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Specific Binding of the Syringolide Elicitors to a Soluble Protein Fraction from Soybean Leaves

Cheng Ji,[a,1] Yasushi Okinaka,[b,1] Yoji Takeuchi,[b] Tetsu Tsurushima,[c] Richard I. Buzzell,[d] James J. Sims,[a] Sharon L. Midland,[a] David Slaymaker,[a] Masaaki Yoshikawa,[b,2] Naoto Yamaoka,[b] and Noel T. Keen[a,3]

[a] Department of Plant Pathology, University of California, Riverside, California 92521
[b] Division of Biological Sciences, Graduate School of Science, Hokkaido University, Sapporo 060, Japan
[c] Faculty of Business, Hannan University, Matsubara City, Osaka 580, Japan
[d] Experiment Station, Canada Department of Agriculture, Harrow, Ontario, Canada

Syringolides are glycolipid elicitors produced by Gram-negative bacteria expressing *Pseudomonas syringae* avirulence gene D. The syringolides mediate gene-for-gene complementarity, inducing the hypersensitive response only in soybean plants carrying the *Rpg4* disease resistance gene. A site(s) for ^{125}I-syringolide 1 was detected in the soluble protein fraction from soybean leaves, but no evidence for ligand-specific binding to the microsomal fraction was obtained. The K_d value for syringolide 1 binding with the soluble fraction was 8.7 nM, and binding was greatly reduced by prior protease treatment or heating. A native gel assay was also used to demonstrate ligand-specific binding of labeled syringolide 1 with a soluble protein(s). Competition studies with ^{125}I-syringolide 1 and several structural derivatives demonstrated a direct correlation between binding affinity to the soluble fraction and elicitor activity. However, differential competition binding studies disclosed no differences in syringolide binding to soluble fractions from *Rpg4/Rpg4* or *rpg4/rpg4* soybean leaves. Thus, the observed binding site fulfills several criteria expected of an intracellular receptor for the syringolides, but it is most likely not encoded by the *Rpg4* gene. Instead, the *Rpg4* gene product may function subsequent to elicitor binding, possibly in intracellular signal transduction.

INTRODUCTION

Gene-for-gene complementarity denotes the occurrence of active disease resistance responses only when a host plant carries a dominant resistance gene and the pathogen harbors a dominant avirulence gene (Flor, 1971). The strict dependence of resistance responses on these genes and their economic importance have stimulated the exploration of underlying molecular mechanisms. The elicitor–receptor model has been proposed to explain gene-for-gene systems (Albersheim and Anderson-Prouty, 1975; Keen and Bruegger, 1977; Gabriel and Rolfe, 1990). In this model, an avirulence gene directs the production of a signal molecule (elicitor), which is recognized by a plant receptor encoded by the complementary disease resistance gene to induce active defense reactions, collectively called the hypersensitive response (Keen, 1990). Several bacterial, viral, and fungal avirulence genes have been identified, and some of them have been shown to direct the production of specific elicitors (reviewed in Boller, 1995; Leach and White, 1996). Recently, disease resistance genes have also been cloned from several plant species (reviewed in Staskawicz et al., 1995; Bent, 1996). Although nucleotide sequence data have provided only limited insight into their function, some molecular genetic evidence strongly suggests that resistance gene proteins may directly interact with pathogen-specific elicitors (Mindrinos et al., 1994; Grant et al., 1995; Scofield et al., 1996; Tang et al., 1996).

The *Rpg4* disease resistance gene in soybean confers resistance to *Pseudomonas syringae* pv *glycinea* expressing the complementary avirulence gene *avrD* (Kobayashi et al., 1990). *Rpg4* is a single, dominant gene (Keen and Buzzell, 1991), but it has not been cloned and characterized. Bacteria expressing *avrD* produce low molecular weight glycolipid elicitors, called syringolides (Figure 1A), that convey gene-for-gene specificity because they are only functional in *Rpg4* plants (Keen et al., 1990; Keen and Buzzell, 1991; Midland et al., 1993). These structures have recently been confirmed, and their absolute stereochemistry has been established by several independent syntheses (Henschke and Rickards, 1996). *avrD* occurs as two distinct classes of functional alleles in various *P. syringae* pathovars (Yucel et al., 1994a). Class I alleles direct the production of two structurally related molecules, syringolides 1 and 2 (Yucel et al., 1994b), whereas class II alleles lead to the production of only syringolides 1 and 3 (Figure 1). The syringolides all elicit hypersensitive cell

[1] The first two authors contributed equally to this work.
[2] Deceased on June 4, 1995.
[3] To whom correspondence should be addressed. E-mail keen@ucrac1.ucr.edu; fax 909-787-4294.

death and defense response gene activation in *Rpg4* soybean plants (Keen et al., 1990; Stayton et al., 1994; Atkinson et al., 1996). Genetic analysis revealed that induction of hypersensitive necrosis in response to the syringolides and disease resistance to bacteria expressing *avrD* always cosegregated at the *Rpg4* locus (Keen and Buzzell, 1991). Therefore, the evidence strongly indicates that the syringolides mediate *avrD–Rpg4* complementarity.

