Only some race 1 isolates of *Fusarium oxysporum* f.sp. *lycopersici* suppress *I*-2- and *I*-3-mediated resistance in tomato

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Abstract

Race 1 isolates of *Fusarium oxysporum* f.sp. *lycopersici* (Fol) are characterized by the presence of avirulence gene 1 (*AVR1*) in their genome. This gene is absent in other races of Fol. *AVR1* encodes avirulence protein 1 (Avr1) that triggers race-specific resistance in tomato plants carrying resistance (*R*) gene *I* or *I*-1. In addition to its avirulence function, *AVR1* suppresses *I*-2- and *I*-3-mediated resistance in tomato cultivars. Here we show that not all race 1 isolates are able to suppress *I*-2- and *I*-3-mediated resistance, even though they all contain *AVR1* in their genome. Lack of suppression can neither be explained by differences in the coding sequence of the *AVR1* gene, nor by its local genetic context or by altered *AVR1* expression levels. We hypothesize that lack of suppression by some race 1 isolates is due to the influence of an unknown factor present in the genome of some *F. oxysporum* f.sp. *lycopersici* isolates.
Introduction

During infection plant pathogens generally secrete small proteins, which are referred to as effector proteins. Many of these have been identified as virulence factors. In turn, host plants have developed resistant (R) proteins to recognize some of these effectors. The effector protein of the pathogen that is recognized by a corresponding R protein in the host is called an avirulence (Avr) protein. The interaction between a R protein and its cognate Avr protein often leads to localized cell death in the host, a phenomenon called the hypersensitive response (HR). Usually an HR restricts further growth of the pathogen in the plant. However, pathogens can evolve to evade R protein recognition through mutating or deleting the corresponding AVR gene. Alternatively, pathogens sometimes employ other effector proteins to suppress R gene-mediated immunity in host plants.

Pathogenic forms of *Fusarium oxysporum*, an asexual soil-borne fungus, cause wilt or root rot disease in many economically important crops. The species is divided into formae speciales depending on their host specificity, and further into physiological races based on cultivar specificity. *F. oxysporum* f. sp. *lycopersici* (Fol) is a wilt pathogen of tomato that causes substantial losses in tomato production worldwide. To date, three physiological races of Fol have been identified, races 1, 2 and 3. So far, four monogenic resistance genes (*I*, I-1, I-2 and I-3) against Fol have been identified in wild type tomatoes and introgressed into tomato cultivars. Race 1 isolates are avirulent on tomato lines containing R gene *I* or I-1, whereas races 2 and 3 are virulent on these lines. Races 2 and 3 are avirulent on tomato plants containing R gene I-2 and I-3, respectively; race 3 is virulent on I-2 plants. *I*-mediated immunity in tomato depends on the presence of the corresponding AVR genes in Fol. Three avirulence genes (*AVR1*, *AVR2* and *AVR3*) have been identified that correspond to matching *I* genes (*I* or I-1, I-2 and I-3, respectively). *AVR1* is present in race 1 isolates only, whereas *AVR2* and *AVR3* are present in all Fol races (Chapter 1). In race 3 isolates *AVR2* is mutated resulting in breaking *I*-2-mediated resistance in tomato.
Previously, we showed that race 1 isolates partially overcome \textit{I}-3-mediated resistance even though they contain a functional \textit{AVR3} in their genome\textsuperscript{12}. Later, working with race 1 isolate Fol004, it was found that the ability of race 1 isolates to infect \textit{I}-3 and also \textit{I}-2 containing plants is due to the capability of \textit{AVR1} to suppress \textit{I}-2- and \textit{I}-3-mediated immunity\textsuperscript{10}. In this study we extend previous experiments with race 1 isolate Fol004 with additional race 1 isolates\textsuperscript{10}. Not all of these isolates were found to suppress \textit{I}-2- and \textit{I}-3-mediated resistance. The observed differences in the suppressive ability of \textit{AVR1} can not be explained by differences in either the coding sequence of the \textit{AVR1} gene or its local genetic context, nor by altered \textit{AVR1} expression levels. We propose that lack of suppression by some race 1 isolates is due to the influence of an unknown fungal factor present in the genome of a subset of Fol isolates.

\textbf{Results}

\textbf{Lack of resistance-suppressive activity in some Fol race 1 isolates}

\textit{AVR1} of Fol race 1 isolate Fol004 suppresses \textit{I}-2- and \textit{I}-3-mediated resistance in tomato\textsuperscript{10}. Here we set out to test some additional race 1 isolates (Table 1) for their virulence on plants containing either \textit{R}-gene \textit{I}-2 (plant line 90E341F) or \textit{I}-3 (E779); plant lines C32 (no \textit{Fusarium} resistance gene) and GCR161 (containing \textit{R} gene \textit{I}) as well as a Fol race 2 (Fol007) and a Fol race 3 isolate (Fol029) were included as controls. The presence of \textit{AVR1}, \textit{AVR2} and \textit{AVR3} in all Fol race1 isolates tested was confirmed by PCR amplification and sequencing (see below)\textsuperscript{8}. In a bioassay, 10-day-old seedlings were inoculated with each of the elven isolates listed in Table 1, approximately 20 plants per treatment. Mock-inoculated plants served as controls. Three weeks after inoculation fresh plant weight and disease index (DI) were measured\textsuperscript{8}. The experiment was repeated twice (Fol001, Fol011, Fol016, Fol021 and
Fol022) or three times (Fol003, Fol004, Fol006, Fol007, Fol009 and Fol029) with very similar results. All isolates showed almost identical virulence on the general sus-

