Plant Physiol. (1996) 110: 365–376

Physiological and Molecular Characteristics of Elicitin-Induced Systemic Acquired Resistance in Tobacco[1]

Harald Keller, Jean-Pierre Blein, Philippe Bonnet, and Pierre Ricci*

Institut National de la Recherche Agronomique, Station de Botanique et de Pathologie Végétale, BP 2078, 06606 Antibes Cedex, France (H.K., P.B., P.R.); and Institut National de la Recherche Agronomique, Laboratoire de Phytopharmacie et Phytobiologie Cellulaire, BV 1540, 21034 Dijon Cedex, France (J.-P.B.)

Elicitins are low molecular weight proteins secreted by all *Phytophthora* species analyzed so far. Application of the purified proteins to tobacco *Nicotiana tabacum* leads to the induction of resistance to subsequent inoculations with the black shank-causing agent, *Phytophthora parasitica* var *nicotianae*. In this paper, we describe the systemic characteristics of elicitin-induced acquired resistance in tobacco. Elicitin application is followed by the rapid translocation of the protein in the plant. The basic elicitin, cryptogein, induces necrosis formation in the leaves, which results from accumulation of the protein in these organs. Necrosis does not seem to be essential for the establishment of systemic acquired resistance (SAR), since resistance induced by the acidic elicitin, capsicein, is not accompanied by the development of visible symptoms on the leaves. Both elicitins trigger the coordinate accumulation of transcripts from nine genes, previously described to be expressed during establishment of SAR. Additionally, elicitin treatment leads to the activation of the multiple response gene *str 246*. In leaves, transcript accumulation was found to be higher in all cases in response to cryptogein compared to capsicein treatment. These results, along with northern hybridization analysis following infiltration of leaves with cryptogein, indicate that SAR genes appear to be expressed locally, corresponding to necrosis formation as well as systemically during induction of resistance. To our knowledge, elicitins are the only well-characterized, pathogen-derived molecules that trigger SAR in a plant.

The incompatible interaction of a plant and a potentially pathogenic microorganism is often associated with tissue necrosis at the site of contact between the avirulent pathogen and the resistant host. This localized and rapid necrosis characterizes the plant's HR (Klement, 1982). Additionally, in several pathosystems HR is accompanied by the induction of local or systemic resistance to infection by challenge pathogens (Ryals et al., 1994). To a large extent, the mechanisms leading to HR and incompatibility involve transcriptional gene activation (Ebel and Scheel, 1992). However, until now, few genes have been identified whose expression specifically correlates to incompatibility. The major part of defense-related genes are activated during incompatible as well as compatible interactions, comprising in tobacco (*Nicotiana tabacum*) the so-called *str* genes (Godiard et al., 1991) and in almost all pathosystems the genes coding for PR proteins (for review, see Linthorst, 1991). Differences in temporal and spatial expression patterns of these genes in a given plant appear to be essential for determining susceptibility or resistance to an invading pathogen (Kombrink et al., 1993). Since antimicrobial activities could be demonstrated for some of the PR proteins in vitro (Woloshuk et al., 1991) and since expression of PR 1a in transgenic plants leads to enhanced pathogen resistance (Alexander et al., 1993), the activation of PR protein genes during plant-pathogen interactions may be a decisive factor in determining resistance or susceptibility. Additionally, the expression of PR protein genes in tobacco appears to be closely correlated with SAR, obtained after preinoculation with TMV or after treatment with chemical inducers such as INA or SA. This led to their classification by some research groups as SAR genes, whose transcriptional activation serves as a valuable marker in analyzing the induction of resistance (Ward et al., 1991b; Ryals et al., 1994).

For a better understanding of the basic cellular and molecular mechanisms that control the outcome of a plant-pathogen interaction, intensive research was performed toward the identification of pathogen-derived molecules (elicitors), which determine early recognition of the microorganism by a given plant. The elicitors analyzed to date vary widely in their chemical nature (Ebel and Cosio, 1994), but are all characterized by their ability to trigger defense responses when applied to a plant, thus mimicking pathogen attack. Elicitins, a family of low molecular weight proteins, are secreted by all analyzed *Phytophthora* species when cultured in vitro. Upon application to tobacco, these proteins elicit necrosis formation and histological disturbances in the plant, which are similar to those observed during HR in nonhost interactions between *Phytophthora* species and tobacco (for review, see Ricci et al., 1993). In addition, acquired resistance to infection by Ppn, the agent that causes black shank of tobacco, is obtained (Ricci et al., 1989). The infection potential of different *Phytophthora parasitica* isolates on tobacco appears to directly correlate with the fungal ability to produce and secrete elicitins. Only the

[1] This work was supported by a grant of the Deutsche Forschungsgemeinschaft (No. KE 501/1-1) to H.K.

* Corresponding author; e-mail ricci@antibes.inra.fr; fax 33-93-67-88-88.

Abbreviations: HR, hypersensitive response; INA, 2,6-dichloroisonicotinic acid; Ppn, *Phytophthora parasitica* var *nicotianae*; PR, pathogenesis related; SA, salicylic acid; SAR, systemic acquired resistance; *str*, sensitivity related; TMV, tobacco mosaic virus.

highly virulent isolates specific to tobacco show no elicitin secretion in vitro (Ricci et al., 1992). In contrast, elicitin-producing tobacco isolates of *P. parasitica* show a reduced virulence on tobacco and a capacity for invading a broader host range (Bonnet et al., 1994). Therefore, in the tobacco-*Phytophthora* interaction, the elicitins appear to function as avirulence factors.

The elicitins of different *Phytophthora* species are holoproteins composed of 98 amino acids (Ricci et al., 1989). A comparison of the amino acid sequences of eight elicitins analyzed to date revealed 68 to 94% homology, but no relation to sequences of other proteins of fungal or plant origin (Huet et al., 1993; Ricci et al., 1993). Despite a high degree of sequence conservation, exchanges of certain charged amino acids to neutral ones result in a strong difference in the overall charge, allowing the classification into acidic and basic elicitins.

When applied to tobacco plants, acidic and basic elicitins are characterized by different degrees of efficiency in eliciting hypersensitive-like responses in the plant leaves. Whereas the basic cryptogein (from *Phytophthora cryptogea*) exhibits a strong necrotizing activity at concentrations as low as 100 pmol per plant, more than 10 nmol of acidic capsicein (from *Phytophthora capsici*) are necessary to trigger a visible reaction (Ricci et al., 1989).

The early effects of elicitins on tobacco cells have been examined using suspension-cultured cells. Addition of cryptogein causes a rapid and persistent alkalization of the extracellular medium (Blein et al., 1991), an increase in the conductivity of the medium correlating with K^+ efflux from the cells (Viard et al., 1994), a transient production of active oxygen species, and the phosphorylation of several proteins (Viard et al., 1994). These reactions are presumed to result from specific interactions between elicitins and receptors on the plasma membranes of the cells. Evidence for the presence of cryptogein-binding sites comes from competitive-displacement experiments using radiolabeled cryptogein (Blein et al., 1991). Furthermore, cryptogein-treated suspension-cultured cells exhibit reactions such as the production of ethylene and the phytoalexin capsidiol (Milat et al., 1991b), the stimulation of lipoxygenase activity, and the accumulation of proteinase inhibitors (Bottin et al., 1994). In contrast, far less is known about the effects of elicitins on whole plants, especially the molecular events leading to elicitin-induced resistance of tobacco plants to Ppn infection. The hypersensitive-like response provoked in tobacco leaves by application of cryptogein is accompanied by ethylene production, capsidiol synthesis (Milat et al., 1991a), and accumulation of PR 1a protein. These responses appear to be preceded by the translocation of the fungal protein within the plant (Devergne et al., 1992).

