Assignment of Amino Acid Residues of the AVR9 Peptide of *Cladosporium fulvum* That Determine Elicitor Activity

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The AVR9 peptide of *Cladosporium fulvum* is an elicitor of the hypersensitive response in tomato plants carrying the *Cf-9* resistance gene (MM-Cf9). To determine the structure-activity relationship of the AVR9 peptide, amino acids important for AVR9 elicitor activity were identified by independently substituting each amino acid of AVR9 by alanine. In addition, surface-exposed amino acid residues of AVR9 were substituted by other amino acids. Activity of the mutant *Avr9* constructs was studied by expressing the constructs in MM-Cf9 tomato plants, using the potato virus X (PVX) expression system and assessing the severity of necrosis induced by each PVX::*Avr9* construct. This allowed direct identification of amino acid residues of AVR9 that are essential for elicitor activity. We identified amino acid substitutions that resulted in AVR9 mutants with higher, similar, or lower elicitor activity compared to the wild-type AVR9 peptide. Some mutants had completely lost elicitor activity. A selection of peptides, representing different categories, was isolated and injected into leaves of MM-Cf9 plants. The necrosis-inducing activity of the isolated peptides correlated well with the necrosis induced by the corresponding PVX::*Avr9* derivatives. Based on the necrosis-inducing activity of the mutant AVR9 peptides and the global structure of AVR9, we assigned sites in AVR9 that are important for its necrosis-inducing activity. We postulate that the “hydrophobic β -loop” region of the AVR9 peptide is crucial for necrosis-inducing activity in tomato plants that carry the *Cf-9* resistance gene.

Additional keywords: alanine scan, cystine knot, gene-for-gene, site-directed mutagenesis.

Host specificity in plant-pathogen interactions has been described by the gene-for-gene model (Flor 1971). This model postulates that resistance is regulated by specific recognition of the avirulence factors of the invading pathogen by the plant (De Wit 1992). Although the interactions between plants and

bacteria, viruses, nematodes, and fungi comply with the gene-for-gene model, details on the molecular interaction between avirulence and resistance gene products remain unclear. Recently, the structure of a number of plant resistance genes has been revealed, and their general features show striking similarities (Bent 1996; Staskawics et al. 1995). The avirulence genes, however, appear more variable, and knowledge of their structure and intrinsic function remains limited (De Wit 1995; Dangl 1994). This has hampered progress on elucidation of the molecular mechanisms involved in gene-for-gene-based interactions.

So far, a number of bacterial avirulence genes have been cloned (Dangl 1994; Pirhonen et al. 1996), but the activity of their encoded products remains elusive. This is most likely due to the fact that they are not extracellular, like fungal elicitor products, but are delivered directly into the plant cell by the type III secretion system (Jones 1997; Leister et al. 1996; Van den Ackerveken et al. 1996; Gopalan et al. 1996). The products of two viral avirulence factors have been studied in more detail. The coat protein (CP) of tobacco mosaic virus (TMV) triggers resistance in tobacco plants expressing the *N* resistance gene. The elicitor-active parts of the CP have been studied extensively, and a domain within the TMV CP has been assigned whose structural integrity is crucial for recognition by the host (Taraporewala and Culver 1996). The CP of potato virus X (PVX) triggers resistance in potato plants expressing the *Rx1* gene (Bendahmane et al. 1995). As yet, few fungal avirulence genes encoding race-specific elicitors have been cloned. The NIP1 elicitor peptide of the barley pathogen *Rhynchosporium secalis* is a small race-specific elicitor that triggers defense responses, including the activation of the *PRHv-1* gene, in barley cultivars carrying resistance gene *Rrs1* (Rohe et al. 1995).

The interaction between the leaf mold pathogen *Cladosporium fulvum* and its only host, tomato (*Lycopersicon esculentum*), is a well-established model system for studying gene-for-gene-based resistance (De Wit 1995). An incompatible *C. fulvum*-tomato interaction is associated with the hypersensitive response (HR), which is thought to play a central role in disease resistance. HR is characterized by the rapid death of host cells adjacent to the site of fungal penetration and the activation of a variety of defense responses in the surrounding area (Dangl et al. 1996). The *C. fulvum* avirulence genes *Avr4* and *Avr9* have been cloned, and their products, AVR4 and

