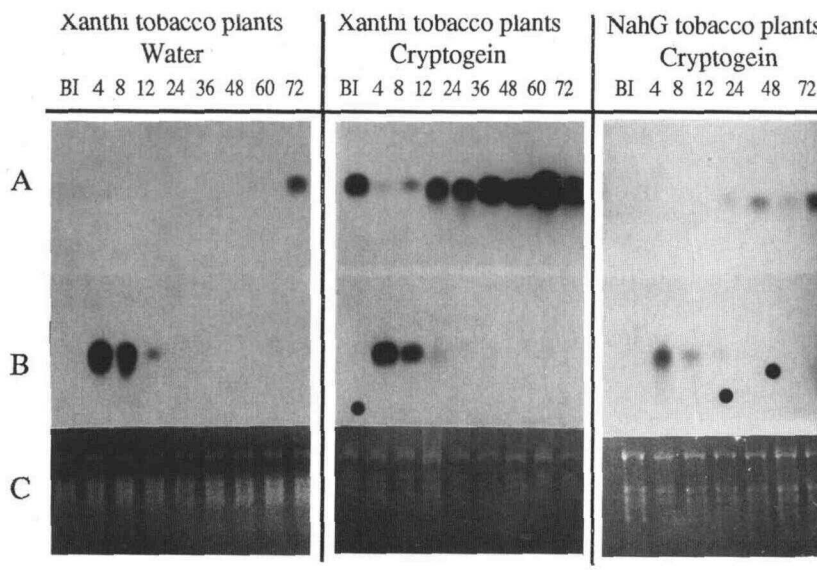

Figure 2. Kinetics of RNase NE and PR-1a mRNA accumulation in Ppn-inoculated leaves. cv Xanthi nc. or NahG plants were stem treated with water or cryptogein (100 pmol) and leaf infiltrated with Ppn zoospores (100 zoospores per infiltration) 48 h later. Total RNA was isolated from leaves just before inoculation (lanes BI) and after inoculation at the indicated time points (in hours). After gel blotting, RNA (5 μg) was hybridized with the PR-1a cDNA probe (A) or with the PCR product corresponding to a fragment of the RNase NE cDNA probe (B). Staining of 28S and 18S RNA was with ethidium bromide (C).

bacco plants were analyzed by agarose-gel electrophoresis and cloned. The sequence determination showed that one of the PCR products presented a very high homology (99%) with the coding sequence of the S-like RNase NE gene of *N. alata* (nucleotides 140–406), expressed in styles, petals, and immature anthers, but not in vegetative tissues (Dodds et al., 1996b). By using another pair of primers (corresponding to the 5′ and 3′ ends, respectively, of *N. alata* NE cDNA) we extended the sequence to the complete coding sequence of *N. tabacum*. The comparison of this sequence with the sequence of *N. alata* NE cDNA indicated that the two cDNAs were nearly identical, with two simple base substitutions in the 3′ noncoding region at nucleotide 747 (A→C) and 769 (C→T). The *N. tabacum* cDNA of 814 nucleotides contains a single open reading frame with a predicted amino acid sequence of 231 residues. This protein would be identical to RNase NE from *N. alata* and 86% identical to the extracellular RNase LE from tomato (Köck et al., 1994). It was inferred that the derived amino acid sequence contained a signal peptide region of 27 amino acids and a mature, secreted RNase region of 204 amino acids (22,391 D).

We examined the NE gene expression by northern-blot analysis, and Figure 2B shows that a 1-kb mRNA was expressed in leaves at very early times after inoculation (4–12 h), regardless of whether the cv Xanthi nc. plant was pretreated with cryptogein or water. In leaves induced to SAR with the elicitin, this transient expression coincided with the inhibition of PR-1a mRNA accumulation. This analysis was also performed with transgenic tobacco plants, which are no longer able to accumulate salicylic acid and to acquire systemic resistance to subsequent inoculations (NahG plants) (Gaffney et al., 1993). An application of cryptogein on the stem of NahG plants induces a HR-like necrosis, but no SAR is expressed in response to root or leaf inoculation with Ppn (Keller et al., 1996b; P. Bonnet, H. Keller, E. Galiana, J.P. Blein, and P. Ricci, unpublished results). The NE mRNA accumulation in NahG plants appeared to be less than in cv Xanthi nc. plants (Fig. 2B), as was the case for PR-1a mRNA (Fig. 2A).

