The AVR4 Elicitor Protein of *Cladosporium fulvum* Binds to Fungal Components with High Affinity

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The interaction between tomato and the fungal pathogen *Cladosporium fulvum* complies with the gene-for-gene system. Strains of *C. fulvum* that produce race-specific elicitor AVR4 induce a hypersensitive response, leading to resistance, in tomato plants that carry the *Cf-4* resistance gene. The mechanism of AVR4 perception was examined by performing binding studies with ¹²⁵I-AVR4 on microsomal membranes of tomato plants. We identified an AVR4 high-affinity binding site ($K_D = 0.05$ nM) which exhibited all the characteristics expected for ligand–receptor interactions, such as saturability, reversibility, and specificity. Surprisingly, the AVR4 high-affinity binding site appeared to originate from fungi present on infected tomato plants rather than from the tomato plants themselves. Detailed analysis showed that this fungus-derived, AVR4-specific binding site is heat- and proteinase K-resistant. Affinity crosslinking demonstrated that AVR4 specifically binds to a component of approximately 75 kDa that is of fungal origin. Our data suggest that binding of AVR4 to a fungal component or components is related to the intrinsic virulence function of AVR4 for *C. fulvum*.

Active disease resistance in plants relies on a highly sensitive and specific defense system that enables recognition and evasion of invading pathogens. Pathogens that produce non-specific elicitors are able to initiate a disease response in most genotypes within a plant species (Knogge 1996). Race-specific elicitors are considered to induce resistance only in genotypes of host plants that carry the corresponding resistance (*R*) gene (De Wit 1997; Flor 1971). Addition of elicitor proteins to suspension-cultured plant cells results in a rapid induction of plant defense-associated responses (Basse et al. 1993; Boller 1995; Felix et al. 1999; Ham et al. 1991; Hanania and Avni 1997; Lee et al. 2001; Meindl et al. 1998; Nürnberger et al. 1994). The high sensitivity of suspension-cultured cells and their extremely quick responses to low amounts of elicitor suggest that the induction of defense responses is mediated by receptors that exhibit high affinities for elicitor proteins. Indeed, specific high-affinity binding sites (HABSs) have been identified for elicitor proteins, peptides, and oligosaccharide signals (Bourque et al. 1999; Cheong and Hahn 1991; Ito et al. 1997; Matsubayashi and Sakagami 1999; Matsubayashi et al. 1997; Meindl et al. 2000; Mithöfer et al. 1996; Nürnberger et al.

1995; Scheer and Ryan 1999). Since the majority of HABSs identified thus far reside in the plasma membrane of plant cells, a role for such binding sites as putative receptors for elicitor molecules has been proposed. A close correlation also has been found between the biological activity of elicitor molecules and the affinity for their binding sites, which further implies that the HABSs for these elicitors act as functional receptors (Hanania and Avni 1997; Meindl et al. 1998, 2000; Thevissen et al. 2000).

The interaction between tomato and the extracellular, biotrophic pathogen *Cladosporium fulvum* is a well-established model system that complies with the gene-for-gene model. Strains of *C. fulvum* that carry avirulence (*Avr*) genes induce an active defense response, including the hypersensitive response (HR), in tomato plants that carry the corresponding *Cf* resistance genes (Joosten and De Wit 1999). Several *Cf* genes and their corresponding *Avr* genes have been cloned (Joosten and De Wit 1999). The *Cf* gene family encodes putative plasma membrane-anchored proteins with a predicted extracellular leucine-rich repeat domain, a transmembrane region, and a short cytoplasmic tail (Thomas et al. 1998). For *Cf-4* and *Cf-9*, the corresponding avirulence genes of *C. fulvum*, *Avr4* and *Avr9*, respectively, have been cloned (Joosten et al. 1994; Van den Ackerveken et al. 1992). AVR4 and AVR9 elicitor proteins are both encoded as pre-proteins that are processed by fungal and plant proteinases within the leaf apoplast into mature proteins of 86 and 28 amino acids, respectively. To date, very little is known about the intrinsic function of AVR4 and AVR9 for the fungus. No clear deleterious effects on virulence have been found in strains of *C. fulvum* in which either the *Avr4* or the *Avr9* gene is truncated or deleted, respectively (Joosten et al. 1997; Marmeisse et al. 1993). Nevertheless, both *Avr4* and *Avr9* gene products may have a role in virulence, development, reproduction, or general fitness of *C. fulvum*.

The predicted extra-cytoplasmic localization of Cf-4 and Cf-9 would fit with a model in which the secreted AVR4 and AVR9 proteins are perceived in the leaf apoplast. Suspension-cultured tobacco cells expressing *Cf-9* rapidly produce defense response-associated active oxygen species in response to AVR9 (Piedras et al. 1998). However, no direct interaction between AVR9 and Cf-9 has yet been demonstrated (Luderer et al. 2001). Furthermore, binding studies with AVR9 have shown that both MM-Cf9 (resistant) and MM-Cf0 (susceptible) plants contain an AVR9-specific HABS in the plasma membrane (Kooman-Gersmann et al. 1996), suggesting an indirect role for Cf-9 in perception of AVR9. Such indirect perception has been referred to as the “guard hypothesis” (Van der Biezen and Jones 1998), whereby it is proposed that the HABS represents a virulence target for AVR9. The Cf-9 protein is thought to act as a “guard” to

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monitor the behavior of one or more host-encoded virulence proteins that are targets for AVR9 (Luderer and Joosten 2001; Van der Hoorn et al. 2002).

