Molecular aspects of the interaction between tomato and Fusarium oxysporum f.sp. lycopersici

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CHAPTER 3

Loss of avirulence and reduced pathogenicity of a gamma irradiated mutant of

Fusarium oxysporum f.sp. lycopersici

Jurriaan J. Mes, Robbert Wit, Christa Testerink, Francis de Groot,
Michel A. Haring and Ben J.C. Cornelissen

Submitted

ABSTRACT

In tomato resistance to races of the fungal wilt pathogen Fusarium oxysporum f.sp. lycopersici is brought about by monogenic dominant resistance genes. The I-2 gene confers resistance to F. oxysporum f.sp. lycopersici race 2 isolates containing a corresponding avirulence gene. To characterize the avrI-2 of F. oxysporum f.sp. lycopersici, a gamma irradiation mutagenesis program was performed to generate an avirulent mutant of F. oxysporum f.sp. lycopersici race 2 that is able to break the I-2 resistance due to loss of an avirulence gene. A race 2 isolate was labelled with a phleomycin resistance gene and a GUS marker gene to distinguish mutants from natural occurring isolates able to infect I-2 plants. From a large scale gamma irradiation and selection program we selected one mutant that was able to infect a tomato line containing the I-2 resistance gene. Besides loss of avirulence this mutant showed reduced pathogenicity towards tomato plants without Fusarium resistance genes as well. To detect genomic alterations the mutant was compared to the original isolate by DNA analysis. Southern analysis on CHEF blots using chromosome specific probes demonstrated a chromosomal translocation in the mutant. RAPD analysis and AFLP analysis have identified several genomic changes in the mutant. The polymorphisms detected could lead to the cloning of the avirulence gene eventually.
INTRODUCTION

Pathogens are specialised in the colonisation of a limited number of host plants only. In general plants try to resist pathogenic invasions and have a whole array of defence mechanisms at their disposal the pathogen has to deal with. Each pathogen has its own characteristic way to cope with such plant defence responses, to enter its host and to use it for its own support. To this end pathogens have developed pathogenicity determinants adjusted to their host and mechanisms to evade or suppress defence responses.

*Fusarium oxysporum* is a fungal pathogen infecting a wide range of plant species. Isolates of the species *F. oxysporum* are divided into at least 80 different formae speciales correlating with the host ranges in which they cause disease (Armstrong and Armstrong, 1981). Within formae speciales isolates are subdivided into races, depending on the cultivars they can infect successfully. We are using the interaction between tomato and *F. oxysporum* f.sp. *lycopersici* as a model system to study wilting diseases (Kroon and Elgersma, 1993; Simons et al., 1998; Mes et al., 1999, chapter 2). The host range of this forma specialis is limited to tomato. Recently, we have gathered evidence for a gene-for-gene relationship (Flor, 1971) between *F. oxysporum* f.sp. *lycopersici* and tomato (Mes et al., 1999, chapter 2). Races of *F. oxysporum* f.sp. *lycopersici* lacking a functional avirulence gene fail to activate host defence in time, resulting in a successful colonisation of the xylem vessels and disruption of the water-conduction system of the plant. Races with a functional avirulence gene are restricted at the site of infection by a resistance gene dependent defence response. Isolation of avirulence genes may give more insight in the early steps of race-specific defence reactions.