The elicitor–receptor model predicts that *Rpg4* but not *rpg4* soybean plants possess a syringolide-specific receptor that initiates resistance responses after infection by pathogenic bacteria expressing *avrD*. To test this hypothesis, we synthesized a radiolabeled syringolide 1 derivative (Tsurushima et al., 1996) that retained high elicitor activity and examined its binding to cell-free fractions from *Rpg4* and *rpg4* soybean leaves. We describe a specific binding site(s) for the syringolides in the soluble protein fraction from soybean leaves; the binding site fulfills several criteria expected of an intracellular receptor but occurs in both *Rpg4* and *rpg4* cultivars.

RESULTS

Binding of ^{125}I-Syringolide 1 to Soybean Cell Fractions

A synthetic derivative of syringolide 1 (^{125}I-syringolide 1; Figure 1B) retained >95% activity in the soybean cotyledon elicitor assay (Tsurushima et al., 1996). In experiments with soybean leaf microsomal fractions, no significant specific binding of ^{125}I-syringolide 1 was detected (Table 1). This somewhat surprising finding encouraged us to examine the soluble (100,000*g*) fraction of soybean leaf extracts. In preliminary experiments, inconsistent results were obtained in attempts to study binding of ^{125}I-syringolide 1 to soluble fractions by using a filter assay. Therefore, we developed a dextran-coated charcoal (DCC) binding assay, as described in Methods. When we used this assay, specific binding of ^{125}I-syringolide 1 that was displaceable by excess unlabeled ligand occurred with the soluble fraction from cultivar Harosoy leaves (Table 1). Prior treatment of the soluble fraction with proteases or heat reduced the specific binding by ≥85%. The optimum for specific binding was pH 7, and binding was greatly inhibited at pH values <6 or >8 (Figure 2). Binding was similar when assays were conducted at 25 or 4°C. These data suggest that syringolide binding involved an intracellular protein(s) and occurred at pH values likely to occur in the cell cytoplasm.

A dose–response study showed that binding of ^{125}I-syringolide 1 to the soluble leaf fraction was saturable (Figure 3A), whereas nonspecific binding was proportional to the ligand concentration. Furthermore, Scatchard plots indicated a calculated K_d value of ~8.7 nM for syringolide binding and a maximum binding site concentration of 2.9 pmol/mg protein (Figure 3B). The binding of ^{125}I-syringolide 1 was reversible—>50% dissociation of bound ligand was observed 9 hr after the addition of a 1000-fold excess of unlabeled syringolide 1 (Figure 4). These results demonstrated the occurrence of a ligand-specific binding site for syringolide 1 in the soluble fraction and suggested a single affinity class of binding site.

^{125}I-syringolide 1 binding to a protein(s) in the soluble fraction was also revealed by a native gel assay. When we used this method, the labeled ligand was incubated with the soluble fraction, the mixture was electrophoresced on a native polyacrylamide gel, and radioactivity was then detected by autoradiography. As shown in Figure 5, two labeled spots were observed with the crude soluble fraction (lane 1), but they were not detected either when 1000-fold unlabeled ligand was present or when the soluble fraction was treated with protease or heat (Figure 5, lanes 2 to 4). Therefore, this

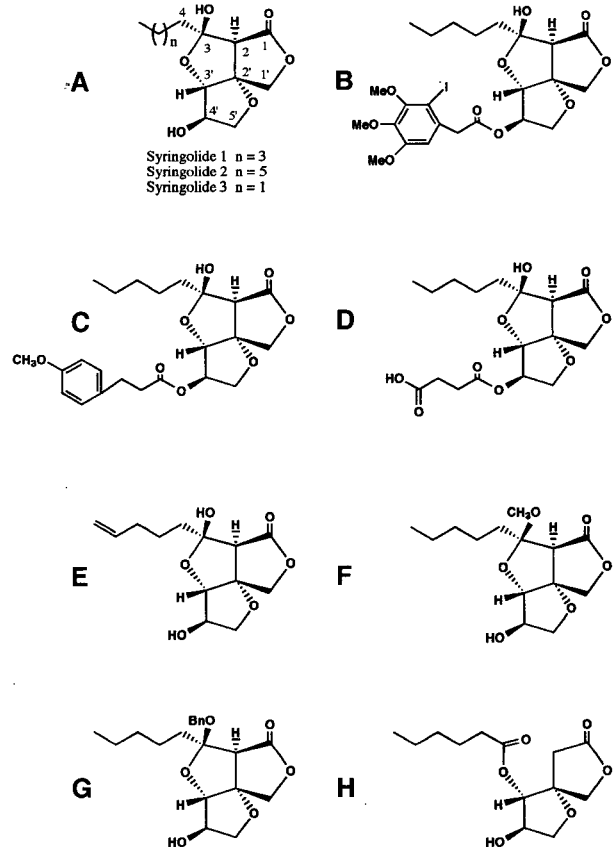

Figure 1. Structures of the Syringolides and Derivatives Used in This Study.