In this study, we have investigated the binding of AVR4 to microsomal membranes of MM-Cf4 (resistant) and MM-Cf0 (susceptible) tomato plants and nonhost plant species. We have identified an AVR4 HABS that appeared to originate from fungi present on infected tomato plants rather than from tomato itself. Interestingly, this fungus-derived AVR4 HABS is heat and proteinase K resistant. In addition, affinity crosslinking demonstrated that AVR4 specifically interacts with a component of approximately 75 kDa that is of fungal origin. Binding of AVR4 to fungal components in relation to its intrinsic virulence function for *C. fulvum* will be discussed.

RESULTS

Production and biological activity of iodinated AVR4.

In order to examine whether a HABS is present for AVR4 on tomato membranes, it was essential to obtain large quantities of pure, elicitor-active AVR4 protein. The methylotrophic yeast *Pichia pastoris* was used as an eukaryotic, heterologous expression system (Romanos 1995) to obtain high levels of correctly folded mature AVR4 protein. AVR4 protein isolated from intercellular fluid (IF) obtained from *C. fulvum*-infected tomato leaves is unglycosylated (Joosten et al. 1997). To prevent yeast-specific glycosylation of AVR4; the potential *N*-linked glycosylation site (NLS) in AVR4 was removed by changing the codon for serine into a codon for alanine in the open reading frame (ORF) of *Avr4*. By using *Potato virus X* (PVX)-mediated expression in MM-Cf4 plants, as described by Joosten and associates (1997), this mutation was found not to affect the necrosis-inducing activity of AVR4 (data not shown). Heterologously expressed AVR4 protein was purified from the culture filtrate by ion exchange and reverse phase high-performance liquid chromatography (HPLC) (Van den Burg et al. 2001). Injection of purified AVR4 into MM-Cf4 leaves resulted in a clear HR, demonstrating that the protein had retained its elicitor activity (Van den Burg et al. 2001).

To test whether iodination affects the biological activity of AVR4, iodination with nonradioactive iodine (^{127}I) was per-

formed using a lactoperoxidase labeling protocol, resulting in mono ^{127}I -labeled AVR4. Unlabeled AVR4 induces a rapid oxidative burst in suspension-cultured tobacco cells expressing *Cf-4*, in a concentration-dependent manner (De Jong et al. 2002). Addition of ^{127}I -labeled AVR4 protein to these cell suspensions resulted in a similar, concentration-dependent response (Fig. 1). This demonstrates that iodination does not affect the biological activity of the AVR4 elicitor. Identical labeling conditions were applied with radioactive iodine (^{125}I) to obtain mono ^{125}I -labeled AVR4 that was used in subsequent binding experiments.

Binding of ^{125}I -AVR4 to microsomal membranes of MM-Cf4 tomato plants.

To examine the presence of AVR4-specific binding sites on microsomal membrane fractions (MFs) isolated from MM-Cf4 tomato leaves, binding of ^{125}I -AVR4 to MFs was followed over time and at various temperatures. Optimal binding was achieved at 37°C (data not shown). An AVR4-specific binding site was found and binding reached equilibrium 4 h after incubation with AVR4 at 37°C (Fig. 2A). Specific binding of ^{125}I -AVR4 remained stable up to 8 h, followed by a slow, partial decrease after 22 h of incubation (data not shown). At lower temperatures (i.e., 4 and 24°C), equilibrium of ^{125}I -AVR4-specific binding was reached after 22 and 10 h of incubation, respectively (data not shown).

Addition of a 500-fold molar excess of unlabeled AVR4 at equilibrium resulted in a decrease of specific binding of ^{125}I -AVR4, demonstrating that AVR4-specific binding is reversible (Fig. 2B). From these data, a dissociation constant (K_D) of 0.09 nM was calculated for AVR4 (Fig. 2C), suggesting that AVR4 binds to microsomal membranes of MM-Cf4 with high affinity.

Saturation of ^{125}I -AVR4 binding to microsomal membranes of MM-Cf4.

Incubation of MFs isolated from MM-Cf4 leaves with increasing concentrations of ^{125}I -AVR4 showed that AVR4 binding is saturable (Fig. 3A). Saturation of AVR4-specific binding was reached at an ^{125}I -AVR4 concentration of approximately 0.25 nM (Fig. 3A). Scatchard analysis performed on these data resulted in a plot with a linear slope, which implies a uniform population of AVR4-specific binding sites. The calculated K_D value of 0.05 nM ($K_D = -1/\text{slope}$) is in the same order of magnitude as the K_D value calculated from the rate constants (Fig. 2C). The total concentration of binding sites ($R_t = 0.04$ pmol/mg of protein) was determined by extrapolation of the slope to the *x*-axis (Fig. 3B). The calculated Hill coefficient (n_H) of 0.91 is close to 1, suggesting that AVR4 binding to microsomal membranes of MM-Cf4 is noncooperative (Fig. 3C).

Specificity of ^{125}I -AVR4 binding to microsomal membranes of MM-Cf4.

