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A High-Affinity Binding Site for the AVR9 Peptide Elicitor of *Cladosporium fulvum* Is Present on Plasma Membranes of Tomato and Other Solanaceous Plants

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The race-specific *Cladosporium fulvum* peptide elicitor AVR9, which specifically induces a hypersensitive response in tomato genotypes carrying the *Cf-9* resistance gene, was labeled with iodine-125 at the N-terminal tyrosine residue and used in binding studies. ^{125}I-AVR9 showed specific, saturable, and reversible binding to plasma membranes isolated from leaves of tomato cultivar Moneymaker without *Cf* resistance genes (MM-Cf0) or from a near-isogenic genotype with the *Cf-9* resistance gene (MM-Cf9). The dissociation constant was found to be 0.07 nM, and the receptor concentration was 0.8 pmol/mg microsomal protein. Binding was highly influenced by pH and the ionic strength of the binding buffer and by temperature, indicating the involvement of both electrostatic and hydrophobic interactions. Binding kinetics and binding capacity were similar for membranes of the MM-Cf0 and MM-Cf9 genotypes. In all solanaceous plant species tested, an AVR9 binding site was present, whereas in the nonsolanaceous species that were analyzed, such a binding site could not be identified. The ability of membranes isolated from different solanaceous plant species to bind AVR9 seems to correlate with the presence of members of the *Cf-9* gene family, but whether this correlation is functional remains to be determined.

INTRODUCTION

Host specificity in plant–pathogen interactions has been described using the gene-for-gene model (Flor, 1971), in which the products of avirulence genes of a pathogen induce a hypersensitive response (HR) in plants that carry corresponding resistance genes. A typical feature of HR is the rapid death of a few cells that surround the infection site, which is thought to prevent further growth of the pathogen. The interaction between the fungal pathogen *Cladosporium fulvum* (syn *Fulvia fulva*) and tomato is a model system to study the molecular basis of gene-for-gene–based resistance (De Wit, 1995).

The avirulence genes *Avr4* (Joosten et al., 1994) and *Avr9* of *C. fulvum* (Van den Ackerveken et al., 1992) have been cloned. Both avirulence genes occur as single-copy genes in the fungal genome. The *Avr9* gene encodes a 63–amino acid peptide, which, after secretion, is processed by fungal and plant proteases to the mature 28–amino acid AVR9 elicitor peptide (Van den Ackerveken et al., 1993). The AVR9 elicitor is the only factor responsible for the induction of active defense responses in tomato plants that carry the complementary *Cf-9* resistance gene. This has been demonstrated by transferring the *Avr9* gene to a race of *C. fulvum* virulent on Cf-9 tomato genotypes, which resulted in avirulence (Van den Ackerveken et al., 1992). Disruption of the *Avr9* gene in a race avirulent on Cf-9 plants resulted in virulence (Marmeisse et al., 1993). Injection of the AVR9 peptide elicitor into leaves of a near-isogenic line of tomato cultivar Moneymaker, carrying the *Cf-9* resistance gene (MM-Cf9), resulted in a local necrotic response at the site of injection. The intensity of necrosis in the injected leaf area correlates with the concentration of the elicitor (Van den Ackerveken et al., 1992).

Recently, the *Cf-9* resistance gene of tomato, which is complementary to the *Avr9* avirulence gene of *C. fulvum*, was cloned and sequenced (Jones et al., 1994). The *Cf-9* gene belongs to a clustered gene family that maps at chromosome 1. Although members of the *Cf-9* gene family are expressed in leaves of both resistant and susceptible tomato genotypes, only genotypes that are resistant to strains of *C. fulvum* harboring the *Avr9* gene carry a functional *Cf-9* resistance gene (Jones et al., 1994). The amino acid sequence deduced from the *Cf-9* gene predicts an extracytoplasmic, membrane-anchored glycoprotein with 28 extracellular leucine-rich repeats (LRRs).

Several studies have shown that the response of plants to non–race-specific fungal elicitors is mediated through receptor proteins that are localized on the plasma membrane of the

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plant. Recently, receptors have been identified for an oligopeptide elicitor from *Phytophthora sojae* (Nürnberger et al., 1994) and for a glycopeptide elicitor from yeast (Basse et al., 1993). The binding of these elicitor peptides to membrane-localized binding sites is species specific and correlates with the induction of defense-related plant responses. This correlation indicates that these receptors play an important role in the initiation of plant defense. Binding proteins also have been identified for several oligosaccharides derived from fungal cell walls (Cosio et al., 1988) and for the fungal toxins victorin from *Cochliobolus victoriae* (Wolpert et al., 1989) and fusicoccin from *Fusicoccum amygdali* (De Boer et al., 1989).

Victorin and fusicoccin have binding sites in all higher plants tested (Marré, 1979; Loschke et al., 1994). Victorin binding proteins have been isolated from oat cultivars that are either susceptible or resistant to the toxin. Thus, although these binding proteins seem to be important for the activity of victorin, they do not determine the specificity of the plant response (Navarre and Wolpert, 1995).

Thus far, no resistance gene–associated receptors for race-specific peptide elicitors have been identified. Although the *Cf-9* resistance gene of tomato and the corresponding *Avr9* avirulence gene of *C. fulvum* have been cloned, the mechanism of perception of the race-specific AVR9 elicitor by *Cf-9*–resistant tomato plants is still unclear. To reveal whether a receptor for the AVR9 race-specific elicitor is present and to characterize this receptor, we performed binding studies with the AVR9 peptide. The peptide was radiolabeled with iodine-125 at its N-terminal tyrosine residue, and binding to plasma membranes of tomato leaves was studied. In this study, we report the presence of a single class of high-affinity binding sites for the AVR9 elicitor on plasma membranes of tomato and other solanaceous plants. AVR9 binding was found not to be restricted to membranes of plants that have a functional *Cf-9* resistance gene. In the plant species tested to date, AVR9 binding seems to be confined to membranes of plants that contain members of the *Cf-9* gene family.