(A) Syringolides 1, 2, and 3.
(B) 4′-(2-Iodo-3,4,5-trimethoxyphenylacetyl) syringolide 1.
(C) 4′-(4-Methoxyphenyl)propionyl syringolide 1.
(D) 4′-Succinyl syringolide 1.
(E) 7,8-Dehydrosyringolide 1.
(F) 3-*O*-methyl syringolide 1.
(G) 3-*O*-benzyl syringolide 1.
(H) Secosyrin 1.

Table 1. *Specific Binding of* ^{125}I-Syringolide 1 *to Soybean* Cell Fractions

Fraction	^{125}I-Syringolide 1 Bound (cpm)[a]		
	Total	Nonspecific	Specific
Microsomal membranes	819 ± 183	807 ± 169	12
Soluble fraction	6388 ± 1186	1597 ± 344	4791
Soluble fraction treated with			
Pronase[b]	2305 ± 412	1607 ± 355	698
Proteinase K[b]	2063 ± 397	1513 ± 197	550
Heat[c]	2211 ± 381	1743 ± 209	477

[a] Soluble fractions (200 μg of protein in 1 mL) or microsomal fractions (100 μg of protein in 1 mL) were incubated with ^{125}I-syringolide 1 (final concentration of 2 nM) for 2 hr. Nonspecific binding was determined in the presence of 1000-fold unlabeled syringolide. Data are means ± SE for three replicates.

[b] The soluble fraction was incubated with the listed enzymes (100 μg/mL) for 15 min at 25°C before the binding assays.

[c] The soluble fraction was boiled for 10 min before it was used in the binding assay.

assay confirmed results obtained with the DCC assay in identifying a ligand-specific binding site(s) in the soluble fraction that was eliminated by treatments expected to degrade or inactivate proteins. Furthermore, nonspecific binding was low in the native gel assay, as shown by the absence of detectable bound radioactivity in preparations incubated with an excess of unlabeled ligand.

Syringolide Binding to the Soluble Fraction from *Rpg4* and *rpg4* Soybean Leaves

Preliminary binding assays with soluble fractions prepared from leaves of several *Rpg4* and *rpg4* soybean cultivars surprisingly showed that ligand-specific binding of syringolide 1 occurred uniformly with cultivars Harosoy, Flambeau, Merit, and Acme (data not shown). However, these cultivars were not isogenic, leaving the possibility that *Rpg4* cultivars might contain an additional binding site specified by *Rpg4*. To test this possibility, S_9 recombinant inbred (RI) lines developed from Merit × Flambeau (*rpg4/rpg4* × *Rpg4/Rpg4*) F_2 plants were used to eliminate cultivar-specific factors. This approach was taken because near-isogenic lines have not yet been developed for the *Rpg4* locus. Using the precedent of bulk segregant analysis (Michelmore et al., 1991), we selected RI lines with either the *Rpg4/Rpg4* or *rpg4/rpg4* genotypes (RR or rr groups) and used them to prepare bulked soluble fractions, as illustrated in Figure 6. The genetic backgrounds of the two groups were expected to be mutually analogous, that is, similar to near-isogenic lines. Soluble fractions extracted from pooled RR (10 RI lines) and rr (nine RI lines) plants were then used for binding assays. No significant differences in ^{125}I-syringolide 1 binding were observed between the two groups either at 4 or 25°C, and the same K_d values were observed at both temperatures (data not shown). These results strongly argue that the same syringolide binding site(s) occurs in *Rpg4* and *rpg4* cultivars.

Competitive Binding Activity and Elicitor Activity of Syringolide Derivatives

Several syringolide derivatives (Figure 1) of varying elicitor activity were previously synthesized (Tsurushima et al., 1996) for use in competition binding assays. Syringolides 1 and 2, as well as derivatives retaining free 3-hydroxyl groups, strongly inhibited the binding of ^{125}I-syringolide 1 (Table 2). Syringolide 2, containing two more methylene units than syringolide 1, had almost the same competitive activity. Derivatives constructed on the 4′-hydroxyl group of syringolide 1 (Figures 1C and 1D) were also good competitors, with half-maximal inhibition (IC_{50}) values ranging from 121 nM (4′-[3,4,5-trimethoxyphenylacetyl] syringolide 1) to 197 nM (4′-succinyl syringolide 1). Somewhat surprisingly, 7,8-dehydrosyringolide 1 (Figure 1E) gave reduced competitor activity (IC_{50} = 378 nM) and less elicitor activity than the earlier compounds, despite greater structural similarity to syringolide 1 (Table 2). Compounds lacking free 3-hydroxyl groups (3-*O*-methyl syringolide 1 and 3-*O*-benzyl syringolide 1; Figures 1F and 1G, respectively) exhibited greatly reduced competitive activities (IC_{50} > 50,000 nM). Even less competitive activity (IC_{50} > 68,000 nM) was observed with

