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Syringolin, a Novel Peptide Elicitor from *Pseudomonas syringae* pv. *syringae* that Induces Resistance to *Pyricularia oryzae* in Rice

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Recognition by rice plants (*Oryza sativa*) of the nonhost pathogen *Pseudomonas syringae* pv. *syringae* leads to an active response ultimately resulting in local acquired resistance against the rice blast fungus *Pyricularia oryzae*. An observable aspect of this defense response is the increased abundance of a set of transcripts. The accumulation of one of these transcripts, *Pir7b*, was dependent on the function of the bacterial *lemA* gene, which encodes part of a two-component regulatory system. This suggested that the *lemA* regulatory system controlled the production of an elicitor of *Pir7b* transcript accumulation. This elicitor, which we name syringolin, was purified to homogeneity and its structure was elucidated. Syringolin is a novel and unusual secreted peptide consisting of a 12-membered ring formed by the two non-proteinogenic amino acids 5-methyl-4-amino-2-hexenoic acid and 3,4-dehydrolysine. The α-amino group of the latter is connected by a peptide bond to a valine that in turn is linked to a second valine via a urea moiety. Application of syringolin onto rice leaves elicited the accumulation of *Pir7b* as well as of other defense-related transcripts and induced resistance toward *P. oryzae*. Thus, syringolin is one of several determinants by which rice plants can perceive the nonhost pathogen *P. syringae* pv. *syringae*.

Plants are exposed to a large number of potential pathogens, and, accordingly, have evolved a variety of effective defense mechanisms that can be passive and preformed or active in nature. Active defense responses often take the form of a hypersensitive reaction (HR) involving localized host cell death at the infection site accompanied by complete resistance. In many systems it has been shown that these local events can induce systemic acquired resistance (SAR) against later infections by a variety of other pathogens to which the plant normally would be susceptible. A prerequisite for active defense responses is the perception of potential pathogens by the plant, and the identification of the molecular structures involved in the recognition process is the focus of extensive research (Baker et al. 1997; Bent 1996; Boller 1995; Hammond-Kosack and Jones 1996). Molecules eliciting active defense responses in plants have been isolated from pathogenic microorganisms. Based on their specificity, elicitors have been divided into two groups. Specific elicitors evoke defense reactions in a cultivar-specific way, as observed in race-cultivar-specific interactions characterized by the "gene-for-gene" concept. In contrast, nonspecific elicitors are involved in general or nonhost resistance and are thought to represent molecular structures widely occurring in pathogens (Boller 1995; Yoshikawa et al. 1993).

In rice, acquired resistance toward *Pyricularia oryzae*, the fungal pathogen that causes rice blast disease, can be triggered by inoculation with the nonhost pathogen *P. syringae* pv. *syringae* (Smith and Métraux 1991). Complementary DNAs have been cloned that correspond to transcripts accumulating concomitantly with resistance induction (Reimmann and Dudler 1993; Reimmann et al. 1995, 1992; Wäspi et al., *in press*). While an HR and resistance induction were caused by a number of nonhost *P. syringae* pv. *syringae* strains that originated from different host plants, we discovered that the set of transcripts that accumulated after inoculation was not identical in all cases. In particular, *Pir7b* transcripts did not accumulate after inoculation with the *P. syringae* pv. *syringae* strain SM, in contrast to all other defense-related mRNA species tested (Reimmann et al. 1995). The *Pir7b* gene encodes an esterase (EC 3.1.1.1) belonging to the large family of "α/β hydrolase fold" proteins (Wäspi et al., *in press*). Although the difference between the SM strain and the other strains with regard to *Pir7b* transcript accumulation was not known, it was found that a functional, bacterial *lemA* gene was a prerequisite for *Pir7b* gene activation in rice (Reimmann et al. 1995). As the *lemA* gene encodes the sensor part of a bacterial two-component regulatory system (Hrabak and Willis 1992), these observations suggested that the *lemA* regulatory system controlled the production of an elicitor of *Pir7b* transcript accumulation. Here we report the isolation of this elicitor, which we refer to as syringolin, the elucidation of its structure, and its biological activity on rice. Syringolin is a novel and unusual peptide that is secreted by the bacteria under the appropriate conditions. Application of syringolin onto rice leaves leads to the accumulation not only of *Pir7b* mRNA but also of

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all other defense-related transcripts, and to enhanced resistance against *P. oryzae*. Thus, syringolin is one of the determinants by which rice plants are able to recognize nonhost *P. syringae* pv. *syringae* strains.