RESULTS

Radiolabeling of the AVR9 Peptide and Optimization of Binding Conditions

The 28–amino acid AVR9 peptide was labeled by lactoperoxidase treatment, introducing radioactive iodine-125 into metapositions of the phenolic hydroxyl group of the Tyr-1 residue. Iodination did not affect the specific necrosis-inducing activity of the AVR9 peptide, as was shown by injection of non–radioactively iodinated AVR9 into leaves of MM-Cf9 plants (results not shown). Incubation of membrane preparations from MM-Cf9 tomato leaves with ^{125}I-AVR9 resulted in specific binding to microsomal membranes and enriched plasma membranes obtained by two-phase partitioning, as shown in Table 1. Plasma membranes showed ~10-fold higher binding capacity per milligram of protein compared with microsomal membranes, which indicates that the binding site is localized on the plasma membrane.

Table 1. Isolation of Tomato Membranes by Two-Phase Partitioning and Binding of AVR9

Fractions	Yield (μg protein/g fresh weight)[a]	Specific H^+-ATPase Activity (nmol Pi/mg protein/min)[a]	Specific Binding of ^{125}I-AVR9[a,b] (cpm)
Microsomal membranes	1,621 ± 402	13 ± 3	1,879[c]
Plasma membranes	30 ± 7	109 ± 13	16,442 ± 2,782
Membranes reextracted from lower phase	36 ± 12	63 ± 35	7,466 ± 2,898

[a] Data represent the average value of four independent membrane isolations.
[b] Binding of ^{125}I-AVR9 (final concentration is 2×10^{-10} M) to 1 μg of membrane protein.
[c] Data from one isolation.

Plasma membranes that were obtained by reextraction of the lower phase of the two-phase system were less pure and showed less binding of ^{125}I-AVR9 than did the plasma membranes from the first extraction. The amount of bound radioligand increased linearly with the amount of plasma membrane in the range of 0 to 50 μg of membrane protein (results not shown). From Figure 1A, it is clear that binding is strongly temperature dependent, with slow binding at 4°C and fast binding at higher temperatures. Equilibrium conditions were reached after ~60 min, when plasma membranes were incubated with ^{125}I-AVR9 at 37°C (Figure 1A), as opposed to 480 min at 24°C. At 45°C, the initial binding was faster, but the maximum binding capacity was lower than at 37°C. At 60°C, the initial binding was similar to the binding at 37°C, but it strongly decreased ~10 min after the addition of AVR9, suggesting that the binding site is proteinaceous and becomes inactivated at high temperatures. The proteinaceous nature of the binding site could be confirmed by incubation of the membranes with different proteinases (e.g., trypsin, proteinase K, and protease XXIII). They all abolished the binding capacity of the membranes after treatment for 2 hr (results not shown). The different proteinases did not affect the AVR9 peptide, as was confirmed by low pH PAGE, where no change in mobility was observed. Also, proteinase-treated AVR9 showed no change in necrosis-inducing activity after injection into MM-Cf9 leaves (data not shown).

Figure 1B shows that binding is optimal between pH 5.5 and 6.0, resembling the pH of apoplastic fluid of *C. fulvum*–infected tomato leaves (results not shown). Increasing the pH of the binding buffer to 7.0 lowered the binding capacity by >50%, whereas at pH 8.0 no binding was observed. Decreasing the

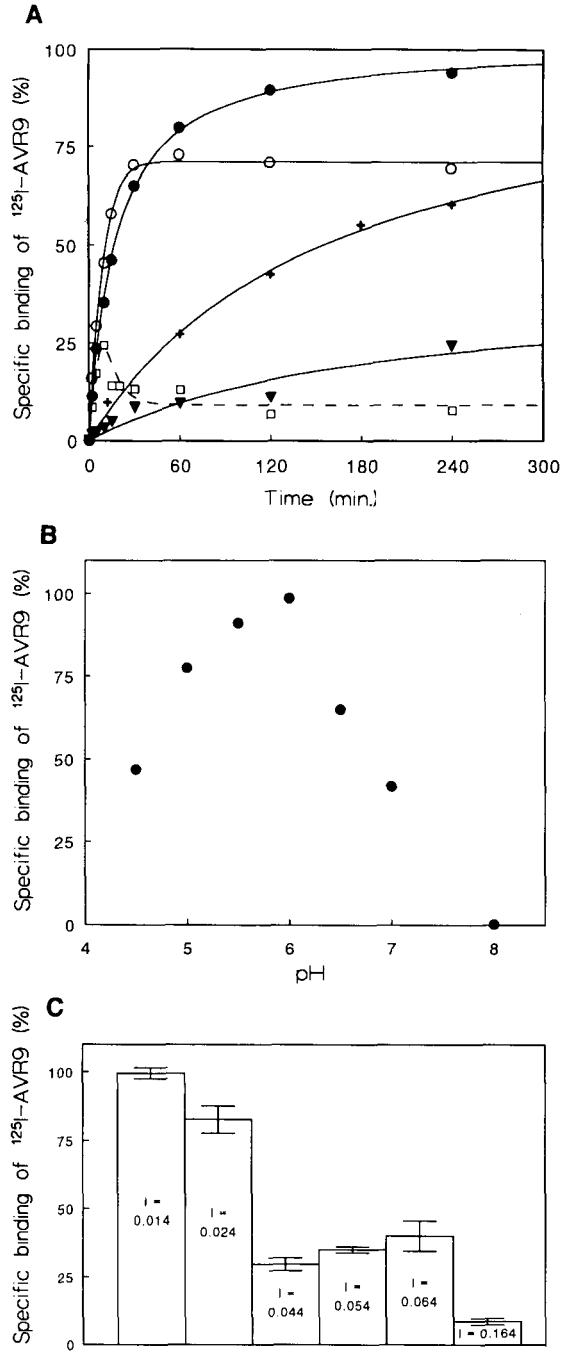

Figure 1. Effects of Temperature, pH , and Ionic Strength on Specific Binding of ^{125}I-AVR9 to 1 μg of Plasma Membrane Protein from Leaves of MM-Cf9.