To determine the specificity of AVR4 binding to MFs isolated from MM-Cf4 leaves, a competitive binding assay with AVR4 and AVR9 was performed. Addition of increasing amounts of unlabeled AVR4 protein resulted in progressive inhibition of ^{125}I -AVR4 binding. A 50% inhibition of specific binding (IC_{50}) was observed at an AVR4 concentration of approximately 0.16 nM (Fig. 4). This value is in the same order of magnitude as the K_D values determined in time course experiments and ligand saturation analysis (Figs. 2 and 3). When ^{125}I -AVR4 binding reactions were performed in the presence of concentrations up to a 1,000-fold molar excess of unlabeled AVR9, no competition for ^{125}I -AVR4 binding could be detected (Fig. 4). Similarly, other extracellular proteins of *C. fulvum*, ECP1 (Joosten and De Wit 1988; Van den Ackerveken et al. 1993) (Fig. 4) and ECP4 (Laugé et al. 2000) (data not shown), also did not compete for ^{125}I -AVR4 binding. Together,

Fig. 1. Oxidative burst of suspension-cultured tobacco cells expressing *Cf-4* after treatment with AVR4 or ^{127}I -AVR4. Suspension-cultured tobacco cells expressing *Cf-4* were supplied with the fluorescent probe pyranin (10 $\mu\text{g}/\text{ml}$) and challenged with buffer (open square) and with two concentrations (0.3 and 1.5 nM) of unlabeled AVR4 (black dot) or ^{127}I -labeled AVR4 (open triangle). Quenching of the pyranin fluorescence, due to production of H_2O_2 by the cells, was measured in time. A.U. = arbitrary units.

these data demonstrate that AVR4 binding to microsomal membranes of MM-Cf4 is specific.

Binding of ^{125}I -AVR4 to microsomal membranes of MM-Cf0 tomato and nonsolanaceous plant species.

We further investigated whether the AVR4 HABS present in MFs isolated from MM-Cf4 leaves is also present in MM-Cf0 leaves. When binding studies were carried out on MFs of MM-Cf0, a similar AVR4-specific HABS was identified ($K_D = 0.08$ nM, $R_t = 0.057$ pmol/mg of protein, and $n_H = 0.93$) (data not shown). In addition, time course experiments were performed with MFs of MM-Cf0 (Fig. 2), which resulted in similar AVR4-specific binding characteristics as found in MFs of MM-Cf4 plants (data not shown).

To determine whether nonsolanaceous plant species also contain AVR4-specific binding sites, MFs isolated from leaves of *Arabidopsis* spp. and barley grown under regular greenhouse conditions were incubated with ^{125}I -AVR4. Interestingly, these nonsolanaceous plant species showed the presence of an AVR4-specific HABS with K_D values of a similar order of magnitude as found in MM-Cf0 and MM-Cf4 plants ($K_D = 0.07$ and 0.12 nM for *Arabidopsis* spp. and barley, respectively).

Further biochemical characterization of the AVR4 HABS.

To further analyze the nature of the AVR4 HABS, MFs isolated from MM-Cf4 leaves were subjected to a number of different treatments, prior to binding assays with ^{125}I -AVR4. In addition, binding experiments with ^{125}I -AVR9, which served as a control, were carried out (Kooman-Gersmann et al. 1996).

To test for heat-stability of the AVR4 HABS, MFs were incubated at 95°C for 15 min, prior to incubation with ^{125}I -AVR4 or ^{125}I -AVR9. Surprisingly, whereas heat treatment completely abolished AVR9 binding, AVR4-specific binding to MFs was reduced by only 15% (Fig. 5A).

In order to assay for proteinase sensitivity of the AVR4 HABS, MFs were treated with proteinase K. The presence of low concentrations of proteinase K during incubation with ^{125}I -AVR4, however, resulted in breakdown of the AVR4 protein (data not shown). Therefore, proteinase K-treated MFs were heated prior to incubation with ^{125}I -AVR4 in order to inactivate the enzyme. Proteinase K did not affect the stability of AVR9 (Kooman-Gersmann et al. 1996); therefore, no heat treatment was required to inactivate proteinase K prior to ^{125}I -AVR9 binding. Although proteinase K-treatment of MFs completely abolished ^{125}I -AVR9-specific binding, no significant effect was found for AVR4 binding (Fig. 5B). Incubation of MFs with heat-treated proteinase K only slightly affected ^{125}I -AVR9-specific binding, whereas the ^{125}I -AVR4-specific binding remained unaffected.

Finally, fractions were isolated from MFs of MM-Cf4 leaves using the two-phase partitioning protocol to enrich for plasma membranes (Larsson et al. 1987). When binding was performed on membranes present in the upper phase, a significant increase in AVR9-specific binding sites, which are localized predominantly in the plasma membrane (Kooman-Gersmann et al. 1996), was found, indicating that plasma membrane enrichment had occurred (Fig. 5C). In contrast to the increase in the amount of binding sites for AVR9, a significant decrease in AVR4-specific binding sites was found in these plasma membrane-enriched fractions (Fig. 5C). Together, our data indicate that the AVR4 HABS is heat and proteinase K resistant and that it is not associated with the plasma membrane.

Inconsistencies in ^{125}I -AVR4-specific binding between different batches of MFs of MM-Cf4 plants.

