The AVR9 Race-Specific Elicitor of Cladosporium fulvum Is Processed by Endogenous and Plant Proteases

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The avirulence gene avr9 of the fungal tomato pathogen Cladosporium fulvum encodes a race-specific peptide elicitor that induces a hypersensitive response in tomato plants carrying the complementary resistance gene Cf9. The avr9 gene is highly expressed when C. fulvum is growing in the plant and the elicitor accumulates in infected leaves as a 28-amino acid (aa) peptide. In C. fulvum grown in vitro, the peptide elicitor is not produced in detectable amounts. To produce significant amounts of the AVR9 elicitor in vitro, the coding and termination sequences of the avr9 gene were fused to the constitutive gpd-promoter (glyceraldehyde 3-phosphate dehydrogenase) of Aspergillus nidulans. Transformants of C. fulvum were obtained that highly expressed the avr9 gene in vitro and produced active AVR9 peptide elicitors. These peptides were partially sequenced from the N terminus and appeared to consist of 32, 33, and 34 aa's, respectively, and are the precursors of the mature 28-aa AVR9 peptide. We demonstrated that plant factors process the 34-aa peptide into the mature 28-aa peptide. We present a model for the processing of AVR9 involving cleavage of a signal peptide during excretion and further maturation by fungal and plant proteases into the stable 28-aa peptide elicitor.

Specific recognition in the gene-for-gene interaction between the fungal pathogen Cladosporium fulvum and tomato is mediated via so-called race-specific elicitors, the primary products of avirulence genes (Van Kan et al., 1991; Van den Ackerveken et al., 1992). Receptors in the resistant plant, which might be the direct products of resistance genes, are thought to recognize the specific elicitors (De Wit, 1992). The subsequent defense reaction is characterized by an HR that restricts growth of the pathogen to the site of penetration.

Race-specific elicitors in the C. fulvum–tomato interaction were first identified by De Wit and Spikman (1982). One such race-specific elicitor, inducing HR on tomato plants carrying the resistance gene Cf9, was purified and characterized (De Wit et al., 1985) and the aa sequence was determined (Scholtens-Toma and De Wit, 1988). The corresponding cDNA was isolated using oligonucleotide probes and designated avr9 (Van Kan et al., 1991). Races of C. fulvum virulent on tomato genotype Cf9 completely lack the avr9 gene and, therefore, do not produce AVR9 peptide elicitor. Transformation of such a virulent race with a genomic clone containing avr9 resulted in transformants that became avirulent on tomato genotype Cf9 (Van den Ackerveken et al., 1992). In addition, disruption of avr9 in wild-type avirulent races of C. fulvum resulted in virulence on tomato genotype Cf9 (Marmeisse et al., 1993). Altogether, these results prove that the avirulence gene avr9 is the only genetic factor responsible for the induction of HR and other defense responses on tomato genotype Cf9 and that this is mediated by its product, the AVR9 race-specific peptide elicitor.

The AVR9 elicitor is an extracellular peptide that is produced by C. fulvum while growing in vivo but not in vitro. The avr9 gene encodes a 63-aa peptide, but in the plant, a 28-aa peptide accumulates predominantly. Expression of avr9 in vitro, as determined by northern blot analysis, has been detected only under conditions of nitrogen starvation. Under these conditions, however, no elicitor activity could be detected in the culture filtrate of in vitro-grown mycelium (G. F. J. M. Van den Ackerveken, unpublished data). High-level expression of avr9 in vitro would enable us to isolate and purify large quantities of AVR9 elicitor, which can be used in future studies on recognition and signal transduction pathways involved in plant defense. Here we report on the construction and analysis of transformants of C. fulvum producing AVR9 in vitro by employing a high-expression promoter of Aspergillus nidulans. Our experiments provide evidence for the involvement of both fungal and plant proteases in the sequential processing of the 63-aa precursor AVR9 into the mature 28-aa race-specific peptide elicitor.

MATERIALS AND METHODS

Subculture of Cladosporium fulvum and AVR9 Bioassay

Cladosporium fulvum Cooke (syn. Fulvia fulva [Cooke] Cif) was grown on potato dextrose agar or in liquid B5-medium in shake cultures (De Wit and Flach, 1979). AVR9 elicitor activity on tomato (Lycopersicon esculentum Mill.) was assayed by injection of 50-μL samples into leaves of cv Moneymaker (no Cf resistance genes) and a near-isogenic line of Money-maker carrying resistance gene Cf9 (genotype Cf9) (De Wit).