(A) Time courses of specific binding of ^{125}I-AVR9 (final concentration is 2 × 10^{-10} M) to MM-Cf9 membranes at 4°C (▼), 24°C (+), 37°C (●), 45°C (○), and 60°C (□) (pH 6.0; *I* of 0.014). Specific binding is total binding minus nonspecific binding, as determined by the addition of a 1000-fold excess of unlabeled AVR9. One hundred percent is 4.5 × 10^{-11} M ^{125}I-AVR9 bound.

pH from 5.5 to 4.5 resulted in a 50% reduction of binding. Binding of the AVR9 peptide also was influenced by the ionic strength (*I*) of the assay buffer, as shown in Figure 1C. An increase in *I* from 0.014 to ~0.05 by the addition of NaCl, KCl, KI, $CaCl_2$, $MgCl_2$, or $MgSO_4$ resulted in a significant decrease in specific binding, whereas an increase of *I* to 0.164 by the addition of $CaCl_2$ or $MgCl_2$ practically abolished binding. Increasing the concentration of the phosphate buffer to 50 mM (*I* of 0.07) caused a decrease in the binding capacity of the membranes by 25% (results not shown). The AVR9 peptide remained stable during incubation with membrane fractions for at least 2 hr, as was determined by nondenaturing, low pH PAGE of the reaction mixture, followed by autoradiography (results not shown). Based on the results described, standard equilibrium experiments were performed by incubating membranes with ^{125}I-AVR9 for 3 hr at 37°C in 10 mM phosphate buffer, pH 6.0, with 0.1% fatty acid–free BSA to reduce background binding.

Characterization of the AVR9 Binding Site in Tomato Membranes

Kinetic analyses of ^{125}I-AVR9 binding to MM-Cf9 tomato membranes showed that at 37°C, binding was half-maximal after 15 min, whereas an equilibrium between association and dissociation was reached after 60 min (Figure 2). Nonspecific binding, as determined by adding a 1000-fold excess of unlabeled AVR9 at the start of the binding experiment (*t* of 0 min), was ~10% of the total binding (results not shown). Dissociation of bound ^{125}I-AVR9 was accomplished by the addition of a 1000-fold excess of unlabeled AVR9 when binding was at equilibrium (*t* of 120 min). Complete dissociation of the binding complex was established after overnight incubation (Figure 2). Specific binding was reduced by ~50% after overnight incubation. This reduction may have been caused by instability of the binding site at 37°C. The slow dissociation rate constant (K_{off} of 5 × 10^{-5}/sec) indicates that the binding site has a high affinity for AVR9.

(B) Effect of pH on the specific binding of ^{125}I-AVR9 (2 × 10^{-10} M) to MM-Cf9 membranes (at 37°C for 2 hr; *I* of 0.014). One hundred percent is 3.5 × 10^{-11} M ^{125}I-AVR9 bound.

(C) Effect of the ionic strength (*I*) of the binding buffer on specific binding of ^{125}I-AVR9 (2 × 10^{-10} M) to MM-Cf9 membranes (at 37°C for 2 hr; pH 6.0). ($I = \frac{1}{2} \times \Sigma_i[m_i \times z_i]$, where m_i and z_i are the molality and the charge of the *i*th ion in the solution, respectively.) Error bars give the standard error of the mean. An *I* of 0.014 represents binding in 10 mM phosphate buffer, no salts added; *I* of 0.024, 10 mM NaCl, KCl, or KI added; *I* of 0.044, 10 mM $CaCl_2$ or $MgCl_2$ added; *I* of 0.054, 10 mM $MgSO_4$ added; *I* of 0.064, 50 mM KCl or KI added; *I* of 0.164, 50 mM $CaCl_2$ or $MgCl_2$ added. One hundred percent is 3.5 × 10^{-11} M ^{125}I-AVR9 bound.

All data represent the average of two measurements. Duplicate experiments gave comparable results.

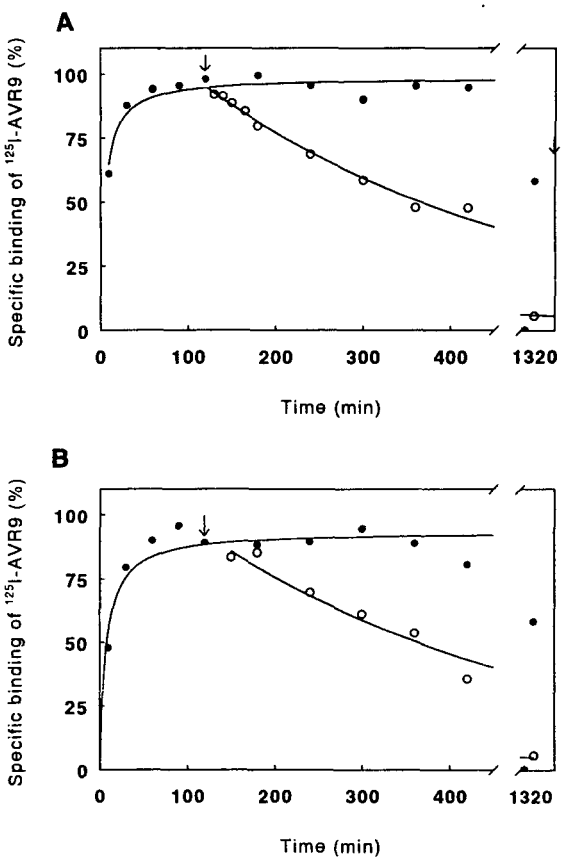

Figure 2. Time Course of Specific Binding and Displacement of ^{125}I-AVR9 to Plasma Membranes from MM-Cf9 and MM-Cf0.