In this paper we describe experiments performed with tobacco plants to assess whether elicitin-induced acquired resistance follows the scheme proposed for the induction of SAR by biological (e.g. TMV) or abiotic (e.g. INA) stimuli. For this purpose, an application/inoculation system is presented that takes the natural infection process by Ppn into account. The use of iodinated cryptogein in translocation experiments allowed a comparison of elicitin migration, elicitin accumulation, and necrosis formation. SAR in tobacco is characterized by a coordinate induction of the so-called SAR genes (Ward et al., 1991b; Ryals et al., 1994). Therefore, the respective cDNA probes were used in northern hybridization experiments to compare the phenotypical occurrence of elicitin-induced acquired resistance with the expression of SAR marker genes. For these experiments, RNA was extracted either from leaf and stem tissue upon treatment of whole tobacco plants, or from tissue at and surrounding the infiltration sites upon direct infiltration of cryptogein into tobacco leaves. The correlation between elicitin migration, SAR gene expression, necrosis formation, and resistance induction is discussed.

MATERIALS AND METHODS

Plant Material and Fungal Cultures

Tobacco plants (*Nicotiana tabacum* cv Xanthi nc) were grown in a growth chamber at 24°C with a 16-h light period. All treatments were performed 3 or 6 weeks after seeding, as indicated in "Results." Mycelia of Ppn (isolate 329), *Phytophthora cryptogea* (isolate 52), and *Phytophthora capsici* (isolate 147) from the *Phytophthora* collection of the Institut National de la Recherche Agronomique (Antibes, France) were cultivated on V_8-agar (Ribeiro, 1978) at 24°C in the dark and transferred weekly to fresh medium. For zoospore production, Ppn mycelia were cultivated on pea liquid medium at 24°C under continuous light. After 1 week, mycelia were transferred to agar plates (3% agar in water), harshly macerated using a scalpel, and further incubated for 3 d. For zoospore release, the dishes were kept at 4°C for 1 h prior to the addition of 10 mL of water (37°C) and further incubated at 37°C for 30 min. Zoospore suspensions were collected and adjusted to 20,000 spores/mL for inoculations.

Purification, Radiolabeling, and Inactivation of Elicitins

Purification of cryptogein and capsicein from fungal culture media, purity controls, and quantitative analysis of the purified proteins were performed as described elsewhere (LeBerre et al., 1994). Cryptogein was labeled with ^{125}I using iodogen as the catalyst (Blein et al., 1991) to a specific activity of 26 Ci/mmol protein. Inactivation of cryptogein was achieved by *S*-carboxymethylation following reduction of the protein (Fernandez-Luna et al., 1985).

Plant Treatments

Three-week-old plantlets were decapitated prior to the application of 5-μL solutions of cryptogein (1.5 μg), capsicein (20 μg), carboxymethylated cryptogein (3 μg), $HgCl_2$ (50 μg), or water to the cutting sites. For treatment of 6-week-old plants, decapitation was performed above the third fully expanded leaf counted from the apex of the plants, and 20-μL solutions containing the different compounds in the quantities indicated above were applied to the fresh wound sites. Alternatively, cryptogein (1 pmol/20 μL) was directly infiltrated into leaves through the abaxial epidermal layer. To analyze elicitin transloca-

tion within the plants, ^{125}I-labeled proteins were diluted with unlabeled elicitins to the same concentrations as above and 5×10^5 cpm and 1.5×10^6 cpm were applied to 3- and 6-week-old plants, respectively. Biological assays for induced resistance to fungal infection were performed by root inoculation of 3-week-old tobacco plants with 1-mL suspensions containing 20,000 zoospores of Ppn, 4 h after the application of elicitins.

Detection of Labeled Elicitins in Plant Tissues

In situ localization of the labeled proteins was analyzed by direct autoradiography/fluorography of the plant organs at −80°C. Soluble proteins were extracted in 30 mM phosphate buffer, pH 7, containing 1 mM 2-mercaptoethanol and 1 mM EDTA. After centrifugation and successive extraction of the insoluble tissue debris with 1 M NaCl, 0.5% SDS (w/v), methanol, acetone, and diethylether, bound proteins were released by treatment of the pellets with a hot solution containing 5% SDS (w/v) and 7 M 2-mercaptoethanol. Final release of covalently bound proteins was achieved by alkaline hydrolysis in 1 M NaOH at 80°C for 1 h. Qualitative and quantitative analyses of the extracts were performed by fluorography after SDS-PAGE and γ-counting, respectively.

RNA Extraction

For each time point after onset of the different treatments, three plants were harvested and separated into stem and leaf material. At the different time points after infiltration of leaves with cryptogein (four infiltration sites per leaf), the infiltrated areas were precisely cut out and separated from tissue surrounding this zone by 1 cm. Total RNA was extracted essentially as described by Logemann et al. (1987), except that an additional $CHCl_3$ washing step was added after phenol:$CHCl_3$ extraction of the guanidine hydrochloride solutions.

RNA Gel and Dot Blot Hybridization Analysis

Total RNA (10 μg) was separated on 1.2% agarose gels containing 6.3% formaldehyde. RNA was blotted onto nylon membranes (Hybond N^+, Amersham) and cross-linked by alkali fixation using 0.05 N NaOH. SAR gene-specific probes (generously provided by Dr. J. Ryals, CIBA-GEIGY Corp., Research Triangle Park, NC) and the *str 246* gene-specific probe (a gift of Drs. Y. Marco and D. Roby, Institut National de la Recherche Agronomique-Centre National de la Recherche Scientifique, Toulouse, France) were labeled to high specific activities using the Multiprime Labeling Kit (Amersham) with 25 μCi of [α-^{32}P]dCTP according to the manufacturer's instructions. Hybridization was performed at 42°C for 15 h in 50% formamide, 5× Denhardt's reagent, 5× SSC, 50 mM sodium phosphate (pH 6.5), 0.1% SDS, and 10% dextran sulfate after addition of 100 μg/mL denatured salmon sperm DNA. Prior to autoradiography, filters were washed with 0.2× SSC, 0.1% SDS at 65°C.

RESULTS

A Model for SAR

In 6-week-old tobacco plants (herein called aged plants) that had been stem treated with elicitins, fungal invasion was restricted when Ppn mycelia were placed either onto the decapitated stem (Ricci et al., 1989) or onto the cutting site of a petiole (Bonnet et al., 1996). However, under natural conditions, epidemiological spreading of the black shank disease occurs through germinating Ppn zoospores infecting the root system of tobacco, which is followed by rapid fungal invasion of the plant. Taking this natural infection route into account, a test system for induced Ppn resistance was designed, consisting of elicitin application to decapitated stems of 3-week-old tobacco plants (herein called young plants) followed by inoculation of the soil with Ppn zoospore suspensions. When young plants were treated in this way with either 150 pmol of the basic cryptogein or 2 nmol of the acidic capsicein prior to inoculation with 20,000 zoospores of Ppn per plant, complete resistance to fungal infection was obtained. In contrast, control plants became rapidly infected by the fungus, leading to a collapse of the plant as soon as 3 to 4 d postinoculation (Fig. 1). Induction of resistance was characterized by the browning of roots and hypocotyls, which did not, however, affect the plants' viability.

Time Course of Elicitin Uptake

As previously demonstrated, elicitin uptake through the roots and translocation to the apical parts of the plant is a rapid process (Devergne et al., 1992). Since cryptogein can be labeled with ^{125}I to high specific radioactivity without losing its biological activity, the migration of the protein could be easily followed upon application to stems of young tobacco plants. Exposure of these plants to direct autoradiography revealed that cryptogein is rapidly translocated to the leaves and the basal parts of the plants. Occurrence in leaves could be detected as early as 40 min after application (Fig. 2A). No further uptake by the plants occurred 6 h after onset of elicitin treatment. At this time a stable elicitin distribution between leaves and stems was achieved (Fig. 2B). Soluble elicitin could be recovered from extracts of both tissues over the whole experimental period, as indicated by the presence of a 10-kD band of nondegraded elicitin after SDS-PAGE (Fig. 3, B and C). In addition, cryptogein could be re-extracted from young shoots formed 4 to 5 d after onset of the treatment (Fig. 3B). Therefore, part of the protein appears to retain a high degree of stability and mobility in planta, making it likely that the stable distribution of elicitin between leaves and stems reflects the establishment of a dynamic equilibrium rather than the end of cryptogein migration. However, a large proportion of the radiolabel in the plant (more than 50% of total extractable cryptogein 8 d after onset of the treatment) became incorporated into the stem cell walls, from which it could be released only under reducing and hydrolyzing conditions (Fig. 3A). This cross-linking seems to involve the whole, nondegraded molecule, which could