Abbreviations: aa, amino acid(s); AVR9, avr9 gene product; gpd, glyceraldehyde-3-phosphate dehydrogenase gene; HR, hypersensitive response; HRLC, high resolution liquid chromatography; IF, intercellular fluid.
and Spikman, 1982). One to 2 d after injection, AVR9 elicitor activity was visible as necrosis (HR) on genotype Cj9 but not on cv Moneymaker.

**Construction of the avr9 Expression Vector**

The *Aspergillus nidulans* gpd promoter (Punt et al., 1988) was employed to constitutively express the *avr9* gene of *C. fulvum* (see Fig. 1). The *avr9* coding and termination region was isolated from plasmid pCF1 (Van den Ackerveken et al., 1992) by digestion with BspHI and HindIII. The uidA gene and trpC terminator were deleted from plasmid pNOM102 (Roberts et al., 1989) by digestion with HindIII and NcoI and replaced by the *avr9* coding and termination region, resulting in plasmid PCF22.

**Isolation of AVR9-Producing Transformants of *C. fulvum***

After race 5 of *C. fulvum* was grown for 48 h in liquid B5 medium, the mycelium was harvested and used for the isolation of protoplasts (Harling et al., 1988). Co-transformation (Oliver et al., 1988) of 10^7 protoplasts with 2 µg of pAN7-1 containing the hygromycin resistance gene *hpt* (Punt et al., 1987) and 4 µg of PCF22 resulted in stable hygromycin-resistant transformants. Five of these transformants were assayed for the production of AVR9 elicitor in vitro and analyzed by Southern blot.

**Purification of AVR9 Elicitor from Culture Filtrate**

Transformants producing AVR9 elicitor in vitro were grown in liquid shake cultures for 7 to 10 d in 50 mL of B5 medium in 300-mL conical flasks at 22°C and 100 strokes min^-1. Cell-free culture filtrate was obtained by filtration of the cultures through filter paper over a Büchner funnel. To 500 mL of culture filtrate, 1 volume of acetone was added and the majority of high mol wt proteins was precipitated overnight at −20°C. After centrifugation, the supernatant was collected and acetone was removed in a rotary evaporator at 50°C. Sodium phosphate buffer (1 M, pH 5.5) was added to the remaining supernatant to a final concentration of 20 mM and adjusted to pH 5.5 with H_3PO_4. The conductivity of the sample was adjusted to <4 mS cm^-1 by dilution with 20 mM sodium-phosphate buffer (pH 5.5). The buffered sample was applied overnight to a CM-Sephadex C-25 column (10 × 2.2 cm), which was preequilibrated with 20 mM sodium phosphate buffer, pH 5.5. The column was subsequently washed with 20 mM sodium phosphate buffer, pH 5.5, until the A_{280} of the effluent returned to baseline level. AVR9 peptides were eluted by washing the column with 20 mM sodium phosphate buffer, pH 5.5. The eluted sample was applied overnight to a CM-Sep-Pak C18 cartridge (Waters), a reversed-phase cartridge for rapid desalting that had been preconditioned with 10 mL of 90% (v/v) acetonitrile, 0.1% (v/v) TFA followed by 10 mL of 0.1% (v/v) TFA. Following application, the cartridge was washed with 10 mL of 0.1% (v/v) TFA. The elutor peptides were eluted from the cartridge with 10 mL of 90% (v/v) acetonitrile, 0.1% (v/v) TFA. Acetonitrile was removed from the eluate in a rotary evaporator at 50°C and the remaining sample was freeze-dried. High-resolution purification was achieved by reversed-phase HRLC (Bio-Rad) using a SuperPac Pep-S column (5 µm, C2/C18, 4 × 250 mm, Pharmacia). The sample (200 µL in 0.1% [v/v] TFA) was applied to the column, which was preequilibrated with 0.1% (v/v) TFA. The column was eluted with a linear gradient of 0.1% (v/v) TFA to 90% (v/v) acetonitrile, 0.1% (v/v) TFA in 30 min at a flow rate of 1 mL min^-1. Fractions of 0.5 mL were collected and freeze-dried. The dried protein fractions in individual peaks were pooled and dissolved in 20 mM Mes, pH 5.5. The sample (200 µL) was applied to a MA7S cation-exchange column (HRLC, 7.8 × 50 mm, Bio-Rad), which was preequilibrated with 20 mM Mes, pH 5.5. Following application, the column was eluted with a gradient of 20 mM Mes, pH 5.5, to 20 mM Mes, pH 5.5, 0.4 M NaCl in 30 min at a flow rate of 1 mL min^-1. Individual peaks containing elicitor activity on genotype Cj9 were analyzed by low pH PAGE.