![Image](Lack of suppression of I-2- and I-3-mediated resistance)

![Image](Fol004)

(Race 1)

![Image](Fol006)

(Race 1)

![Image](Fol003)

(Race 1)

![Image](Fol009)

(Race 1)

![Image](Fol007)

(Race 2)

![Image](Fol029)

(Race 3)

![Image](Water)

(Mock)

**Figure 1. Some race 1 isolates of *F. oxysporum f.sp. lycopersici* suppress *I*-2- and *I*-3-mediated resistance while others do not.** Ten-day-old seedlings of tomato lines C32 (- no *I* gene), GCR161 (*I*), 90E341F (*I*-2) and E779 (*I*-3) were inoculated with race 1 isolates Fol003, Fol004, Fol006 and Fol009, race 2 isolate Fol007 and race 3 isolate Fol029. Mock-inoculated plants served as control. The experiment was repeated three times with similar results. Representative plants are shown three weeks after inoculation. Isolates Fol004 and Fol006 are virulent on both *I*-2 and *I*-3 plants and suppress *I*-2- and *I*-3-mediated resistance. In contrast, isolates Fol003 and Fol009 are not or hardly virulent on *I*-2 and *I*-3 plants and thus unable to suppress *I*-2- and *I*-3-mediated resistance. All four race 1 isolates shown here are avirulent on *I* plants.
Figure 2. Quantification of disease symptoms. Three weeks after inoculation fresh plant weight above the cotyledons (grams) was measured (green bars) and the disease index (orange bars) of the same plants was scored ranging from 0 (no symptoms) to 4 (severely wilted, fully brown vessels, death). The mean fresh plant weight and mean disease index were subjected to analysis of variance (ANOVA). The virulence activity of strains on tomato represented by same letter does not differ significantly according to $F$ test ($P=95\%$). Error bars indicate standard error of the mean. The experiment was repeated three times with consistent results. A. C32 plants (no $I$ gene), B. GCR161 plants (R gene $I$), C. 90E341F plants (R gene $I$-2), D. E779 tomato lines (R gene $I$-3).

ceptible line C32, except for Fol003 that was found to be less virulent (Figures 1 and 2A, and data not shown). All race 1 isolates were avirulent on $I$ plants (Figures 1 and
Lack of suppression of \( I-2 \)- and \( I-3 \)-mediated resistance

2B, and data not shown), confirming the presence of a functional \( AVR1 \) in the genome of those isolates and hence their correct classification as race 1. Both Fol004 and Fol006 were found to be virulent on \( I-2 \) plants although slightly less than race 3 isolate Fol029 (Figure 1 and 2C). On \( I-3 \) plants, these two race 1 isolates were virulent as well (Figures 1 and 2D), in line with our earlier finding that \( AVR1 \) suppresses \( I-2 \)- and \( I-3 \)-mediated resistance\(^{10}\). In contrast, \( I-2 \) plants inoculated with Fol003 and Fol009 showed only slight vascular browning (disease index 1; Figure 2C) and fresh plant weight did not differ significantly from control lines (plant lines inoculated with race 2 isolate Fol007 or water) and therefore Fol003 and Fol009 can be considered avirulent on \( I-2 \) plants (Figures 1 and 2C). Likewise, \( I-3 \) plants inoculated with Fol003 showed only slight vascular browning (disease index 1;

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**Table 1. \( F. \) oxysporum f.sp. lycopersici isolates used in this study**

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Original name</th>
<th>Origin</th>
<th>Race</th>
<th>VCG</th>
<th>Length fragment with ( AVR1 ) locus sequenced(^a)</th>
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<tr>
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<td>Netherlands</td>
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<td>0030</td>
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<td>France</td>
<td>1</td>
<td>0030</td>
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<tr>
<td>Fol007(^b)</td>
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<td>France</td>
<td>2</td>
<td>0030si</td>
<td>-</td>
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<td>0030si</td>
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<tr>
<td>Fol021</td>
<td>FOL1 (66044)</td>
<td>Israel</td>
<td>1</td>
<td>0030</td>
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<td>FOL-650 B</td>
<td>Israel</td>
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<td>0030</td>
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<tr>
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<td>Florida, USA</td>
<td>3</td>
<td>0030</td>
<td>-</td>
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<tr>
<td>Fol078</td>
<td>MAFF 305121</td>
<td>Japan</td>
<td>1</td>
<td>-</td>
<td>Not sequenced in this study</td>
</tr>
</tbody>
</table>

\(^a\) Positions relative to the \( AVR1 \) start codon are shown between brackets.

\(^b\) Isolate does not contain \( AVR1 \).

Isolate MAFF 305121 was kindly provided by Dr. Kawase, director of the NIAS Genebank, National institute of Agrobiological Sciences in Japan. The donors of other isolates are mentioned in Rep et al.\(^{12}\)
Figure 2D) and no significant loss in weight. Fol009 caused no symptoms at all on I-3 plants (Figures 1 and 2D). The other race 1 isolates listed in Table 1 showed intermediate virulence phenotypes on I-2 and I-3 plants (data not shown). We conclude that not all Fol race 1 isolates have the ability to suppress I-2- and I-3-mediated resistance in tomato plants, even though they all contain a functional \textit{AVR1} in their genome: Fol004 and Fol006 show a suppressive phenotype, whereas Fol003 and Fol009 do not, or at least to a much lesser extent.