To assess whether the induction of RNase NE gene expression was a specific response to the inoculation with Ppn, or a general response to the stress caused by the infiltration of the zoospore suspension, we analyzed the accumulation of NE mRNA after direct leaf infiltration with water or cryptogein. Cryptogein or water infiltrations led to low mRNA inductions; high-level transcript accumulations were detectable after inoculation with Ppn (Fig. 3). Therefore, the stress caused by the infiltration procedure induces a basic level of expression of the NE gene, which is increased after infection with Ppn.

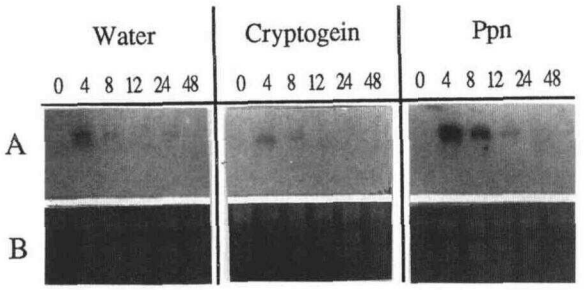

Figure 3. RNase NE mRNA induction in correlation with HR and pathogen infection. cv Xanthi nc. leaves were infiltrated with water, with 10 pmol of cryptogein, or with a suspension containing 100 zoospores of Ppn. In each case the volume of the infiltrated solution was 100 μL. Total RNA was isolated from leaves just before infiltration (lanes 0) and after infiltration at the indicated time points (in hours). RNA (5 μg) was separated on agarose gel, blotted onto nylon membranes, and probed with the RNase NE cDNA (A). Staining of 28S and 18S RNA was with ethidium bromide (B).

Characterization of Extracellular RNase Activities in SAR-Induced Plants after Challenge Inoculation

The induction of tobacco RNases was first studied by the analysis of the kinetics of the total enzymatic activity in crude extracts from nonnecrotic leaf tissue (devoid of cryptogein) 2 d after the elicitin treatment on the stem. RNase activity was determined before the inoculation with the fungus (Fig. 4A, lane BI) and at different times after Ppn infection. In plants induced to SAR with cryptogein RNase activity started to increase rapidly after inoculation, reaching maximal values 12 h later (Fig. 4A). The data also indicate that, in time, the evolution of RNase activity coincided with the accumulation of NE mRNA (compare with Fig. 2B). In leaves from water-treated control plants the increase of RNase activity was delayed and seemed to follow the invasion of inoculated areas by the fungal pathogen. These results show that the early up-regulation of RNase activity is an event occurring during the expression of the SAR phenotype after challenge inoculation.

We then used a gel assay to detect RNase activity in protein extracts from leaves before and at different times after the inoculation with the fungus. This analysis was performed with ICFs from cv Xanthi nc. leaves to characterize RNase activities in the extracellular space, where RNase NE is supposed to be localized. After SDS-PAGE, renaturation, and negative staining, three RNase activities were detected, with apparent sizes of about 28, 22, and 18 kD (Fig. 4B). The second band had an apparent size of 22 kD, consistent with that expected for the extracellular form of RNase NE. Its activity was increased in leaves from 4 h after inoculation onward and to a higher level in plants induced to SAR with cryptogein. The 18-kD RNase activity was detected 8 h after Ppn inoculation, but only upon induction of SAR with cryptogein. These results show that the expression of the SAR phenotype in response to pathogen inoculation is correlated with the induction of specific extracellular RNase activities.