RESULTS

Production and purification of syringolin.

To determine whether the *Pir7b* elicitor was secreted by the bacteria, *P. syringae* pv. *syringae* strain B 301D-R was grown overnight in LB (Luria-Bertani) medium under aerated conditions or, alternatively, under conditions optimized for the production of the phytotoxin syringomycin, i.e., for 8 days in SRM_{AF} medium without shaking (Gross 1985; Mo and Gross 1991a, 1991b). The conditioned media were filter sterilized and assayed for the ability to elicit *Pir7b* transcript accumulation in rice leaves. These experiments showed that, under the latter conditions, the elicitor was indeed secreted into the medium. Ultrafiltration experiments with Amicon filters with exclusion limits of 500 and 3,000 Da, respectively, indicated that the molecular mass of the eliciting activity was between these two values (data not shown).

To isolate the *Pir7b* elicitor, conditioned SRM_{AF} medium from 200-ml cultures was concentrated five times by ultrafiltration with an Amicon filter with a 500-Da exclusion limit. Five milliliters of the concentrate was run on a Superdex 30 preparative gel filtration column. Fractions were tested for *Pir7b* eliciting activity by droplet application onto rice leaf segments, extracting total RNA 24 h later, and probing an RNA gel blot with a *Pir7b* probe. The activity eluted between 86 and 95 min and coincided with the peak indicated in Figure 1A. Peaks of several runs were pooled and aliquots were further separated by high-performance liquid chromatography (HPLC) with a C_{18} column (Fig. 1B). *Pir7b* eliciting activity was found in peaks 1 and 2. Peak 1 was collected from 50 HPLC runs, yielding approximately 3 mg of material. We refer to the active compound contained in peak 1 as syringolin. For the structural characterization of syringolin, this material was subjected to mass spectrometry and nuclear magnetic resonance (NMR) analysis.

This material was also used to relate peak 1 areas of HPLC runs to absolute amounts of injected syringolin and thus to estimate syringolin concentrations of test solutions. With this procedure, syringolin concentrations of 20 to 60 mg per liter of conditioned medium were typically reached with strain B 301D-R after 5 to 8 days of culture. Since earlier experiments revealed that the SM strain and *lemA* mutant strains did not induce *Pir7b* transcript accumulation upon infiltration into rice leaves (Reimmann et al. 1995), syringolin production was measured in cultures of the SM strain and the *lemA* mutant NPS3136 (Willis et al. 1990). In agreement with the earlier results, no syringolin was detected in culture supernatants of these strains (data not shown). Conversely, strains 548 and B728a, two other strains previously shown to elicit *Pir7b* mRNA accumulation (Reimmann et al. 1995), are both syringolin producers (data not shown).

Elucidation of the structure of syringolin.

The structure of syringolin as derived from consideration of its spectral data is given in Figure 2A. The mass spectra show its mass to be 493 amu, corresponding to a molecular formula of $C_{24}H_{39}N_5O_6$. The NMR chemical shifts of the C atoms of syringolin and the correlation to the protons directly attached to them were obtained from heteronuclear, single quantum, correlation experiments (data give in Materials and Methods). The configuration of the two double bonds is *trans*, as shown by the vicinal coupling constant of 16 Hz in both cases. The

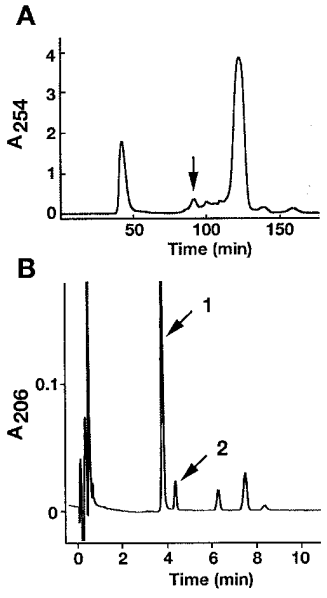

Fig. 1. Isolation of the *Pir7b* elicitor. **A,** Gelfiltration chromatogram of conditioned medium. Peak containing elicitor activity indicated by arrow. **B,** Reverse-phase high-performance liquid chromatography profile of fractions corresponding to elicitor-active peak indicated in **A**. Peaks labeled with 1 and 2 contained *Pir7b* elicitor activity.