In tomato resistance against *F. oxysporum* f.sp. *lycopersici* race 2 is brought about by the I-2 resistance gene. This gene shows the typical characteristics of resistance genes of the NBS-LRR class (Ori et al., 1997; Simons et al., 1998; Ellis and Jones, 1998). While resistance genes share similar basic elements, no common features have been identified in the corresponding avirulence genes thusfar (Parker and Coleman, 1997). Therefore cloning of avirulence genes by PCR, as currently is performed for resistance genes (Leister et al., 1996), is not an option. So far, avirulence genes of plant pathogenic bacteria have been cloned by complementation approaches (Dangl, 1994). Fungal avirulence genes have been isolated by product based cloning (Van Kan et al., 1991; Joosten et al., 1994; Rohe et al., 1995) and by map based cloning (Valent and Chumley, 1994; Kang et al., 1995; Sweigard et al., 1995). For *F. oxysporum* neither of these two methods is feasible. Often fungal avirulence genes are expressed *in planta* only (Van den Ackerveken et al., 1994; Joosten et al., 1994). Therefore, attempts to isolate the avirulence gene product are complicated by the localization of the specificity of the interaction, notably the xylem vessels. Map based cloning is not applicable because the life-cycle of *F. oxysporum* does not include a sexual stage (Fungus Imperfecti), and hence the fungus can not be use in classical genetic studies. Furthermore, a shotgun
complementation strategy is not practical due to the size of the genome and the low efficiency of transformation. Transposon tagging is a promising method for *F. oxysporum* f.sp. *lycopersici* but the system is still in a developmental phase (Daboussi and Langin, 1994). Like in the program to isolate avirulence genes from the flax rust fungus (Timmis et al., 1990), we have chosen for a random deletion mutagenesis approach and subsequent selection for mutants that have lost avirulence: such mutants have gained virulence on plants that carry the resistance gene corresponding to the lost avirulence gene. Subtraction techniques (Straus and Austubel, 1990; Lisitsyn et al., 1993), RNA comparison analysis (Liang and Pardee, 1992) and marker based selection methods like RAPD and AFLP analysis (Williams et al., 1990; Vos et al., 1995) can then be used to trace the deletion and to clone the avirulence gene.

Here we report on the efficiency of a deletion mutagenesis approach by gamma irradiation to induce mutants of *F. oxysporum* f.sp. *lycopersici* and on the selection of a mutant able to break the resistance brought about by the I-2 gene. In a next step towards the isolation of the avirulence gene, RAPD and AFLP analysis were performed to identify genomic changes.

**MATERIALS AND METHODS**

**Fungal strains.** Isolates of *F. oxysporum* f.sp. *lycopersici* used in this study are listed in Table 1.

**Table 1.** Number, source, race and vegetative compatibility group of used isolates of *Fusarium oxysporum* f.sp. *lycopersici*.

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Original number</th>
<th>Origin and donator&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Race</th>
<th>VCG</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fol002</td>
<td>WCS862</td>
<td>Netherlands, a</td>
<td>2</td>
<td>0030</td>
</tr>
<tr>
<td>Fol004</td>
<td>IPO1530</td>
<td>Netherlands, b</td>
<td>1</td>
<td>0030</td>
</tr>
<tr>
<td>Fol007</td>
<td></td>
<td>France, c</td>
<td>2</td>
<td>0030</td>
</tr>
<tr>
<td>Fol015</td>
<td>BFOL-53</td>
<td>USA, Louisiana, d</td>
<td>2</td>
<td>0030</td>
</tr>
<tr>
<td>Fol026</td>
<td>14844</td>
<td>Australia, e</td>
<td>3</td>
<td>0030</td>
</tr>
<tr>
<td>Fol029</td>
<td>5397</td>
<td>USA, Florida, f</td>
<td>3</td>
<td>0030</td>
</tr>
<tr>
<td>Fol035</td>
<td></td>
<td>Netherlands, b</td>
<td>3</td>
<td>0030</td>
</tr>
<tr>
<td>Fol036</td>
<td></td>
<td>USA, Florida, g</td>
<td>3</td>
<td>0030</td>
</tr>
</tbody>
</table>

<sup>a</sup>donator is not necessarily the original collector: a, N.A.M. v Stekelenburg; b, IPO-DLO, Research Institute for Plant Protection, Wageningen, the Netherlands; c, L. Davidse; d, K.S. Elias; e, D.J. McGrath; f, J.P. Jones; g, J.W. Scott