Exogenous Application of RNase Activity in the Extracellular Space of Leaves Prevents the Growth of Ppn in Planta

The increase in the activity of at least two different extracellular RNases upon challenge inoculation in SAR-induced leaves led us to test the effect of the introduction of RNase activity in the extracellular space on the growth of the fungus. To artificially reconstitute the increase of RNase activity at the early step of Ppn inoculation, leaves

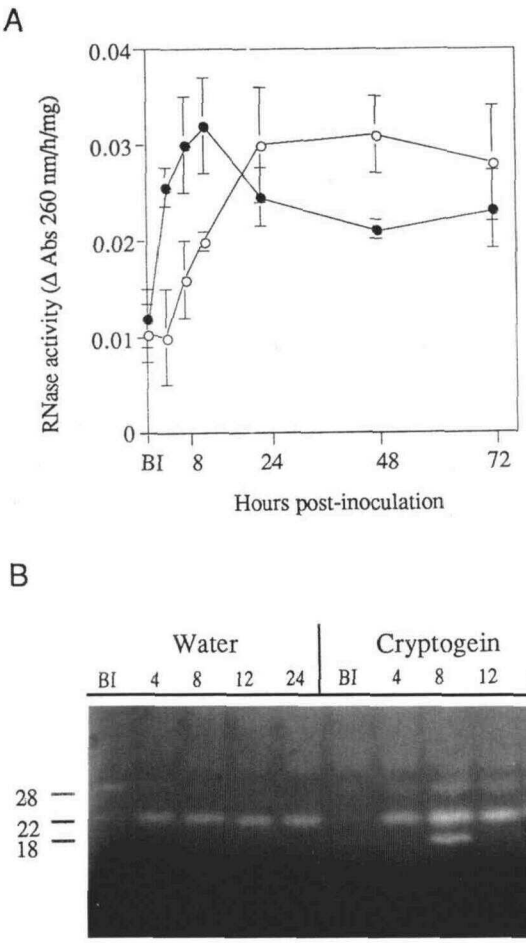

Figure 4. A, Effect of the induction of SAR with cryptogein on total RNase activity in Ppn-inoculated leaves. cv Xanthi nc. plants were stem treated with water (○) or cryptogein (●) and leaf infiltrated with Ppn zoospores 2 d later. Protein extracts (5 μg) were isolated from leaves after inoculation at the indicated time points and assayed for RNase activity. Values are the means ± SD of duplicates from three different experiments. B, SDS-PAGE of RNases activities from the ICF of cv Xanthi nc. leaves. Tobacco plants were stem treated with water or cryptogein. Two days later, the ICF was prepared from leaves just before (BI) and at different times (4, 8, 12, and 24 h) after inoculation with Ppn. After gel electrophoresis in the presence of RNA and renaturation, the gel was stained for RNA (negatively stained for RNase activity). In these experiments the level of SAR induction had been measured for at least two leaves for each plant by the determination of resistance to Ppn, as described in Figure 1.

of tobacco plants were inoculated by infiltrating a 100-μL suspension containing 100 zoospores into the parenchymatic tissues in the presence of RNase A from bovine pancreas (10 μg/mL, 1 Kunitz unit/mL). In this way, RNase activity was brought into the extracellular space, where specific RNase activities were induced upon induction of SAR with elicitin. Figure 5, A and B, shows that under these conditions leaves were protected against Ppn in a very effective manner, independently of any cryptogein treatment.

In control experiments Ppn zoospores were infiltrated in the presence of myoglobin at 10 μg/mL (left part of each leaf in Fig. 5A), and a typical latent phase of 48 h was observed before the beginning of leaf invasion by Ppn. This was followed by a growth period during which the invaded areas expanded with a mean increase of 3.3 cm^2/d (Fig. 5B). In the presence of RNase A, the appearance of