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AVR9, have been characterized (Joosten et al. 1994; Van Kan et al. 1991; Van den Ackerveken et al. 1992). The AVR4 and AVR9 elicitors trigger HR in tomato plants carrying resistance genes *Cf-4* and *Cf-9*, respectively. The mature AVR4 elicitor consists of 86 amino acids, of which 8 are cysteine residues (Joosten et al. 1997). The AVR9 elicitor is encoded as a 63 amino acid pre-propeptide. Like the AVR4 peptide, AVR9 is secreted by the fungus when it infects tomato plants. The mature AVR9 elicitor peptide consists of 28 amino acids (Van den Ackerveken et al. 1993). Injection of the AVR9 peptide into leaves of cv. MoneyMaker (MM-Cf0) tomato plants and leaves of a near isogenic line carrying the *Cf-9* resistance gene (MM-Cf9) results in necrosis only in the MM-Cf9 genotype. The tomato *Cf* resistance genes, *Cf-2* (Dixon et al. 1996), *Cf-4* (Jones et al. 1996), *Cf-4A* (F. Takken, *personal communication*), *Cf-5* (Jones et al. 1996), and *Cf-9* (Jones et al. 1994), recently have been cloned and characterized. All CF proteins are predicted to be extracytoplasmic, with a transmembrane domain and a small cytoplasmic domain. *Cf-9* encodes an 863 amino acid protein containing 27 imperfect leucine-rich repeats and 22 potential glycosylation sites (Jones and Jones 1996). Although the *Cf-9* resistance gene only confers resistance to races of *C. fulvum* expressing the corresponding *Avr9* gene, a binding site for the AVR9 elicitor was found in both MM-Cf0 and MM-Cf9 genotypes, as well as in other solanaceous plant species (Kooman-Gersmann et al. 1996). The definite role of the described binding site in AVR9-induced resistance in tomato plants carrying the *Cf-9* genes is not yet understood.

¹H nuclear magnetic resonance studies have resolved the global fold of the AVR9 peptide (Vervoort et al. 1997). The AVR9 peptide consists of three antiparallel β -sheets and one extended loop, as is schematically represented in Figure 1. The AVR9 peptide has a very compact and stable structure due to three disulfide bridges that are arranged in a knot. The elicitor has structural homology to peptides belonging to the group of cystine-knotted peptides (Pallaghy et al. 1994; Isaacs 1995). A generally applied strategy to decode the structure-activity relationships of peptide or protein domains is alanine-scanning mutagenesis (Cunningham and Wells 1989; Sahm et al. 1994; Reidhaar-Olson et al. 1996). This involves a systematic study in which each single amino acid residue of a peptide is independently substituted by alanine. More detailed information on the amino acid residues that are important for biological activity of a peptide can be obtained by selectively substituting solvent-exposed amino acids by other amino acid residues. We have performed a combination of alanine-scanning mutagenesis and selective amino acid substitutions to study the structure-activity relationships of the AVR9 peptide.

The PVX expression system has been used to transiently express heterologous genes in solanaceous plants (Chapman et al. 1992). Expression of the wild-type *Avr4* or the wild-type *Avr9* gene of *C. fulvum* by the PVX expression system causes systemic necrosis only in tomato plants carrying the *Cf-4* or *Cf-9* resistance gene, respectively (Joosten et al. 1997; Hammond-Kosack et al. 1995), but not in MM-Cf0 or tobacco plants. In this study, mutants of the *Avr9* gene were produced by site-directed mutagenesis (Horton and Pease 1991) and expressed in MM-Cf9 plants by the PVX expression system. The elicitor activity of mutant AVR9 peptides was analyzed by detailed assessment of the severity of systemic necrosis induced in the

mutant PVX::*Avr9*-infected plants compared to the necrosis induced by wild-type PVX::*Avr9*. This approach allowed the assignment of amino acid residues within the AVR9 peptide that are important for its elicitor activity.

RESULTS

Necrosis induced by PVX::*Avr9* constructs on MM-Cf9 plants.

MM-Cf9 plants infected with mutant PVX::*Avr9* derivatives were screened for development of systemic necrotic lesions. The development of necrosis was followed over time for a period of 21 days, and symptoms were divided into six classes: 1 (–), 2 (+), 3 (++), 4 (+++), 5 (++++), and 6 (+++++). Figure 2A shows typical examples of plants with various degrees of necrosis. In class 1 (–), only mild mosaic symptoms caused by the PVX::*Avr9* derivatives were visible on the infected MM-Cf9 tomato plants. In class 2 (+), the inoculated cotyledons of the plant died, and necrotic spots were observed at the tips of the inoculated compound leaves. Plants continued to grow, and occasionally, small necrotic spots appeared on the older leaves. In class 3 (++), the cotyledons and inoculated leaves showed necrosis, and systemic spread of small necrotic spots was observed on all leaves of the plant. In class 4 (+++), spreading of both smaller and larger necrotic spots was observed throughout the plant, and occasionally, leaves turned completely necrotic. The apical meristem of these plants was not affected, and therefore, the plants continued to grow, while the older leaves eventually died. In class 5 (++++), plants showed complete systemic necrosis and died approximately 2 weeks