**Fungal transformation.** Plasmid pAN8.1 containing the phleomycine resistance gene fused to the glyceraldehyde phosphate (gpd) promoter was obtained from Dr. P. Punt, TNO, Zeist, the Netherlands (Punt and Van den Houdel, 1992). Plasmid pCF20 which contained the uidA gene (GUS) controlled by the same gpd promoter was obtained from Dr. G. Honee, Agricultural University Wageningen, the Netherlands (Roberts et al., 1989). Protoplast isolation and fungal transformation were essentially according to Kistler and Benny (1988) with some modifications. *Fusarium* was grown in liquid potato dextrose broth (PDB) for 5
days at 25°C by continuous shaking. Conidia were collected by centrifugation and then washed with sterile water. The conidia were used to inoculate an overnight culture by adding 5.10^8 conidia to 40 ml PDB. After approximately 15 hours, mycelium was collected by centrifugation and washed with 1.2 M MgSO_4 (pH5.8). Digestion of cell walls was performed with Glucanex (25 mg/ml) in 1.2 M MgSO_4 (pH 5.8) at 30°C. Protoplasts were separated from undigested mycelium by filtration through three layers of MiraCloth (Calbiochem Corp., La Jolla, Calif., USA). Four volumes of 1 M sorbitol solution were added and protoplasts were collected by centrifugation. Protoplasts were washed once in sorbitol, counted and diluted to 2.10^8 pps/ml. For transformation 200 μl protoplasts and 10 μg plasmid was used. During transformation DNase inhibitor aurintricarboxylic acid was added to a final concentration of 10 mM (Punt and Van den Hondel, 1992).PEG (60%) was added dropwise to the protoplast/DNA mixture. After incubation for 30 minutes on ice, protoplasts were washed in 0.5 M MgSO_4 containing 0.1% PDB and protoplasts were recovered in the same solution. After transformation the protoplasts were plated on Czapek Dox agar (CDA) osmostabilised with 0.5 M MgSO_4 (Schuren and Wessels, 1994) containing 100 μg/ml phleomycine (Cayla, Toulouse, France).

Irradiation mutagenesis. Conidia scraped from F. oxysporum f.sp. lycopersici cultures grown two weeks on potato dextrose agar (PDA) plates were counted, diluted and then plated on agar plates (200 conidia per plate). Plates containing the conidia were irradiated in a 137Cs-unit H 622 at different dosage levels (0.9 Gy/min) and then incubated at room temperature for several days until colonies grow from the surviving conidia.

Irradiation efficiency was tested by screening for chlorate resistant mutants of which some have a mutation at the nitrate reductase gene. Therefore conidia were plated on CDA supplemented with 5% chlorate before and during gamma irradiation. Chlorate resistant colonies were transferred to CDA in order to confirm the nitrate nonutilizing deficiency, visible as thin growing mycelium. Which of the chlorate resistant colonies had a mutation in the nitrate reductase gene was tested by media complementation, only mutants which morphology is restored by CDA media supplementation with NO_3 (0.5 g/l) or hypoxanthine (0.2 g/l) have a mutation at the nitrate reductase locus (Correll et al., 1987).

Mycelium of nitrate reductase mutants was grown for two weeks in PDB and collected by filtration. Mycelium was lyophilised and ground with the use of liquid nitrogen. DNA isolation and DNA gelblot analysis were performed as reported previously (Mes et al., 1994). Plasmid pNE24 that carries the nitrate reductase (nia) gene of F. oxysporum f.sp. melonis (Diolez et al., 1993) was obtained from M. Daboussi (University Paris-Sud, 91405 Orsay, France). A 4 kb HindIII fragment of F. oxysporum, containing the complete nitrate reductase gene was used as probe.