**PAGE**

Low pH PAGE was performed on 15% (w/v) polyacrylamide slab gels under nondenaturing conditions using pyronine Y as a front marker (Reisfeld et al., 1962). Following electrophoresis at 200 V, the gels were stained and fixed according to Steck et al. (1980). Omitting formaldehyde in the staining and destaining solutions resulted in loss of AVR9 peptides from the gel.

Under denaturing conditions, PAGE was performed on Tricine-SDS-PAGE gels as described by Schägger and Von Jagow (1987).

**Peptide Sequencing**

Purified peptides were sequenced on a gas-phase-sequenator (SON, sequence facility, Leiden, The Netherlands). At least five N-terminal aa’s were determined for all peptides. Twenty amino acids were determined for a mixture of 32- and 33-aa peptides. The 28-aa peptide elicitor was completely sequenced.

**Radioiodination of AVR9 Peptides**

AVR9 peptides of 28 and 34 aa’s were radiolabeled by iodination with Na^125I (Amersham) using Iodobeads (Pierce) as a catalyst. One Iodobead was washed with PBS before incubation with 19.5 MBq Na^125I in 95 µL of PBS for 5 min in an Eppendorf vial. AVR9 peptide was added (5 µL, 5 nmol) and incubated for an additional 15 min at room temperature. Following incubation, the reaction mixture (100 µL) was transferred to a new vial. The remaining Iodobead was washed with 100 µL of PBS. The washing fluid was combined with the reaction mixture (200 µL) and applied to a Sep-Pak C18 cartridge to remove free ^125I. The labeled peptides were eluted from the column with 90% (v/v) acetonitrile, 0.1% (v/v) TFA. The eluted fractions were freeze-dried and redissolved in milli-Q water. The specific activity of the labeled 34-aa AVR9 was 1 to 2 MBq nmol^-1 as determined by liquid scintillation counting.
Processing of the AVR9 Elicitor of Cladosporium fulvum

RESULTS

Isolation of AVR9-Producing Transformants of C. fulvum

For the development of transformants of C. fulvum, which constitutively produced high amounts of the AVR9 elicitor in vitro, a chimeric gene was constructed (designated pCF22) that consisted of the A. nidulans gpd promoter fused to the coding and termination region of the C. fulvum avr9 gene (Fig. 1). Co-transformation of race 5 of C. fulvum with pAN7-avr9, which contained the hygromycin resistance marker and pCF22, resulted in stable hygromycin-resistant transformants. Five transformants were grown in liquid shake culture and the culture filtrate was assayed for elicitor activity.

While some of these transformants produced the AVR9 elicitor in vitro. Southern analysis of these AVR9-producing transformants indicated that 5 to 20 copies of pCF22 were integrated into their genomes.

Isolation, Purification, and Characterization of AVR9 from Culture Filtrate

The AVR9 race-specific elicitor isolated from IF of C. fulvum-infected tomato leaves is a 28-aa peptide (Scholtens-Toma and De Wit, 1988), which migrates on low pH PAGE because of its slightly basic nature (Fig. 2, lane 1). Analysis of the culture filtrates of in vitro-grown C. fulvum transformants producing AVR9 by low pH PAGE showed that the filtrates contained peptides with an electrophoretic mobility different from AVR9 isolated from IF (Fig. 2, lane 2). These same peptides were produced by all three transformants. Although these peptides had a lower mobility on low pH gel, they had similar necrosis-inducing activity on tomato genotype C9 as the 28-aa peptide. Purification of these peptides from culture filtrates by preparative cation-exchange chromatography and reversed-phase chromatography resulted in an enriched mixture of several peptides with necrosis-inducing activity on tomato genotype C9. These peptides were subsequently purified by HRLC employing a combination of cation-exchange chromatography and reversed-phase chromatography. The purified peptides were sequenced from the N terminus and appeared to be precursors of the 28-aa peptide elicitor. The obtained sequence of 20 N-terminal aa's of a mixture of 32- and 33-aa AVR9 peptides overlapped with the sequence of the 28-aa elicitor, which has been sequenced completely three times. The 33- and 34-aa pep-
Table 1. Characteristics of different AVR9 peptides (size, sequence, calculated $M_r$, and isoelectric point [pI]) and the supporting experimental data