Fig. 1. Schematic representation of the structure of the *Cladosporium fulvum* AVR9 peptide, based on the common structure of cystine-knotted peptides (adapted from Pallaghy et al. 1994).

after inoculation. The first necrotic symptoms appeared at 4 to 5 days after inoculation. In class 6 (++++), plants died within 10 days after inoculation, whereas the first necrotic symptoms appeared approximately 3 days after inoculation.

Symptoms were observed on plants inoculated 3 to 4 weeks after sowing. Inoculations with all mutant PVX::Avr9 constructs were repeated three to six times and were reproducible, i.e., the necrosis observed was always typical for each particular mutant compared to wild-type PVX::Avr9-infected plants. Growth conditions affected necrosis to some extent, but the differences in necrosis-inducing activity between the mutants were consistent. Also, variation was observed when plants of a different age were used. Young plants died more rapidly than

older plants, but again, the differences between the various mutant PVX::Avr9 constructs remained consistent.

Effect of amino acid substitutions on necrosis-inducing activity.

The cystine-knotted structure of the AVR9 peptide, schematically represented in Figure 1, shows three antiparallel β -sheets, a short loop (F21 to L24), and an extended loop (T07 to R18). Disruption of the cystine-knotted structure by modification of any of the three disulfide bonds (C02-C16, C06-C19, or C12-C26) led to complete loss of necrosis-inducing activity of the peptide. Residues C16, C19, and C26 were not modified, because they were expected to give results identical to

Fig. 2. Necrosis-inducing activity of wild-type and mutant *Cladosporium fulvum* AVR9 peptides. **A**, Classes of necrosis induced by wild-type (class 5, +++) and mutant potato virus X (PVX)::Avr9 derivatives. A representative of each class was selected, and their respective PVX::Avr9 constructs were inoculated on MM-Cf9 tomato plants. Class 1 (-) is represented by both a cysteine mutant (C02A) and the PVX⁻ vector, without the Avr9 gene. Photographs were taken at day 10 postinoculation. **B**, Necrosis-inducing activity of wild-type and mutant AVR9 peptides isolated from PVX::Avr9-inoculated *Nicotiana clelandii*. Ten microliters of different concentrations of peptide was injected near the midvein of the leaf. Necrosis was visible 1 day after injection, and photographs were taken when the necrotic spot had dried 7 days after injection. Injections were repeated twice with similar results.

their counterparts C2, C6, and C12. Table 1 shows the symptoms observed after inoculation of MM-Cf9 plants with mutant PVX::Avr9 derivatives coding for AVR9 peptides with single alanine substitutions. Most alanine substitutions did not result in substantial differences in necrosis-inducing activity (+++ or +++) compared to the wild-type PVX::Avr9 (++++). Substitution of the amino acids in the N-terminal part of the extended loop region, from position T07 to D11, greatly affected the necrosis-inducing activity of the AVR9 elicitor. The mutant PVX::Avr9 constructs, encoding amino acid residues T07, R08, A09, F10, or D11 substituted by alanine or valine (A09V), all had decreased necrosis-inducing activity (+ or ++) (Table 1). The short loop of AVR9, which comprised amino acids F21 to L24, was localized in the same region of the peptide as amino acid residues T07 to D11 (Vervoort et al. 1997; Fig. 1). Within this short loop, two mutants (F21A and L24A) had completely lost necrosis-inducing activity. Furthermore, two mutants outside the loop regions (N03A and H28A) had decreased necrosis-inducing activity. Substitution of glutamine (Q25A), which is located in the third β -sheet, resulted in an increase in necrosis-inducing activity (+++++) compared to wild-type AVR9 (++++).