(A) Binding to 2 μg of protein from plasma membrane fractions of MM-Cf9. Binding was initiated by adding ^{125}I-AVR9 to a final concentration of 7×10^{-10} M. Displacement (○) was initiated by the addition of 7×10^{-7} M unlabeled AVR9 at a *t* of 120 min (arrow), whereas binding (●) indicates no addition of unlabeled AVR9.
(B) Binding to 2 μg of protein from plasma membrane fractions of MM-Cf0. Binding and displacement are as given in **(A)**.

To characterize further the AVR9 binding site, microsomal membranes or plasma membranes were incubated with increasing amounts of ^{125}I-AVR9. As shown in Figure 3A, saturation of the binding sites was achieved at a concentration of ~0.5 nM AVR9. Linearization of the data in a Scatchard plot showed the presence of a single class of binding sites (Figure 3B). The dissociation constant (K_d) was calculated to be 0.07 nM by Scatchard analysis. The apparent concentration of the binding sites (R_t) was calculated to be 0.8 pmol/mg membrane protein for microsomal membrane fractions and 7 pmol/mg protein for enriched plasma membrane fractions. Enriched plasma membrane fractions also have a 10-fold higher specific H^+-ATPase activity compared with microsomal membranes (Table 1). These results confirm that the binding site is localized on the plasma membrane.

Specificity of AVR9 Binding to Membranes of Tomato Genotypes with and without the *Cf-9* Resistance Gene

No significant difference in the binding kinetics or binding capacity was observed between membranes of the resistant (MM-Cf9) and susceptible (MM-Cf0; without *Cf* resistance genes) tomato genotypes. Saturation experiments were performed with membranes of each of the two genotypes by using varying binding conditions. Significant differences in K_d or R_t between MM-Cf9 and MM-Cf0 plants were never observed (Figures 3A and 3B). Also, association and dissociation kinetics of binding of AVR9 to MM-Cf0 membranes, shown in Figure

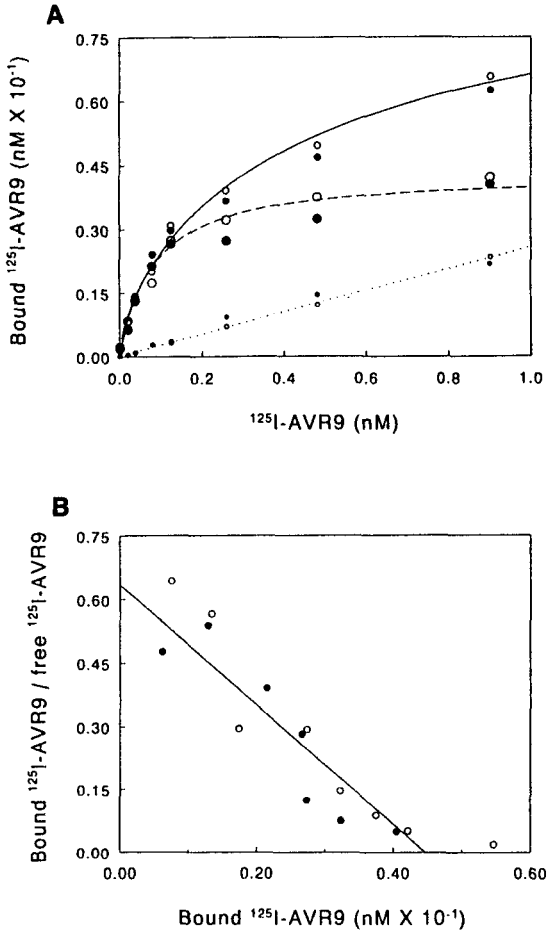

Figure 3. Saturation of the AVR9 Binding Site in Tomato Membranes.

(A) Microsomal membranes of MM-Cf0 (○) and MM-Cf9 (●) tomato plants were incubated with increasing amounts of ^{125}I-AVR9. The solid line represents total binding, and the dotted line represents nonspecific binding as determined by the addition of a 1000-fold excess of unlabeled AVR9. The dashed line represents specific binding (total minus nonspecific binding). The data represent one experiment, which is representative of six independent experiments.
(B) Scatchard plot of specific binding data derived from **(A)**.

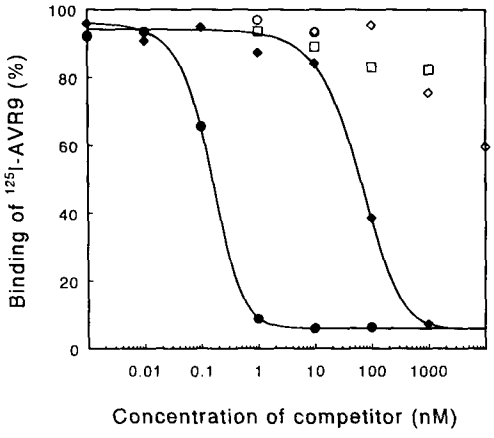

Figure 4. Competition of ^{125}I-AVR9 Binding to Plasma Membrane Fractions of MM-Cf9 Tomato Plants.

Binding was performed in 0.5 mL of binding buffer with a final concentration of 1×10^{-11} M ^{125}I-AVR9. Competitor peptides are as follows: AVR9 (●), reduced AVR9 (♦), AVR4 (○), RsAFP1 (◇), and MjAMp2 (□).

2B, were identical to the kinetics of AVR9 binding to MM-Cf9 membranes (Figure 2A).