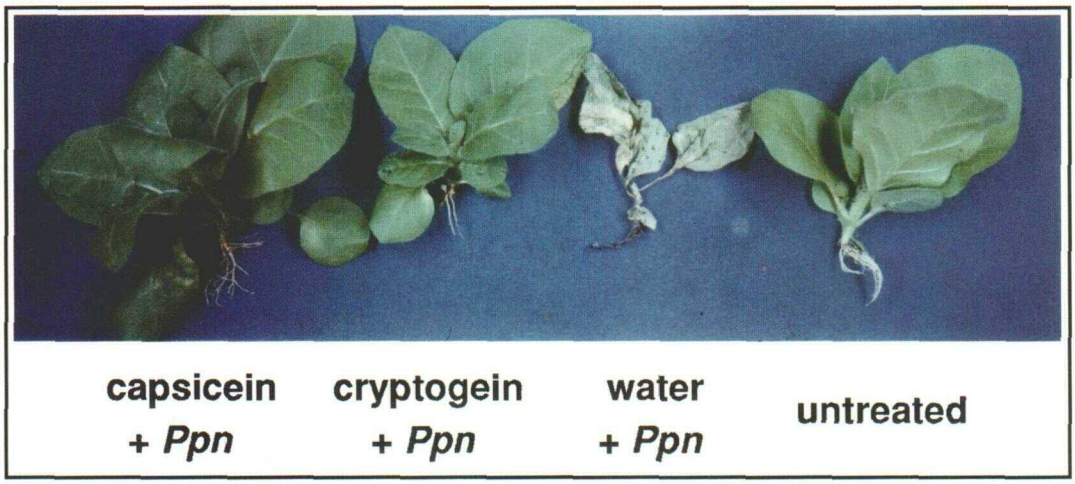

Figure 1. SAR of young tobacco plants to infection with Ppn. Four hours prior to soil inoculation with zoospore suspensions (20,000 spores/mL), plants were treated with either capsicein (2 nmol), cryptogein (150 pmol), or water by application to the apical stem cutting site. A control plant was decapitated (untreated) but neither treated with elicitins nor infected with Ppn. Plants were incubated for 7 d in the contaminated soil. Whereas water-treated plants were completely rotted by the black shank disease after this period, elicitin-treated plants remained healthy. Hypocotyl browning occurred as the visible symptom of the arrested invasion process.

be visualized by autoradiography, yielding the intact 10-kD protein (Fig. 3D).

Elicitin Presence Determines Necrosis Formation

Application of cryptogein to decapitated aged tobacco plants also led to the downward translocation of the protein. However, in contrast to young tobacco plants, in which cryptogein treatment led to the formation of small necrotic spots on the leaves, old plants developed large, coherent necrotic areas. When these plants were used in translocation analysis, the occurrence of necrosis correlated with the presence of elicitin in the leaf tissue. In comparison, leaves or part of leaves that did not show any symptoms also did not contain any traces of elicitin (Fig. 4). These results suggest that the immediate trigger for hypersensitive-like cell death may be the protein itself.

However, for translocation within the plant, elicitins must enter the vascular system. When cryptogein was directly infiltrated into parenchymatic tissue of tobacco leaves, it remained restricted to the infiltrated area, which necrotized 16 to 24 h later (not shown).

Elicitins Induce SAR Gene Expression in Tobacco

In vitro translation studies using mRNA from elicitin-treated plants had already demonstrated an overall complex pattern of gene induction in comparison to control plants (Keller et al., 1994). To compare elicitin-dependent acquired resistance to SAR at the molecular level, induced transcript accumulation was analyzed in northern hybridization experiments using SAR gene-specific probes from tobacco. In addition, a probe for the *str 246* gene, whose expression after fungal, bacterial, and viral infection was reported to be systemic (Gough et al., 1995), was included. For these experiments, stem and leaf tissues were analyzed separately after migration of elicitins via the vascular system, as was the infiltrated tissue and the zone surrounding it after infiltration of cryptogein in tobacco leaves. Specific attention was given to differences in mRNA steady-state levels after treatments with the necrotizing cryptogein and the nonnecrotizing capsicein. Elicitin-induced transcript accumulation was compared to that induced by a chemical stress mediator and lesion inducer, $HgCl_2$, and that triggered by the linearized and biologically inactive cryptogein (Ricci et al., 1989).

Application of cryptogein to decapitated tobacco stems and subsequent translocation of the protein into the leaves led to the accumulation of SAR gene transcripts in both stems and leaves (Fig. 5). In comparison, a generally weaker mRNA accumulation was detected upon application of capsicein. Similar results were obtained for *str 246* transcript accumulation. In contrast, no increased mRNA levels were detected following wounding (decapitation) in control plants. In leaves, treatment with carboxymethylated cryptogein did not lead to the accumulation of mRNA of any of the genes analyzed, whereas in stems low transcript levels for the basic PR 1, PR 3, PR 4, and *str 246* genes were detectable. Stem application of $HgCl_2$ led to a strong expression of the PR 3 gene as well as to the accumulation of transcripts encoding class III chitinases, basic PR 1, PR 2, PR 4, PR 5, and *str 246*. With the exception of basic PR 1 mRNA, induced transcript levels after $HgCl_2$ treatment were detectable exclusively in the leaves. In comparison, *str 246* mRNA accumulated at almost equal amounts after cryptogein and $HgCl_2$ application, even in the stem (Fig. 5).

In general, steady-state levels of mRNA from all analyzed genes were higher in leaves after cryptogein treatment than after application of capsicein. In contrast, in stem tissues, no such striking differences in transcript levels were detected after cryptogein versus capsicein appli-

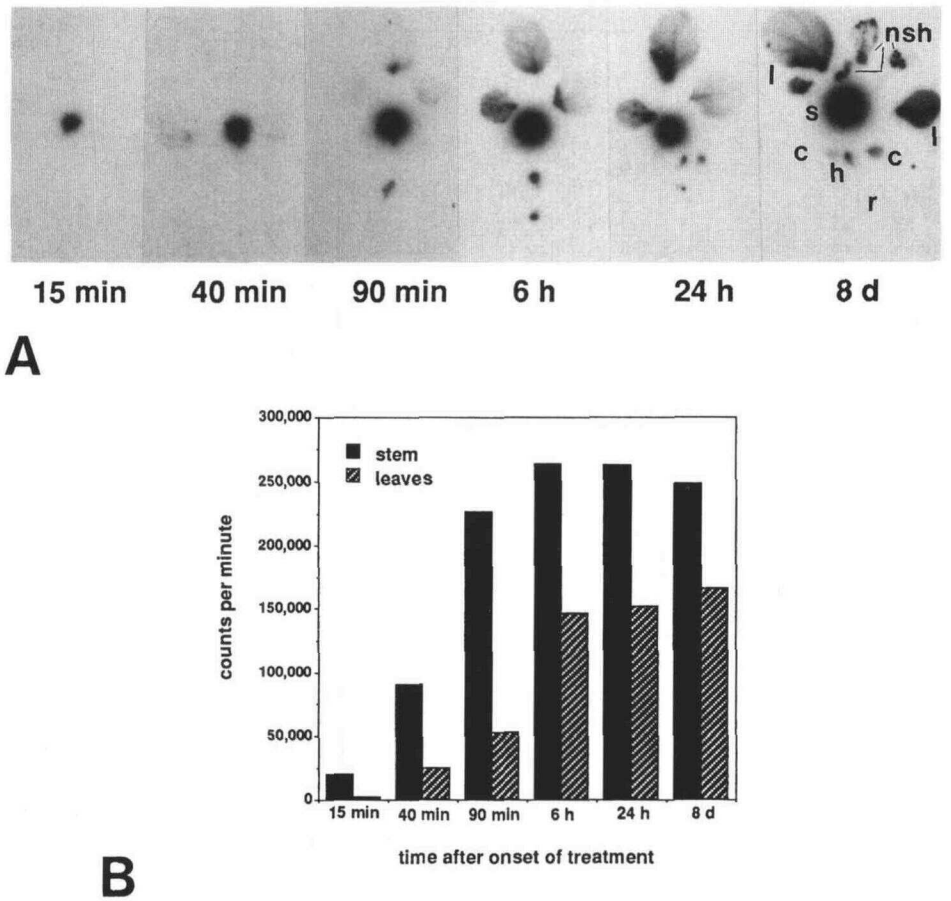

Figure 2. A, Translocation of radiolabeled cryptogein within young tobacco plants. [^{125}I]cryptogein (5×10^5 cpm/1.5 μg protein) was applied in a volume of 5 μL to the apical stem cutting site of a plant. Plants were collected, washed, and separated into stem and leaf parts before exposure to autoradiography. Radiolabeled proteins in the leaves were found as early as 40 min after onset of treatment. Six hours after elicitin application, no further accumulation of labeled proteins in the leaves was detectable. r, Roots; h, hypocotyls; c, cotyledons; s, stem; l, leaves; nsh, new shoots formed several days after onset of treatment. B, Accumulation of radiolabeled cryptogein in extracts from stems and leaves of two plants, as analyzed by direct γ-counting.