During the assays described above, we observed inconsistencies in ^{125}I -AVR4-specific binding activities between differ-

Fig. 2. Binding of ^{125}I -AVR4 to microsomal membranes of MM-Cf4 tomato. Microsomal membrane fractions of MM-Cf4 leaves were incubated with 0.07 nM ^{125}I -AVR4 at 37°C over different time points. Each data point represents the average of six independent binding assays. **A**, Time course of AVR4 binding was assayed in the absence (open diamond) or in the presence ($\times$) of a 500-fold molar excess of unlabeled AVR4, which was added at $t = 0$ min. Specific binding (black dot) was determined by subtracting aspecific binding ($\times$) from total binding (open diamond). **B**, Reversibility of AVR4 binding was assayed by adding a 500-fold molar excess of unlabeled AVR4 at $t = 240$ min (black triangle). **C**, Kinetics of specific association ($-\ln [1 - B/B_{\max}]$) (black dot) and dissociation ($\ln [\text{cpm of B}]$) (black triangle) calculated from A and B (Hulme 1999). The dissociation rate constant of the receptor-ligand complex ($k_{\text{off}} = -\text{slope}$) is $5.4 \times 10^{-3} \text{ min}^{-1}$, and the dissociation half-time ($t_{1/2} = \log_e(2)/k_{\text{off}}$) is 128 min. The association rate constant ($k_{\text{on}} = [\text{slope} - k_{\text{off}}]/[^{125}\text{I}\text{-AVR4}]$) is $0.063 \text{ nM}^{-1} \text{ min}^{-1}$, and the half-time ($t_{1/2} = \log_e(2)/[k_{\text{on}} \times (^{125}\text{I}\text{-AVR4}) + k_{\text{off}}]$) is 72 min. The ratio $k_{\text{off}}/k_{\text{on}}$ yields a dissociation constant (K_D) of 0.09 nM. B = specific binding and $B_{\max}$ = specific binding at equilibrium.

ent batches of MFs. In some MFs that were isolated from leaves of MM-Cf4 plants, as well as from *Arabidopsis* spp., no ^{125}I -AVR4-specific binding sites could be detected. Interestingly, all of these MFs were isolated from plants that were grown in confined compartments. The MFs that exhibited ^{125}I -AVR4-specific binding sites, however, were isolated from MM-Cf4 and *Arabidopsis* plants grown under regular greenhouse conditions.

This observation, together with the predicted nonproteinaceous character of the AVR4 HABS, prompted us to investigate the origin of the AVR4 HABS further. When tomato plants are grown under regular greenhouse conditions, it appears that these plants are often infected with *Oidium lycopersicum*, thereby showing some sporulating colonies on the leaflets. To investigate whether a correlation exists between the presence of fungal contaminants and binding of AVR4 to its HABS, binding assays were performed on MFs isolated from

Fig. 3. Saturation of ^{125}I -AVR4 binding to microsomal membranes of MM-Cf4 tomato. **A**, microsomal membranes isolated from MM-Cf4 tomato leaves were incubated for 4 hr at 37°C with increasing concentrations of ^{125}I -AVR4. Aspecific binding was determined by addition of a 500-fold molar excess of unlabeled AVR4 at $t = 0$ min ($\times$). Specific binding (black dot), which was determined by subtracting aspecific binding ($\times$) from total binding (open diamond), was saturated at a free ligand concentration of about 0.25 nM. Free ligand concentration was calculated by subtracting the initially added ^{125}I -AVR4 from total bound ^{125}I -AVR4. Each data point represents the average of six independent binding assays. **B**, Scatchard plot analysis of data obtained for specific binding of ^{125}I -AVR4 as presented in **A**. From the slope of the Scatchard plot, a dissociation constant ($K_D = -1/\text{slope}$) of 0.05 nM was calculated, while extrapolation of the slope to the x-axis allowed a calculation of the total receptor concentration ($R_t = 0.02 \text{ nM} = 0.02 \text{ pmol ml}^{-1}$ per 48 μg of protein per 0.1 ml of assay) of protein at 0.04 pmol/mg. **C**, Hill plot of data obtained for specific binding (**B**) of ^{125}I -AVR4 as presented in **A**. $B_{\max}$ represents the amount of specific AVR4 binding at saturation level. From the slope of the Hill plot the Hill coefficient ($n_{\text{H}} = 0.91$) was calculated.

MM-Cf4 leaves that were severely infected with *O. lycopersicum* (i.e., leaves of which 50 to 75% of the surface was infected). Interestingly, the total concentration of binding sites ($R_t = 0.09 \text{ pmol/mg}$ of protein) which exhibit a K_D value of 0.11 nM was higher in these MFs (data not shown) compared with the concentration in the MFs isolated from MM-Cf4 plants grown under regular greenhouse conditions ($R_t = 0.04 \text{ pmol/mg}$ of protein) (Fig. 3).

To discriminate between fungus- and plant-specific binding, as well as fungus-induced, plant-specific binding, we intended to test *O. lycopersicum* for the presence of an AVR4-specific binding site. However, the requirement of plant material for the obligate pathogen *O. lycopersicum* to grow and reproduce hampered the isolation of sufficient fungal biomass. Therefore, we performed binding assays on *C. fulvum* mycelium extracts instead. Significantly, an AVR4-specific HABS was detected, which exhibited a K_D value of 0.26 nM, in the absence of plant material (Fig. 6). The total concentration of binding sites in these fractions ($R_t = 9.2 \text{ pmol/mg}$ of protein) was significantly higher than the R_t in MFs isolated from MM-Cf4 plants (Fig. 3). Moreover, the AVR4 HABS present in the mycelium extracts also appeared to be heat resistant (data not shown), as demonstrated for the AVR4 HABS present in MFs isolated from MM-Cf4 plants (Fig. 5A). Together, these data demonstrate that the AVR4 HABS is of fungal rather than of plant origin.

Chemical crosslinking of ^{125}I -AVR4 to a distinct compound is dependent on the presence of a fungal infection of tomato.