For the large scale selection of an avirulent mutant, conidia were plated on PDA (500 conidia per plate), irradiated and grown for two days at room temperature. Small colonies were transferred to tubes containing three ml of liquid Czapek Dox. Liquid cultures were grown for 4 days at 25°C by continious motion. The whole mixture of mycelia and conidia was used to inoculate 7-days-old seedlings of tomato line C295. Inoculated plants were grown in the greenhouse at 25°C for three weeks and then examined for Fusarium wilt symptoms. Infected plants were used for re-isolation of the mutagenised isolate causing the symptoms. Single spored cultures of the re-isolate were tested the same way as described above to distinguish real avirulent mutants from false positives.

Plant material. The previously described near-isogenic lines of tomato cultivar Moneymaker, viz. C32, GCR161 and C295 (Mes et al., 1999, chapter 2) were used. C32 is susceptible to races 1 and 2; GCR161 containing the resistance to race 1; C295 (also known as Mobox, Laterrot, INRA, France) is resistant to both races 1 and 2. Scott (University of Florida, USA) provided us with tomato line 90E341F containing the race 2 resistance of the I-2 locus of PI126915 (Stall and Walter, 1965) without the linked resistance against race 1, tomato line 90E402F which contains the race 1 resistance (I-1) obtained from LA716 (Scott and Jones, 1989), and tomato line 90E218F containing the I-3 locus of LA716 giving resistance against race 2 and 3. The biotech company KeyGene (Wageningen, the Netherlands) provided a transgenic lines (KG324) of susceptible tomato line KG52201 transformed with cosmid B22.
Loss of avirulence containing the sequence of the \( I-2 \) gene (Simons et al., 1998). The original transformant was selfed and a homozygous T2 line selected.

**Plant infection.** Twelve-day-old seedlings were infected by a standard root-dip inoculation method (Mes et al., 1999, chapter 2). All plant inoculation experiments were performed in 10 replicates which were randomly arranged in equal blocks, each block containing all treatments once. After 3 weeks, weight of the upper cotyle part of the plant was measured. Data were statistically analysed with ANOVA.

**CHEF analysis.** Protoplasts, isolated as described above, were suspended in STE (1 M Sorbitol, 25 mM Tris-HCl, 50 mM EDTA, pH7.5) at a concentration of \( 2 \times 10^8 \) pps/ml. Protoplast solution was mixed with equal volume STE containing 1.2% InCert agarose (FMC Bioproducts, Rockland, USA) and mounted in mold chambers. Plugs were incubated in NDS (0.1 M Tris, 0.5 M EDTA, 1% lauroylsarcosyl, pH9.5) containing 2 mg/ml pronaseE at 50°C overnight and washed three times in 50 mM EDTA (pH8.0). Electrophoresis was performed using a CHEF-DRII, Biorad. Chromosomes were separated in a ten-days run in 1% SeaKem gold agarose (FMC Bioproducts, Rockland, USA) at 4°C using switch times between 20 to 80 minutes at 1.5 V/cm. Running buffer (0.5 x TBE) was refreshed every two days. Gels were stained with etidium bromide and destained with water. CHEF gel blot analysis were basically as described in Sambrook et al. (1989).

**RAPD and AFLP analysis.** RAPD analysis were performed as described previously (Mes et al., 1999, chapter 2) using primers obtained from kit A, B, C, F and K from Operon Technologies, Inc. (Alameda, CA).

Amplified fragment length polymorphism analysis was performed according to Vos et al (1995) with some modifications. Standard adapters were ligated to EcoRI and Msel digested DNA. Preamplifications were performed using primers containing no selective nucleotides. Preamplifications were diluted 50 times before selective AFLP amplifications were performed using primer combinations with a total of 3 or 4 selective nucleotides.