In addition, a subset of amino acids of the AVR9 peptide, which are solvent-exposed, was substituted by different amino acid residues. The effect of these substitutions and some double substitutions on the necrosis-inducing activity of their respective PVX::Avr9 derivative is shown in Table 2. Most of the additional PVX::Avr9 mutants showed similar effects on necrosis-inducing activity, as was found for the alanine mutants (Table 1). The significance of the extended loop region for AVR9 necrosis-inducing activity was confirmed by the R08S and F10S PVX::Avr9 constructs, because both resulted in a decrease in necrosis-inducing activity compared to wild-type AVR9. The R08K mutation resulted in a PVX construct with higher necrosis-inducing activity than wild-type AVR9. This also was observed for the R18K mutation. Not only the F21A and L24A mutants but also PVX::Avr9 derivatives encoding the F21S and L24S mutant peptides were inactive, confirming the importance of the F21 and L24 residues for AVR9 necrosis-inducing activity. The basic amino acids H22 and K23, which are located between these two crucial residues, were not important for necrosis-inducing activity of the AVR9 peptide. Both alanine substitutions (H22A and K23A) and substi-

tutions by neutral amino acid residues (H22L and K23Q) retained activity (Tables 1 and 2). The necrosis-inducing activity of the double mutants, which showed up as cloned polymerase chain reaction (PCR) products during sequencing, confirmed the results obtained with the single amino acid substitutions.

Virus titer and elicitor activity of AVR9 peptides isolated from PVX::Avr9-infected *Nicotiana clelandii*.

Several control experiments were performed to prove that the observed necrosis was not caused by a difference in PVX::Avr9 virus titer but by a difference in the necrosis-inducing activity of the encoded peptides. A dilution series was made of sap of PVX::Avr9-infected *N. clelandii* plants, including PVX derivatives containing the wild-type Avr9 gene (+++++) and the R08K (+++++) and H22L (+++) Avr9 mutants. After inoculation of MM-Cf9 plants with 10- to 1,000-fold-diluted sap, the severity of necrosis was assessed over time. Necrosis induced by the various dilutions, containing different virus titers, was similar, and differences between the mutants remained (results not shown). This indicates that the virus titer initially used in the undiluted sap was sufficient to ensure optimal infection of MM-Cf9 plants and that the necrosis-inducing activity was independent of the PVX virus titer. For several of the mutants, two independent PVX::Avr9 constructs were made and inoculated on MM-Cf9 plants. Identical, independently made constructs always gave comparable results.

A selection of mutant AVR9 peptides was isolated from PVX-infected *N. clelandii* plants. These included R08K, L24S, and H28L, representing peptides with, respectively, higher, no, or lower elicitor activity compared to wild-type AVR9. The peptides were purified, and the last purification step on reversed-phase high-performance liquid chromatography (HPLC) resulted in single peaks for all the peptides (data not shown). Approximately 1 to 2 μ g of wild-type or mutant AVR9 peptide could be isolated per inoculated *N. clelandii* plant, even of the inactive mutant peptide L24S. Necrosis-inducing activity of the mutant AVR9 peptides was assayed by injection of a dilution series into leaflets of MM-Cf9 tomato plants. Figure 2B shows an injection series of the mutant and wild-type AVR9 peptides. Wild-type AVR9, isolated from PVX::Avr9-infected tobacco leaves, showed necrosis at a concentration of $\sim 0.3 \mu$ M. The more active peptide in the PVX-based assay, R08K (+++++), induced necrosis at concentrations as low as $\sim 0.1 \mu$ M, whereas the less active mutant in the PVX-based

Table 1. Effect of alanine substitutions on the necrosis-inducing activity (NIA) of the AVR9 peptide of *Cladosporium fulvum*, tested by inoculation of MM-Cf9 tomato plants with potato virus X::Avr9 derivatives^a

Mutant	NIA	Mutant	NIA
Wild-type	++++	L13A	+++
Y01A	+++	G14A	++++
C02A	–	Q15A	++++
N03A	++	G17A	+++
S04A	+++	R18A	++++
S05A	+++	D20A	+++
C06A	–	F21A	–
T07A	++	H22A	+++
R08A	+	K23A	++++
A09V	+	L24A	–
F10A	++	Q25A	+++++
D11A	++	V27A	++++
C12A	–	H28A	++

^a The different classes of necrosis-inducing activity are 1 (–), 2 (+), 3 (++), 4 (+++), 5 (++++), and 6 (+++++) (shown in Figure 2A).