To identify the fungus-derived AVR4 HABS, ^{125}I -AVR4 was chemically crosslinked to *C. fulvum* mycelium extracts and MFs of *O. lycopersicum*-infected MM-Cf4 leaves. ^{125}I -AVR4 was crosslinked to a single band that migrates as a molecule of approximately 85 kDa upon sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) in MFs isolated from *O. lycopersicum*-infected MM-Cf4 leaves (Fig. 7A). No AVR4-specific crosslinking was detected in MFs isolated from MM-Cf4 plants grown in confined compartments (Fig. 7B). Significantly, ^{125}I -AVR4 was crosslinked to a band of the same size when binding was performed with *C. fulvum* mycelium extracts (Fig. 7C). In the presence of a 500-fold molar excess

Fig. 4. Competition of ^{125}I -AVR4 binding by unlabeled AVR4, AVR9, or ECP1. Various concentrations of unlabeled AVR4 (black dot), AVR9 (black triangle), or ECP1 (open circle) were mixed with 0.07 nM ^{125}I -AVR4 and added to microsomal membrane fractions of MM-Cf4 tomato leaves (20 μg of protein per assay). Binding was performed at 37°C for 4 h. Aspecific binding was determined in the presence of a 500-fold molar excess of unlabeled AVR4. Maximal specific binding, determined by subtracting aspecific binding ($= 2.2 \pm 0.1 \text{ pM}$) from total binding ($= 8.7 \pm 0.6 \text{ pM}$), was referred to as 100%. Data of six independent binding experiments are presented as the percentage of maximal specific binding.

of unlabeled AVR4, no crosslinked band could be detected, suggesting that crosslinking to this molecule is specific for AVR4 (Fig. 7A and C). These data demonstrate that the AVR4-specific crosslinked component is of fungal origin rather than of plant origin. Use of glutaraldehyde, *m*-maleimidobenzoyl-*N*-hydroxysuccinimide ester (MBS), and photo-reactive *N*-hydroxysulfosuccinimidyl-4-azidobenzoate (sulfo-HSAB) as crosslinkers, which all react through different crosslinking mechanisms, gave similar results (data not shown). Presuming that one ^{125}I -AVR4 molecule (molecular mass of 10 kDa) is crosslinked to a single molecule, the predicted molecular mass of the fungus-derived AVR4-specific crosslinked band is approximately 75 kDa.

DISCUSSION

An AVR4-specific HABS that is of fungal origin.

To characterize the mechanism of specific perception of AVR4 by MM-Cf4 plants, MFs from leaves of such plants were analyzed for the presence of an AVR4 HABS. Although an AVR9-specific HABS was detected in the plasma membranes of MM-Cf9 plants (Kooman-Gersmann et al. 1996), no such binding site was found for AVR4. However, an AVR4-specific HABS was found that appeared to originate from fungi present on infected tomato plants rather than from the tomato plant itself. This fungus-derived AVR4 HABS exhibited all the characteristics expected for ligand-receptor interactions, such as

saturability, reversibility, and specificity. Furthermore, whereas the AVR9 HABS has been described as proteinase K and heat sensitive (Kooman-Gersmann et al. 1996), the AVR4-specific HABS proved to be proteinase K and heat resistant, suggesting that the HABS might have a nonproteinaceous nature.

Previously, proteinase- and heat-resistant binding sites have been identified in tobacco plasma membranes for harpin and cryptogin, which are elicitor proteins secreted by *Pseudomonas syringae* pv. *syringae* and *Phytophthora cryptogea*, respectively (Bourque et al. 1999; Lee et al. 2001). Lee and associates (2001) reported that the proteinase-resistant binding site for harpin is nonproteinaceous. The heat-resistant binding site for cryptogin, however, was shown to be a glycoprotein (Bourque et al. 1999). Indeed, glycosylated proteins repeatedly have been demonstrated to be more resistant against thermal or chemical denaturation and proteolytic degradation compared with their nonglycosylated counterparts (Tanner and Lehle 1987). Thus, the heat- and proteinase K-resistant fungal AVR4 HABS might represent either a stable glycoprotein or a non-proteinaceous polysaccharide, like glucan or chitin fragments (Bartnicki-Garcia 1968; Kollar et al. 1997). The latter is consistent with high-affinity binding interactions between (plant) proteins and oligosaccharide elicitors that have been identified before (Asensio et al. 2000; Baureithel et al. 1994; Brandhorst and Klein 2000; Cheong et al. 1991; Cosio et al. 1988; Day et al. 2001; Ito et al. 1997; Shibuya et al. 1993). Interestingly, we identified an AVR4-specific crosslinked molecule of approxi-

Fig. 5. Biochemical characterization of the AVR4 high-affinity binding site. Heat treatment and proteinase K treatment, respectively, were carried out on microsome membrane fractions (MFs) isolated from MM-Cf4 tomato leaves. Binding was performed with ^{125}I -AVR4 and ^{125}I -AVR9 at final concentrations of 0.07 nM. Depicted values correspond to specific binding measured 30 min after incubation at 37°C. Each data point represents the average of three binding assays. **A**, binding of ^{125}I -AVR4 (upper part) and ^{125}I -AVR9 (lower part) to MFs without (–) or with (+) heat treatment. **B**, Binding of ^{125}I -AVR4 and ^{125}I -AVR9 to MFs that were not treated (–), treated with inactivated proteinase K (–*), or treated with 5 mg/ml (+) or 15 mg/ml (++) of proteinase K. Prior to incubation with ^{125}I -AVR4, proteinase K-treated MFs were incubated at 95°C for 15 min to inactivate proteinase K. **C**, Binding of ^{125}I -AVR4 and ^{125}I -AVR9 to MFs of MM-Cf4 leaves (MF) and to plasma membrane-enriched fractions isolated from MM-Cf4 leaves (PM).