\textbf{Difference in I-2 and I-3 suppressive ability is not associated with mutation(s) in \textit{AVR1}.}

Since race 1 isolates can be divided into two groups based on the ability to suppress I-2- and I-3-mediated resistance, we set out to test whether this difference is associated with polymorphisms in \textit{AVR1}. Previously, the \textit{AVR1} open reading frames in Fol001, Fol003, Fol004, Fol006, Fol010 and Fol016 were sequenced and no polymorphisms were found\textsuperscript{8}. Using primer pair 1091 and 1033 (Table 2) we sequenced the coding region of \textit{AVR1} in Fol009, Fol021 and Fol022 and these were also found to be 100\% identical to that of Fol004-\textit{AVR1} (data not shown).

In the genome of Fol004, the \textit{AVR1} locus is located on an 1944 bp fragment between a copy of a Tfo1-like transposable element and a copy of \textit{Fot5} (Figure 3; Chapter 3)\textsuperscript{10}. Using primer pairs 1197/1163, 1165/1079, 1063/1080, and 1084/1198 (Table 2) the sequence of the \textit{AVR1} locus between the two transposable elements of all nine Fol race 1 isolates used in this study was determined (Table 1); no sequence polymorphisms were detected. This excludes the possibility that mutations in the \textit{AVR1} locus affect the suppression ability of \textit{AVR1}.

\textit{AVR1} expression during colonisation of tomato is similar between I-2 and I-3-suppressing and non-suppressing race 1 isolates.

Next, we questioned whether differences in \textit{AVR1} gene expression levels could be responsible for the difference in the ability to suppress I-2- and I-3-mediated
Lack of suppression of I-2- and I-3-mediated resistance by some race 1 isolates. Levels of AVR1 transcripts in the general susceptible tomato line inoculated with either Fol009 (non-suppressing) or Fol004 (suppressing) were determined 8 and 12 days post inoculation (dpi). Using the AVR1 specific primer pair 2579/2162 (Table 2) RT-PCR experiments were conducted using cDNA synthesized on RNA isolated from infected plants as template. As control, transcript levels of FEM1 were determined using primers 157 and 158 (Table 2). FEM1 is a constitutively expressed gene encoding a cell wall glyco-protein and serves as a measure for fungal biomass. Figure 4 shows amplified fragments after 27, 28 and 29 PCR cycles at both 8 and 12 dpi. At 8 dpi, the transcript level of Fol009-AVR1 seems to be lower than that of Fol004-AVR1. However, the same holds true for Fol009-FEM1 compared of Fol004-FEM1, suggesting that the amount of fungal biomass is somewhat higher in the Fol004-inoculated plants than in Fol009-inoculated plants. We conclude that the expression of AVR1 relative to the expression of FEM1 is similar in both Fol isolates at 8 dpi. At 12 dpi, a smaller difference in AVR1 transcript levels was detected between Fol004 and Fol009 infected plants. Also here the difference can be accounted for by the observed difference in fungal biomass (FEM1 mRNA levels). Based on these results we conclude that the lack of the ability
to suppress \(I-2\)- and \(I-3\)-mediated resistance by Fol009 is not explained by (strongly) reduced expression of \(AVR1\) during host colonization.

![PCR amplification results](image)

**Figure 4.** *AVR1* expression during plant colonization is similar between a suppressive and a non-suppressive isolate. Total RNA was isolated from Fol004- (“4”) and Fol009- (“9”) infected tomato line C32 at 8 and 12 days post inoculation. cDNA was synthesized using an oligo-dT primer and Revert Aid H minus reverse transcriptase (Fermentas, Thermo Scientific, Pittsburgh PA, U.S.A.). PCR amplification was carried out in a reaction volume of 30 μl using cDNA as template and gene specific primer pairs (Table 2). To determine the relative amount of amplified fragment, a 5μl aliquot was taken out from the reaction mixture after 27, 28, 29 cycles and analyzed on a 1% agarose gel and stained with ethidium bromide.

<table>
<thead>
<tr>
<th>Dpi</th>
<th>PCR cycles</th>
</tr>
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<tbody>
<tr>
<td>8</td>
<td>27 28 29</td>
</tr>
<tr>
<td>12</td>
<td>27 28 29</td>
</tr>
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</table>

4-Fol004 inoculated plants, 9-Fol009 inoculated plants, Mo1-mock inoculated plants 8dpi and Mo2-mock inoculated plants at 12 dpi; M-Mass ruler DNA ladder, low range; G-PCR product amplified from genomic DNA; W-water (no DNA) control.