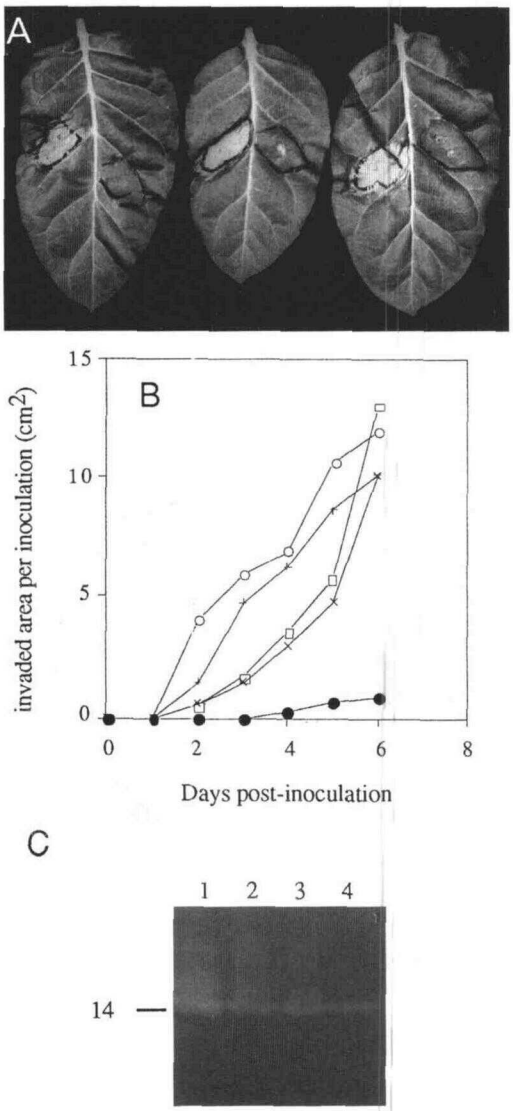

Figure 5. A, cv Xanthi nc. leaves were inoculated with Ppn zoospores in the presence of 10 μg/mL myoglobin (left part of each leaf) or RNase A at the same concentration (right part of each leaf). Leaves were photographed 3 d after inoculation. B, Kinetics of leaf invasion by Ppn. Invaded areas were measured at the indicated time points after infiltration of a suspension of Ppn zoospores in water (×), and in the presence of RNase A (●), RNase A oxidized with performic acid and devoid of RNase activity (○), myoglobin (+), or DNase I (□). Values are the mean of four replicates from one representative example of three experiments. A replicate corresponds to four inoculated areas on four leaves of one plant. C, Detection of RNase activity after co-infiltration of RNase A with Ppn into tobacco leaves. The amount of 0.1 μg of purified RNase A was used as a control (lane 1). Protein extract (20 μg) was isolated from leaves 2 d (lane 2), 4 d (lane 3), and 6 d (lane 4) after inoculation with the fungus. After electrophoresis the gel was negatively stained for RNase activity.

disease symptoms was delayed and their development drastically slowed down (right part of each leaf in Fig. 5A). The latent phase exceeded 3 d in all cases (Fig. 5B), and reached 4 d in 85% of the leaves. The spread of the invaded surface was limited to 0.3 cm^2/d, leading to a mean reduction of 90% compared with the inoculated zones in the presence of myoglobin. Actually, in the presence of RNase A, 80% of the inoculated areas had not developed any disease symptoms after 6 d, and 20% had developed symptoms with an average increase of 1.5 cm^2/d.

To test the specificity of RNase activity on the reduction of disease symptoms, Ppn zoospores were infiltrated into leaves in the presence of DNase I or RNase A previously inactivated with performic acid. The treatment with DNase I did not alter the development of the fungus. No growth restriction was observed after inoculation of Ppn into leaves in the presence of inactivated RNase A. Thus, the growth curve observed in the presence of RNase A contrasted strongly to those obtained in the different controls in the absence of active RNase (Fig. 5B). A 14-kD RNase activity with the same apparent size as RNase A could be detected in protein extracts from the leaves 2, 4, and 6 d after the inoculation procedure (Fig. 5C). This indicated that during this period RNase A remained active, although it appeared to decrease with time. In these experiments the development of disease symptoms was strictly correlated with the development of the fungus. Trypan blue stained the part of leaves where disease symptoms were observed (Fig. 6A), and Ppn mycelium was detected only in these areas (Fig. 6B). Fungal structures were also observed in the presence of RNase A, but only in leaves that developed limited disease symptoms. The mycelium was always confined in these areas and could not be detected in the healthy surrounding tissues. Therefore, the treatment with RNase A seems to inhibit the development of disease symptoms by restricting Ppn growth in planta. Altogether, these results demonstrate that an increase of RNase activity in the extracellular space is sufficient to prevent the development of Ppn in tobacco leaves.