mately 75 kDa that migrates as a distinct band on SDS-PAGE instead of as a smear, suggesting that it represents a proteinaceous compound rather than a polysaccharide. Significantly, this AVR4-specific molecule of approximately 75 kDa proved to be of fungal rather than of plant origin. Thus, the fungal heat- and proteinase K-resistant AVR4 HABS might represent a molecule of approximately 75 kDa. Alternatively, the fungal AVR4 HABS might represent a polysaccharide. In that case, binding of AVR4 to this polysaccharide might be required for subsequent crosslinking of AVR4 to the molecule of 75 kDa, which in turn exhibits polysaccharide-binding properties.

Our data demonstrate that the AVR4 HABS is present in at least two different classes of fungi (i.e., Fungi Imperfecti and Ascomycetes). Although the exact nature of the HABS is unknown, binding of AVR4 to *C. fulvum* mycelium extracts might indicate that AVR4 in some way contributes to virulence of *C. fulvum* on tomato. It could be possible that AVR4 binds to the surface of *C. fulvum* and protects the fungus against attack by extracellular cell wall-degrading enzymes of tomato. Natural strains that lack a functional AVR4 protein, however, do not exhibit a reduced virulence under laboratory conditions (Joosten et al. 1997). These strains might have compensated for the lack of AVR4 by the production of another secreted protein that protects the fungus, or might indeed exhibit a reduced virulence that is not detected under laboratory conditions. To further elucidate the intrinsic function of AVR4, we are currently identifying the fungal component or components to which AVR4 binds.

The mechanism underlying AVR4 perception by MM-Cf4 plants.

Although AVR4 binds to fungal components with high affinity, this interaction is not required for elicitor activity of AVR4 in MM-Cf4 plants, because AVR4 exhibits necrosis-inducing activity when injected into MM-Cf4 leaves in the absence of fungal components. Growth of *C. fulvum* is restricted to the apoplastic space of tomato; therefore, AVR4 perception by MM-Cf4 plants most likely occurs extracellularly. However, an AVR4-specific binding site in microsomal membranes of MM-Cf4 plants has not yet been detected. This implies that the mechanism of perception of AVR4 by MM-Cf4 plants is different from the perception of several other fungus-derived elicitors, including AVR9, for which a HABS has been found in the

plasma membrane of solanaceous plants. The mechanism of AVR4 perception by MM-Cf4 plants remains to be unraveled.

MATERIALS AND METHODS

Plant material.

Plants were grown either under regular greenhouse conditions or, to obtain pathogen-free plants, in confined compartments. Leaves were harvested from 4-week-old tomato (*Lycopersicon esculentum*) cv. Moneymaker (MM-Cf0), a near-isogenic line of MM-Cf0 carrying the *Cf-4* resistance gene (MM-Cf4), an *Arabidopsis* sp. (*Arabidopsis thaliana* ecotype Columbia), and barley (*Hordeum vulgare* cv. Triumph).

Isolation of microsomal MFs and plasma membranes.

Microsomal MFs were obtained as described by Sandstrom and associates (1987). Briefly, leaves were homogenized in a blender (Snijders Scientific, Tilburg, The Netherlands) in MB1 buffer (25 mM Tris-HCl, pH 7.5, 250 mM sucrose, 3 mM EDTA, fatty-acid-free bovine serum albumin [BSA] at 10 µg/ml, and 1 mM phenylmethylsulfonyl fluoride [PMSF]), following filtration through one layer of cheese cloth and two layers of Miracloth (Calbiochem, La Jolla, CA, U.S.A.) and differential centrifugation steps. Plasma membranes were purified from these microsomal membranes by aqueous two-phase partitioning (Larsson et al. 1987). In brief, 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 potassium phosphate buffer, pH 7.5. After isolation, the MFs and the plasma membrane-enriched fractions were resuspended in MB3 buffer (10 mM Tris-HCl, pH 7.5, 250 mM sucrose, and 1 mM PMSF) and stored at -80°C. The protein concentration of these fractions was determined using a BCA protein assay reagent with BSA as a standard (Pierce Chemical, Rockford, IL, U.S.A.).

Isolation of *Cladosporium fulvum* mycelium extracts.

Cladosporium fulvum race 4 was grown on potato dextrose agar (PDA) or in liquid B5 medium in shake cultures at 22°C and 90 rpm (De Wit and Flach 1979). *C. fulvum* mycelium extracts were obtained, based on the method as described by Sandstrom and associates (1987), as follows: liquid B5 medium was inoculated with conidia from 10-day-old PDA cultures of *C. fulvum* (1×10^7 conidia ml⁻¹) and the culture was allowed to grow for 72 h in a rotary shaker at 22°C. The mycelium was harvested and 400 mg of fresh weight was transferred to a 1.5-ml Eppendorf tube. To remove remaining liquid B5 medium, mycelium was pelleted by centrifugation at 9,000 × g, resuspended in 500 µl of MB1 buffer, and pelleted again. Subsequently, the mycelium was crushed with a potter. Following centrifugation at 3,000 × g for 3 min, the supernatant was transferred to a new Eppendorf tube and centrifuged at 9,000 × g for 20 min. The pellet was resuspended in 50 µl of MB3 buffer. These fractions, which are referred to as mycelium extracts, were used in binding assays (0.5 µg of protein per assay) and were used for crosslinking with ¹²⁵I-AVR4 (1 µg of protein per assay).