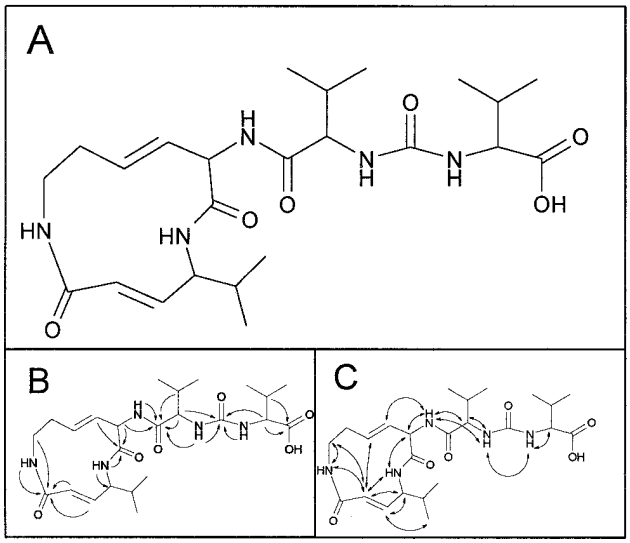

Fig. 2. A, Structure of syringolin. The ring structure is composed of 5-methyl-4-amino-2-hexenoic acid and 3,4-dehydrolysine. The α-amino group of the latter is joined by a peptide bond to a valine that in turn is linked to a second valine via a urea moiety. **B,** Heteronuclear multiple bond correlations (HMBC) (in DMSO-d_6) used to establish the structure of syringolin are indicated by arrows. **C,** Rotating frame Overhauser enhancement spectroscopy (ROESY) correlations (in DMSO-d_6). The correlation between the two urea NHs was obtained in H_2O, since their chemical shift difference is larger in H_2O than in DMSO-d_6.

chemical shifts of the protons within each amino acid moiety were correlated with $^{1}H,^{1}H$ correlation spectroscopy experiments (data not shown), whereas the connectivity of the amino acid parts was established by heteronuclear multiple bond correlations (HMBC; Fig. 2B) and by rotating frame Overhauser enhancement spectroscopy (ROESY; Fig. 2C). The results of these NMR experiments completely define the structure of syringolin as presented in Figure 2A.

Syringolin increases resistance in rice against rice blast.

As evident from Figure 3A, Pir7b protein accumulation was detectable by gel blot analysis with specific antibodies at 8 h and reached a plateau at 16 h after a 40 µM syringolin solution was sprayed onto rice leaves. Figure 3B shows the result of a dose response experiment. Eight 5-µl droplets of syringolin solution of different concentrations were applied per leaf and Pir7b protein content was visualized 72 h later by gel blot analysis. The minimal syringolin concentration at which Pir7b was detectable was 6.25 nM, corresponding to 250 fmol of syringolin applied per leaf (Fig. 3B). Pir7b concentration increased approximately in proportion to the syringolin concentration up to 500 nM (20 pmol per leaf). Application of a 10-fold higher concentration (5 µM; 200 pmol per leaf) only slightly increased Pir7b concentration further and apparently saturated the response, as no significant further increase was observed with a 100-fold higher concentration (50 µM; 2 nmol per leaf). Syringolin did not induce necrosis and, even at the highest concentration applied, no visible phytotoxic effects were observed. This was also the case when syringolin was applied to leaves of wheat (*Triticum aestivum*) and *Arabidopsis thaliana*.