Table 2. Effect of nonalanine substitutions on the necrosis-inducing activity (NIA) of the AVR9 peptide of *Cladosporium fulvum*, tested by inoculation of MM-Cf9 tomato plants with potato virus X::Avr9 derivatives^a

Mutant	NIA	Mutant	NIA
Wild-type	++++	D20N	++++
S04A + G17E	+++	F21S	–
R08S	+	H22L	+++
R08K	+++++	K23Q	+++
F10S	+	L24S	–
D11N	+++	Q25E	++++
Q15A + R18S	++++	Q25E + V27I	++++
L13A + C16stop	–	C26G	–
R18K	+++++	H28L	++

^a The different classes of necrosis-inducing activity are 1 (–), 2 (+), 3 (++), 4 (+++), 5 (++++), and 6 (+++++) (shown in Figure 2A).

assay, H28L (++) , required a concentration of ~3 μ M or higher to induce necrosis. The inactive L24S peptide (–) did not induce necrosis at concentrations as high as 10 μ M. Hence, the results of the injection series with purified wild-type and mutant AVR9 peptides are comparable to the results obtained with the respective PVX::Avr9 inoculations on MM-Cf9 plants.

DISCUSSION

The PVX expression system: Ideal for structure-function analyses of AVR9.

Progress in determining active sites of cystine-knotted peptides has been hampered by the lack of a good expression system for these peptides, combined with the lack of a convenient bioassay (Olivera et al. 1994; Nielsen et al. 1996). We show that the PVX expression system is well suited for mutational analysis of the AVR9 peptide and for testing necrosis-inducing activity of AVR9 mutants on MM-Cf9 tomato plants.

We determined structure-activity relationships of the AVR9 peptide by alanine-scanning mutagenesis and expressed the mutant peptides directly in MM-Cf9 tomato plants by the PVX expression system. During systemic spread of PVX, we did not observe a shift in the necrosis-inducing activity of any of the mutant PVX::Avr9 constructs. Therefore, we conclude that the PVX constructs remain stable during viral spread through the plant and that no loss of insert occurs, as has been described by Hammond-Kosack et al. (1995). Furthermore, between 1 and 2 μ g of wild-type or mutant AVR9 peptide could be isolated per inoculated *N. clevelandii* plant, even for the inactive mutant peptide L24S. This confirms that the PVX expression system provides a reliable and reproducible method to determine elicitor-active regions of AVR9.

Effect of amino acid substitutions on necrosis-inducing activity of AVR9.

The PVX::Avr9 derivatives carrying alanine substitutions in residues T7 to D11 showed a decrease in necrosis-inducing activity. This indicates that these first five amino acid residues of the large extended loop of AVR9 are important for necrosis induction. These results are confirmed by the decrease or increase (R08K) in necrosis-inducing activity of the additional mutants in this region. Furthermore, the hydrophobic residues F21 and L24 in the small loop of AVR9 are crucial determinants of elicitor activity. This was shown by the complete loss of necrotic symptoms when these residues were substituted by alanine or serine. The two important sites in AVR9, T7 to D11 and F21 and L24, are in close proximity. This is shown in more detail in Figure 3, which represents the global structure of AVR9. AVR9 has a hydrophobic region, consisting of the three residues F10, F21, and L24, which are in solvent-exposed positions. On one side, the hydrophobic amino acids are flanked by the basic residues H22 and K23, which do not seem essential for necrosis induction. The acidic residue D11 is located on the opposite side of the hydrophobic core. The hydrophilicity of the side chain of D11 may be important for the structure or necrosis-inducing activity of AVR9, because substitution by asparagine (D11N) produced fairly strong necrosis (+++), whereas substitution by an alanine residue showed further reduction of necrosis (++) . The basic R08 also flanks F10 and F21 (Fig. 3). The R08K mutant showed an increase in necrosis-inducing activity compared to the wild-type AVR9, indicating the hydro-

phobic part of this residue and its positive charge are involved in necrosis induction. Substitution of R08 to A or S led to a decrease in necrosis-inducing activity, confirming the importance of the characteristics of this residue. Even small changes in the extended loop of AVR9 influenced activity, as shown by the decrease in necrosis-inducing activity of the A09V mutant. Three mutant PVX::Avr9 derivatives, coding for amino acid substitutions outside the hydrophobic region of AVR9 (N03A, H28A, and H28L), also showed reduced necrosis. Possibly, these substitutions led to perturbation of the global fold of AVR9. As expected, preventing formation of the cystine-knotted structure by disruption of one of the three disulfide bonds (C02-C16, C06-C19, or C12-C26) led to a complete loss of necrosis-inducing activity of the AVR9 peptide.

Comparison of AVR9 with structurally related peptides.