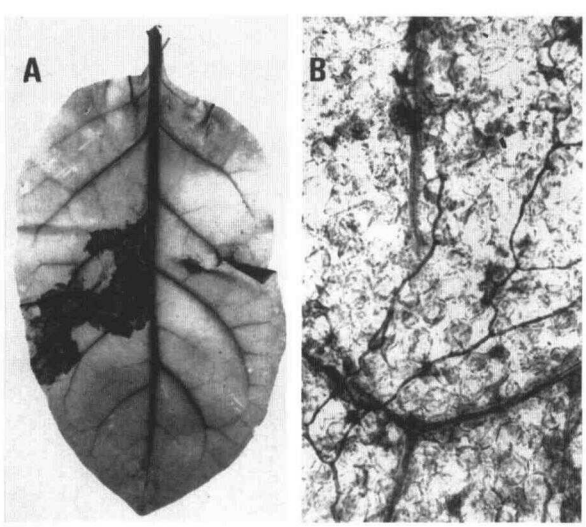

Figure 6. A, A cv Xanthi nc. leaf was inoculated with Ppn zoospores in the presence of myoglobin (left part of leaf) or RNase A (right part of leaf). The leaf was stained with trypan blue 3 d after inoculation. B, Light micrograph of tobacco leaf tissues expressing disease symptoms 3 d after Ppn inoculation. Trypan blue staining allows visualization of branched fungal hyphae (×115).

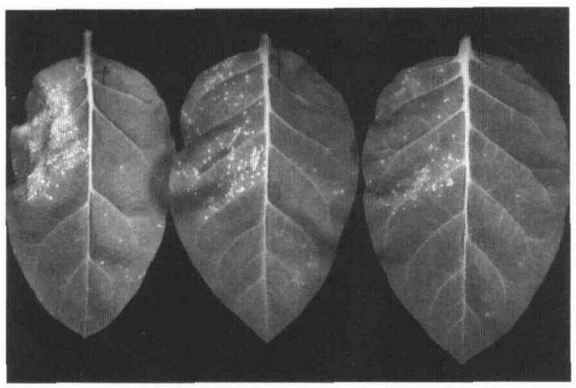

Figure 7. cv Xanthi nc. leaves were inoculated with TMV in the presence of myoglobin (left part of each leaf) or RNase A (right part of each leaf). Leaves were photographed 6 d after inoculation.

RNase activity was also highly efficient in inhibiting the formation of local lesions caused by TMV (Fig. 7). In these experiments leaves of cv Xanthi nc. plants were inoculated with TMV by mechanical injury of the upper epidermis. The number of local lesions was reduced by at least 80% in the presence of active RNase A compared with control experiments in the absence of RNase A (Table I). Thus, the growth control by RNase activity seems to be effective against fungal and viral pathogens.

RNase A Does Not Act as an Elicitor of Tobacco Defense Reactions

The restriction of Ppn growth in RNase A-treated leaves did not seem to be associated with an induction of the classic HR. The inoculation of zoo-spores in the presence of RNase A did not induce macroscopic necrosis on leaves

Table I. *Inhibition of TMV lesion formation by RNase activity*

Leaves from cv Xanthi nc. plants were inoculated with TMV (control), with TMV in the presence of RNase A (10 μg/mL), or with TMV in the presence of RNase A oxidized with performic acid (inactive RNase A, 10 μg/mL). The number of necrotic spots 6 d postinoculation are shown. Values are the means ± SD of 12 inoculated leaves from 3 plants. The percentage of inhibition for TMV lesion formation by RNase A is expressed relative to the control experiments in the absence of RNase A or in the presence of inactive RNase A.