DNA manipulation.

Standard molecular biological techniques were carried out as described by Sambrook and associates (1989). Polymerase chain reactions (PCRs) were performed with *pfu* polymerase (Stratagene, La Jolla, CA, U.S.A.), according to the manufacturer's instructions. PCR-based cloning was performed with *Avr4* cDNA (Joosten et al. 1997) as a template, to obtain construct pPIC-A4 that allowed production of mature AVR4 by *Pichia pastoris* strain GS115. To introduce a single amino acid

Fig. 6. Saturation of ¹²⁵I-AVR4 binding to *Cladosporium fulvum* mycelium extracts. *C. fulvum* extracts were incubated with increasing amounts of ¹²⁵I-AVR4 alone (open triangle) or in the presence of a 500-fold molar excess of unlabeled AVR4 (×). Specific binding (black dot) was determined by subtracting aspecific binding from total binding. Data points are fitted to a hyperbolic curve and each data point represents an average of three independent binding assays.

change (S78A) at the position of the putative *N*-glycosylation site in the ORF of *Avr4*, a PCR was performed with forward primer cgctcgagaaaagagagctgaagctaaggccca (underlined: *XhoI* site) and reverse primer cgcgaattctacgtatcattgcggcgtcttaccggacacgtggccaggtttg (underlined: *EcoRI* site; bold: mutation that introduces the Ser-78 to Ala-78 substitution). The amplified PCR product was digested with *XhoI/EcoRI* and ligated into the *XhoI/EcoRI*-digested expression vector pPIC9 (Invitrogen Corp., Groningen, The Netherlands), resulting in construct pPIC-A4. In this way, the ORF encoding AVR4 was cloned in frame downstream of the α -factor secretion signal, which is fused to the methanol-inducible alcohol oxidase (AOX1) promoter (Ellis et al. 1985). To increase transformation efficiency and to facilitate targeted integration at the *His*⁺ locus, resulting in *P. pastoris* His⁺ transformants (Cregg and Russell 1998), pPIC-A4 was linearized with *SalI* and transformed to *P. pastoris* spheroplasts.

Purification and iodination of AVR4.

P. pastoris GS115 His⁺/mut⁺ transformants transgenic for *Avr4* were selected for production of the AVR4 protein in BMMY medium as described in the Invitrogen manual (version L). Large-scale AVR4 production and purification was performed essentially according to Van den Burg and associates (2001). Briefly, a fermentation protocol was used employing FM22 medium (Stratton et al. 1998), which resulted in high cell densities of *P. pastoris* and high levels of heterologous protein production. Cell-free culture filtrate containing the AVR4 protein was applied to an equilibrated Phenyl Sepharose Fast Flow column (Amersham Pharmacia, Roosendaal, The Netherlands). Following extensive washing with buffer B (10 mM Tris-HCl, pH 8.6, 1 mM EDTA, and 45% saturated (NH₄)₂SO₄), the AVR4 protein was eluted with a linear gradient of 300 ml from 100% buffer B to 100% buffer A (10 mM Tris-HCl, pH 8.6, and 1 mM EDTA). The AVR4-containing fractions were pooled, dialyzed against buffer A, and loaded on a Q-Sepharose Fast Flow column (Amersham Pharmacia). The flow through, which contained the AVR4 protein, was acidified with trifluoroacetic acid (TFA) and injected

on a C4 reverse phase-HPLC column (Waters Chromatography Div., Etten-Leur, The Netherlands). Following elution at 30% acetonitril and 0.1% TFA in water, fractions containing AVR4 were collected and lyophilized prior to storage.

The purified AVR4 protein was nonradio-iodinated with ¹²⁷I and radio-iodinated with ¹²⁵I following a lactoperoxidase labeling protocol (ANAWA Trading SA, Zürich, Switzerland). AVR9 was iodinated as described by Kooman-Gersmann and associates (1996). Iodination of AVR4 resulted in monolabeled ¹²⁷I-AVR4 and ¹²⁵I-AVR4 proteins, as determined by HPLC and MALDI-TOF mass spectrometry (H. A. Van den Burg, unpublished data). These proteins were lyophilized from a solution containing 5% D-mannitol, 0.5% BSA, 1 mM methionine, 21 mM butanesulfonic acid, and 80 KIU aprotinin in 225 mM sodium phosphate buffer (pH 5.0). Stock solutions of the proteins were prepared in water and stored at -20°C. The specific radioactivity of mono-iodinated ¹²⁵I-AVR4 and ¹²⁵I-AVR9 was 2,130 and 2,200 Ci/mmol, respectively.

Oxidative burst assay.

Oxidative burst assays on suspension-cultured tobacco cells expressing *Cf-4* were performed according to De Jong and associates (2000). Briefly, cell suspensions were grown for 4 days after subculturing at 25°C on a gyratory shaker at 120 rpm. Subsequently, cells were harvested and washed with assay buffer (50 mM MES/NaOH, pH 5.7, 175 mM mannitol, 0.5 mM K₂SO₄, and 0.5 mM CaCl₂). Aliquots of the cells (250 mg of cells per 250 μ l of assay buffer) were supplied with the fluorescent probe pyranin (10 μ g/ml) (Apostol et al. 1989) and cells were challenged with unlabeled AVR4 or ¹²⁷I-AVR4 at final concentrations of 0.3 and 1.5 nM. Directly after addition of the elicitor, the quenching of pyranin fluorescence was recorded at an excitation wavelength of 405 nm and an emission wavelength of 512 nm.