To correlate specific binding to the biological activity of the ligand (the AVR9 elicitor only induces HR in leaves of tomato genotypes that contain the *Cf-9* resistance gene), competition analyses were performed using non-radioactively iodinated AVR9, native and reduced AVR9, AVR4, and two small antifungal peptides. The latter four peptides do not induce necrosis when injected into leaves of MM-Cf9. In competition assays using either native AVR9 or non-radioactively iodinated AVR9 (data not shown) as competitors, an apparent K_d of ~0.2 nM was observed for both peptides. Thus, iodination barely affects the binding properties of AVR9. In heterologous competition experiments, the antifungal cysteine-rich peptides of radish (RsAFP1) and *Mirabilis jalapa* (MjAMP2) (Cammue et al., 1992; Terras et al., 1992) and the AVR4 elicitor of *C. fulvum* (Joosten et al., 1994) did not compete for the AVR9 binding sites, as shown in Figure 4. Reduction of the disulfide bonds of the AVR9 peptide abolished the HR-inducing activity of the peptide and resulted in an ~1000-fold decrease in affinity (Figure 4). The observed K_d values vary somewhat between different competition experiments. This may have been due to loss of the very pure AVR9 competitor peptide at low concentrations, caused by nonspecific binding of AVR9 to incubation vials. Results of the binding experiments with different competitor peptides were similar for membranes isolated from leaves of MM-Cf9 and MM-Cf0 tomato plants (results not shown).

Binding of AVR9 to Membranes of Other Solanaceous and Nonsolanaceous Plant Species

Because no differences were found in AVR9 binding between membranes of MM-Cf0 and MM-Cf9 tomato leaves, membranes from the solanaceous species potato, petunia, and tobacco and from the nonsolanaceous plant species wheat, Brussels sprouts, and Arabidopsis were tested for specific binding of the AVR9 elicitor. Injection of the AVR9 elicitor into leaves of these species did not induce necrosis (results not shown). Microsomal membrane preparations of the nonsolanaceous species did not show significant binding of ^{125}I-AVR9 (results not shown), whereas Figure 5 shows that microsomal membranes of the solanaceous species potato, petunia, and tobacco did bind ^{125}I-AVR9. The apparent K_d values for potato, petunia, and tobacco were similar to the K_d values for tomato. The R_t values of potato and tobacco varied between different membrane isolations but were in the same range as the R_t of tomato. For petunia, the R_t value was lower, namely, 0.2 pmol/mg microsomal membrane protein. This may have been due to a lower concentration of plasma membranes in the membrane preparation, as was determined by the specific H^+-ATPase activity (results not shown).

Correlation between AVR9 Binding and Presence of *Cf-9* Homologs

Because binding of AVR9 seemed to be restricted to membranes of solanaceous plants, we investigated whether binding is correlated with the presence of members of the *Cf-9* gene family within these species. The plant species tested in the binding experiments were therefore screened for the presence of *Cf-9* homologs. A DNA gel blot containing BglII-digested DNA from several plant species showed that the solanaceous species tomato, potato, and tobacco, which specifically bind AVR9,

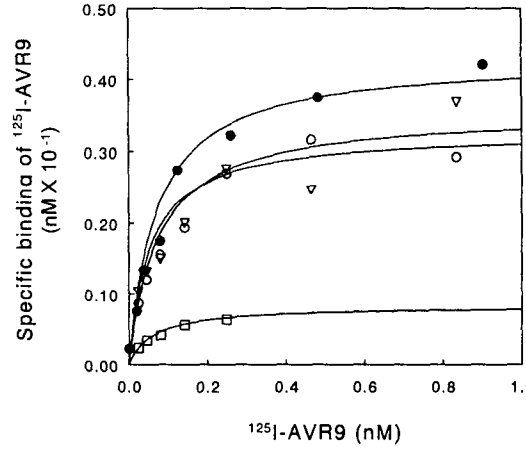

Figure 5. Saturation of AVR9 Binding Sites on Membranes of Solanaceous Plant Species.

Microsomal membranes of potato (▽), petunia (□), tobacco (○), and tomato (●) MM-Cf0 were incubated with increasing amounts of ^{125}I-AVR9 under standard conditions. The specific binding data represent total minus nonspecific binding. The saturation curves were obtained by linearization of the data in a Scatchard plot.

contain several fragments that hybridized with a *Cf-9* probe (Figure 6). Only weakly hybridizing fragments were observed for petunia, indicating that the *Cf-9* homologs in petunia may be more distantly related to *Cf-9* than those of potato, for instance. No hybridizing fragments were detected in the lanes containing DNA of the nonsolanaceous species Brussels sprouts or Arabidopsis (ecotype Landsberg *erecta*), which did not bind AVR9, whereas in the lane containing DNA of wheat, of which the membranes also did not bind AVR9, no discrete bands were visible.

DISCUSSION

A Binding Site for AVR9

Although the AVR9 elicitor only induces HR in tomato plants that carry the *Cf-9* resistance gene, high-affinity binding sites for AVR9 are present on plasma membranes of both the resistant (MM-Cf9) and susceptible (MM-Cf0) tomato genotype. No difference in K_d or R_t was observed between membranes of genotypes with or without the *Cf-9* resistance gene. The observed K_d value of 0.07 nM is low compared with K_d values of ligands binding to other plasma membrane–localized receptors in plants. Chitin fragments and a glycopeptide derived from yeast invertase bind to tomato membranes with K_d values of 23 and 3.3 nM, respectively (Basse et al., 1993; Baureithel et al., 1994), whereas a hepta-β-glucoside from *P. sojae* was found to bind to soybean membranes with a K_d of 0.75 nM (Cheong and Hahn, 1991). A 13–amino acid peptide derived from the 42-kD glycoprotein elicitor from *P. sojae* binds to a receptor in parsley membranes with a K_d of 2.4 nM (Nürnberger et al., 1994).