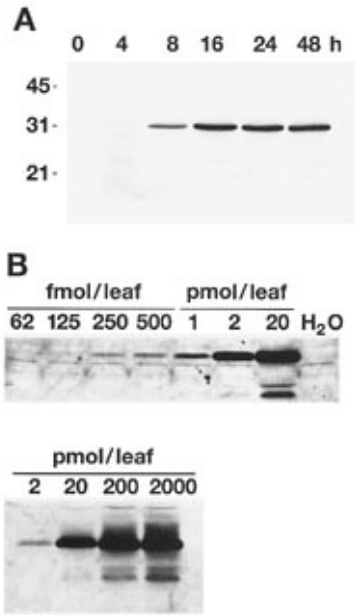

Fig. 3. Gel blot analysis of Pir7b protein accumulation in rice leaves in response to syringolin application. **A,** Proteins were extracted from rice leaves at indicated time points after treatment with 1.6 nmol syringolin per leaf (i.e., eight 5-µl droplets of a 40 µM syringolin solution). Twenty micrograms of protein was loaded per slot. Pir7b protein was detected with a specific antiserum and phosphatase-coupled secondary antibody with nitroblue tetrazolium chloride (NBT) and 0.38 mM 5-bromo-4-chloro-3-indolylphosphate (BCIP) as substrates. **B,** Gel blot of proteins extracted from rice leaves 48 h after treatment with indicated amounts of syringolin (1 pmol per leaf corresponds to eight 5-µl droplets of a 25 nM syringolin solution). Twenty micrograms of protein was loaded per slot. Blot was developed with the ECL chemiluminescent immunodetection system (Amersham International, Amersham, U.K.).

As shown in Figure 4, syringolin induced the transient accumulation not only of *Pir7b* mRNA but also of transcripts corresponding to the *Rir1, Pir2,* and *Pir3* genes that have previously been shown to accumulate upon inoculation of rice leaves with *P. syringae* pv. *syringae* (Reimmann and Dudler 1993; Reimmann et al. 1995, 1992). *Rir1* encodes a homologue of the wheat defense-related *Wir1* gene (Bull et al. 1992; Franck and Dudler 1995), *Pir2* a thaumatin-like protein (Reimmann and Dudler 1993), and *Pir3* a peroxidase (Reimmann et al. 1992). The *Pir7b* gene encodes an esterase belonging to the family of proteins containing an "α/β hydrolase fold" (Wäspi et al., *in press*).

Pir7b transcript accumulation reached a maximum 8 h after spray application of a 40 µM syringolin solution. *Pir2* and *Pir3* transcripts reached maximal levels after 16 h, while accumulation of *Rir1* transcripts was delayed and maximal levels were observed only after 24 h.

To test whether syringolin application was able to increase protection of rice from infection by *P. oryzae*, 3-week-old plants were sprayed with a 40 µM solution of syringolin and spray inoculated with conidiospores 24 h later. The number of blasts on the fourth leaf of each plant was scored 5 days later. As shown in Figure 5, syringolin application significantly reduced the number of blasts, to about 25% of that on control leaves. No phytotoxic effects were observed on the treated plants.

Next it was tested whether syringolin had antifungal activity against *P. oryzae* in vitro. Filter disks were soaked in a 250 µM syringolin or control solution and placed on agar plates that were inoculated with conidiospores. No inhibition of

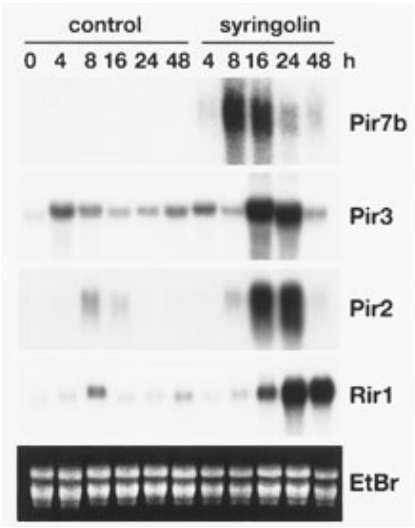

Fig. 4. Gel blot analysis of defense-related transcript accumulation. Rice leaves were sprayed with control solution (0.05% Tween 20 in water) or with a 40 µM syringolin solution. Total RNA was extracted at indicated time points and used to prepare identical blots. Ten micrograms of RNA was loaded per slot. Blots were probed with radiolabeled probes indicated on right. Equal loading was monitored on the ethidium bromide-stained gels, of which the one corresponding to the Rir1-probed blot is shown (EtBr).

germination or growth could be observed around disks soaked with syringolin. Similarly, no significant difference in the number of fungal colonies was found between plates sprayed with a 250 μM solution of syringolin or with control solution before inoculation of the plates (data not shown). In addition, in filter disk assays identical to the ones performed with *P. oryzae*, syringolin did not inhibit growth of the yeast fungus *Rhodotorula pilimanae*, an organism sensitive to the phytotoxin syringomycin (Hrabak and Willis 1993). These results, together with the fact that syringolin induced the accumulation of a set of defense-related genes, suggest that the increased protection of rice plants from rice blast infection is due to elicitation of rice defense responses resulting in acquired resistance and not due to a direct fungitoxic activity of syringolin.