Binding assay.

A number of experiments have been carried out to minimize aspecific binding and optimize specific binding. The effects of pH (pH 5.0 to 9.0), ionic strength (5 mM up to 1 M MgCl₂ or

Fig. 7. Chemical crosslinking of ¹²⁵I-AVR4. The affinity crosslinking experiment was performed on microsomal membrane fractions (MFs) isolated from **A**, *Oidium lycopersicum*-infected MM-Cf4 leaves **B**, leaves of MM-Cf4 plants that were grown in confined compartments, and **C**, on fractions containing *C. fulvum* mycelium extracts. Aliquots of MFs (**A** and **B**, 48 μ g of protein per assay) and *C. fulvum* extracts (**C**, 1 μ g of protein per assay) were treated with 0.07 nM ¹²⁵I-AVR4 alone (-) or in combination with a 500-fold molar excess of unlabeled AVR4 (+). Prior to crosslinking, unbound ¹²⁵I-AVR4 was removed by centrifugation. Crosslinking was initiated by adding different concentrations of the crosslinker bis-sulphosuccinimidyl-suberate (BS³) (**A** and **B**: final concentrations are indicated) or by adding BS³ at a final concentration of 33.5 mM (**C**). The crosslinked proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis under reducing conditions and analyzed by autoradiography.

NaCl), and the concentration of MFs (up to 100 µg of protein per assay) were analyzed. MFs were incubated with an appropriate concentration of ^{125}I -AVR4 (up to 0.2 nM final concentration) and unlabeled AVR4 (up to 2,000-fold molar excess) at different temperatures. Eventually, the following experimental conditions were established as optimal. MFs were resuspended in binding buffer (Bb) (10 mM sodium phosphate buffer [NaPi], pH 6.0, 5 mM MgCl_2 , and 0.1% BSA). Subsequently, aliquots of MFs (48 µg of protein in a final volume of 100 µl) were incubated with ^{125}I -AVR4 or ^{125}I -AVR9 (0.07 nM final concentration) alone (total binding) or in combination with a 500-fold molar excess of unlabeled AVR4 or AVR9 (aspecific binding), respectively. The same conditions were used when binding was performed with ^{125}I -AVR4 or ^{125}I -AVR9 on plasma membrane-enriched fractions (48 µg of protein per reaction). In competition experiments, aliquots of MFs (20 µg of protein per assay) were incubated with ^{125}I -AVR4 and various concentrations of different competitor proteins. Specific binding was determined by subtracting aspecific binding from total binding. Binding assays were terminated by filtration through glass fiber filters (Multiscreen-FB, 1.0-µm glass fiber, Millipore Corp., Etten-Leur, The Netherlands) that were impregnated with 0.5% (wt/vol) polyethylenimine for 30 min and subsequently washed with 100 µl of wash buffer (Bb supplied with 1 M NaCl, pH 6.0) (Bruns et al. 1983). Following filtration, filters were washed three times with 100 µl of wash buffer. The radioactivity retained on the filters was measured by liquid scintillation counting (model LS-6000 TA; Beckman Instruments, Irvine, CA, U.S.A.).

Proteinase treatment.

MFs (48 µg of protein/reaction) were incubated in MB3 buffer for 2 h at 25°C with proteinase K from *Tritirachium album* (Merck) at 5 or 15 mg/ml. In control assays, MFs were incubated with heat-treated proteinase K (incubated for 15 min at 100°C) at similar conditions at 15 mg/ml. Prior to incubation with ^{125}I -AVR4, the mixture containing the microsomal membranes and proteinase K was heated for 15 min at 95°C.

Chemical crosslinking of ^{125}I -AVR4.

MFs (48 µg of protein per reaction) were incubated with ^{125}I -AVR4 at a final concentration of 0.07 nM, either alone or in combination with a 500-fold molar excess of unlabeled AVR4. Following incubation with ^{125}I -AVR4 for 4 h at 37°C, the incubation mixture was centrifuged for 30 min at 100,000 × g to remove unbound ^{125}I -AVR4. The pellet was resuspended in 1 mM NaPi buffer, pH 6.0, and crosslinking was initiated by adding increasing amounts (0.05 up to 33.5 mM final concentration) of the crosslinking reagents bis-sulphosuccinimidyl-suberate (BS^3), MBS, sulfo-HSAB (Pierce Chemical), or glutaraldehyde (Sigma, Zwijndrecht, The Netherlands). Crosslinking reactions were performed at 25°C for 30 min and reaction mixtures containing sulfo-HSAB were subsequently irradiated for 5 min with UV light (254 nm). In the case of *C. fulvum* mycelium extracts, 1 µg of total protein was used per reaction and BS^3 was used at a concentration of 33 mM. All crosslinking reactions were terminated by adding SDS loading buffer (50 mM Tris-HCl, pH 6.8, 5% [wt/vol] SDS, 10% [vol/vol] glycerol, 5% 2-mercaptoethanol, and 0.0025% bromophenol blue). Samples were incubated at 95°C for 5 min and separated by SDS-PAGE on gels containing 7.5% (wt/vol) acrylamide (Laemmli 1970). Gels were dried and analyzed by autoradiography.

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