**Karyotype variability within Fol race 1 isolates**

The genome of Fol4287 (a race 2 isolate) has been sequenced, assembled and annotated, and is publically available (www.broadinstitute.org). In Fol4287 *AVR2* and *AVR3* are located on chromosome 14. Since this isolate lacks *AVR1* it is still unclear which chromosome in race 1 isolates harbours *AVR1*. In 2012, working with two Japanese race 1 isolates (MAFF 305121 and MAFF 103036), Inami and coworkers showed that *AVR1* is not always present on the same chromosome on which *AVR2* and *AVR3* are located (Chromosome 14 in Fol4287). We set out to investigate the chromosomal localization of *AVR1*, *AVR2* and *AVR3* in isolates Fol003, Fol004, Fol006 and Fol009 in a pulsed field electrophoresis and blotting
Lack of suppression of \( I-2 \)- and \( I-3 \)-mediated resistance

experiment. Isolate MAFF 305121 was used as reference isolate. The Contour-clamped homogeneous electric field (CHEF)-gel analysis revealed great karyotype variability within the Fol race 1 isolates (Figure 5). Furthermore, preliminary CHEF-blot hybridization results indicates that \( AVR2 \) and \( AVR3 \) are always located on the same chromosome, the size of which may vary between isolates (data not shown). However, the localization of \( AVR1 \) varies: in MAFF 305121 and Fol009 \( AVR1 \) is localized on the same chromosome that contains \( AVR2 \) and \( AVR3 \), in Fol004 and Fol006 \( AVR1 \) is found on a separate, bigger chromosome, and in Fol003 on an even bigger (approx. 3.5 Mb) chromosome (data not shown). These (preliminary) results confirm and extend the previous observation that in different Fol isolates \( AVR1 \) may be located on different chromosomes\(^{13} \).

**Discussion**

In this study we confirm previous observations that Fol race 1 isolates evade \( I-2 \)- and \( I-3 \)-mediated resistance, despite the presence of the matching avirulence genes, \( AVR2 \) and \( AVR3 \)\(^{10,12} \). However, not all race 1 isolates are able to do so. Among the nine race 1 isolates tested on either \( I-2 \) or \( I-3 \) plants, Fol004 and Fol006 showed a high level of suppression of \( I-2 \)- and \( I-3 \)-mediated resistance, whereas Fol003 and Fol009 hardly showed any suppression (Figures 1 and 2).

All race 1 isolates tested were avirulent on tomato plants containing \( R \) gene \( I \), indicating that the avirulence function of \( AVR1 \) was retained in all isolates. One could speculate that distinct structural features are involved in the avirulence and the resistance -suppressive function of Avr1 as in the case of bacterial AvrPtoB and oomycete AVR3a . AvrPtoB is a type III effector protein from *Pseudomonas syringae* pv. tomato DC3000, of which the N-terminal region is required for triggering resistance in tomato carrying \( R \) gene *Pto*; the C-terminal region is required
Figure 5. Karyotype of Fol race 1 isolates by CHEF-gel electrophoresis. The Following isolates were used; 1 – Fol004, 2 – Fol006, 3 – Fol009, 4 – MAFF 305121, 5 – Fol003. The chromosomes of Saccharomyces pombe (6) and Schizosaccharomyces cerevisiae (7) were used as CHEF DNA size markers.

for suppression of Pto/AvrPto or Cf9/Avr9-initiated cell death in Nicotiana benthamiana. Mutations in the C-terminal region abolished its suppressive function but not its avirulence function. The virulence and avirulence functions of the Phytophthora infestans effector protein AVR3a can also be uncoupled at the structural level. However, sequencing of AVR1 from all race 1 isolates used (Table 1), revealed no polymorphisms. Apparently, the lack of the ability to suppress resistance is not caused by a specific structural feature. A tight linkage between avirulence and virulence functions at the structural level has been found for AvrPt2 and AvrB as well. No significant reduction in AVR1 transcript levels in Fol004 and Fol009 infected C32 tomato lines was found, excluding the possibility that a change in AVR1 transcript (and most likely protein) level affects the suppression ability.
Lack of suppression of I-2- and I-3-mediated resistance

Although race 1 isolates Fol003, Fol004, Fol006 and Fol009 show distinct resistance suppressing abilities, they all are belong to the same vegetative compatibility group (VCG), notably VCG0030 (Table 1), indicating the same clonal origin. So far, the only difference we found among these isolates is the difference in their karyotype pattern. Fol004 and Fol006 show a very similar karyotype pattern. Moreover, in these isolates AVR1 is located on a chromosome of the same size (~2.9 Mb). The lack of obvious differences in karyotypes and the same chromosomal location of AVR1 in Fol004 and Fol006 could reflect their close evolutionary relationship. This is consistent with previous RAPD analysis. The different karyotype pattern in Fol003 and Fol009 implies that genetic re-arrangements have occurred within VCG0030 during evolution and this may somehow correlate with the suppressive function of AVR1.

Recent studies have shown that suppression of effector triggered immunity (ETI) and PAMP triggered immunity (PTI) is a key virulence strategy employed by a broad range of phyto-pathogens. For instance, the Phytophthora infestans suppressor of necrosis 1 (Sne1) suppresses programmed cell death (PCD) induced by Nep1-like protein (Nlp) genes PsojNIP and PiNPP1.1. Sne1 has also been shown to suppress AVR/R-induced HR in a broad spectrum of model systems, including oomycetes (Avr3a/R3a), bacteria (AvrPto/Pto), fungi (Avr9/cf9) and viruses (CP/Rx2). Recently, Six6, an effector protein from Fol, has been shown to suppress I-2/AVR2-induced cell death in Nicotiana benthamiana leaves, suggesting that Fol secretes more effector proteins that can suppress R gene-mediated resistance responses. However, SIX6 does not suppress I-2- and I-3-mediated resistance during infection.