Experiment and Treatment	Necrotic Spots	Inhibition
	n	%
1 Control	177 ± 31	
RNase A	14 ± 8	92
2 Control	237 ± 34	
RNase A	6 ± 4	98
3 Control	216 ± 40	
RNase A	6 ± 3	98
4 Inactive RNase A	328 ± 22	
RNase A	64 ± 18	80

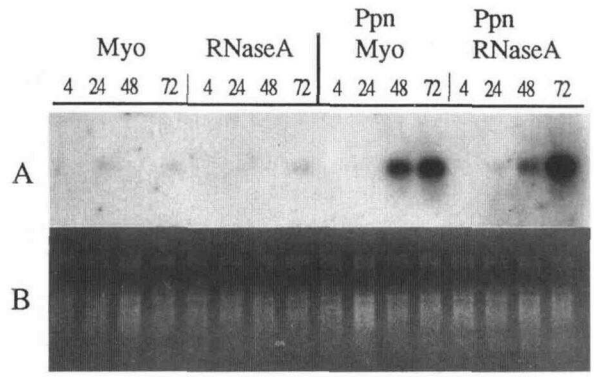

Figure 8. cv Xanthi nc. leaves were infiltrated with myoglobin (Myo) or with RNase A alone or simultaneously inoculated with 100 zoospores of Ppn. Total RNA was isolated from leaves at the indicated time points (in hours) after treatment. PR-1a mRNA accumulation was analyzed by RNA gel-blot hybridization with a PR-1a cDNA probe (A). Staining of the 28S and 18S RNA was with ethidium bromide (B).

except at the site of infiltration, as was the case in the different controls (the infiltrated zones are defined with black ink in Fig. 5A). We sought to determine if the protection against Ppn with RNase A was associated with PR-1a gene expression. After leaf infiltration of RNase A or myoglobin, we analyzed the accumulation of PR-1a mRNA by northern-blot analysis at different time points. In myoglobin- or RNase A-treated leaves, the transcripts were hardly detectable and only after 72 h (Fig. 8). An earlier and much higher accumulation was observed when Ppn was simultaneously inoculated regardless of the protein used. In these cases PR-1a gene expression appears as a response to Ppn invasion. This tends to indicate that the RNase A treatment does not prevent the interaction between the fungus and the plant. Therefore, according to the above criteria, the restriction of Ppn growth induced by RNase A in leaves cannot be ascribed to an induction of tobacco defense reactions. RNase A does not seem to have a direct antimicrobial effect. RNase A at the same concentration as was infiltrated in planta did not affect zoospore germination and germ-tube elongation in water for at least 1 d (Fig. 9) or on malt-agar medium for at least 6 d (data not shown).

DISCUSSION

Pathogenic microorganisms show reduced virulence on plants that have acquired systemic resistance. To study molecular events leading to the establishment of SAR we induced tobacco plants to systemic resistance with the elicitin cryptogein (Ricci et al., 1993). Cryptogein elicits a HR-like necrosis in tobacco plants. In stem-treated plants the protein triggers a resistance that is not restricted to the site of cryptogein application, but can be observed after petiole, root (Bonnet et al., 1996; Keller et al., 1996b), or leaf inoculation (P. Bonnet, H. Keller, E. Galiana, J.P. Blein, and P. Ricci, unpublished results). The experiments reported here support the hypothesis of an involvement of tobacco extracellular RNases in the establishment of elicitin-induced SAR.