cation. A comparison of gene induction in stems and leaves upon cryptogein or capsicein treatment allowed us to classify SAR gene expression into three groups: (a) similar transcript levels in stems and leaves after cryptogein treatment but higher levels in stems after capsicein application (PR 1a, basic PR 1, PR 2, and the class III chitinases); (b) predominant mRNA accumulation in leaves after cryptogein treatment but similar induction in stems and leaves following capsicein application (PR 3 and PR 4); and (c) almost exclusive mRNA accumulation in leaves (PR 5 and PR Q'). Based on this classification, *str 246* falls into the first class of inducible genes (Fig. 5).

Elicitin-induced acquired resistance appears to involve the coordinated activation of SAR genes, a mechanism that was already demonstrated with biological (TMV) and chemical (SA, INA) inducers of SAR. However, transcript accumulation of SAR genes in leaves appears to reflect, at least in part, the necrotizing activity of cryptogein.

As already indicated, local infiltration of cryptogein into the parenchymatic leaf tissue did not lead to a translocation of the protein within the plant. Instead, it remained restricted to the infiltrated region, which subsequently necrotized within 16 to 24 h. Inside this region, high levels of transcripts from all SAR genes as well as from *str 246* were detected (Fig. 6, zone 1). Following elicitin infiltration, transcripts started to accumulate 3 to 6 h (PR 2, PR 4, and the class III chitinases), 9 h (PR 1 basic and PR 3), or 12 h (PR 1a and PR 5) after onset of treatment, either reaching maximal intensities around 12 to 18 h (PR 2, class III chitinases, and *str 246*) or increasing steadily until 24 h posttreatment (PR 1a, PR 1basic, PR 3, PR 4, and PR 5). No or low background mRNA levels (PR 2, class III chitinases) were detectable after water infiltration. Cryptogein-induced transcript accumulation occurred predominantly in the future necrotic region, with a time course preceding lesion formation (collapse of the infiltrated region after 24 h). In general, lower mRNA levels could be detected in the parenchymatic tissue closely surrounding the infiltration zone (Fig. 6, zone 2). However, single SAR genes were induced differentially in and around the infiltration zone.

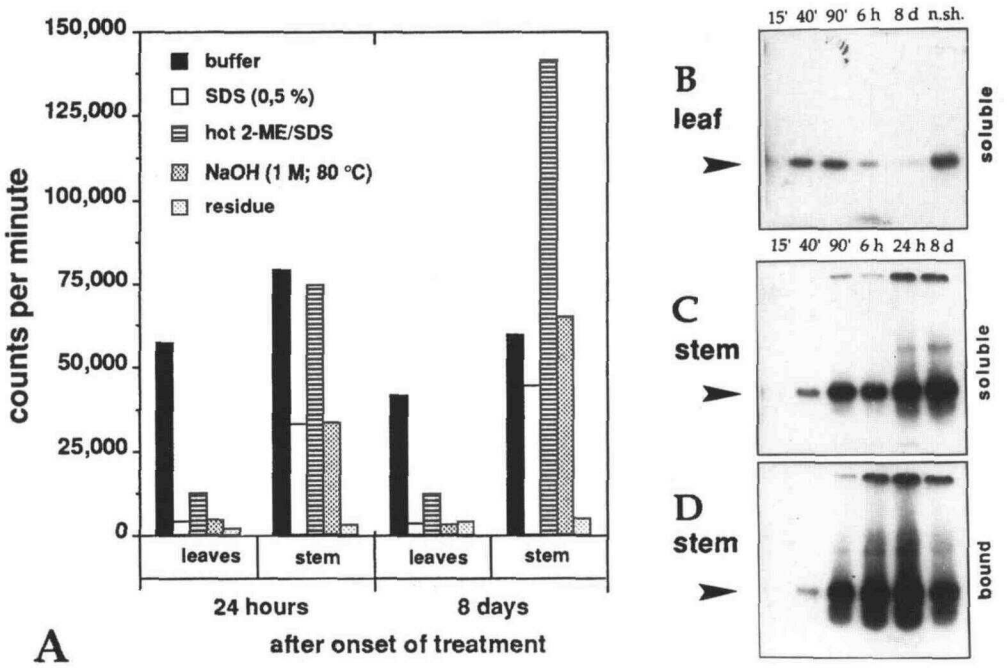

Figure 3. Differential extraction of leaves and stems from [^{125}I]cryptogein-treated young tobacco plants. Labeled elicitin (5×10^5 cpm/plant) was applied to the stem, and soluble proteins were extracted in phosphate buffer, followed by successive extraction of the insoluble tissue debris with different solvents. A, Analysis of plant extracts 24 h and 8 d after cryptogein application by direct γ-counting. Prior to treatment with a hot 2-mercaptoethanol/SDS solution and alkaline hydrolysis, the insoluble material was successively washed with 1 M NaCl, methanol, acetone, and diethylether. These treatments released a total of 10,000 cpm (not shown). B to D, SDS-PAGE (15% acrylamide) and fluorography of buffer extracts of leaf (B) and stem (C) material, as well as proteins released by 2-mercaptoethanol-SDS treatment (D) at different time points after onset of treatment. n.sh., New shoots that formed 4 to 5 d after elicitin application; 2-ME, 2-mercaptoethanol. The arrowheads indicate the relative mobility of authentic cryptogein.

The most extreme example was basic PR 1, whose transcript accumulated almost exclusively inside the future necrotic region, whereas high PR 2 mRNA levels were also detected in the zone surrounding this area (Fig. 6).

Table I summarizes the results from the analysis of induced mRNA accumulation using specific probes for the tobacco SAR genes and *str 246*.

DISCUSSION

Induced plant resistance has become a field of increasing importance in the analysis of plant-microbe interactions. Since the first systematically performed experiments revealing acquired resistance of tobacco plants to TMV infection after preinoculation with the virus (Ross, 1961), this phenomenon has been demonstrated in both mono- and dicotyledons, which are able to acquire resistance to viral, bacterial, and fungal microorganisms (for review, see Kessmann et al., 1994). Therefore, acquired resistance can be considered a part of a plant's strategies to oppose pathogen attack, which can appear either in a limited region at the site of inoculation with an inducing agent (local acquired resistance) or in plant parts distant and different from this site (SAR) (Kloepper et al., 1992). In addition to biological inducers of acquired resistance, several chemicals have been characterized during the last years, which, to a different extent, also have resistance-inducing properties. Among these, the most prominent molecules are SA (White, 1979), INA (Metraux et al., 1991), and 3-aminobutyric acid (Cohen, 1994).

In this context, the *Phytophthora* elicitins represent molecules with particularly interesting characteristics: (a) they are the only known and well-characterized pathogen-derived elicitors of acquired resistance, (b) in addition, they possess the capacity to induce hypersensitive-like cell death, and (c) in contrast to other exogenous elicitors of defense responses with limited mobility (Ebel and Cosio, 1994), elicitins appear to be translocated within the plant. These features make them valuable tools for analyzing HR and its relation to biologically induced resistance, independent of inoculations with microorganisms.