Although suppression of I-2- and I-3-mediated resistance has been well documented, the mechanism of suppression by AVR1 is still unclear. Avr1 is a 242 amino acid protein with a molecular weight 24.3 kda (mature part) with an N-terminal signal peptide and no known functional domains in the mature part. Deletion of AVR1 does not reduce virulence on general susceptible tomato lines, suggesting that AVR1 has no general virulence function. However, recently a virulence function has been assigned to a close homologue of AVR1 in Arabidopsis-infecting F.
oxysporum isolate Fo5176\textsuperscript{23}. The strong conservation of Fol-\textit{AVR1} (\textit{SIX4}) and Fo-5176-\textit{SIX4} extends outside the coding region (99\% nucleotide identity across a region of around 2339 bp), suggesting that they have quite recently evolved from a common ancestor\textsuperscript{23}.

Fol-\textit{AVR1} may suppress \textit{I-2}- and \textit{I-3}-mediated resistance in tomato cultivars through (1) inhibition of \textit{AVR2} and \textit{AVR3} gene expression, (2) blocking the uptake of \textit{AVR} gene products by plants cell, (3) interference, directly or indirectly, with the recognition of Avr2 and Avr3 by corresponding \textit{R} gene products. However, for none of those three mechanisms indications are available. The expression of \textit{AVR2} and \textit{AVR3} is unaltered during infection of tomato plants in the presence of \textit{AVR1} (data not shown). Furthermore, Avr2 is secreted into xylem sap during colonization of tomato by Fol, but is recognized intracellularly\textsuperscript{11}, implying uptake by host cells. Since \textit{I-2} plants show resistance to Fol009, the possibility that Avr1 inhibits Avr2-uptake by host cells can be ruled out. Finally, as yet a direct interaction of Avr1 with either Avr2 or Avr3, or with \textit{I-2} or \textit{I-3} has been observed neither in yeast-two-hybrid experiments nor in \textit{in planta} pull down assays\textsuperscript{11}. Alternatively, the mechanisms of \textit{I-2}- and \textit{I-3}-mediated resistance may share conserved components and Avr1 may interact with one of them. We hypothesize that Avr1 acts on a target downstream of a point where the two \textit{R} gene-signaling pathways converge. This hypothesis leads to models to explain the lack of suppression in some race 1 isolates, summarized in Figure 6. According to the ‘stimulatory model’ (Figure 6A), an unknown factor (Factor X) is needed for Avr1 to successfully manipulate the host target to suppress \textit{I-2}- and \textit{I-3}-mediated resistance in tomato. Factor X might be deleted or mutated in Fol003 and Fol009 leading to the lack of the ability to suppress resistance. According
Figure 6. Models for the suppression of $I_2$- and $I_3$-mediated resistance by $AVR1$.

Suppression of both $I_2$- and $I_3$-mediated resistance by $AVR1$ in tomato suggests that both resistance mechanisms share some common downstream signaling component(s) leading to resistance. Avr1 may act on this unknown host component (blue hexagon) to suppress resistance. (A) The stimulatory model that predicts that in tomato Avr1 requires another effector protein from Fol (Factor X) to manipulate its host target that leads to suppression. We speculate that this factor might be deleted or mutated in isolates not showing suppression. (B) The inhibitory model predicts that Avr1 is able to suppress $I_2$- and $I_3$-mediated resistance. However, another factor present in some Fol isolates inhibits its suppressive function. We speculate that this factor might be deleted or mutated in isolates showing suppression.

to the ‘inhibitory model’ (Figure 6B), an unknown factor is present in Fol003 and Fol009 that inhibits the $AVR1$ suppressive function (suppressor of $AVR1$ suppression). Since the presence of such a factor is not beneficial for Fol, it might have been lost in Fol004 and Fol006. If this is the case, we speculate that the identification of such factors that inhibit $AVR1$-mediated suppression may offer novel strategies for developing disease resistance against Fol.

In summary, we here show that the lack of the suppressive function of $AVR1$ in some race 1 isolates cannot be explained by either a mutation in $AVR1$ or a reduced $AVR1$ expression level. We hypothesize that an as yet unidentified factor is involved in $AVR1$-mediated suppression in planta. Sequencing, assembly and comparison of
the genomes of suppressive and non-suppressive race 1 isolates may reveal new components involved in suppression of \(R/AVR\) induced defense.

**Material and Methods**

**Plant lines, fungal strains**
The following plant lines were used: C32, which is carrying no \(I\) gene and is susceptible to all races of *Fusarium oxysporum* f.sp. *lycopersici*, GCR161, which is resistant only to race 1 isolates and contains \(I\) resistance gene introgressed from *S. Pimpinellifolium*, 90E341F, which is resistant only to race 2 isolates and contains \(I-2\) resistance gene introgressed from *S. pimpinellifolium* and E779, which is resistant to race 2 and race 3 isolates and contains \(I-3\) resistance gene introgressed from *S. Pennelli*\(^5,11,24,25\). The Fol isolates used for this study are listed in Table 1. All isolates were cultured on Czakap Dox Agar (CDA, Oxoid) and incubated in darkness at 25\(^0\)C.