DISCUSSION

We have isolated syringolin, an elicitor secreted by the phytopathogenic bacterium *P. syringae* pv. *syringae* that activates defense-related genes in the nonhost plant *O. sativa* and induces resistance toward the rice blast fungus *P. oryzae*. Syringolin production was shown to be under the control of the *lemA*/*gacA* two-component regulatory system. In vitro, syringolin is produced only if the bacteria are grown in a nutrient-poor medium that also contains D-fructose and arbutin as plant signal molecules (Mo and Gross 1991b).

Syringolin contains a 12-membered ring formed by the two non-proteinogenic amino acids 5-methyl-4-amino-2-hexenoic acid and 3,4-dehydrolysine, which both have hitherto not been found in nature. The α-amino group of the latter is connected to a valine that in turn is linked to a second valine by a urea moiety. This urea moiety has only been rarely detected in natural products (for a recent example see Schmidt et al. 1997, and references therein). It is likely that this structure is the product of a peptide synthetase, as are many other non-ribosomally synthesized bioactive peptides. The structure of syringolin is most similar to a group of closely related antitumor antibiotics termed glidobactins and cepafungins that have been isolated from the gram-negative bacteria *Polyangium brachysporum* and *Pseudomonas* spp., respectively (Oka et al. 1988b; Shoji et al. 1990). These compounds contain a 12-membered ring consisting of 4-amino-2-pentenoic acid and 4-hydroxylysine that is linked to an L-threonine residue that in turn is acylated by different unsaturated fatty acids (Oka et al. 1988b; Terui et al. 1990). The acyl group appears to be important for the antifungal activity, as its removal essentially eliminated toxicity to fungi (Oka et al. 1988a). Syringolin is not acylated, and we did not observe antifungal activity in vitro.

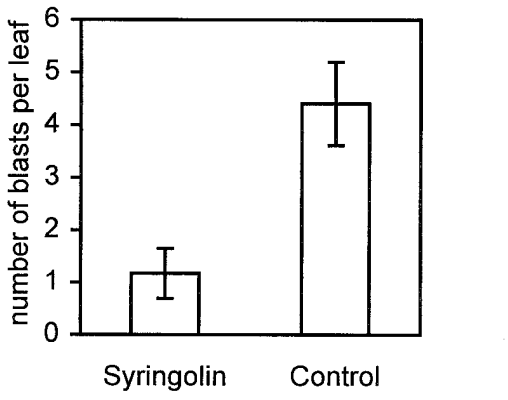

Fig. 5. Reduction of blast disease of rice by syringolin. Three-week-old rice plants were sprayed with a 40 μM solution of syringolin or a control solution (0.05% Tween 20) and inoculated with spores of *Pyricularia oryzae*. Number of blast lesions on the fourth leaf was determined after 5 days. Mean number ± standard deviation from the mean is given from two independent experiments encompassing 6 pots with approximately 15 plants per pot.

The accumulation of transcripts that occurs upon application of syringolin is extremely transient in nature, indicating that they have a relatively short half life. It also shows that the activity of syringolin must be correspondingly transient. This could be explained by rapid inactivation of syringolin itself or of elements in the signal transduction pathway leading from elicitor perception to transcript accumulation.

Syringolin is produced not only by *P. syringae* pv. *syringae* strain B301D-R, but also by other wild-type strains such as 548 and B728a that were shown to elicit *Pir7b* transcript accumulation upon infiltration (Reimmann et al. 1995). Thus, syringolin production appears to be widely occurring among *P. syringae* pv. *syringae* strains, although not ubiquitously, as strain SM, which failed to elicit *Pir7b* activity (Reimmann et al. 1995), does not produce detectable amounts of it. At present it is unknown why the SM strain does not produce syringolin. Possible causes may be the lack of functional genes encoding the necessary biosynthetic enzymes, or, alternatively, the absence of signals, both in vitro and after infiltration into rice plants, necessary for induction of syringolin production.