Resistance to Ppn infection induced by cryptogein and capsicein appears to be systemic, since application of these proteins to the apical parts of a tobacco plant leads to the inhibition of fungal infection through the roots. The phenotypic symptoms, like browning of plant roots and hypocotyls, strongly resemble those observed during incompatible interactions of tobacco with *P. cryptogea* or *P. capsici*. After inoculation of tobacco roots, the development of both fungi becomes restricted to roots and hypocotyls without further affecting the normal growth of the plant (N. Maïa,

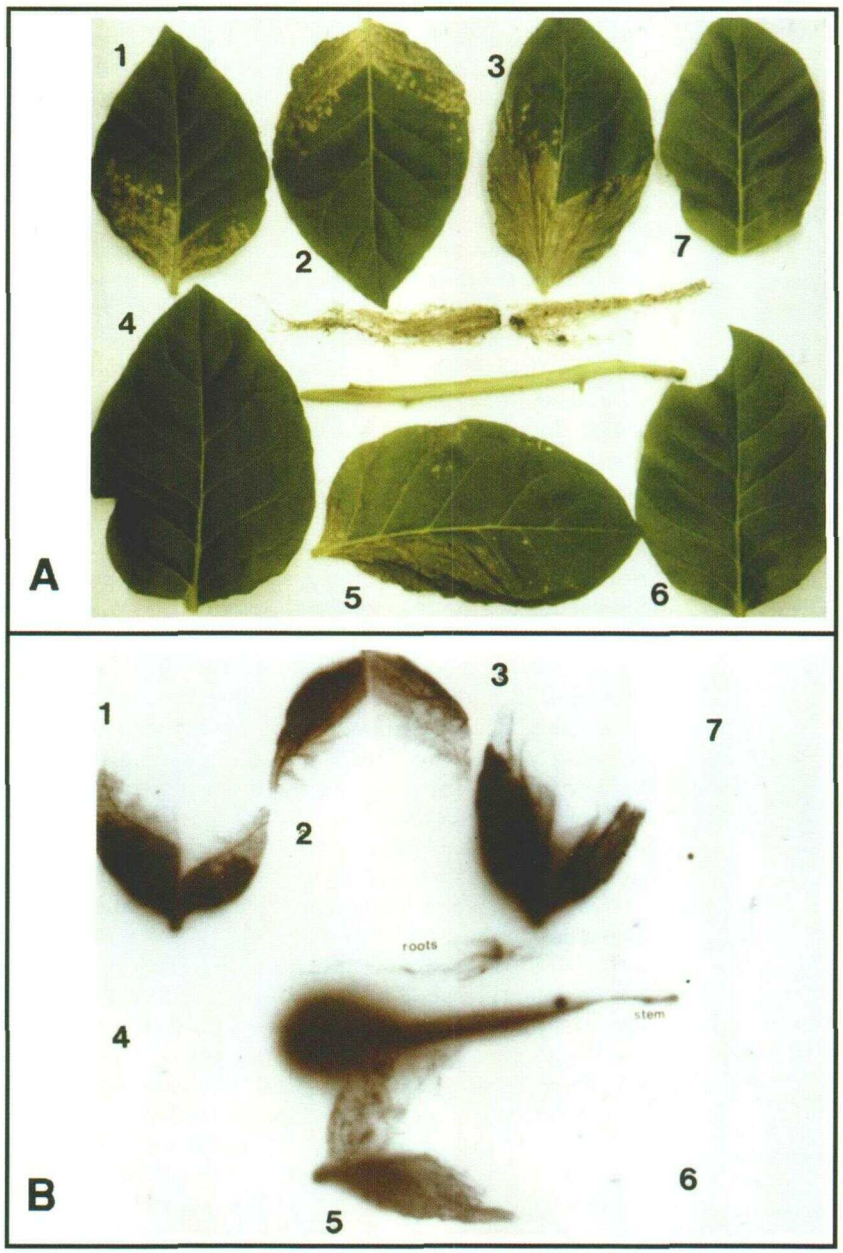

Figure 4. Leaves, longitudinal stem section, and roots of a 6-week-old tobacco plant 3 d after the application of [^{125}I]cryptogein (1.5×10^6 cpm/1.5 μg protein). Organs were photographed (A) and exposed to an x-ray film (B). Numbers 1 to 7 indicate the order of leaves from the apical (1, first leaf following the site of elicitin application) to the basal part of the plant.

personal communication). Systemic resistance induced by elicitins is persistent, since inoculation of treated plants through contaminated soil over a period of at least 7 d did not lead to the development of the black shank disease. Similar results were obtained when elicitin-treated plants were inoculated with Ppn mycelia on petiolar cutting sites, giving rise to acquired resistance over a period of at least 15 d posttreatment. In addition, the treated plants showed enhanced resistance to challenge inoculations with several other microorganisms, including *Sclerotinia sclerotiorum* (Bonnet et al., 1996), *Botrytis cinerea*, and *Rhizoctonia solani* (C. Coubard and D. Blancard, unpublished results).

Elicitin application to decapitated plants was followed by the immediate migration of the proteins to the lower plant parts, including leaves and roots. Elicitin uptake and translocation appeared to be a rapid process over the first 6 h after application. After this time, no further uptake of the applied protein was detected. Simultaneously, translocation of the elicitin between stems and leaves reached a stable distribution in both organs. Although the major portion of elicitin in the leaves remained soluble, in the stems the equilibrium of distribution was accompanied by an increased fixation of the proteins to the cell wall. The processes of uptake, translocation, and cross-linking to wall material maintained the intact protein, thus pointing to a high degree of elicitin stability against degradation. As indicated by experiments using tobacco cell-suspension cultures, the biological activity of elicitins is mediated through binding sites on the host cell membranes (Blein et al., 1991; Ricci et al., 1993). How cross-linking of elicitins

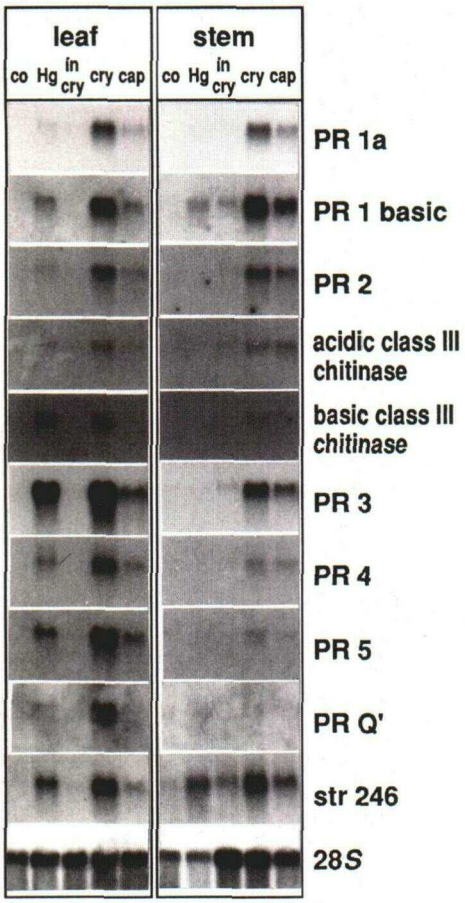

Figure 5. Accumulation of SAR gene and *str 246* transcripts in young tobacco as analyzed by northern blot hybridization using specific cDNA probes. Plants were treated by application of water (co), 50 μg of $HgCl_2$ (Hg), 3 μg of *S*-carboxymethylated cryptogein (in cry), 1.5 μg of cryptogein (cry), or 20 μg of capsicein (cap) to the decapitated stem. Plants (three individuals for each treatment and time point) were collected 6, 18, 27, and 43 h after onset of treatment. RNA was extracted separately from leaf and stem material and for each treatment; 2.5 μg from each time point were mixed prior to electrophoresis to yield a total of 10 μg. Loading of RNA was controlled by hybridization of the filters with a 257-bp fragment corresponding to the extreme 3′ region of the *P. parasitica* 28S rDNA (generous gift of I. Lacourt, Institut Nationale la Recherche Agronomique, Antibes, France), which cross-hybridizes to tobacco rRNA (stringency of washing, 0.2× SSC, 0.1% SDS, 50°C).

with host cell walls may interfere with their biological activity needs to be elucidated. Incorporation into cell walls may be the result of a detoxification process by the host plant. Alternatively, it may reflect a dynamic storage of the native protein, which thus remains accessible for reentry into vascular circulation. The latter hypothesis is supported by the finding that 1 week after treatment the entire protein can be reextracted from shoots that formed several days after treatment of the plants. Therefore, maintenance of the state of induced resistance may be achieved by successive endogenous supply with the inducer.