**Plant inoculation**
A standard root dip inoculation method was used to test the virulence of Fol isolates on the different tomato cultivars. Briefly, 10 days old tomato seedlings were uprooted, the root was cut about 1 cm from the root tip and dipped in an inoculum with a spore density of \(1\times10^7\) spores/ml for about 5 minutes. The seedlings were then potted individually. The plant lines dipped in water were used as negative control (mock inoculation). 20 plants per isolate were used to assess the virulence. Disease symptoms were measured 21 days post inoculation. To score the result, plant weight above the cotyledons and the degree of vascular browning were determined. Disease index was scored on a scale of 0–4 [0, no symptoms; 1, one or two brown vascular bundles in the stem below the level of the cotyledons; 2, one or two brown vascular bundles at the level of the cotyledons (no strong growth distortion, but plants can be smaller); 3, at least three brown vascular bundles and growth distortion (strong bending of the stem and asymmetric development); 4, all vascular bundles are brown,
plant either dead or small and wilted]. The mean fresh plant weight and mean disease index were subjected to analysis of variance (ANOVA) using Statview 5.0.

DNA isolation, PCR analysis and sequencing
For sequencing the \textit{AVR1} locus fungal genomic DNA (gDNA) was extracted using the following method. A patch of mycelium was scraped from the margin of a colony and suspended in 400 µl Tris-EDTA buffer (10 mM Tris pH 8, 1 mM EDTA pH 8) together with 300 µl phenol:chloroform (1:1) and approximately 300 µl glass beads (about 400 µm). The mycelium was mechanically disrupted by vortexing for 2 minute. The supernatant (150 µl) was collected after centrifugation (5 minute) at maximum speed and mixed with an equal volume of chloroform. Again, the supernatant (100 µl) was collected after vortexing and centrifugation (5 minute) and stored in -20°C for further use. 1 µl of genomic DNA was used for PCR experiments. Primers used for amplification of the \textit{AVR1} locus are given in Table 2. The amplified products were resolved electrophoretically in a 1% agarose gel. PCR products were sequenced and analyzed using Seqbuilder (www.dnastar.com). Clustal algorithm was used for sequence alignment\textsuperscript{26}.

RT-PCR analysis
Ten-days-old C32 tomato seedlings were inoculated with race 1 isolates Fol004 and Fol009 and with water as control. The seedlings were potted in vermiculite. For the isolation of RNA 20 plants per treatment were used. Plant material below the hypocotyls was harvested at 8 and 12 days post inoculation. The root samples were pooled and ground in liquid nitrogen. Total RNA from the samples was extracted with TRIzol LS reagent (Invitrogen, Life Technologies, Grand Island, NY, U.S.A.) and subsequently purified with RNeasy Mini kit (Qiagen). DNA was removed by on-column treatment with RNase-free DNase (Qiagen). cDNA was synthesized using the M-MuLV reverse transcriptase RNase H minus kit (Fermentas, Thermo Scientific, Pittsburgh PA, U.S.A.). PCR was used to amplify 1 µl cDNA or DNA using gene specific primers (Table 2). For the relative quantification of the PCR products, a 5 µl
aliquot was taken out after 27, 28, 29 cycles and analyzed on 1% agarose gels, and stained with ethidium bromide.

**Table 2. Primers used in this study**

<table>
<thead>
<tr>
<th>No.</th>
<th>Sequence (5’-3’)</th>
<th>Target position relative to ATG</th>
</tr>
</thead>
<tbody>
<tr>
<td>1197</td>
<td>TATTCAGTTGCCAGCAATG</td>
<td><em>AVR1</em> -1013 to -585</td>
</tr>
<tr>
<td>1163</td>
<td>GACTTGCGCTGTAAGGCTA</td>
<td><em>AVR1</em> -1013 to -585</td>
</tr>
<tr>
<td>1165</td>
<td>ACTAGGCTTACCAGCCAAG</td>
<td><em>AVR1</em> -605 to +86</td>
</tr>
<tr>
<td>1079</td>
<td>GTTCCGATGATGTCACCCT</td>
<td><em>AVR1</em> -605 to +86</td>
</tr>
<tr>
<td>1091</td>
<td>TCAGGCTTACCTTAGCATA</td>
<td><em>AVR1</em> -72 to +895</td>
</tr>
<tr>
<td>1033</td>
<td>GCCGACCGAAAAACCTAA</td>
<td><em>AVR1</em> -72 to +895</td>
</tr>
<tr>
<td>1063</td>
<td>CGACCAGACGTAGTCGCTC</td>
<td><em>AVR1</em> +718 to +1181</td>
</tr>
<tr>
<td>1080</td>
<td>GTGAATACATATGGAAGAGGAC</td>
<td><em>AVR1</em> +718 to +1181</td>
</tr>
<tr>
<td>1084</td>
<td>CTTGACCCTTTTGGCCTTC</td>
<td><em>AVR1</em> +1126 to +1763</td>
</tr>
<tr>
<td>1198</td>
<td>ATCCTCGAAGCAGCCTCT</td>
<td><em>AVR1</em> +1126 to +1763</td>
</tr>
<tr>
<td>2579</td>
<td>AAAAGGCGCCCATGAATCTCAAGGCACTGT</td>
<td><em>AVR1</em> +1 to 793</td>
</tr>
<tr>
<td>2162</td>
<td>AGAAAGCTGGGTCCCTAAGCTAAGTTAGT</td>
<td><em>AVR1</em> +1 to 793</td>
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<tr>
<td>157</td>
<td>ATGAAGTACACTCTCGCTACC</td>
<td><em>FEM1</em> +1 to +274</td>
</tr>
<tr>
<td>158</td>
<td>GGTGGAAGTGAAAGAGTCACC</td>
<td><em>FEM1</em> +1 to +274</td>
</tr>
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</table>