As we have shown, syringolin induces accumulation of defense-related transcripts and increases resistance against *P. oryzae*. Thus, rice cells must be able to sense the compound, most likely by means of a receptor. However, because local acquired resistance against *P. oryzae* is induced by *Pseudomonas* strains that produce syringolin as well as by the SM strain that does not, syringolin is redundant in this respect. Rice cells obviously are able to recognize the nonhost pathogen by molecular structures other than syringolin, and thus must have a multitude of perception systems that enable them to recognize different molecular structures associated with nonhost pathogens. However, the nonhost responses evoked by recognition events apparently can depend on the particular elicitor structure that is recognized, as indicated by the fact that syringolin is necessary for *Pir7b* transcript accumulation. Thus, although both the syringolin nonproducer strain SM as well as syringolin induce resistance, the signal transduction pathways triggered by the different molecular structures are not identical and lead to responses that are not congruent. Our results also show that the occurrence of necrotic lesions is not a necessary prerequisite for resistance induction in rice.

Since resistance of rice toward rice blast is triggered by inoculation of strains that produce syringolin as well as by the SM strain that does not, the *Pir7b* gene product does not appear to contribute significantly to the resistance against *P. oryzae* (Reimmann et al. 1995). Pir7b was recently shown to encode an α/β hydrolase fold-containing protein exhibiting esterase activity toward specific naphthol AS esters (Wäspi et al., *in press*), which may suggest a role in a detoxification process. However, no hydrolytic activity of the enzyme toward syringolin and syringomycin, which as cyclic peptides

both contain intramolecular ester bonds resembling the ones in naphthol AS esters, was observed (Wäspi et al., *in press*). Of course, these results do not exclude a role of the Pir7b esterase in other plant-pathogen interactions.

Syringolin is a low-molecular-weight compound whose structure is not directly encoded by a gene. Although low-molecular-weight membrane constituents of microbes such as arachidonic acid and ergosterol have been found to be potent elicitors, most of the well-characterized, non-cultivar-specific elicitors are high-molecular-weight polysaccharides, glycoproteins, and proteins (Boller 1995; Hahn 1996; Yoshikawa et al. 1993), or well-defined fragments derived from these polymers, such as the classical branched β-1,3-(1,6-heptaglucoside from *Phytophthora megasperma* f. sp. *glycinea* (Sharp et al. 1984) and a short peptide fragment derived from a glycoprotein of *P. megasperma* (Nürnberger et al. 1994). Specific elicitors whose action depends on the cultivar carrying a specific resistance gene have also been identified. Examples are the AVR9 peptide, the processed product of the *Avr9* avirulence gene of the tomato pathogen *Cladosporium fulvum* (Van den Ackerveken et al. 1993), and NIP1 from the barley pathogen *Rhynchosporium secalis* (Rohe et al. 1995). In contrast, most bacterial avirulence gene products have no elicitor activity when applied exogenously, but must be transported into the host cell by type III secretion systems (see Alfano and Collmer 1996, for a review). An interesting exception is the *avrD* avirulence gene of *P. syringae* pv. *tomato* that indirectly specifies the production of the low-molecular-weight compound syringolide via the condensation of D-xylulose and a β-ketoalkanoic acid (Midland et al. 1993). Syringolide is secreted and triggers an HR in soybean cultivars harboring the *Rpg4* resistance gene, even though soybean is not a host for *P. syringae* pv. *tomato* (Keen et al. 1990). Although syringolin does not cause an HR on rice and there is no evidence for a cultivar-specific action, in its other aspects it is most reminiscent of the AvrD elicitor. Indeed, it is not excluded that syringolin may cause defense reactions, including cultivar-specific ones, on other nonhost species.

Although the function of syringolin is not known, it seems likely that it may contribute in some way to the virulence of *P. syringae* pv. *syringae* in its interactions with host plants. The fact that the production of syringolin is under the control of the *lemA* global regulator, which has been shown to be important for pathogenicity in some systems (Hrabak and Willis 1992), is compatible with this view. Evolution of a perception system in plants for recognition of such virulence factors may then be the result of the evolutionary "arms race" between plants and pathogens (Alfano and Collmer 1996). Whether such a perception system is cultivar-specific or not may not be a fundamental difference. However, this will only be clarified once the molecular mechanisms underlying nonhost and cultivar-specific resistance are understood and can be compared. Use of isolated syringolin for experiments aimed at the identification of a receptor in rice cells to answer such questions is an attractive strategy that we currently pursue.

MATERIALS AND METHODS

Biological material.