AVR9 belongs to a family of structurally related peptides that share a cystine-knot motif. The cystine-knot motif has been observed in many apparently unrelated peptides, including growth factors (McDonald and Hendrickson 1993), ω -conotoxins (Sevilla et al. 1993), the squash-seed family of protease inhibitors (Bode et al. 1989; Heitz et al. 1989), the sweet-taste suppresser gumarin (Arai et al. 1995), and kalata B1, a cyclic plant peptide (Saether et al. 1995). Although some family members display similar biological activities and have comparable three-dimensional structures, they share very little sequence similarity. Where known, the peptides have a high affinity with their target proteins. Hence, it appears that this motif is able to accommodate a wide range of sequences and biological activities. For some of the cystine-knotted peptides, the active sites have been identified by chemical synthesis of analogues, followed by activity and binding analyses (Kim et al. 1994; Sato et al. 1993). Where known, the active sites of the peptides, as of AVR9, are located in the solvent-exposed regions, never in the core cystine region (Kim et al. 1994; Le-Nguyen et al. 1993).

AVR9 structurally is most closely related to carboxy peptidase inhibitor (Vervoort et al. 1997). The active site of carboxy peptidase inhibitor is located in the C-terminal tail of the peptide and comprises Y-V-G, a sequence that is not present in AVR9. For the cystine-knotted trypsin inhibitors, the trypsin-binding loop comprises the amino acid residues P-R-I-L-M (Cliche et al. 1993), a site that also is absent in the AVR9 peptide. The P, I, and M residues of the trypsin inhibitors form a hydrophobic site. Peptidase inhibitors of *N. alata* are structurally somewhat less related to AVR9 (Nielsen et al. 1994). The typical trypsin-inhibiting region of these peptides comprises amino acid residues R and N, whereas the chymotrypsin-inhibiting region comprises amino acid residues L and N, which are present on an exposed loop of the protein. Again, these sites are not present in AVR9. So although the structure of AVR9 closely resembles that of many peptidase inhibitors, AVR9 does not contain amino acid residues identical to the known inhibitory sites. In preliminary experiments with wild-type AVR9, no protease-inhibiting activity was found (results not shown). Additional assays are required to ascertain the lack of protease-inhibiting activity of AVR9.

In addition to protease inhibitors, AVR9 is structurally related to several ion-channel blockers or toxins. As yet, no toxic activity of AVR9 has been reported. The ω -conotoxin GVIA (ω -CTX GVIA) from *Conus geographus* venom binds to Ca²⁺

channels, with very high affinity. For ω -CTX GVIA, the amino acid residues important for binding and channel-blocking activity have been assigned by alanine scanning (Kim et al. 1994). Most alanine substitutions do not affect the inhibiting activity of the peptide, except for substitution of K2, which leads to a 40-fold decrease of Ca^{2+} channel-inhibiting potential (Sato et al. 1993), and substitution of Y13, which leads to a dramatic 1,000-fold decrease in Ca^{2+} channel binding (Kim et al. 1994). The cystine-knotted structure of ω -CTX GVIA is required for its activity, because linearization renders the molecule inactive (Kim et al. 1994). This also holds for AVR9, in which linearization abolishes necrosis-inducing activity of the peptide (Kooman-Gersmann et al. 1996).

For the cyclic cystine-knotted peptide kalata B1, no structure-activity data are available. One of the features of this peptide is the relatively large hydrophobic surface present on one side of the peptide (W02-P03-V04 and V18-G19, representing two flanking loops). The elicitor-active site of AVR9 also has an exposed hydrophobic region comprising two flanking loops that contain the two phenylalanines and a leucine (F10, F21, and L24). For kalata B1, it is speculated that the flexible loop (residues 8 to 12, N-G-L-P-V) may be important for activity (Saether et al. 1995). Several other channel-blocking peptides have exposed, hydrophobic amino acid residues flanked by positively charged residues, which are proposed to be part of the channel-binding surface (Manoleras and Norton 1994). These features also are found in AVR9, in which the F10, F21, and L24 residues are solvent-exposed and flanked by the positively charged R08, H22, and K23 residues.