**RESULTS**

**Marking the wild type isolate**

Wild type race 2 isolate Fol007 of *F. oxysporum* f.sp. *lycopersici* (Table 1) is avirulent on plants containing the \( I-2 \) gene and pathogenic on plants lacking this race-specific resistance trait (Fol007wt). Circumstantial evidence suggests that for expression of the \( I-2 \) specific resistance the presence of a corresponding avirulence gene (avr\( I-2 \)) in the fungus is required (Mes et al., 1999, chapter 2). To be able to distinguish Fol007wt mutants lacking a functional avr\( I-2 \) gene from naturally occurring race 3 isolates that infect \( I-2 \) gene containing tomato, the wild type race 2 isolate was marked with a phleomycine resistance gene and the \( \beta \)-glucuronidase (GUS) marker gene. The latter was introduced by cotransformation with the former. Phleomycine resistant colonies were tested for GUS activity (Jefferson et al., 1987). Transformants that were both phleomycine resistant and GUS positive were single-spored, and characterized by Southern blot analysis. Three stable, phleomycine (p) resistant and GUS (g) positive transformants with a single copy insertion of either gene were selected and tested for their pathogenicity on different tomato lines. All three showed a pathogenic profile identical to Fol007wt (data not shown). One of them was designated Fol007pg and used in our further studies.
Induction of mutants

A gamma irradiation approach was followed to create avirulent mutants. Conidia spores of Fol007wt were irradiated for different periods of time, and allowed to germinate and grow for two days on PDA plates. Emerging colonies were counted and taken as a measure of survival of the treatment. Figure 1 shows the dose-dependent sensitivity of conidia spores to $^{137}$Cs irradiation. In the dose-range used a linear relationship is observed between survival and exposure. In all further experiments an exposure dosage of 130 Gy was used resulting in approximately 20% survival of irradiated conidia spores.

To establish the mutagenic effect of gamma irradiation, the induction of mutations in the nitrate reductase gene was examined. This gene is required for nitrate utilization and mutants can be detected easily by selection for chlorate resistance and subsequent media complementation analysis (Correll et al., 1987). Nitrate reductase mutants were found at a frequency of one per 2600 surviving conidia, a factor 140 times higher than found spontaneously. Twenty-four mutants were further characterised by Southern blot analysis using a 4 kb DNA fragment containing the whole nitrate reductase gene as probe (Diolez et al., 1993). Nine mutants showed an altered hybridization pattern compared to wild type (data not shown), suggesting a deletion in or close by the nitrate reductase gene. One mutant did not hybridize at all with the probe indicating a deletion of at least 4 kb. The genome of the remaining 14 mutants probably contain point mutations or deletions in the nitrate reductase gene too small to detect by Southern blot analysis under the conditions used.

![Graph showing dose-response of conidia of F. oxysporum f.sp. lycopersici exposed to $^{137}$Cs irradiation. Dose is expressed in Grays.](image-url)
In a large scale experiment conidia of Fol007pg were irradiated (130 Gy) and allowed to grow. To select out mutants affected in their growth (e.g. auxotrophic mutants) or spore production, emerging colonies were transferred to liquid minimal medium. Approximately 2.2% of the irradiated surviving colonies displayed mutations that affected growth and/or spore formation. Full grown cultures were individually used to inoculate seedlings of C295 tomato. Efforts to test pools of mutants were unsuccessful probably due to the induction of resistance by avirulent isolates in a mixture of spores. In total 21,712 colonies that survived the gamma irradiation and the selection procedures were tested for virulence on tomato seedlings. In 37 cases plants showed Fusarium wilt symptoms. From these diseased plants Fusarium was reisolated, purified and used to inoculate C295 in a second screen. Only one of the 37 re-isolates showed repeatedly Fusarium disease symptoms on C295 tomato and hence seemed to be affected in a function required for full expression of resistance brought about by the 1-2 gene. This isolate was designated Fol007avr.