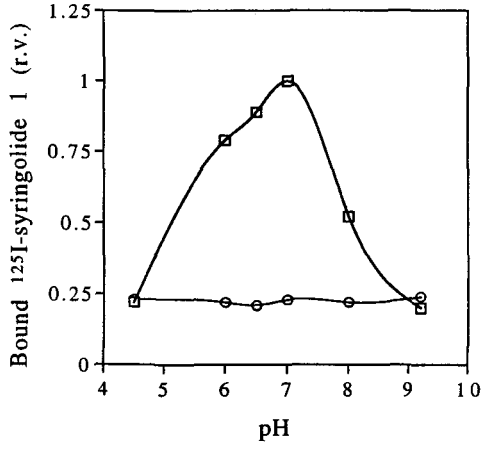

Figure 2. Effect of pH on ^{125}I-Syringolide 1 Binding to the Soluble Fraction from Cultivar Harosoy Soybean Leaves.

Binding of ^{125}I-syringolide 1 was measured under equilibrium conditions at the indicated pH values. The pH of reaction mixtures containing 50 mM sodium phosphate was adjusted with HCl or NaOH to the given values. Data are expressed as relative values (r.v.), with 1.0 = 4980 cpm. (□), specific binding; (○), nonspecific binding.

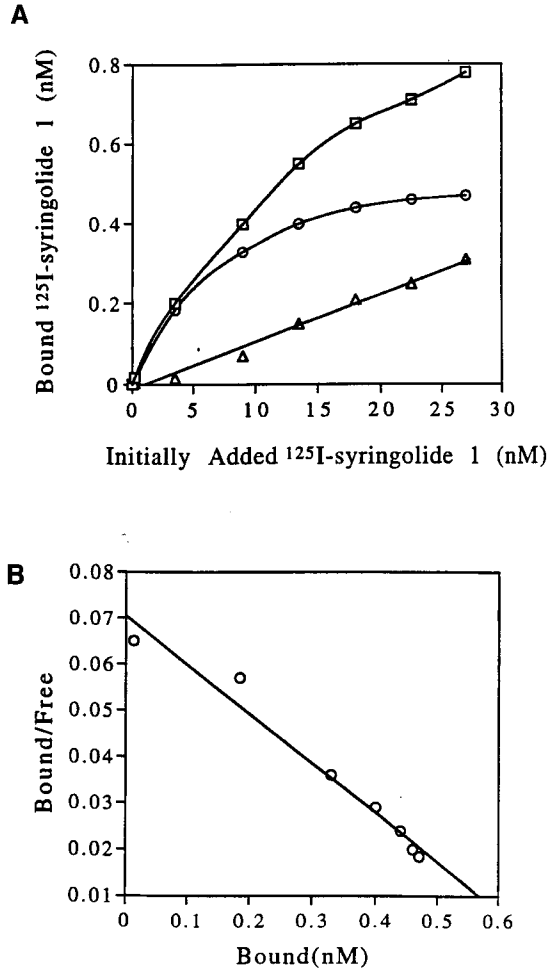

Figure 3. Saturability of the Binding of ^{125}I-Syringolide 1 to the Soybean Soluble Fraction.

(A) Increasing amounts (0.1 to 27 nM) of ^{125}I-syringolide 1 were added to the soluble fraction and incubated, as described in Methods. Nonspecific binding of ^{125}I-syringolide 1 (△) was determined in the presence of 1500-fold unlabeled syringolide 1. Specific binding (○) was determined by subtraction of nonspecific binding from total binding (□).

(B) Scatchard plot of specific binding data in **(A)**.

secosyrin 1 (Figure 1H), a compound with a similar lipid glycoside structure but without detectable elicitor activity (Table 2).

Syringolides and several chemical derivatives were compared for ability to elicit hypersensitive cell death in cultured soybean cells (Table 2). The compounds yielded EC_{50} values (concentrations that gave half-maximal cell death in the elicitor activity assay) comparable to those previously determined by using a soybean cotyledon assay (Tsurushima et al., 1996). Compounds that were efficient competitors of ^{125}I-syringolide 1 binding also gave high elicitor activity on *Rpg4* cells (Table 2). On the other hand, all compounds with greatly reduced competitive binding activity (e.g., 3-*O*-methyl syringolide 1 and secosyrin 1) gave very low or undetectable elicitor activity on *Rpg4* cells (Table 2). Therefore, these results demonstrate a good correlation between elicitor activity with the *Rpg4* cell line and competitive binding activity for all of the compounds tested. The results show that a free 3-hydroxyl group was required for both efficient syringolide binding to the soluble protein fraction and elicitor activity.