<table>
<thead>
<tr>
<th>Size (aa)</th>
<th>aa Sequence</th>
<th>$M_r$</th>
<th>pI</th>
<th>Experimental Data</th>
</tr>
</thead>
<tbody>
<tr>
<td>63</td>
<td>MKLSSVLALL1ATTPLCWAALAPVGLVGVLDCYNSCICRACFDCLQCGRCDFHKLQCYH</td>
<td>6741</td>
<td>7.07</td>
<td>Derived from cDNA sequence</td>
</tr>
<tr>
<td>40</td>
<td>AALPVGLVGVLDCYNSCICRACFDCLQCGRCDFHKLQCYH</td>
<td>4259</td>
<td>7.11</td>
<td>Predicted (based on probability)</td>
</tr>
<tr>
<td>34</td>
<td>LGVGLDCYNSCICRACFDCLQCGRCDFHKLQCYH</td>
<td>3751</td>
<td>7.11</td>
<td>Purified and partially sequenced</td>
</tr>
<tr>
<td>33</td>
<td>GVGLDCYNSCICRACFDCLQCGRCDFHKLQCYH</td>
<td>3637</td>
<td>7.11</td>
<td>Purified and partially sequenced</td>
</tr>
<tr>
<td>32</td>
<td>VGGLDCYNSCICRACFDCLQCGRCDFHKLQCYH</td>
<td>3580</td>
<td>7.11</td>
<td>Purified and partially sequenced</td>
</tr>
<tr>
<td>28</td>
<td>YCNSSCICRACFDCLQCGRCDFHKLQCYH</td>
<td>3195</td>
<td>7.62</td>
<td>Purified and completely sequenced</td>
</tr>
</tbody>
</table>

Figure 3. Proteolytic cleavage of the 34-aa AVR9 peptide by plant factors. Autoradiograph of 125I-labeled AVR9 peptides separated by low pH PAGE. A, Untreated 34-aa (lane 1, major lower band) and 34-aa (lane 2) peptides can be separated by low pH PAGE because they significantly differ in their isoelectric points. A, Labeled 34-aa peptide was incubated with 200 μl of IF from healthy tomato cv Moneymaker and genotype Cf9 for 2 h at 37°C at different pH values. The proteolytic activity is optimal at acidic to neutral pH and marginal at alkaline pH. B, The processing of the 34-aa peptide into the 28-aa form at pH 4 (lane 3) is not inhibited by pepstatin (40 μg ml⁻¹, lane 4) nor by ethanol, a solvent for pepstatin (lane 5). Boiling the IF prior to incubation with the 34-aa peptide resulted in loss of proteolytic activity of IF (lane 6).

DISCUSSION

The avirulence gene *avr9* is highly expressed in *C. fulvum* when growing inside tomato leaves (Van Kan et al., 1991), whereas *C. fulvum* grown in vitro does not produce any tides (Fig. 2, lanes 3 and 4) occur most abundantly, whereas the 32-aa peptide is present in relatively small quantities but co-migrates with the 33-aa peptide on low pH PAGE. The minor peptide band in lane 2 (indicated with an asterisk) was characterized as a mixture of 32, 33, and 34 aa's. Using the Tricine-SDS-PAGE system (Schägger and Von Jagow, 1987), which is recommended for the separation of proteins with a molecular mass <10 kD, the 28- and 32- to 34-aa peptides co-migrated and could not be separated.