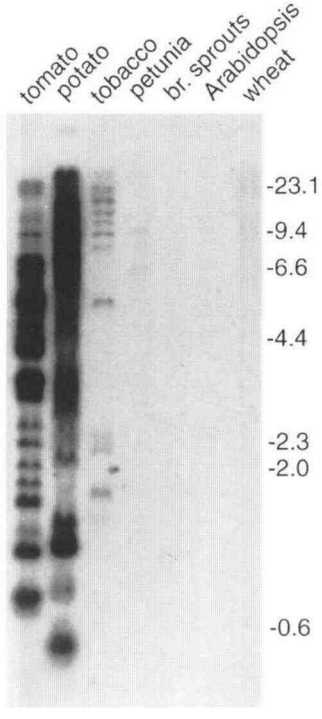

Figure 6. DNA Gel Blot Containing BglII-Digested Genomic DNA Hybridized with a BglII-EcoRV Fragment of the *Cf-9* Gene.

All lanes contain 4.5 μg of DNA. Tomato indicates MM-Cf9; br. sprouts, brussels sprouts. Molecular length markers are given at right in kilobases.

In mammalian systems, high-affinity binding sites with K_d values in the picomolar range have been reported frequently. K_d values of 1 to 60 pM have been observed for binding of transforming growth factor-β (TGF-β) to cell-surface receptors (Segarini et al., 1987). Very high affinities also have been found for the binding of inhibitors to enzymes. The ribonuclease inhibitor, a protein built entirely of LRRs, binds ribonuclease with a K_d of 1 to 70 fM (Hofsteenge, 1994). Binding of AVR9 is slow, with half-maximal binding after ~15 min at 37°C. A slow association rate is often found in combination with a low K_d (K_{off}/K_{on}) value and is in agreement with the very low K_{off} of 5×10^{-5}/sec. The strong effect of temperature on binding of AVR9 to its binding site suggests the involvement of hydrophobic interactions, whereas the effects of pH and ionic strength suggest the involvement of electrostatic forces on binding as well. Histidine, of which two residues are present in the AVR9 peptide, has a pK value of 6. At pH values above 6, the histidine residues become deprotonated, which may cause the negative effect on AVR9 binding.

The concentration of AVR9 required to cause necrosis when injected into leaves of MM-Cf9 tomato plants is $\sim 3 \times 10^{-7}$ M, which is ~4000-fold higher than the K_d value we have calculated from the binding studies. The apparent discrepancy between the in vitro and in vivo results may be due to the fact that the appearance of necrosis can be seen as a final step in the active defense response, which depends on physiological and environmental conditions. A similar phenomenon has been observed for fusicoccin, which invokes proton excretion in higher plants at a concentration of $\sim 10^{-7}$ M, whereas its high-affinity receptors have reported K_d values of $\sim 10^{-9}$ M (Basel et al., 1994). It is possible that the observed discrepancy occurs because binding of AVR9 to membranes is highly influenced by salt concentration and is optimal at low ionic strength. Not much is known about the ionic strength of the apoplast, and apparent K_d values may therefore be different from intrinsic values. Also, the cell wall could function as an adsorbent and diffusion barrier for AVR9, resulting in a low actual concentration of AVR9 near the plasma membrane receptor.

Although the affinity of AVR9 to its binding site is much higher than the affinities found for other plant receptors, the R_t of 0.8 pmol/mg microsomal protein is in the same range as that found for other membrane-bound receptors present in plants. In tomato, the reported R_t values vary from 19 fmol/mg microsomal protein for a glycopeptide elicitor (Basse et al., 1993) to 2.45

pmol/mg microsomal protein for chitin fragments (Baureithel et al., 1994).

The observed binding site specifically binds native AVR9, barely binds reduced AVR9, and does not bind other small cysteine-rich peptides. Therefore, we suggest that the binding site is a functional receptor for AVR9 in resistant tomato genotypes. Because plasma membranes from MM-Cf0 plants also bind, this implies there is a population of AVR9 binding sites with similar binding characteristics but with potentially different functions.

Recently, the three-dimensional structure of the AVR9 peptide has been resolved by ^{1}H–nuclear magnetic resonance (J.J.M. Vervoort, A. Berg, P. Vossen, R. Vogelsang, M.H.A.J. Joosten, and P.J.G.M. De Wit, submitted manuscript). The AVR9 peptide consists of a triple-stranded antiparallel β-sheet with three disulfide bridges. Based on its tertiary structure, the AVR9 peptide can be grouped into a superfamily of structurally related peptides with a common cystine knot motif, which is found in several proteinase inhibiting peptides, various ion channel blockers (Pallaghy et al., 1994), and mammalian growth factors, such as TGF-β (Isaacs, 1995). The tertiary structure of the AVR9 peptide is most closely related to the potato carboxypeptidase inhibitor (Chang et al., 1994). Whether this common motif also has functional implications remains to be determined.

Does CF-9 Bind AVR9?

Tomato plants containing the *Cf-9* gene are resistant to AVR9-producing races of *C. fulvum*. Furthermore, the CF-9 protein contains 28 LRRs of 24 amino acids and is likely to be localized on the outer surface of the plasma membrane, with only 21 amino acids of the protein located intracellularly (Jones et al., 1994). LRRs were shown to be involved in protein–protein interactions, including binding of different types of protein ligands (Kobe and Deisenhofer, 1994). The suggestion that the CF-9 protein is directly involved in AVR9 perception therefore seems plausible. Because *Cf-9* belongs to a gene family, the CF-9 protein as well as its homologs could be involved in AVR9 binding.