DISCUSSION

We have shown that the soluble fraction of soybean leaf extracts contains a ligand-specific, saturable binding site(s) for the *avrD*-specific syringolide elicitors. Ligand saturation analysis revealed a single class of binding sites for ^{125}I-syringolide 1 with a K_d of ~8.7 nM (Figure 3). This value is somewhat higher than those previously observed for several nonspecific elicitors (reviewed in Boller, 1995; Hahn, 1996) or for the *avr9* peptide-specific elicitor (Kooman-Gersmann et al., 1996). However, it is noteworthy that the described binding sites for all of these latter elicitors are associated with the plant cell plasma membrane, whereas the observed syringolide binding site(s) is the first to be biochemically identified in the soluble fraction. This suggests that an intracellular receptor initiates or is required for signaling events leading to defense responses. Such a mode of action is reminiscent of the host-selective toxin victorin, which was shown to have a binding site(s) in the mitochondrial matrix of oats (Navarre and Wolpert, 1995). In addition, recent evidence

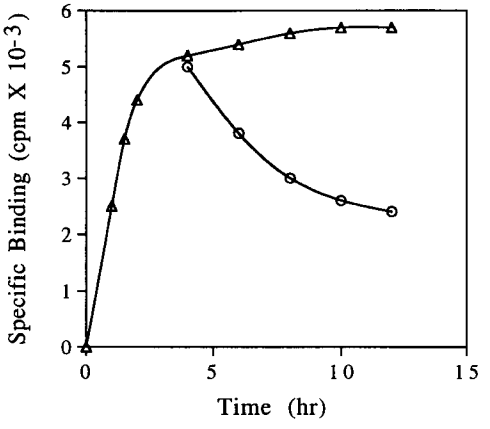

Figure 4. Kinetics of ^{125}I-Syringolide 1 Binding and Dissociation to the Soybean Soluble Fraction.

Binding to the soluble fraction was initiated by adding ^{125}I-syringolide 1 to a final concentration of 2.0 nM. (○), addition of 1000-fold unlabeled syringolide 1 at 3.5 hr; (△), no addition of unlabeled syringolide 1.

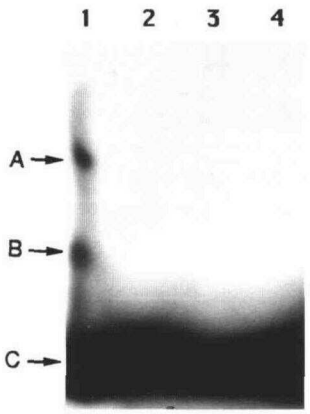

Figure 5. Native Gel Assay of Syringolide Binding to Components of the Soluble Fraction.

Crude soluble fractions were incubated with ^{125}I-syringolide 1 for 1.5 hr and then subjected to electrophoresis on a 10% native gel, as described in Methods (20 μg of total protein was loaded per lane). The gel was dried and exposed to x-ray film. Lane 1 contains the soluble fraction; lane 2, the soluble fraction plus unlabeled syringolide 1 (1000-fold); lane 3, the soluble fraction treated with proteinase K; and lane 4, the soluble fraction boiled for 10 min. A and B denote the putative syringolide binding protein(s); C indicates free ^{125}I-syringolide 1.

suggests that recognition of certain bacterial avirulence proteins may occur inside host plant cells (Yang and Gabriel, 1995; Gopalan et al., 1996; Scofield et al., 1996; Tang et al., 1996; Van den Ackerveken et al., 1996). One curiosity that remains to be explained is how an intracellular receptor mediates syringolide responses at the plasma membrane, such as generation of active oxygen species and the opening of specific ion channels (Atkinson et al., 1996).

The apparent intracellular location of the syringolide binding site may at least partially explain the considerable difference between binding efficiency (K_d of 8.7 nM) and the relatively high syringolide 1 concentrations required for elicitor activity in the cell death assay (EC_{50} of 30 μM). One possible explanation may lie in the fact that different cells were used for binding and elicitor assays. Whereas binding assays utilized soluble fractions from soybean leaves and the syringolides are active in leaves, we have not devised a highly quantitative elicitor assay using leaves. However, Tsurushima et al. (1996) showed that the syringolides and most of the derivatives used in this study gave similar activity in the soybean cotyledon elicitor assay, as was observed here with the suspension cell assay. It is possible that cell death may not occur unless intracellular signaling induced by the syringolides reaches a critical threshold level, as proposed previously by Gross et al. (1993). If this is the case, cell death is a relatively insensitive indicator of elicitor activity and only caused by high signal flux. However, in studies with cultured soybean cells, extracellular alkalization and calcium influx (Atkinson et al., 1996) were elicited in *Rpg4* soybean cells by similar concentrations of syringolide 1, as determined by the cell death assay used in this study. Finally, it should be remembered that the syringolides must pass through at least the cell wall and plasma membrane before reaching the intracellular binding site(s). Thus, the efficiency of uptake by plant cells as well as possible degradation and nonspecific sequestering may contribute to the requirement of relatively high external syringolide concentrations.