A controversial exists around the question of whether hypersensitive necrosis is correlated with, or even necessary for, SAR. Until recently, it was presumed that both processes are closely linked, since biologically induced resistance could only be achieved by preinoculation with necrotizing pathogens. However, recent research on *Arabidopsis thaliana* has demonstrated that HR is not always connected with SAR. Although mutants of *A. thaliana*, which are characterized by the constitutive formation of leaf lesions, show a generally enhanced resistance to pathogen attack (Dietrich et al., 1994; Greenberg et al., 1994), some mutant lines exhibiting constitutive immunity do not show any leaf defects (Delaney et al., 1994). In addition, the *A. thaliana RPS2* disease resistance gene mutant, *rps2–201*, which does not exhibit a local HR upon infection with *Pseudomonas syringae* pv *tomato*, retains the capability to acquire systemic resitance (Cameron et al., 1994). Of the chemical SAR inducers, neither INA nor 3-aminobutyric acid induce leaf necrosis at concentrations sufficient for resistance induction (Metraux et al., 1991; Cohen, 1994). Similar results are obtained with tobacco plants after application of capsicein, which is also able to induce SAR at concentrations where no effects on leaf integrity can be observed. In addition, although cryptogein and capsicein show the same distribution behavior in the plant as well as similar accumulation patterns in the leaves (not shown), only cryptogein triggers local hypersensitive-like leaf necrosis at the sites of its accumulation. These results support the hypothesis that hypersensitive necrosis may contribute to, but is not a prerequisite for, the induction of SAR.

Analysis of gene expression has become an integral part of studies on the establishment of SAR (Ward et al., 1991b; Ryals et al., 1994). In the present paper, it is shown that tobacco plants respond to elicitin application by the expression of all analyzed SAR genes in a manner very similar to their response to TMV infection or treatment with INA or SA (Ward et al., 1991b). In general, however, leaf tissues accumulated SAR gene transcripts to higher levels after treatment of the plants with the necrotizing cryptogein than after application of the nonnecrotizing capsicein. The different induction potentials of these elicitins appear to reflect their different necrotizing activities. Lesion formation could also be observed in leaves after treatment of the plants with heavy metal ions such as Hg^{2+}. However, although treatment of tobacco with $HgCl_2$ also led to detectable SAR transcript accumulation in the leaves (and of basic PR 1 and *str 246* mRNA in stems), no induction of resistance was obtained. Therefore, SAR gene activation appears, at least to a certain extent, to correlate with the necrotizing activities of the different inducers. In stem tissues, elicitin treatment led to visible necrotic symptoms only in the close vicinity of the application site (compare Fig. 4), regardless of whether basic or acidic elicitin was used. In this organ, the less profound differences in mRNA accumulation between capsicein and cryptogein treatment appear to reflect the less-pronounced differences in necrosis induction. Additional evidence for a link between SAR gene expression and necrosis formation derives from leaf infiltration experiments using cryptogein, where elicitin

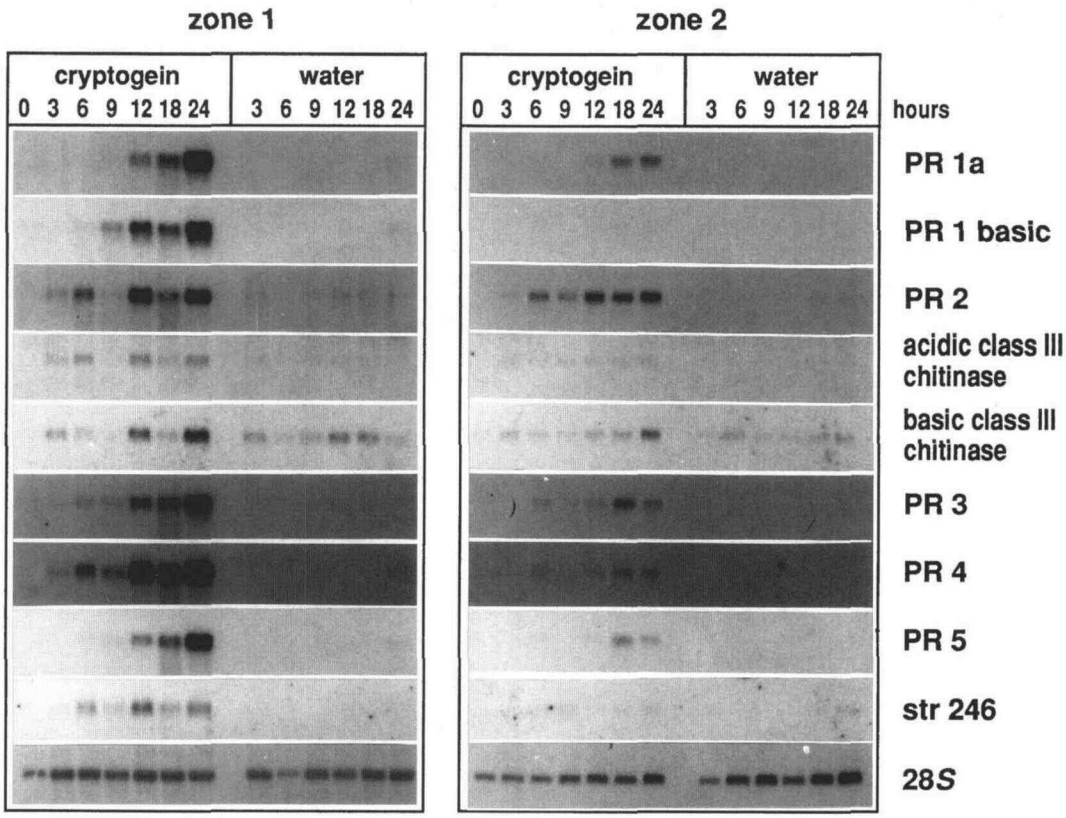

Figure 6. Northern blot analysis of transcripts accumulating in leaves that were infiltrated with water or 1 pmol of cryptogein. Material was collected at time point 0 and 3, 6, 9, 12, 18, and 24 h after onset of treatment. RNA was extracted from the infiltration area (zone 1) and the area surrounding it by 1 cm (zone 2). Loading of RNA was controlled as described for Figure 5.

distribution and necrosis formation were restricted to the infiltrated regions. Early accumulation of SAR gene and *str 246* transcripts occurred predominantly in the future necrotic zone, suggesting the involvement of gene expression in the hypersensitive-like response. However, it should be noted that the mode of activation of single SAR genes appears to depend on different stimuli. The gene encoding the basic isoform of PR 1 is strongly activated by ethylene (Eyal et al., 1993) but responds weakly to SA treatment (Ward et al., 1991b). In contrast, PR 2 is strongly activated by SA (Ward et al., 1991b) but responds weakly to ethylene (Brederode et al., 1991). Ethylene production is known to be one of the early responses triggered by cryptogein in tobacco cells (Blein et al., 1991; Milat et al., 1991b), and SA is required for the establishment of SAR in tobacco (Gaffney et al., 1993). Therefore, strictly localized basic PR 1 mRNA accumulation may be the result of local ethylene production, whereas the occurrence of PR 2 transcript in tissue surrounding the future necrotic zone may reflect a SA-mediated systemic induction.