**Biological characterization of the mutant.**

On phleomycine containing agar plates re-isolated Fol007avr showed the same radial growth and colony morphology as Fol007pg, whereas none of the four race 3 isolates in our collection (Table 1) tested was resistant to phleomycine (Fig. 2). GUS activity was detected in Fol007avr as well, confirming that the mutant was derived from Fol007pg. Conidia production in liquid CD and PDB was equal for Fol007wt, Fol007pg and Fol007avr, and comparable to Fol029, a
race 3 isolate of \textit{F. oxysporum} f.sp. \textit{lycopersici} (Table 1). All these observations are in accordance with the notion that the primary metabolism of the mutant was not affected and that no genes are mutated responsible for growth and spore production.

To further characterize Fol007avr a range of tomato lines containing various race-specific resistance genes against \textit{F. oxysporum} f.sp. \textit{lycopersici} were inoculated. Experiments were repeated several times and the results were all comparable. The outcome of one experiment is shown in Table 2. Tomato lines susceptible to race 2 (C32, GCR161 and 90E402F) are heavily infected by Fol007wt and Fol007pg resulting in significant loss of plant weight. Fol007avr infects these lines as well although much less effective then the isolate it originates from. This suggests that the mutant is affected in its pathogenicity. Tomato lines containing the 1-2 locus (90E341F, C295) or 1-3 locus (90E218F) are infected by neither Fol007wt nor Fol007pg; none of the plants showed Fusarium wilt symptoms although in some cases the mean weight of plants infected with Fol007pg differed significantly from the mean weight of the water control plants. However, this weight reduction was not found consistently (see Table 3). In contrast, both 1-2 containing lines 90E341F and C295 were infected by Fol007avr: all ten infected plants of 90E341F and seven out of ten infected C295 plants showed Fusarium wilt symptoms (Fig. 3). In addition, weight reduction was significant compared to Fol007wt and Fol007pg plants. This result confirms the suggestion that Fol007avr is impaired in facilitating expression of resistance brought about by the 1-2 gene. Resistance of the 1-3 locus (line 90E218F) was not broken by any of the isolates nor by the mutant, indicating that functional expression of \textit{avrI-3} is not affected by the mutation(s) in Fol007avr. Isolate Fol029 is clearly virulent on all lines that lack the I-3 resistance gene, supporting its classification as a race 3 isolate.

\textbf{Table 2.} Mean plant fresh weight (g) of tomato plants 3 weeks after inoculation with isolates of \textit{Fusarium oxysporum} f.sp. \textit{lycopersici}.

<table>
<thead>
<tr>
<th>Tomato lines</th>
<th>C32 I</th>
<th>GCR161 I-1-2</th>
<th>90E402F I-1</th>
<th>90E341F I-2</th>
<th>C295 I-I-2</th>
<th>90E218F I-2, I-3</th>
</tr>
</thead>
<tbody>
<tr>
<td>race isolate</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>007wt</td>
<td>0.1 ± 0.2 c*</td>
<td>1.1 ± 1.4 c</td>
<td>0.9 ± 0.8 c</td>
<td>11.4 ± 1.3 a</td>
<td>10.2 ± 1.9 a</td>
</tr>
<tr>
<td>2</td>
<td>007pg</td>
<td>1.3 ± 1.5 a</td>
<td>0.9 ± 1.7 c</td>
<td>2.2 ± 2.6 c</td>
<td>8.4 ± 1.2 c</td>
<td>8.7 ± 0.7 ab</td>
</tr>
<tr>
<td>?</td>
<td>007avr</td>
<td>3.9 ± 2.6 b</td>
<td>6.4 ± 4.5 b</td>
<td>7.8 ± 4.0 b</td>
<td>3.8 ± 1.5 d</td>
<td>7.0 ± 3.7 b</td>
</tr>
<tr>
<td>3</td>
<td>029</td>
<td>0.1 ± 0.2 c</td>
<td>0.0 ± 0.0 c</td>
<td>0.1 ± 0.1 d</td>
<td>0.1 ± 0.1 e</td>
<td>0.1 ± 0.1 c</td>
</tr>
<tr>
<td>H2O cont.</td>
<td></td>
<td>9.8 ± 1.5 a</td>
<td>10.5 ± 1.7 a</td>
<td>10.2 ± 2.3 a</td>
<td>9.9 ± 2.0 b</td>
<td>9.3 ± 3.5 a</td>
</tr>
</tbody>
</table>