The binding of ^{125}I-syringolide 1 in the DCC assay with soluble leaf fractions was saturable and reversible by subsequent addition of excess unlabeled ligand (Figures 3 and 4). These observations suggest a classical binding site that facilitates noncovalent ligand recognition. In the DCC assay, nonspecific binding was relatively high (Figure 3). However, the DCC assay has also been observed to produce significant nonspecific binding with crude preparations in animal systems

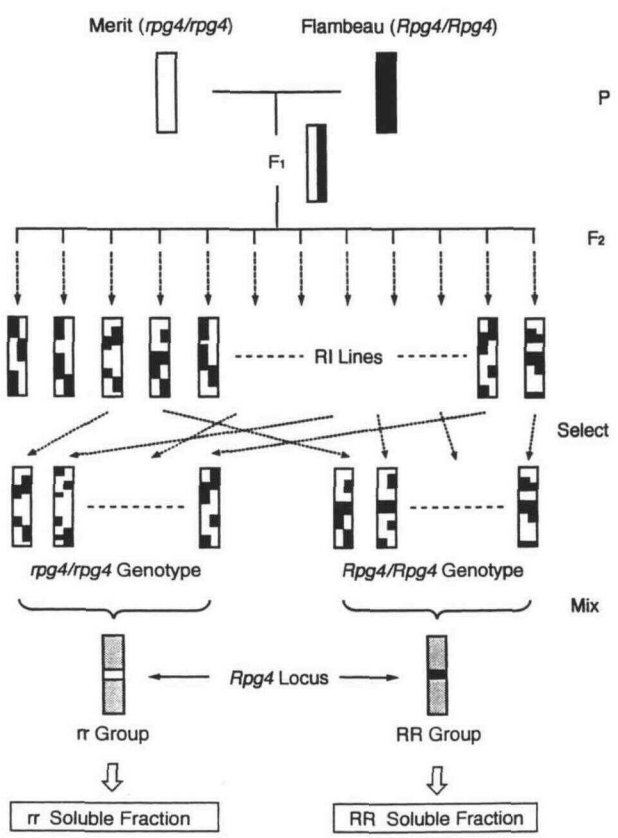

Figure 6. Genetic Construction of RI Lines Used in the Differential Competition Assays.

Each bar indicates either parent (P), F_1, or RI lines (plant populations) derived from F_2 plants. The pattern represents genetic construction of RR and rr groups with backgrounds (stippled regions) that are nearly identical on average.

Table 2. Competitive Binding Ability of Syringolides and Several Derivatives Compared with Their Elicitor Activity

Substance[a]	Binding Activity[b] (IC_{50}, nM)	Elicitor Activity[c] (EC_{50}, μM)
Syringolide 1	96	30
Syringolide 2	105	19
4′-(4-Methoxyphenyl)propionyl syringolide 1	182	89
4′-(3,4,5,-Trimethoxyphenylacetyl) syringolide 1	121	94
4′-Succinyl syringolide 1	197	NA[d]
7,8-Dehydrosyringolide 1	378	249
3-O-methyl syringolide 1	52,442	8,790
3-O-benzyl syringolide 1	56,967	9,120
Secosyrin 1	68,256	>10,000

[a] Structures are shown in Figure 1, and syntheses were described previously (Tsurushima et al., 1996; Zeng et al., 1997).
[b] IC_{50} against ^{125}I-syringolide 1 (20 nM) binding to the soluble fraction from cultivar Harosoy leaves.
[c] Concentrations (EC_{50}) required to give half-maximal induction of hypersensitive cell death with cultivar Harosoy suspension cells.
[d] NA, not assayed.

(Odell, 1980; Strange, 1992). The amphipathic nature of the syringolides may contribute to nonspecific binding, although crude microsomal preparations did not show extremely high nonspecific binding (Table 1). Nonspecific binding might be due to binding of iodine-125 to proteins as well as any residual radioligand not removed by charcoal adsorption. Under our DCC assay conditions, the residual radioactivity in no-protein buffer controls was 2 to 8% of total binding compared with experimental tubes containing plant proteins (data not shown). The use of excess unlabeled ligand in the native gel binding assay (Figure 5) gave complete reversal of ^{125}I-syringolide 1 binding to the crude soluble fraction. Therefore, this assay appears to provide low nonspecific binding. Significantly, the native gel binding assay disclosed two radioactive bands, both presumably proteins, based on protease sensitivity. It is currently impossible to determine whether these bands represent the presence of two different proteins in the crude soluble fraction with similar binding affinities or whether a single binding protein exists in different complexes with other macromolecules. This last issue is worthy of further study because very little is known about receptor complexes in plants or the presentation of elicitors to plant cells, particularly their intracellular presentation.