We conclude that although the cystine-knot peptides share structural similarities their functional similarities are less obvious. The cystine-knot structure seems to be an energetically favorable structure that is well suited to exhibit inhibitory or channel-blocking activities. Next to their common structural features, the only additional similarity between AVR9 and the known cystine-knotted peptides is the presence of hydrophobic surface regions. The hydrophobic surfaces of the channel-inhibiting proteins are thought to embody the inhibiting regions. Although no inhibitory function of AVR9 is known yet, we speculate that the elicitor-active hydrophobic surface of AVR9 may not be involved only in necrosis induction but may reflect its intrinsic biological function as a pathogenicity factor. Analogous to other cystine-knotted peptides, AVR9 may exhibit an inhibitory or stimulating function in plant metabolism by interfering with a specific ion channel or nutrient pump. The *Avr9* gene is induced under nitrogen-limiting conditions (Van den Ackerveken et al. 1994), suggesting a role for AVR9 in amino acid metabolism or uptake. Although no pathogenicity function of AVR9 has been demonstrated yet, various reported extracellular elicitor peptides produced by *C. fulvum* in planta are likely to play a role in pathogenicity. This has been demonstrated for the ECP1 and ECP2 peptides of *C. fulvum* (Laugé et al. 1997). Possibly, these different extracellular peptides provide the fungus with a large array of possibilities to retrieve nutrients from the plant. The specific recognition of the AVR9 peptide by tomato plants carrying the *Cf-9* resistance gene shows the versatility of plants to recognize foreign peptides through a process of coevolution.

Fig. 3. Surface views of the global structure of the *Cladosporium fulvum* AVR9 peptide (Vervoort et al. 1997). The left panel shows the effect of amino acid substitutions on the necrosis-inducing activity of AVR9. Residues representing the necrosis-inducing activity of classes 4, 5 and 6 (+++, +++++, and +++++, respectively) are in light blue, cystines are in lime, residues representing the necrosis-inducing activity of classes 2 and 3 (+ and ++, respectively) are in pink, and the F21 and L24 residues representing mutants that have lost elicitor activity (-) are in red. Residues T7 to D20 in Vervoort et al. (1997), because they used a 33 amino acid AVR9 peptide in their studies. The right panel shows the charge distribution of the AVR9 molecule. Positively charged residues are in blue, negatively charged residues are in red, polar residues are in green, hydrophobic residues are in yellow, and glycine residues are in white.

MATERIAL AND METHODS

Cloning and mutagenesis.

Standard molecular biological techniques were performed as described by Sambrook et al. (1989). Primer-directed mutagenesis of the *Avr9* gene was performed by PCR overlap extension, essentially according to the protocol of Horton and Pease (1991). The wild-type PVX::*Avr9* plasmid vector (Hammond-Kosack et al. 1995) was used as template. Primers were designed to introduce the chosen mutations into the target codons, as listed in Table 3 for alanine substitutions and in Table 4 for other substitutions. All primers were 20 to 23 bp long, with a minimal calculated melting temperature (T_m) of 50°C ($T_m = 2(A-T) + 4(G-C)$, excluding the mutational mismatches). In the first mutagenesis step, two PCR amplification reactions were performed, with the PVX::*Avr9* plasmid vector as template DNA. In the first reaction, we used the OX10 primer (5'-CAA TCA CAG TGT TGG CTT GC-3', upstream of the *Avr9* insert in the PVX vector) as a general primer and a mutagenic primer that created the required mutation. The second reaction included the general primer N31 (5'-GAC CCT ATG GGC TGT GTT G-3', downstream of the *Avr9* insert in the PVX vector) and a mutagenic primer that carried the mismatch complementary to the mutagenic primer used in the first PCR reaction. PCR amplifications were carried out with AmpliTaq DNA Polymerase (Perkin Elmer-Cetus, Norwalk, CT), in a Perkin Elmer GeneAmp 9600 thermocycler at 25 to 40 cycles of 30 s at 94°C, 30 s at 40°C, and 20 s at 72°C. After these PCR reactions, the amplified fragments were isolated, and corresponding fragments were mixed and subjected to overlap extension PCR amplification, using the OX10 and N31 primers. The latter PCR reaction was performed with Ultra DNA Polymerase (Perkin Elmer-Cetus), with PCR conditions identical to the first PCR amplifications. After the two-step mutagenesis procedure, the resulting PCR fragment was digested with *Clal*, and the *Clal* fragment was isolated and ligated into the *Clal* site of the PVX expression vector pTXΔGC3A (Hammond-Kosack et al. 1995). Transformants of *Escherichia coli* DH5α carrying the mutant *Avr9* gene in the correct orientation in the PVX expression vector were selected by PCR screening, and the nucleotide sequence of the PVX construct was verified by DNA sequencing.