High-affinity AVR9 binding sites were found to be present on membranes of MM-Cf0 and MM-Cf9 tomato plants and on membranes of all other solanaceous species tested. No binding to membranes isolated from nonsolanaceous species was observed. DNA gel blot analysis showed that binding of AVR9 to plasma membranes correlates with the presence of members of the *Cf-9* gene family in all plant species tested to date. Based on this correlative evidence, we suggest that the observed high-affinity binding sites are CF-9–like proteins. However, it should be taken into account that the *Cf-9* homologs observed in petunia showed only weak hybridization, indicating that the *Cf-9* homologs in petunia may be more distantly related to *Cf-9* than the homologs observed in other solanaceous plants, such as potato. Furthermore, some background hybridization can be observed in the lane (Figure 6) containing wheat DNA, whereas wheat membranes did not bind AVR9. Whether the observed correlation is functional remains to be determined. Because we cannot conclude that the functional CF-9 protein binds AVR9, additional molecules or proteins may be involved in perception of AVR9.

Several systems have been described in which partly homologous proteins are required to give a biological response. The self-incompatibility systems of Arabidopsis and *Brassica* species have two partly homologous genes, the *S* locus glycoprotein (*SLG*) gene and the *S* locus receptor kinase (*SRK*) gene, which share sequence homology. Both *SLG* and *SRK* are required to give the incompatible phenotype (Nasrallah et al., 1994). A similar mechanism might be required for CF-9 and its homologs. Mechanisms for dimerization of partly homologous receptors also have been described for mammalian receptors, such as the TGF-β receptors I and II, two distantly related receptors (Wrana et al., 1994). TGF-β binds directly to receptor II and is then recognized by receptor I, which is recruited into the complex. Possibly, CF-9 homologs are required for AVR9 binding but do not give the HR without the presence of the functional CF-9 protein (De Wit, 1995).

The involvement of the CF-9 protein in AVR9 perception will be studied in more detail by performing AVR9 binding experiments with membranes from tobacco and Arabidopsis plants, which have been transformed with the *Cf-9* resistance gene, made available by the research group of J.D.G. Jones (Sainsbury Laboratory, Norwich, UK). Tobacco and Arabidopsis plants transformed with *Cf-9* will be used as representatives of a solanaceous plant containing *Cf-9* homologs and a nonsolanaceous plant without *Cf-9* homologs, respectively. These transgenic plants need to be analyzed in detail to study the production of the CF-9 protein in heterologous systems. Arabidopsis, transformed with *Cf-9*, will confirm or exclude the involvement of the CF-9 protein in AVR9 binding, because Arabidopsis itself does not bind AVR9.

All known *C. fulvum* races that are virulent on Cf-9 tomato genotypes lack the *Avr9* gene, and accordingly, they lack the AVR9 peptide. No virulent races of *C. fulvum* producing inactive or less active AVR9 peptides, which might be used in binding studies, have yet been found in nature. Therefore, modified AVR9 peptides with increased or decreased necrosis-inducing activity are being designed now. They will be used in heterologous competition experiments to characterize the AVR9 binding site in more detail.

METHODS

Plant Material

Leaves from the tomato (*Lycopersicon esculentum*) cultivar Moneymaker without *Cf* resistance genes (designated MM-Cf0) and the near-isogenic genotype MM-Cf9 were harvested from 3-week-old plants that were grown in peat soil at 25°C under 16-hr-light conditions. Microsomal fractions and plasma membranes were isolated essentially according to Sandstrom et al. (1987). All isolation steps were performed at 4°C. Briefly, large veins were removed from tomato leaves, and the leaves were ground in ice-cold buffer containing 25 mM Tris-HCl, pH 7.5, 250

mM sucrose, 3 mM EDTA, 10 μg/mL fatty acid–free BSA, and 1 mM phenylmethylsulfonyl fluoride. Following filtration through two layers of Miracloth (Calbiochem, La Jolla, CA) and differential centrifugation, plasma membranes were purified from the microsomal membranes by aqueous two-phase partitioning (Larsson et al., 1987). For partitioning, three successive extraction steps were performed in a 6.5% polyethylene glycol–6.5% dextran separating system containing 4 mM KCl, 250 mM sucrose, and 5 mM KPi, pH 7.5.

After isolation, both the microsomal membrane fractions and the plasma membrane–enriched fractions were dissolved in 10 mM Tris-HCl, pH 7.5, and 250 mM sucrose and stored at −80°C. Table 1 shows that a 10-fold enrichment of the plasma membranes could be reached in three subsequent extraction steps of the microsomal membranes in the 6.5% dextran–polyethylene glycol two-phase system. By re-extraction of the three lower phases of the system, more plasma membranes could be obtained, but in general these membranes were less pure, as determined by the specific activity of the K^+- and Mg^{2+}-dependent H^+-ATPase. Based on these results, the standard purification procedure of plasma membranes was performed by three consecutive extractions on the 6.5% dextran–polyethylene glycol phase system, without reextraction of the lower phase. By using this procedure, ~70 to 80% of plasma membranes were lost during partitioning (Table 1). Microsomal membrane fractions from potato (*Solanum tuberosum*), petunia (*Petunia hybrida*), tobacco (*Nicotiana tabacum*), wheat (*Triticum aestivum*), brussels sprouts (*Brassica oleracea*), and *Arabidopsis thaliana* ecotypes Columbia and Landsberg *erecta* were isolated following the same procedure as described for tomato, except that tobacco membranes were isolated from frozen leaf material without removal of the large veins.

To determine the purity of the plasma membranes, the plasma membrane–localized, vanadate-sensitive, K^+- and Mg^{2+}-dependent H^+-ATPase activity was determined according to Ames (1966) in the presence of 0.0075% Triton X-100 and 1 mM ammonium molybdate. Background activity was determined in the presence of 1 mM vanadate and subtracted from the total activity.