The observed syringolide binding site(s) showed high ligand selectivity, as demonstrated by using several syringolide derivatives and structural analogs (Figure 1 and Table 2). Furthermore, a strong correlation occurred between competitive binding activity and elicitor activity for all the substances used. This suggests that ligand binding is required for the induction of *Rpg4*-dependent defense responses; in other words, the identified binding site(s) is a prime candidate for the syringolide receptor. Particularly noteworthy in these studies was the greatly reduced elicitor activity and competitor activity of all derivatives built off of the 3-hydroxyl group of syringolide 1. This result clearly suggests that the 3-hydroxyl group is crucial for receptor recognition. Because chemically this is a hemiketal hydroxyl group, the possibility of ring opening for generation of an active binding species cannot be formally excluded. Derivatives of the 3-hydroxyl effectively lock the hemiketal ring closed. Opening the ring in the native syringolides could uncover the 3-ketone and 3′-hydroxyl functionalities for interaction with a binding site.

Surprisingly, no significant difference was observed in syringolide binding to the soluble fractions from *Rpg4* or *rpg4* soybean lines by using several named cultivars and recombinant inbred lines (Figure 6). Kooman-Gersmann (1996) also could not show differences in binding of the labeled AVR9 peptide elicitor to plasma membranes from *Cf-9* or *cf-9* tomato plants. These results argue against the prediction by the elicitor–receptor model that disease resistance gene products necessarily act directly as receptors. However, as noted earlier, yeast two-hybrid data have suggested that AvrPto may interact directly with the complementary resistance gene protein Pto (Scofield et al., 1996; Tang et al., 1996). Accordingly, the probability emerges that plants do harbor receptors for specific elicitors but that they may or may not be the protein products of disease resistance genes. In our case, instead of facilitating initial syringolide perception, the *Rpg4* gene product may be involved in a subsequent signal transduction pathway leading to the induction of defense responses.

METHODS

Chemicals

Syringolides 1 and 2, secosyrin 1, and 3-methoxysyringolide 1 were purified or synthesized as previously described (Midland et al., 1993, 1995). 7,8-Dehydrosyringolide 1 was synthesized (Zeng et al., 1997). The synthesis of ^{125}I-syringolide 1 was described previously (Tsurushima et al., 1996), and the product was usually used within 20 days. The initial specific radioactivity produced with a 1:10 molar ratio of ^{125}I (specific activity of 2183 Ci/mmol) to 4′-(3,4,5-trimethoxyphenylacetyl) syringolide 1 was typically ~190 Ci/mmol. Structures of all synthesized chemicals were confirmed with nuclear magnetic resonance and mass spectrometry spectra. Evans blue (Waco Pure Chemicals, Waco, TX), proteinase K, pepstatin A, leupeptin, Norit-A charcoal (Sigma), pronase (Calbiochem), and dextran T70 (Pharmacia) were purchased.

Plant Material

Soybean (*Glycine max*) plants used in this study were cultivar Harosoy (*Rpg4/Rpg4*), cultivar Acme (*rpg4/rpg4*), or recombinant in-

bred lines (RI lines) from a cross of cultivar Merit × cultivar Flambeau (*rpg4/rpg4* × *Rpg4/Rpg4*) (Keen and Buzzell, 1991). Single F_2 plants were selfed to the F_9 generation for production of the RI lines. Soybean seedlings were grown in soil overlaid with vermiculite in a growth chamber at 22°C with a 14-hr photoperiod.

Preparation of Soybean Microsomal Membranes

Primary and trifoliate leaves from soybean plants (~140 g fresh weight) that were ~2 weeks old were homogenized with 800 mL of extraction buffer in a blender at 0°C. The extraction buffer was composed of 24 mM Tris-HCl, pH 7.4, 10 mM $MgCl_2$, 0.3 M sucrose, 2 mM DTT, 1 mM phenylmethylsulfonyl fluoride, 1 μg/mL pepstatin A, and 1 μg/mL leupeptin (Yoshikawa et al., 1983). The slurry was filtered through a nylon screen (pore size of 4.5 μm) and centrifuged at 9000*g* for 10 min at 4°C. Membranes were then sedimented at 100,000*g* for l hr at 1°C. The protein content of the resuspended microsomal membrane fraction was determined with a protein assay kit (Bio-Rad), using bovine γ globulin as a standard.