**Protoplast isolation and Contour-clamped homogeneous electric field (CHEF)-gel analysis**

For protoplast isolation, the fungal isolates were grown in NO₃ medium for 5 days at 25°C with continuous shaking. Conidia were harvested by filtration through double layer of Miracloth (Calbiochem Corp., La Jolla, CA) and 5*10⁸ spores were used to inoculate 40 ml of potato dextrose broth (PDB) (Becton Dickinson, Sparks, MD., USA) medium. After 15 h of growth, mycelium was collected by centrifugation and washed with MgSO₄ solution (1.2 M MgSO₄, 50 mM sodium citrate (pH 5.8)). Digestion of cell walls was performed with Glucanex (100 mg/ml; Sigma) in 1.2 M MgSO₄ solution at 30°C (shaking). Protoplasts were separated from undigested mycelium by filtration through two layers of Miracloth. Four volumes of 1 M sorbitol solution (1 M Sorbitiol, 50 mM Cacl₂.2H₂O, 10 mM Tris pH 7.4) were added, and protoplasts were collected by centrifugation. Protoplasts were resuspended in 10
Lack of suppression of I-2- and I-3-mediated resistance

ml SNT (1 M Sorbitol, 0.1 M NaCl, 10 mM Tris pH 7.4) and collected by centrifugation.

For CHEF analysis, protoplasts were suspended in STE (1 M sorbitol, 25 mM Tris-HCl, and 50 mM EDTA, pH 7.5) at a concentration of 2*10^8 pps/ml. Protoplast suspensions were mixed with an equal volume of 1.2% InCert agarose (FMC BioProducts, Rockland, ME) in STE and mounted in mold chambers. Plugs were incubated in NDS (0.1 M Tris, 0.5 M EDTA (pH 9.5), 1 % lauroylsarcosine). Electrophoresis was performed using a CHEF-DRII (Bio-Rad Laboratories, Veenendaal, the Netherlands). Chromosomes were separated in a 10-day run in 1% SeaKem gold agarose (FMC bio products) at 4°C using switch times between 20 to 80 min at 1.5 V/cm. Running buffer (0.5x Tris-borate-EDTA (TBE) was refreshed every two to three days. Gels were stained with ethidium bromide.

Acknowledgement

We thank Ludek Tikovsky, Harold Lemereis and Thijs Hendrix for taking care of the plants. We are grateful Dr. Makoto Kawase, director of the NIAS Genebank, National Institute of Agrobiological Sciences in Japan who provided us with Fol isolate MAFF 305121. We would like to thank Ido Vlaardingerbroek (Swammerdam Institute for Life Science, University of Amsterdam, Amsterdam, The Netherlands) for his technical support in Contour-clamped homogeneous electric field (CHEF)-gel analysis.

References


Lack of suppression of I-2- and I-3-mediated resistance.


Appendix (Chapter 2)

*SIX4* is restricted to some *formae speciales* of the *Fusarium oxysporum* species complex
Abstract

In *Fusarium oxysporum* f.sp. *lycopersici*, the product encoded by *SECRETED-IN-XYLEM4* (*SIX4*) functions as an avirulence protein (hence also known as Avr1) corresponding to R protein I as well as a suppressor of I-2- and I-3-mediated immunity. Recently, homologues of *SIX4* were found in Arabidopsis infecting *F. oxysporum* isolate Fo5176 where it functions as a virulence factor. In this study, we show that homologues of *SIX4* are also present in the genome of some other strains of *F. oxysporum*. 
**SIX4 of Fusarium oxysporum f.sp. lycopersici**

During infection of tomato by *Fusarium oxysporum* f.sp. *lycopersici* (Fol), the fungus secretes small proteins in the xylem\(^1\). These proteins are collectively referred to as Six (secreted in xylem) proteins. To date over 14 Six proteins have been identified\(^2,3\). Some of them have been shown to be virulence factors that promote Fol infection\(^4\).

Three Six proteins, notably Six1, Six3 and Six4, are recognized – either directly or indirectly – by resistance (R) gene products of tomato: Six1 (Avr3) is recognized I-3, Six3 (Avr2) by I-2 and Six4 by I\(^4\). Recognition triggers resistance. Six4 does not contribute to the virulence of Fol. However, besides acting as an avirulence factor, Six4 suppresses I-2- and I-3-mediated resistance, at least in some cases (Chapter 2)\(^5\).

Six4 is encoded by a single gene (*SIX4*) that is present in race 1 isolates only. In the genome of isolate Fol004, *SIX4* is preceded by both a partial miniature impala (Mimp 4) and a Tfo1-like repetitive element and followed by a Fot5 transposable element (Figure 1).

**Figure 1. Conservation of the SIX4 / SIX4a genomic region in three formae speciales of Fusarium oxysporum.** In Fol004, *AVR1* is flanked by a partial miniature impala repetitive element (Mimp 4) 485 bp upstream the start codon and a Tfo1-like repetitive element 714 bp upstream the start codon. Downstream, *AVR1* is flanked by a Fot5 transposable element (541 bp from the stop codon)\(^5\). IR – inverted repeat.
Occurrence of SIX4 homologues in the *F. oxysporum* species complex

Recently two homologues of SIX4 have been identified in the *Arabidopsis*-infecting *F. oxysporum* isolate, Fo5176. These were named *Fo5176-SIX4a* (FOXB_04209) and *Fo5176-SIXb* (FOXB_15628). *Fol-SIX4* and *Fo5176-SIX4a* show 99.7% identity at the nucleotide level. In the as yet not fully assembled genome sequence of this *Arabidopsis* isolate, *Fo5176-SIX4a* localizes on a small (2339 bp) contig (supercontig 01435; GenBank: AFQF01001368.1) that aligns completely to the corresponding SIX4 genomic region in *Fol* (GenBank: AM234064.2) with a 99.4% nucleotide identity (Figure 1). *Fo5176-SIXb* is found at the end of another contig (1624 bp; supercontig 04359; GenBank: AFQF01004115.1), and appears to lack the coding sequence for the N-terminal signal peptide. The *Fo5176-SIXb* ORF shows 84.3% identity at the nucleotide level to that of *Fol-SIX4*.