We have studied early events taking place after challenge inoculation of leaves with Ppn in tobacco plants induced to SAR with cryptogein. Two days after the application of the elicitin on the stem, a suspension of Ppn zoospores was infiltrated into the lamina of nonnecrotic leaf areas devoid of cryptogein. Under these conditions the fungal development was severely reduced. We first followed the time course of PR-1a gene expression, which is known to be correlated with the induction of SAR (Ryals et al., 1994) and associated with the reduction of infection by oomycetes in transgenic plants (Alexander et al., 1993). The inoculation with Ppn led to a dramatic suppression of the systemic cryptogein-induced PR-1a mRNA accumulation. Down-regulation of PR-1a transcript accumulation might correspond to a very early response of these tissues to the infection with the pathogen. This transient inhibition was followed by a fast and intensive accumulation of PR-1a mRNA. The tight control of PR-1a mRNA level after challenge inoculation could be an important step for SAR establishment.

We have characterized and cloned a complete cDNA from *N. tabacum* corresponding to the RNase NE gene, which is expressed precisely when the down-regulation of PR-1a transcript accumulation was observed in tobacco plants induced to SAR with cryptogein. The early NE gene expression in response to Ppn inoculation was also observed in untreated plants. The experiments performed with NahG plants indicate that this expression is in part salicylic acid dependent. The NE cDNA has been previously cloned from *N. alata* and is induced in response to phosphate limitation (Dodds et al., 1996b). Its activation after Ppn inoculation could be indicative of some similarities in the regulation of S-like RNase gene expression in response to pathogen inoculation and during phosphate starvation. It would be interesting to determine whether the two signals (pathogen inoculation and phosphate limitation) could stimulate common signal transduction mechanisms. The analysis of NE and PR-1a gene expression, in correlation with the susceptibility of tobacco to Ppn during phosphate limitation, may help to evaluate this hypothesis

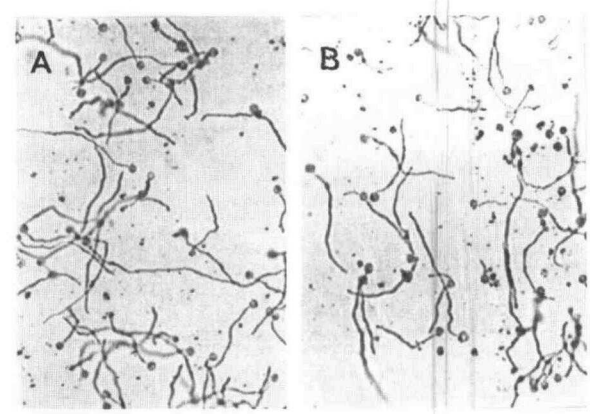

Figure 9. Morphology of Ppn hyphae growing in water supplemented with myoglobin (A) or RNase A (B) 12 h after zoospore germination (×60). Similar observations were made after 24 h of growth.

and the precise involvement of the NE gene in tobacco defense reactions.

The induction of NE gene expression coincided with a rapid increase in RNase activity, specifically after cryptogein treatment and upon challenge inoculation. Tobacco plants induced to SAR seem to have gained a systemic potential for an instant increase in RNase activity in response to the infection by Ppn. Such a dynamic phase involving RNases in the expression of the SAR phenotype after challenge inoculation was first suggested by Lusso and Kuc (1995). These authors reported a long-lasting increase of RNase activity in tobacco plants induced to SAR with TMV and after challenge inoculation with TMV or *Peronospora tabacina*. The increase was observed 3 d after TMV application or after spraying spores of *P. tabacina*. After the triggering of SAR with cryptogein, the induction of RNase activity was observed at an earlier time after Ppn inoculation by infiltration. This difference could be ascribed to the different modes of inoculation, leading to delayed or rapid plant-pathogen interactions. For example, TMV necrotic lesions appear 3 to 4 d after application of viral particles, whereas disease symptoms emerge within 1 to 2 d after leaf infiltration of Ppn zoospores. Thus, the expression of the SAR phenotype would be faster when leaves are inoculated by direct infiltration because of the ability of the fungus to immediately invade the apoplastic compartment.