Preparation of the Soluble Fraction from Soybean Leaves

Soybean leaves (10 g fresh weight) from ~2-week-old plants were harvested and homogenized at 0°C in 40 mL of sodium phosphate buffer (30 mM, pH 7.2, containing 10 mM DTT, 1 mM phenylmethylsulfonyl fluoride, 2 μg/mL pepstatin A, and 2 μg/mL leupeptin) in a blender. The homogenate was filtered through four layers of cheesecloth and centrifuged at 3000*g* for 30 min at 4°C. The supernatant was then centrifuged at 100,000*g* for 1.5 hr at 4°C. The resulting supernatant was mixed with an equal volume of a dextran-coated charcoal (DCC) suspension. This was prepared by mixing equal volumes of an aqueous 20% suspension of Norit-A charcoal and 1.0% dextran T70. The mixture of DCC and soluble fraction was incubated at 0°C for 3 hr to adsorb small molecules in the soluble fraction. The mixture was then centrifuged at 10,000*g* for 10 min. The resulting supernatant was passed through a 0.2-μm nitrocellulose filter to remove any remaining DCC. Protein content was determined as described above, and the soluble leaf fraction was stored at −80°C until use.

^{125}I-Syringolide 1 Binding Assays

A DCC adsorption method was used to measure binding of ^{125}I-syringolide 1 to the soluble fraction (Mester et al., 1970; Ho and Ofner, 1986; Strange, 1992). These assays were performed in binding buffer (50 mM sodium phosphate, pH 7.2, 0.3 M KCl, and 2 mM DTT). The soybean microsomal fraction was suspended in binding buffer (20 mM Tris-HCl, pH 7.2, 5 mM $MgCl_2$, 1 μg/mL pepstatin A, and 1 μg/mL leupeptin). For measuring total binding, 950 μL of soluble leaf fraction (200 μg of protein) or microsomal fraction (100 μg of protein) in the respective binding buffers was mixed with ^{125}I-syringolide 1, dissolved in 10 μL of 95% ethanol (2.0 nM final concentration, unless otherwise indicated), and incubated at 4°C for 2 hr. Nonspecific binding was determined in the presence of 1000- or 1500-fold excess unlabeled syringolide 1. Assays were terminated by addition of 100 μL of ice-cold DCC to the reaction mixtures, followed by incubation on ice for 10 min. Unbound ^{125}I-syringolide 1 adsorbed to the DCC and was pelleted by centrifugation at 7000*g* for 5 min at 4°C. Supernatant fluid (0.5 mL) was then transferred to a scintillation vial containing 10 mL of scintillation fluid, and radioactivity was determined in a Beckman DP550 γ counter.

Membrane filtration binding assays were conducted according to methods described by Bruns et al. (1983).

Native Gel Binding Assay

The crude soluble fraction was incubated with ^{125}I-syringolide 1 or competitors, as described above. These reaction mixtures were then applied to wells of a 10% (w/v) native polyacrylamide gel (0.75 mm thick, prepared in a Bio-Rad Mini-gel apparatus). The gel was run for ~2 hr at 4°C under constant current (13 mA) with a Tris-HCl running buffer (5 mM Tris-HCl and 38.4 mM glycine, pH 8.3). The gel was dried with a slab gel dryer and clamped to Kodak OG-1 diagnostic film. After 48 to 72 hr of exposure at −80°C, the film was developed by an x-ray film processor.

Elicitor Assay

The ability of syringolides and their derivatives to elicit hypersensitive death of cultured soybean cells was measured by Evans blue uptake. Suspension cell cultures of soybean cultivar Harosoy were initiated from cotyledons and maintained on a rotary shaker at 25°C and 120 rpm. Cells were grown in B5 medium (Gamborg et al., 1968) with 2 mg/L 2,4-D and routinely subcultured every 12 days. Two hundred and fifty microliters of a 10-day-old suspension culture (0.3 g of cells per mL) was added to a 1.5-mL microcentrifuge tube, including the compound to be assayed, and incubated for 3 hr on a rotary shaker at 25°C and 120 rpm. The cells were then washed three times with 1 mL of B5 medium, resuspended in 300 μL of B5 medium, and incubated for an additional 12 hr. Cells were pelleted and resuspended in 1 mL of 0.1% Evans blue solution. After incubation for 15 min at room temperature, the mixture was passed through a glass fiber filter (GF/C; Whatman). The filter was washed with 5 mL of water, placed in a 1.5-mL Eppendorf tube with 1 mL of 1% SDS, and boiled for 10 min. After centrifugation, the supernatant fluid was collected, and absorbance was measured at 610 nm. Greater absorbance was taken as indicative of higher elicitor activity. Concentration curves were drawn for the various compounds, and Evans blue absorbance values were plotted. Elicitor activity was then expressed as the concentration giving half-maximal cell death (EC_{50}).

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