Purification and Iodination of the AVR9 Peptide

The 28–amino acid AVR9 peptide was isolated from apoplastic fluid, which was obtained from leaves of plants of the tomato genotype MM-Cf5 infected by race 5 of *C. fulvum*, as described by De Wit and Spikman (1982). Purification of the peptide was performed essentially according to the method described for the 34–amino acid AVR9 peptide from culture filtrate of a transformant of *C. fulvum* that constitutively produced AVR9, omitting the reverse-phase HPLC purification step (Van den Ackerveken et al., 1993). Briefly, high–molecular weight proteins were pelleted by overnight precipitation in 50% acetone and then centrifugation. The supernatant was collected, and acetone was removed in a rotary evaporator at 40°C. The sample was further purified by cation exchange chromatography on a CM-Sephadex C-25 column (Pharmacia Biotechnology), which was eluted using a pH gradient, followed by desalting of the active fractions on a Sep-Pak C18 cartridge (Waters, Milford, MA). Finally, cation exchange chromatography was performed on an MA7S column (7.8 × 50 mm; Bio-Rad), which was eluted with an NaCl gradient, and fractions were desalted once more on a Sep-Pak C18 cartridge before further use. The peaks were analyzed by low pH PAGE (Reisfeld et al., 1962), and the amount of purified 28–amino acid AVR9 peptide was determined by OD_{280} measurement by using a molar extinction coefficient of 1640, as determined by the Genetics Computer Group (Madison, WI) sequence analysis software package.

The N-terminal tyrosine residue of the purified elicitor was radiolabeled with iodine-125 by lactoperoxidase treatment (ANAWA Laboratories Inc., Zurich, Switzerland), and the monoiodinated peptide was isolated by reverse-phase HPLC, lyophilized, and stored at −20°C. The specific activity was 2200 Ci/mmol.

Competitor Peptides

The 36–amino acid antifungal peptide MjAMP2 from *Mirabilis jalapa* (Cammue et al., 1992) and the 45–amino acid peptide RsAFP1 from radish (*Raphanus sativus*) (Terras et al., 1992) were a kind gift of W.F. Broekaert (F.A. Janssens Laboratory of Genetics, Catholic University of Leuven, Belgium). The 106–amino acid AVR4 elicitor peptide from *C. fulvum* (Joosten et al., 1994) was kindly provided by M.H.A.J. Joosten (Wageningen Agricultural University, Wageningen, The Netherlands). Reduction of the AVR9 peptide was performed by incubating 100 μg of AVR9 peptide in 0.1 M Tris-HCl, pH 8.8, 1 mM EDTA, 6 M guanidine-HCl, and 0.1 M DTT. The denatured peptide was reisolated by reverse-phase HPLC.

Binding of AVR9 to Microsomal Membranes and Plasma Membranes

Membranes were resuspended in 90 μL of binding buffer (10 mM phosphate buffer, pH 6.0, 0.1% BSA) and preincubated at 37°C for 20 min. Binding was initiated by the addition of 10 μL of different concentrations of ^{125}I-AVR9, followed by incubation under gentle shaking at 37°C in a water bath for 3 hr. Nonspecific binding was determined in the presence of a 1000-fold excess of unlabeled AVR9. For competition experiments, membranes were suspended in 480 μL of binding buffer to which 10 μL of 5×10^{-9} M ^{125}I-AVR9 and 10 μL of the competitor peptide were added. The membranes were not preincubated at 37°C but were kept on ice until both the iodinated AVR9 and the competitor peptide were added to ensure that no binding occurred until both peptides were present.

Glass fiber filters (Whatman GF/F) were soaked for 1 to 2 hr in 0.5% polyethylenimine, transferred to a filtration manifold (Millipore, Bedford, MA), and washed with 5 mL of H_2O and 2 mL of binding buffer. Filtration of the samples was carried out at 10^4 pascals, and filters were subsequently washed with 12 mL of binding buffer. The filters were transferred to scintillation vials, and 3 mL of LumaSafe Plus (LUMAC. LSC B.V., Groningen, The Netherlands) was added. The radioactivity was counted in a scintillation counter (model LS-6000 TA; Beckman Instruments).

Proteinase Treatment of Membranes

Enriched plasma membrane fractions were incubated with 0.1 mg of proteinase K from *Tritirachium album* (Merck 124568), protease XXIII from *Aspergillus oryzae* (Sigma P4032), or trypsin from bovine pancreas (Sigma T8642). For this, 1 μg of membrane protein was incubated for 2 hr at 25°C at pH 7.5, with the various active or heat-inactivated (control) proteinases, and immediately tested for binding of AVR9. Binding was performed as described above. Incubation of 1 μg of the AVR9 peptide with 0.1 mg of the proteinases for 3 hr did not affect the stability of the peptide. Proteinase-treated AVR9 showed the same specific necrosis-inducing activity after injection into MM-Cf9 leaves as untreated AVR9 peptide. Also, migration patterns on a low-pH polyacrylamide gel were similar for both untreated and proteinase-treated AVR9.

DNA Gel Blot Analysis

Genomic DNA was isolated from leaves of tomato (MM-Cf9) and from potato, petunia, tobacco, wheat, brussels sprouts, and Arabidopsis ecotype Landsberg *erecta*, as previously described (Van der Beek et al., 1992; wheat, Rogers and Bendich, 1985). The DNA was digested with the restriction enzyme BglII, and the fragments were separated on a 1% agarose gel, blotted onto Hybond N^+ membranes (Amersham, Buckinghamshire, UK), and hybridized according to the manufacturer's instructions with a labeled BglII-EcoRV fragment (0.7 kb) of the *Cf-9* gene, encompassing the coding sequence of three leucine-rich repeats (LRRs) and the complete C-terminal part of the protein (Jones et al., 1994). The BglII-EcoRV DNA fragment was labeled with α-^{32}P, using the Ready.To.Go labeling kit from Pharmacia (Woerden, The Netherlands), according to the manufacturer's instructions. Blots were washed at 55°C in 1 × SSC (0.15 M NaCl and 0.015 M sodium citrate).

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