The purification of the 28-aa peptide as described by Scholtens-Toma and De Wit (1988) involved preparative low pH PAGE, which precluded the possible isolation of other peptides such as those isolated from culture filtrate as described above. We wanted to know whether these AVR9 precursors were also present in IF of *C. fulvum*-infected tomato leaves. Therefore, we fractionated and analyzed proteins present in IF by a procedure similar to the one used for the purification of AVR9 peptides from culture filtrate. Following the preparative purification steps of cation-exchange and reversed-phase chromatography, the fractions containing elicitor activity were pooled and analyzed by low pH PAGE. In addition to the major 28-aa peptide, the IF contains several other peptides that behave similarly on low pH PAGE and cation-exchange HRLC as the AVR9 peptides isolated from culture filtrate. Following the preparative purification steps of cation-exchange and reversed-phase chromatography, the fractions containing elicitor activity were pooled and analyzed by low pH PAGE.

In culture filtrates of in vitro-grown *C. fulvum* transformants constitutively expressing the *avr9* gene, the 28-aa elicitor could not be detected. The smallest necrosis-inducing peptide isolated from culture filtrate is 32 aa’s in size. The final processing of AVR9 into the 28-aa peptide, which is the most abundant AVR9 peptide in IF of *C. fulvum*-infected tomato leaves, is therefore thought to take place in the plant. Purified 28- and 34-aa peptide elicitors were radioactively labeled with 125I, which is incorporated into the amino acid Tyr and results in mono- or diiodotyrosine (Fig. 3B, lanes 1 and 2, respectively). Iodinated AVR9 remains biologically active as tested in a pilot experiment using unlabeled NaI (results not shown). The labeled peptides were incubated with IF from healthy tomato plants at 37°C for 2 h. Analysis of the reaction mixtures by low pH PAGE revealed that factors present in the IF of both tomato cv Moneymaker and genotype Cf9 were able to process the 34-aa elicitor into the 28-aa peptide elicitor (Fig. 3A). Proteolytic activity was optimal at acidic pH (2–7) and marginal at alkaline pH (>7) (Fig. 3A, and data not shown). The proteolytic activity in IF was destroyed by boiling IF for 5 min prior to incubation (Fig. 3B, lane 6). No reduced proteolytic activity was observed when pepstatin, an inhibitor of acidic aspartyl endopeptidase of tomato (Rodrigo et al., 1989), was added to the reaction mixture containing the 34-aa peptide (Fig. 3B, lane 4).
AVR9 elicitor. High-level expression of avr9 in vitro has been accomplished by expression of avr9 under control of the heterologous gpd promoter of A. nidulans. In A. nidulans, the gpd promoter constitutively expresses the gpd gene encoding glyceraldehyde-3-P dehydrogenase (Punt et al., 1988). In Saccharomyces cerevisiae, glyceraldehyde-3-P dehydrogenase may account for 5% of total cellular proteins (Krebs et al., 1953). Transformants of C. fulvum containing the gpd promoter-avr9 fusion produced and excreted a set of AVR9 race-specific elicitors in culture filtrates of in vitro-grown mycelium; these filtrates induce HR in tomato plants carrying the resistance gene Cf9.

The AVR9 peptides from culture filtrate were purified to homogeneity using a combination of cation-exchange and reversed-phase chromatography, and sequenced from the N terminus. Instead of the 28-aa AVR9 peptide, as found in C. fulvum-infected tomato leaves, the culture filtrate contained a mixture of 32-, 33-, and 34-aa peptides. The same peptides could also be detected in IF of C. fulvum-infected tomato leaves. These peptides were not isolated before, because the purification method described previously (Scholtens-Toma and De Wit, 1988) involved preparative low pH PAGE followed by excision of the band containing the 28-aa peptide, and thus precluded the isolation of the 32-, 33-, and 34-aa peptides. The reduced mobility of the larger forms of AVR9 on low pH PAGE is due to the presence of an additional aspartic acid residue (D) reducing the isoelectric point. The peptides (indicated with asterisks, Fig. 2, lanes 2 and 5) with reduced mobility on low pH PAGE but with identical N-terminal sequences might be explained by deamination of Asp or Gln residues, resulting in a less-positive charge (Ahern and Klibanov, 1985).