The increase of total RNase activity was correlated with an accumulation of at least two RNases in the apoplastic compartment a few hours after Ppn inoculation. An 18-kD band was detected only in the ICF of cryptogein-treated plants, indicating a specific activation of this activity during the expression of SAR. A 22-kD band was detected after inoculation and was increased in SAR-induced plants. This activity began to be detectable at the same time point (4 h) as NE mRNA, suggesting that the S-like RNase NE could contribute to this 22-kD activity. NE protein is predicted to be localized in the apoplastic compartment, with an expected size of 22 kD. The increase of the 22-kD activity during SAR could be subsequent to an up-regulation of RNase NE activity, and it should imply a posttranscriptional regulation because NE gene expression is activated in response to Ppn infiltration independently of cryptogein treatment. The SAR 22-kD activity could also be the result of the activation of different RNases. These data indicate that tobacco extracellular RNases could be involved in tobacco defense reactions during the expression of SAR after challenge inoculation.

The accumulation of at least two different RNases in the extracellular space suggests that the RNase activity, rather than one single specific RNase, could be important in SAR. To mimic the early increase of RNase activity observed in Ppn-inoculated areas in SAR-induced plants, we have delivered Ppn zoospores in the presence of RNase A into the apoplastic fluid of leaves from cryptogein-untreated tobacco plants. Using this method we found that increasing the extracellular RNase activity prevents the growth of the fungus in planta. This protection was durable, since the pathogen growth was completely inhibited for at least 6 d in 80% of the inoculated leaves, a phenotype reminiscent of that expressed by SAR-induced leaves in cryptogein-treated plants. Moreover, the same treatment inhibited the formation of local lesions caused by TMV, indicating that the protection of tobacco leaves by RNase activity is effective toward different, unrelated pathogens. Our data cannot explain how RNase activity exercises its control on pathogen growth in planta. The absence of control on Ppn in vitro growth tends to indicate that RNase activity affects a pathogenic function of the fungus that is only necessary for the development of the fungus in plant tissues. RNase treatment of leaves does not lead to the triggering of typical defense reactions such as PR-1a gene expression or HR-like necrosis induction. Therefore, according to the few criteria analyzed, RNase A cannot be considered as an elicitor of defense reactions, at least in tobacco leaves.

The long-lasting prevention of the growth of two different pathogens by RNase activity, taken together with the expression of RNase NE gene in response to Ppn inoculation and the early increase of apoplastic RNase activities after induction of SAR with cryptogein, support the hypothesis that extracellular RNases of plants are involved in the control of pathogen development during the expression of SAR. To our knowledge, to date no RNase function has been demonstrated for proteins induced in plant-pathogen interactions. The amino acid sequence homology between a parsley PR-10 protein and a ginseng RNase (Moiseyev et al., 1994) has led to the classification of intracellular PR-10 proteins as RNase-like proteins (Van Loon et al., 1994). A 30-kD protein with a broad-spectrum antifungal activity has been purified from *Engelmannia pinnatifida* (Huynh et al., 1996). The determination of the N-terminal amino acid sequence indicated that this protein presents significant homology with style extracellular RNases. This work provides evidence for the existence of antifungal proteins that show similarity with RNases.

Our results suggest that some extracellular RNases of plants are involved in the defense against pathogen infection, although the involved mechanisms are not elucidated. An important step to understanding how RNase activity could achieve the prevention of pathogen growth would be to define the involvement of individual tobacco RNases and how they might act in concert during the SAR process. To this aim, the characterization and purification of RNase activities specifically activated upon induction of SAR will be necessary. In solanaceous plants with gametophytic self-incompatibility, the RNase activity of extracellular S-RNases is required for rejection of the pollen with the same genotype (Huang et al., 1994; Royo et al., 1994). Putative SAR-RNase(s) may induce pathogen growth restriction by using similar mechanisms as S-RNases, arresting the growth of pollen tubes. Nevertheless, in contrast to S-RNases in self-rejection, such RNases should act with a low specificity, since SAR is effective toward a broad range of pathogens. This property could explain our ability to reproduce the SAR phenotype by the introduction of exogenous RNase activity into the apoplastic compartment.

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