Table 3. Codon exchanges made in potato virus X::*Avr9*, encoding alanine and valine (A09V) substitutions

Mutant ^a	Codon exchange	Mutant ^a	Codon exchange
Wild-type	...	L13A	CTT::GCT
Y01A	TAC::GCC	G14A	GGA::GCA
C02A	TGT::GCT	Q15A	CAA::GCA
N03A	AAC::GCC	G17A	GGA::GCA
S04A	AGT::GCT	R18A	AGA::GCA
S05A	TCT::GCT	D20A	GAC::GCC
C06A	TGT::GCT	F21A	TTT::GCT
T07A	ACA::GCA	H22A	CAT::GCT
R08A	AGA::GCA	K23A	AAG::GCG
A09V	GCT::GTT	L24A	CTC::GCC
F10A	TTT::GCT	Q25A	CAA::GCA
D11A	GAC::GCC	V27A	GTA::GCA
C12A	TGT::GCT	H28A	CAT::GCT

^a *Cladosporium fulvum* AVR9 peptide mutants.

In vitro transcriptions and plant inoculations.

For in vitro transcription, 1 µg of *Sst*I-linearized PVX plasmid DNA was prepared and used in the mMACHINE in vitro transcription kit (Ambion, Austin, TX). Infectious 5'-capped mRNA was produced according to the manufacturer's instructions. The reaction mix (10 µl) was mixed with 10 µl of H₂O, containing 5 µg of bentonite per µl and 20 µl of 50 mM sodium phosphate buffer, pH 7.0, was added. Leaves of 4-week-old *N. clelandii* plants (four leaves per plant) were inoculated with 10 µl of the mRNA sample. Ten to fourteen days after inoculation, leaves showing systemic mosaic symptoms were harvested for sap preparation. Sap was made by grinding the leaves in a mortar with sand (1 g fresh weight/ml of buffer, 50 mM sodium phosphate, pH 7.0) and stored at -20°C. Three- to four-week-old tomato plants were inoculated with 10 µl of sap on both cotyledons and on the top leaflets of the primary and secondary compound leaf. Plants were grown in a day-night regime of 16 h, 25°C light and 8 h, 20°C dark at 60 to 80% relative humidity. All inoculations were performed at least three times.

Purification of mutant AVR9 peptides and injections.

Several AVR9 peptides were purified from the apoplastic fluid (AF) of *N. clelandii* leaves inoculated with sap containing the mutant PVX::*Avr9* construct of choice. For purification of a mutant AVR9 peptide, 25 5-week-old *N. clelandii* plants were inoculated with sap of leaves from the PVX-infected plants. After 2 weeks, AF was isolated from leaves showing mosaic symptoms, following the method described by De Wit and Spikman (1982). Samples were incubated at 90°C for 10 min to inactivate PVX and denature heat-labile proteins. High molecular weight proteins in the AF were precipitated by adding acetone to a final concentration of 50% (vol/vol) and overnight incubation at -20°C. After centrifugation, the supernatant containing the heat-stable mutant AVR9 peptide was collected, and acetone was removed in a rotary evaporator at 50°C. The mutant peptides were purified further by a combination of cation exchange chromatography on a CM-Sephadex C-25 column (Pharmacia Biotech AB, Uppsala, Sweden), followed by reversed-phase HPLC on a SuperPac Pep-S column (Pharmacia Biotech), essentially as described by Van den Ackerveken et al. (1993). The peptides were quantified by comparing the intensity of the AVR9 bands on silver-stained, low-pH polyacrylamide gels with a defined quantity of AVR9 isolated from *C. fulvum*-infected leaves as a standard (Reisfeld et al. 1962).

Table 4. Codon exchanges made in potato virus X::*Avr9*, encoding non-alanine substitutions

Mutant ^a	Codon exchange(s)	Mutant ^a	Codon exchange(s)
Wild-type	...	R18K	AGA::AAA
S04A + G17E	AGT::GCT + GGA::GAA	D20N	GAC::AAC
R08S	AGA::TCA	F21S	TTC::TCT
R08K	AGA::AAA	H22L	CAT::CTT
F10S	TTT::TCT	K23Q	AAG::CAG
D11N	GAC::AAC	L24S	CTC::TCC
Q15A + R18S	CAA::GCA + AGA::AGC	Q25E	CAA::GAA
L13A + C16 stop	CTT::GCT + TGT::TGA	Q25E + V27I	CAA::GAA + GTA::ATA
		C26G	TGT::GGT
		H28L	CAT::CTT

^a *Cladosporium fulvum* AVR9 peptide mutants.

Dilutions series of the purified peptides were prepared, and 20 µl of each concentration was injected into leaflets of MM-Cf9 near the main vein. Control injections were performed in leaflets of MM-Cf0. All injections were performed twice.

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