Plant factors mediate the processing of the 34-aa AVR9 peptide, produced by C. fulvum in vitro, into the 28-aa form (Fig. 3). Radiolabeled (32P) 34-aa AVR9 elicitor was incubated with IF isolated from healthy plants and subsequently analyzed by low pH PAGE. The processing was inactivated by boiling the IF before incubation with the labeled peptide substrate, suggesting enzymatic cleavage by plant proteases. Alkaline (Vera and Conejero, 1988) and acidic proteinases (Rodrigo et al., 1989) of tomato have been reported to be involved in degradation of plant proteins. The processing of the 34-aa peptide is optimal at acidic pH, suggesting the involvement of an acidic aspartyl proteinase. However, pepstatin, which was reported to inhibit a tomato aspartyl proteinase (Rodrigo et al., 1989), did not inhibit cleavage of the 34-aa peptide. The pH in the apoplast is approximately 5.5, indicating that processing can occur in the tomato leaf.

The results described here allow us to propose a model for the processing of AVR9 (Fig. 4). The primary avr9 translation product of 63 aa’s is excreted by C. fulvum into the intercellular space of tomato leaves during infection, after removal of a 23-aa signal peptide. The 40-aa precursor protein is subsequently cleaved by fungal proteases into 32-, 33-, and 34-aa peptides. Plant proteases mediate the final cleavage to form the stable 28-aa race-specific peptide elicitor. The model does not exclude the possibility that the excreted 40-aa precursor is directly cleaved by plant proteases to form the mature 28-aa peptide in one single step.

Figure 4. Model for the processing of the AVR9 precursor in vivo. The primary avr9 translation product of 63 aa’s is excreted by C. fulvum into the intercellular space of tomato leaves during infection, after removal of a 23-aa signal peptide. The 40-aa precursor protein is subsequently cleaved by fungal proteases into 32-, 33-, and 34-aa peptides. Plant proteases mediate the final cleavage to form the stable 28-aa race-specific peptide elicitor. The model does not exclude the possibility that the excreted 40-aa precursor is directly cleaved by plant proteases to form the mature 28-aa peptide in one single step.

Experimentaly, Extracellular proteases produced by C. fulvum are most probably directly processing the 40-aa peptide into the intermediate forms of 32 to 34 aa’s.

The final step in the processing is mediated by plant factors and results in a stable, mature 28-aa race-specific peptide elicitor. We do not know whether the predicted 40-aa peptide can be directly processed into the 28-aa peptide by the plant or whether fungal proteases play an essential role in the formation of the 28-aa race-specific peptide elicitor in vivo. The specific necrosis-inducing activity of the 32-, 33-, and 34-aa peptides is indistinguishable from that of the 28-aa peptide. Whether processing of 32-, 33-, and 34-aa peptides into the 28-aa form by the plant is essential for elicitor activity is not known.

Our results indicate that several processes occur either before or during the recognition of the AVR9 elicitor by tomato genotype Cf9. Once the pathogen enters the leaf, the expression of the avirulence gene avr9 is induced, possibly by the nutritional environment in the leaf or by unknown plant factors (G.F.J.M. Van den Ackerveken, unpublished data). The extracellular AVR9 peptide is instantaneously processed by both fungal and plant proteases. However, we do not know whether the maturation is essential for elicitor activity, because processing of AVR9 peptides occurs too quickly to be studied separately from biological activity of the peptides.

The involvement of both host and pathogen in the formation of elicitors as described here is not unique. In many other host-pathogen interactions, nonspecific elicitors are formed that are released from cell walls. For example, β-1,3-glucanases from soy bean were shown to release elicitor-active carbohydrates from cell walls of Phytophthora megasperma (Keen and Yoshikawa, 1982). In another system, the polygalacturonase-inhibiting protein from Phaseolus vulgaris in-

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hibits the activity of fungal polygalacturonases, prolonging the lifetime of oligogalacturonide elicitors (Cervone et al., 1989). Oligogalacturonides with a degree of polymerization higher than nine act as elicitors of phytoalexin accumulation (Nothnagel et al., 1983). In the absence of polygalacturonase-inhibiting protein, pectic fragments are quickly degraded by polygalacturonases to monomeric forms, which have no elicitor activity.

The \(^{125}\)I-labeled AVR9 peptides are currently used as ligands in receptor-binding studies. A hypothetical receptor in the resistant host plant, which might be the primary product of the complementary resistance gene C\(\epsilon\)9, is proposed to recognize the AVR9 peptide elicitor. Isolation of the C\(\epsilon\)9 gene is a major goal of future studies.

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