* Vertical row values followed by the same letter do not differ significantly according to \textit{F}-test (\(p = 95\%\)). For all genotypes \(p=0.0001\).
Loss of avirulence

Fig. 3. Plant growth 3 weeks after infection with *F. oxysporum* f.sp. *lycopersici* Fol007pg and Fol007avr.

To corroborate that Fol007avr is affected in the promotion of I-2 mediated resistance, experiments were carried out with the *F. oxysporum* f.sp. *lycopersici* race 2 susceptible tomato line KG52201 and the same line transgenic for the I-2 gene (Table 3). As expected, stains Fol007wt, Fol007pg and Fol007avr could infect line KG52201 and control line GCR161. And again Fol007avr showed a reduced pathogenicity. After inoculation with Fol007wt or Fol007avr.

Table 3. Mean plant fresh weight (g) of I-2 transgenic lines KG324 and control lines, weeks after inoculation with isolates of *Fusarium oxysporum* f.sp. *lycopersici*.

<table>
<thead>
<tr>
<th>race isolate</th>
<th>KG52201</th>
<th>GCR161</th>
<th>KG324</th>
<th>C295</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>I locus</td>
<td>cosmid B22</td>
<td>I2 locus</td>
<td></td>
</tr>
<tr>
<td>2 007wt</td>
<td>0.0 ± 0.1 b</td>
<td>1.1 ± 1.8 c</td>
<td>7.7 ± 1.4 a</td>
<td>10.3 ± 1.5 a</td>
</tr>
<tr>
<td>2 007pg</td>
<td>0.6 ± 2.5 b</td>
<td>1.8 ± 3.0 c</td>
<td>7.7 ± 1.4 a</td>
<td>10.4 ± 1.4 a</td>
</tr>
<tr>
<td>2 007avr</td>
<td>1.6 ± 1.9 b</td>
<td>4.5 ± 3.8 b</td>
<td>5.5 ± 2.1 b</td>
<td>7.3 ± 3.8 b</td>
</tr>
<tr>
<td>2 029</td>
<td>0.3 ± 0.8 b</td>
<td>0.1 ± 0.2 c</td>
<td>0.1 ± 0.2 c</td>
<td>0.1 ± 0.2 c</td>
</tr>
<tr>
<td>H2O cont.</td>
<td>10.1 ± 1.3 a</td>
<td>10.8 ± 1.4 a</td>
<td>8.0 ± 1.5 a</td>
<td>11.4 ± 1.7 a</td>
</tr>
</tbody>
</table>

* Vertical row values followed by the same letter do not differ significantly according to F-test (p = 95%). For all genotypes p=0.0001.
Fol007pg, no Fusarium wilt disease symptoms were found in control line C295 nor in line KG324, a KG52201 derivative transgenic for the I-2 gene (Simons et al., 1998). However, both lines are susceptible to Fol007avr resulting in a significant reduction in mean weight. All lines are susceptible for race 3 isolate Fol029, indicating the specificity of the I-2 gene. The results re-confirm that Fol007avr is affected in its pathogenicity and is able to break the I-2 dependent resistance.