To further investigate the presence of homologues of *Fol-SIX4* in other strains of *F. oxysporum*, Fol-Six4 was used as query to search against the genomes of several strains by blastp and tblastn. To date the Fusarium comparative genomics database (www.broadinstitute.org) contains the (draft) genome sequences of over ten *formeae speciales*, including a non-pathogenic isolate (Fo47), an isolate pathogenic to humans and a Fol race 3 isolate. This search resulted in the identification of two Fol-Six4 homologues in the genome of *F. oxysporum* f.sp. conglutinans strain PHW808, a pathogen that specifically infects Brassica species. In addition, a single truncated copy of SIX4 was identified in the genome of *F. oxysporum* f.sp. cubense strain II5 (tropical race 4), a pathogen of banana. In PHW808, one Six4 homologue (FOPG_19729.1) shows 99.1% and 100% identity at the protein level to Fol-Six4 and Fo5176-Six4a, respectively (Figure 2). The gene encoding this protein (hereafter referred to as *PHW808-SIX4a*) is located on supercontig 1807 (2358 bp), which aligns completely to the SIX4 genomic region in *Fol* showing 99.4% nucleotide identity (Figure 1). The gene encoding the second SIX4 homologue (*PHW808-SIXb*, unannotated) is truncated due to an unknown transposable element insertion between nucleotides 60 and 61 (from the start of the ORF). This transposable element possesses 21 bp inverted repeats and is flanked by 8 bp target site duplications. The nucleotide sequence identity does not extend outside the ORF. PHW808-Six4b shows
76% and 100% identity to Fol-Six4 and Fo5176-Sixb, respectively (Figure 2). Both Fo5176 and strain PHW808 infect Arabidopsis, suggesting a close relationship between those two isolates. The 100% sequence identity between Fo5176-SIX4a and PHW808-SIX4a, and between Fo5176-SIX4b and PHW808-SIX4b, (the transposon insertion in PHW808-SIX4b not taken into account) is in line with this observation.

Figure 2. Alignment of the amino acid sequences of Fol-Six4 and its homologues from other formae speciales of F. oxysporum. The protein sequence of F. oxysporum f.sp. conglutinans is deduced after removing the transposable element that was found to be inserted in the gene.

In F. oxysporum f.sp. cubense, the SIX4 homologue (called hereafter II5-SIX4) is located on supercontig 102 (www.broadinstitute.org) and shares 94% nucleotide identity to Fol-SIX4. However, the 5’ region corresponding to nucleotides 1-98 of the ORF of Fol-SIX4 is absent in the II5 SIX4 homologue. The II5-Six4 protein shows 90% identity to that Fol-Six4 (Figure 2). Figure 3 shows a phylogenetic tree based on the alignment of Six4 sequences. Interestingly, we also found a homologue of SIX6, another effector gene from Fol7, located 563 bp upstream of the truncated copy of SIX4 in II5. The homologue of SIX6 from II5 shares 62% identity to that Fol-SIX6. In Fol004, SIX6 is located approximately 94.4 kb downstream SIX4 (data not shown). This suggests that originally in Fol SIX6 and SIX4 might have been located close to each other and an insertion of a large genomic region may caused the separation of these genes.
**Figure 3. A maximum likelihood tree based on the alignment of Six4 sequences.** Protein sequence alignments were constructed and edited using Unipro UGENE bioinformatic software\(^8\). Phylogenetic tree reconstruction and bootstrapping (shown near to branches) were performed using PhyML v3.0\(^9\).

**SIX4 homologues are not found outside the F. oxysporum species complex**

Recently, the genomes of 21 isolates from three *forme speciales* of *F. oxysporum*, notably f.sp. *cucumerinum*, f.sp. *melonis* and f.sp. *radicis-cucumerinum* were sequenced. In none of them a SIX4 homologue was identified (Peter van Dam, unpublished results). We also looked for SIX4 homologues outside the *F. oxysporum* species complex. To this end, the amino acid sequence of Fol-Six4 was used as a query in a tblastn search against all sequences in the Fungal Genome Initiative (FGI) database (www.broadinstitute.org) and the NCBI nucleotide database (www.ncbi.nlm.nih.gov). The search did not result in the identification of additional homologues of SIX4 in other fungal species or in other organisms.

In summary, homologues of SIX4 have been identified in three *forme speciales* of *F. oxysporum*. The absence of the gene outside the *F. oxysporum* species complex and the strong conservation of *Fol*-SIX4, *Fo*-5176-SIX4a and *PHW808*-SIX4a extending outside the coding region (99.4% nucleotide identity across a region of around 2339 bp), suggest that the SIX4 genomic region was obtained from a common ancestor.
References