The results presented here could reflect a dual mechanism of SAR gene activation. Thus, genes may be, first, expressed in response to cell death-inducing agents such as $HgCl_2$ and cryptogein, and, second, independent from cell death in response to systemic signals involved in SAR induction. Accumulation of mRNAs after $HgCl_2$ treatment may reflect the local cell death-inducing process alone, whereas capsicein-induced mRNA accumulation may be correlated with systemic SAR induction. From this point of view, the strong transcript accumulation in leaves after cryptogein application would result from the superposition of both processes, of local gene activation at sites where the elicitin accumulates and causes necrosis (also observed after leaf infiltration), and of systemic gene expression. Although this interpretation would explain the high level of cryptogein-induced resistance, it also implies that expression of the genes involved in the establishment of SAR is regulated by multiple stimuli. Regulatory elements have been defined for the potato PR protein gene *prp1–1* (Martini et al., 1993) and for the PR 1a gene of tobacco (Van de Rhee and Bol, 1993). A similar multiple component responsiveness is discussed for the basic β-1,3-glucanase gene of *Nicotiana plumbaginifolia* (Alonso et al., 1995). Recently, activation of *str 246* expression in tobacco either by infection with *Pseudomonas solanacearum* or by treatment with auxin was correlated with multiple regulatory elements in the promoter region of this gene (Gough et al., 1995). The further dissection of promoter regions exhibiting specific responsiveness to defined stimuli will clearly help to extend our current knowledge about the regulation of gene expression during resistance induction.

The results presented in this paper further support the hypothesis that hypersensitive necrosis is not indispens-

Table I. *Summary of the results of transcriptional activation analysis performed by hybridization between RNA from tobacco exposed to elicitins or other stimuli and specific cDNA probes*

The table also indicates some characteristics of the used probes, the corresponding transcripts, and the resulting translation products. n.d., Not determined. −, No transcript accumulation; +, little transcript accumulation; ++, +++, strong transcript accumulation.

Probe	Origin (by reference)	Size (bases) (approximately)		Function and Characteristics of Gene Products[a]	Activated by					Expression Differences in Response to Elicitin Treatment between Stem and Leaves of a Plant	Transcript Accumulations after Leaf Infiltration with Cryptogein		Transcript Accumulations (h after cryptogein infiltration)
		Probe	mRNA		Decapitation	Mercuric chloride	Cryptogein carboxymethylated	Cryptogein	Capsicein		Inside Infiltrated area	Surrounding Infiltrated area (1 cm)	
str 246	Godiard et al. (1991) Gough et al. (1995)	750	800	Unknown	+	++	+	+++	++	Predominantly in stems due to capsicein treatment	++	+	6
PR 1a	Payne et al. (1988b)	800	800	Unknown; acidic; extracellular	−	+	−	++	+	Predominantly in stems due to capsicein treatment	+++	+	12
PR 1 basic	Payne et al. (1989)	550	800	Unknown; basic isoform of PR 1	−	+	+	+++	++	Predominantly in stems due to capsicein treatment	+++	+	9
PR 2	Ward et al. (1991a)	900	1400	β-1,3-Glucanase, acidic; extracellular	−	+	+	++	+	Predominantly in stems due to capsicein treatment	++	+	3–6
Acidic class III chitinase	Ward et al. (1991b)	1000	1200	Chitinase; acidic; extracellular; identity to basic isoform 60%	−	+	+	++	+	Predominantly in stems due to capsicein treatment	+	+	3–6
Basic class III chitinase	Ward et al. (1991b)	1050	1200	Chitinase; basic; homologous to cucumber chitinase; unrelated to PR 3	−	++	−	++	+	Predominantly in stems due to capsicein treatment	++	+	3–6
PR 3	Payne et al. (1990a)	1100	1100	Chitinase; acidic; extracellular	−	+++	−	+++	++	Predominantly in leaves due to cryptogein treatment	+++	+	6
PR 4	Friedrich et al. (1991)	650	700	Unknown; acidic; extracellular; homologous to C terminus of Win1 and Win2 of potato	−	+	−	++	+	Predominantly in leaves due to cryptogein treatment	+++	+	6
PR 5	Payne et al. (1988a)	900	1000	Unknown; extracellular; homologies to thaumatin and amylase/proteinase inhibitor of maize	−	+	−	+++	+	Expression almost exclusively in leaves	+++	+	12
PR Q′	Payne et al. (1990b)	1150	1300	β-1,3-Glucanase; acidic; 55% identical to PR 2 glucanase	−	+	−	++	+	Expression in leaves only	n.d.	n.d.	n.d.

[a] Reference to Godiard et al., 1991; Ward et al., 1991b.

able for the establishment of SAR, although it might enhance the level of induced resistance. According to the classical understanding of SAR in tobacco, treatment with a biological inducer (such as TMV) triggers a cascade of events that finally leads to acquired resistance. In such a sequence of events starting with biological induction and resulting in SAR, chemical inducers such as SA or INA would act downstream from the primary event. Since SAR induction by elicitins is dependent on SA (Keller et al., 1994), the fungal proteins appear to enter the cascade downstream from biological, but upstream from chemical, induction. Whether elicitins trigger the production of a putative soluble, translocated endogenous plant signal molecule (Vernooij et al., 1994), or whether these proteins induce SAR by simulating the function of such a molecule in the plant signaling pathway, needs to be answered in future investigations.

ACKNOWLEDGMENTS

We gratefully acknowledge Drs. Leslie Friedrich and John Ryals for providing cDNA probes for the SAR genes and Drs. Dominique Roby and Yves Marco for the *str 246* cDNA probe. The authors thank Drs. Yves Marco and Wolfgang Knogge for carefully reading the manuscript and for editing.

Received June 20, 1995; accepted October 21, 1995.

LITERATURE CITED

Alexander D, Goodman RM, Gut-Rella M, Glascock C, Weymann K, Friedrich L, Maddox D, Ahl Goy P, Luntz T, Ward E, Ryals J (1993) Increased tolerance to two Oomycete pathogens in transgenic tobacco expressing pathogenesis-related protein 1a. Proc Natl Acad Sci USA **90:** 7327–7331

Alonso E, de Carvalho Niebel F, Obregon P, Gheysen G, Inzé D, Van Montagu M, Castresana C (1995) Differential in vitro DNA binding activity to a promoter element of the gn1 β-1,3-glucanase gene in hypersensitively reacting tobacco plants. Plant J **7:** 309–320

Blein JP, Milat ML, Ricci P (1991) Responses of cultured tobacco cells to cryptogein, a proteinaceous elicitor from *Phytophthora cryptogea*. Plant Physiol **95:** 486–491

Bonnet P, Bourdon E, Ponchet M, Blein J-P, Ricci P (1996) Acquired resistance triggered by elicitins in tobacco and other plants. Eur J Plant Pathol (in press)

Bonnet P, Lacourt I, Venard P, Ricci P (1994) Diversity in Pathogenicity to tobacco and in elicitin production among isolates of *Phytophthora parasitica*. J Phytopathol **141:** 25–37

Bottin A, Véronési C, Pontier D, Esquerré-Tugayé MT, Blein JP, Rusterucci C, Ricci P (1994) Differential responses of tobacco cells to elicitors from two *Phytophthora* species. Plant Physiol Biochem **32:** 373–378

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

Cameron RK, Dixon RA, Lamb CJ (1994) Biologically induced systemic acquired resistance in *Arabidopsis thaliana*. Plant J **5:** 715–725

Cohen Y (1994) 3-Aminobutyric acid induces systemic resistance against *Peronospora tabacina*. Physiol Mol Plant Pathol **44:** 273–288

Delaney TP, Friedrich L, Kessmann H, Uknes S, Vernooij B, Ward E, Weymann K, Ryals J (1994) The molecular biology of systemic acquired resistance. *In* MJ Daniels, JA Downie, AE Osbourn, eds, Advances in Molecular Genetics of Plant-Microbe Interactions, Vol 3. Kluwer Academic Publishers, Dordrecht, The Netherlands, pp 339–347

Devergne JC, Bonnet P, Panabières F, Blein JP, Ricci P (1992) Migration of the fungal protein cryptogein within tobacco plants. Plant Physiol **99:** 843–847

Dietrich RA, Delaney TP, Uknes SJ, Ward ER, Ryals JA, Dangl JL (1994) Arabidopsis mutants simulating disease resistance response. Cell **77:** 565–577

Ebel J, Cosio E (1994) Elicitors of plant defense responses. Int Rev Cytol **148:** 1–36