P. syringae pv. *syringae* strains: The *P. syringae*. pv. *syringae* strain designated SM by us was obtained from J.-P. Métraux, University of Fribourg. It is identical to the one used in the studies of Smith and Métraux (1991) and is pathogenic on wheat (Vincent and Fulbright 1983). Strain 548 was obtained from the collection of Novartis, Basel, and was originally isolated from pears. Strain B728a is a bean pathogen (Hrabak and Willis 1992), and strain B 301D-R originated from pear (Xu and Gross 1988). All strains cause an HR when infiltrated into rice leaves. If not otherwise stated, *Pseudomonas* strains were grown in LB medium (10 g of Bacto-tryptone, 5 g of Bacto-yeast extract, and 10 g of NaCl per liter) overnight in shaking cultures at 28°C with the appropriate antibiotics.

P. oryzae was maintained on plates containing 2 g of yeast extract per liter, 10 g of starch per liter, 30 g of oat grain meal per liter, and 2% agar, according to Smith and Métraux (1991). Growth conditions and infection of rice plants (*Oryza sativa* cv. Nohrin 29) with *P. oryzae* were as described (Schweizer et al. 1997).

Rhodotorula pilimanae (DSM 70825; German Collection of Microorganisms and Cell Cultures, Mascheroderweg 1b, D-38124, Braunschweig, Germany) was maintained on DSMZ 186 medium plates (3 g of Bacto-yeast extract per liter, 3 g of malt extract per liter, 5 g of Bacto-tryptone per liter, 10 g of glucose per liter, and 15 g of Bacto-agar per liter). For toxicity assays, 1.5 ml of a 3-ml culture grown in LB medium overnight was plated on a DSMZ plate. After drying, filter disks soaked with test solutions were placed on the plate, which was incubated overnight at 30°C and then transferred to room temperature. Plates were evaluated after development of the typical reddish color had occurred.

Pir7b elicitor tests.

Leaves of 14-day-old rice plants were cut into 4-cm-long segments that were wiped with a linsoft tissue to remove surface wax and placed on plates (5 cm diameter; four leaf segments per plate) containing 0.5% phytagar (Gibco BRL, Gaithersburg, MD) and 0.17 mM benzimidazole, with the cut ends stuck into the agar. Test solutions containing 0.05% Tween 20 were applied by placing 8 regularly spaced 5-µl droplets onto the surface of a leaf segment. After the droplets were air dried, the petri dishes were closed and incubated in the greenhouse at 28°C. If not otherwise indicated, RNA or proteins were extracted 24 h after application of test solutions and subjected to gel blot analysis.

Production and purification of syringolin.

P. syringae pv. *syringae* strain B 301D-R was grown as described (Gross 1985; Mo and Gross 1991a, 1991b). For syringolin production, 3 ml of SRM_{AF} medium (1% D-glucose, 0.1% fructose, 100 µM arbutin, 0.4% L-histidine, 0.8 mM $MgSO_4$, 10 µM $FeCl_3$, 0.8 mM potassium phosphate, pH 7) was inoculated with a colony picked from a 2× tryptone-yeast (TY) agar plate and incubated on a rotary shaker (250 rpm; Lab-Shaker, Kühner, Basel, Switzerland) overnight at 25°C. Two hundred milliliters of fresh SRM_{AF} medium was inoculated with 2 ml of the overnight culture and incubated for 5 to 8 days at 25°C without shaking. Two hundred milliliters of conditioned medium was centrifuged at 12,000 × *g* for 20 min and sterile filtrated (0.22 µm pore size). For ultrafiltration, an Amicon cell (model 202) equipped either with a Diaflo YC05 (500 Da cut-off) or a Diaflo YM3 (3,000 Da cut-off) filter (Amicon, Beverly, MA) was used.

Gel filtration chromatography was performed on a LCC-501 Plus FPLC system (Pharmacia, Uppsala, Sweden) equipped with a Superdex 30 16/60 column (Pharmacia). Five milliliters of concentrated medium was loaded and eluted with a running buffer containing 150 mM NaCl and 50 mM sodium phosphate, pH 7 at a flow rate of 1 ml/min. Absorption was monitored at 254 nm and 3-ml fractions were collected. All fractions were assayed for *Pir7b* eliciting activity by droplet application onto leaf segments.