Ebel J, Scheel D (1992) Elicitor recognition and signal transduction. In T Boller, F Meins, eds, Plant Gene Research: Genes Involved in Plant Defense Response, Vol 8. Springer Verlag, Vienna, Austria, pp 183–205

Eyal Y, Meller Y, Levyadun S, Fluhr R (1993) A basic-type PR-1 promoter directs ethylene responsiveness, vascular and abscission zone-specific expression. Plant J **4:** 225–234

Fernandez-Luna JL, Lopez-Otin C, Soriano F, Méndez E (1985) Complete amino acid sequence of the *Aspergillus* cytotoxin mitogillin. Biochemistry **24:** 861–867

Friedrich L, Moyer M, Ward E, Ryals J (1991) Pathogenesis-related protein 4 is structurally homologous to the carboxy-terminal domains of hevein, Win-1 and Win-2. Mol Gen Genet **230:** 113–119

Gaffney T, Friedrich L, Vernooij B, Negrotto D, Nye G, Uknes S, Ward E, Kessmann H, Ryals J (1993) Requirement of salicylic acid for the induction of systemic acquired resistance. Science **261:** 754–756

Godiard L, Froissard D, Fournier J, Axelos M, Marco Y (1991) Differential regulation in tobacco cell suspension of genes involved in plant-bacteria interactions by pathogen-related signals. Plant Mol Biol **17:** 409–413

Gough C, Hemon P, Tronchet M, Lacomme C, Marco Y, Roby D (1995) Developmental and pathogen-induced activation of an *msr* gene from tobacco, *str*246C, involves multiple regulatory elements. Mol Gen Genet **247:** 323–337

Greenberg JT, Guo A, Klessig D, Ausubel FM (1994) Programmed cell death in plants: a pathogen-triggered response activated coordinately with multiple defense functions. Cell **77:** 551–563

Huet JC, Mansion M, Pernollet JC (1993) Amino acid sequence of the α-elicitin secreted by *Phytophthora cactorum*. Phytochemistry **34:** 1261–1264

Keller H, Blein JP, Bonnet P, Bourdon E, Panabières F, Ricci P (1994) Responses of tobacco to elicitins, proteins from *Phytophthora* spp. eliciting acquired resistance. *In* MJ Daniels, JA Downie, AE Osbourn, eds, Advances in Molecular Genetics of Plant-Microbe Interactions, Vol 3. Kluwer Academic Publishers, Dordrecht, The Netherlands, pp 327–332

Kessmann H, Staub T, Ligon J, Oostendorp M, Ryals J (1994) Activation of systemic acquired resistance in plants. Eur J Plant Pathol **100:** 359–369

Klement Z (1982) Hypersensitivity. **In** MS Mount, GH Lacy, eds, Phytopathogenic Prokaryotes, Vol 2. Academic Press, New York, pp 150–177

Kloepper JW, Tuzun S, Kuc JA (1992) Proposed definitions related to induced disease resistance. Biocontrol Science and Technology **2:** 349–351

Kombrink E, Beerhues L, Garcia-Garcia F, Hahlbrock K, Müller M, Schröder M, Witte B, Schmelzer E (1993) Expression patterns of defense-related genes in infected and uninfected plants. **In** B Fritig, M Legrand, eds, Mechanisms of Plant Defense Responses. Kluwer Academic Publishers, Dordrecht, The Netherlands, pp 236–249

LeBerre JY, Panabières F, Ponchet M, Denoroy L, Bonnet P, Marais A, Ricci P (1994) Occurrence of multiple forms of elicitins in *Phytophthora cryptogea*. Plant Physiol Biochem **32:** 251–258

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

Logemann J, Schell J, Willmitzer L (1987) Improved method for the isolation of RNA from plant tissues. Anal Biochem **163:** 16–20

Martini N, Egen M, Rüntz I, Strittmatter G (1993) Promoter sequences of a potato pathogenesis-related gene mediate transcriptional activation selectively upon fungal infection. Mol Gen Genet **236:** 179–186

Metraux JP, Ahl Goy P, Staub T, Speich J, Steinemann A, Ryals J, Ward E (1991) Induced systemic resistance in cucumber in response to 2,6-dichloro-isonicotinic acid and pathogens. *In* H Hennecke, DPS Verma, eds, Advances in Molecular Genetics of Plant-Microbe Interactions, Vol 1. Kluwer Academic Publishers, Dordrecht, The Netherlands, pp 432–439

Milat ML, Ducruet JM, Ricci P, Marty F, Blein JP (1991a) Physiological and structural changes in tobacco leaves treated with cryptogein, a proteinaceous elicitor from *Phytophthora cryptogea*. Phytopathology **81:** 1364–1368

Milat ML, Ricci P, Bonnet P, Blein JP (1991b) Capsidiol and ethylene production by tobacco cells in response to cryptogein, an elicitor from *Phytophthora cryptogea*. Phytochemistry **30:** 2171–2173

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

Payne G, Middlesteadt W, Desai N, Williams S, Dincher, S, Carnes M, Ryals J (1989) Isolation and sequence of a genomic clone encoding the basic form of pathogenesis-related protein 1 from *Nicotiana tabacum*. Plant Mol Biol **12:** 595–596

Payne G, Middlesteadt W, Williams S, Desai N, Parks TD, Dincher S, Ryals J (1988a) Isolation and nucleotide sequence of a novel cDNA clone encoding the major form of pathogenesis-related protein R. Plant Mol Biol **11:** 223–224

Payne G, Parks TD, Burkhart W, Dincher S, Ahl P, Metraux JP, Ryals J (1988b) Isolation of the genomic clone for pathogenesis-related protein 1a from *Nicotiana tabacum* cv. Xanthi-nc. Plant Mol Biol **11:** 89–94

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

Ribeiro OK (1978) A Source Book of the Genus Phytophthora. J Cramer Verlag, Vaduz, Liechtenstein, p 75

Ricci P, Bonnet P, Huet JC, Sallantin M, Beauvais-Cante F, Bruneteau M, Billard V, Michel G, Pernollet JC (1989) Structure and activity of proteins from pathogenic fungi *Phytophthora* eliciting necrosis and acquired resistance in tobacco. Eur J Biochem **183:** 555–563

Ricci P, Panabières F, Bonnet P, Maia N, Ponchet M, Devergne JC, Marais A, Cardin L, Milat ML, Blein JP (1993) Proteinaceous elicitors of plant defense responses. *In* B Fritig, M Legrand, eds, Mechanisms of Plant Defense Responses. Kluwer Academic Publishers, Dordrecht, The Netherlands, pp 121–135

Ricci P, Trentin F, Bonnet P, Venard P, Mouton-Perronnet F, Bruneteau M (1992) Differential production of parasiticein, an elicitor of necrosis and resistance in tobacco, by isolates of *Phytophthora parasitica*. Plant Pathol **41:** 298–307

Ross FA (1961) Systemic acquired resistance induced by localized virus infections in plants. Virology **14:** 340–358

Ryals J, Uknes S, Ward E (1994) Systemic acquired resistance. Plant Physiol **104:** 1109–1112

Van de Rhee MD, Bol JF (1993) Induction of the tobacco PR-1a gene by virus infection and salicylate treatment involves an interaction between multiple regulatory elements. Plant J **3:** 71–82

Vernooij B, Friedrich L, Morse A, Reist R, Kolditz-Jawhar R, Ward E, Uknes S, Kessmann H, Ryals J (1994) Salicylic acid is not the translocated signal responsible for inducing systemic acquired resistance but is required in signal transduction. Plant Cell **6:** 959–965

Viard MP, Martin F, Pugin A, Ricci P, Blein JP (1994) Protein phosphorylation is induced in tobacco cells by the elicitor cryptogein. Plant Physiol **104:** 1245–1249

Ward ER, Payne GP, Moyer MB, Williams SC, Dincher SS, Sharkey KC, Beck JJ, Taylor HT, Ahl-Goy P, Meins F, Ryals J (1991a) Differential regulation of β-1,3-glucanase messenger RNAs in response to pathogen infection. Plant Physiol **96:** 390–397

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

White RF (1979) Acetylsalicylic acid (aspirin) induces resistance to tobacco mosaic virus in tobacco. Virology **99:** 410–412

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