Active fractions were pooled and acidified by addition of trifluoroacetic acid to a final concentration of 0.3%. One-milliliter aliquots were subjected to reverse-phase-HPLC with a Nucleosil 100 5 C_{18} 250/4 column (Macherey-Nagel, Düren, Germany) on an LKB system equipped with 2150 pumps, a 2158 UV monitor, and a 2152 controller. Elution was done isocratically with 20% acetonitrile and 0.06% trifluoroacetic acid in water at a flow rate of 1 ml/min. Absorption was monitored at 206 nm. Peaks were collected and the solvent was evaporated in a Speed Vac concentrator (Savant, Farmington, NY). For tests of *Pir7b* eliciting activity, samples were dissolved in distilled water containing 0.05% Tween 20 and applied to the surface of leaf segments. Concentrations were calculated by weighing the dry substance and dissolving it in an appropriate volume.

Extraction and gel blot analysis of RNA and proteins.

RNA was extracted from at least four leaf segments by the "hot phenol" method as described (Dudler and Hertig 1992), separated on formaldehyde gels, blotted onto Hybond-N membranes (Amersham International, Amersham, U.K.), and hybridized to radiolabeled probes according to standard procedures (Maniatis et al. 1982). The probes consisted of the inserts of the *Rir1*, *Pir2*, *Pir3*, and *Pir7b* cDNA clones (Reimmann and Dudler 1993; Reimmann et al. 1995, 1992) that were cut out with *Eco*RI and *Xho*I.

Proteins were extracted by grinding leaf tissue in 10 vol of extraction buffer (5 mM 2-mercaptoethanol, 50 mM sodium phosphate, pH 7) with mortar and pestle at 4°C. The homogenates were centrifuged at 10,000 × *g* for 10 min. Protein concentrations in the supernatant were determined with the Bio-Rad protein assay kit (Bio-Rad, Richmond, CA). Proteins were separated on 12% sodium dodecyl sulfate polyacrylamide gels and blotted onto nitrocellulose membranes by standard methods (Maniatis et al. 1982). Pir7b protein was detected with a 1:1,000 dilution of a specific antiserum obtained by immunizing rabbits with a recombinant Pir7b fusion protein (described elsewhere) in combination with a 1:2,000 dilution of a secondary alkaline phosphatase-coupled anti-rabbit IgG antibody (Boehringer, Mannheim, Germany). Blots were finally developed either in a solution containing 3 mM 4-nitroblue tetrazolium chloride (NBT), 0.38 mM 5-bromo-4-chloro-3-indolylphosphate (BCIP), 100 mM NaCl, and 100 mM Tris-HCl pH 9.5, or with a chemioluminescent immunodetection system (ECL, Amersham International) according to the manufacturer's description.

Methods of spectroscopic structure analysis.

Fast atom bombardment mass spectra (FAB-MS) were measured with a MAT 90 mass spectrometer (Finnigan, Bremen, Germany) and electro spray ionization mass spectra (ESI-MS) with a Quattro II instrument (Micromass, Manchester, UK). The NMR spectra were measured with a Unity 500 NMR spectrometer (Varian, Palo Alto, CA) in DMSO-d6 at 27°C, and in H_2O at 25°C and pH 7.5.

Spectroscopic data of syringolin.

High resolution FAB-MS: $(M-H)^-$ 492.2814 (C24H38N5O6) requires 492.2822. ESI-MS: 494 $(M+H)^+$, 492 $(M-H)^-$.

The NMR data (DMSO-d_6) are sorted according to the amino acid moieties and presented as follows: proton chemical shift, carbon chemical shift, assignment: 5-methyl-4-amino-2-hexenoic acid moiety: –, 166.3, C(1); 6.09, 121.4, C(2); 6.67, 143.2, C(3); 4.07, 55.4, C(4); 1.72, 31.1, C(5); 0.95 19.5, C(6); 0.90 19.0, C(7); 8.00, NH. 3,4-dehydrolysine moiety: –, 168.9, C(1); 4.85, 53.5, C(2); 5.41, 125.9, C(3); 5.59, 133.0, C(4); 2.27 and 1.96, 34.9, C(5); 3.19 and 3.13, 42.4 C(6); 7.42, C(6)NH; 7.96 C(2)NH. valine moiety chain: –, 171.5, C(1); 4.05, 57.4, C(2); 1.91, 30.9, C(3); 0.83 19.0, C(4); 0.77, 17.4, C(5); 6.25, NH. valine moiety end: 12.38, 174.4, C(1); 3.96, 57.6, C(2); 1.98, 30.1, C(3); 0.85 19.1, C(4); 0.82, 17.5, C(5); 6.28, NH; –, 157.6, urea CO.

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