**Karyotyping *F. oxysporum f.sp. lycopersici* isolates**

Gamma irradiation may change the chromosome organisation of a cell. To examine the mutant for major DNA rearrangements, contour-clamped homogeneous electrophoretic field (CHEF) analysis was carried out. Mutant Fol007avr (Fig. 4A, lane 5) was compared with the original isolate Fol007wt (lane 4), with Fol007pg (not shown) and with other isolates from different races that all belong to the same genetic group VCG0030 (lanes 2-4 and 7-9). All isolates showed different karyotypes, reflecting the plasticity of the *Fusarium* genome. The number of chromosomes that could be separated was estimated at 12, ranging in size from 1.3 Mb to at least 6 Mb. Fol007wt and Fol007pg showed an identical karyotype (data not shown). As compared to Fol007wt, Fol007avr misses a chromosome of 3.75 Mb (Fig. 4A, lanes 4-5). This could be due to either a translocation to one of the larger chromosomes that are difficult to separate, or to a loss of sequences. To further investigate this, probes were developed specific for the chromosome of 3.75 Mb and these were used in Southern analysis.

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**Fig. 4.** CHEF analysis of isolates of *F. oxysporum f.sp. lycopersici*. **A**, Gel stained with etidium bromide. **B**, Gel blotted and hybridized with a chromosome specific probe. lane M, marker; lane 1, Fol004; lane 2, Fol002; lane 3, Fol015; lane 4, Fol007wt; lane 5, Fol007avr; lane 6, Fol026; lane 7, Fol029; lane 8, Fol035; lane 9, Fol036.
On genomic DNA none of the probes resulted in a polymorphism between Fol007wt and Fol007avr, suggesting that the chromosome was not lost. Subsequently, one cloned DNA fragment was used to probe the blot of the CHEF gel (Fig. 4B). In Fol007wt and all other isolates the probe hybridised to the chromosome of 3.75 Mb, confirming the chromosome specificity of the cloned fragment and conservation of this chromosome among the isolates. However, in Fol007avr (Fig. 4B, lane 5) hybridisation was found to the largest chromosome only, suggesting a translocation of the 3.75 Mb chromosome to the largest chromosome.

**RAPD and AFLP analysis**

In an approach to find polymorphisms between Fol007pg and Fol007avr a RAPD analysis was performed. Hundred primers were tested generating more than 1000 fragments reflecting approximately 2% of the genome. All amplified fragments except for one were found in both Fol007pg and Fol007avr, supporting the hypothesis of a chromosome translocation rather than a deletion. Only primer OPA-14 reproducibly gave a polymorphism between Fol007pg and Fol007avr: a fragment of approximately 1300 bp (A141300) present in Fol007pg was not found in Fol007avr; reciprocally, a fragment of 2000 bp (A142000) was present in Fol007avr but not in Fol007pg. Both fragments were cloned and used in Southern analysis. Total genomic DNA of Fol007pg and Fol007avr, digested with either EcoRI or HindIII, was probed with either the A141300 fragment or the A142000 fragment. The latter probe hybridized with numerous bands in both Fol007pg and Fol007avr, suggesting that it contains a repetative sequence (data not shown). In contrast, hybridization with A141300 revealed a single copy polymorphism between Fol007pg and Fol007avr (Fig. 5).

To test for more polymorphisms in Fol007avr, AFLP analysis was carried out. In a first screen all 64 primer combination with 3 selective nucleotides were used in the amplification reactions. Upto 125 bands per primer combination were generated and approximately 12.5% of the genome was covered this way. When a (potential) polymorphism was found, the reaction was repeated in four amplification mixtures with primers containing an additional selective nucleotide. Each polymorphism found in this way was check at least once more. Nine AFLP polymorphisms were identified that all were generated by the same Eco primer and appeared as extra fragments in the mutant Fol007avr. Southern blot analysis using the cloned polymorphic AFLP fragments as probes revealed multiple hybridizing fragments in both Fol007pg and Fol007avr for each fragment. Among the smear of hybridizing fragments it was difficult to determine whether the fragments detected real polymorphisms in the DNA of the mutant.
Fig. 5. Polymorphism between Fol007pg and Fol007avr detected by Southern blot analysis using A141300 as probe. M, marker; Lane 1, Fol007pg digested with EcoRI; Lane 2, Fol007avr digested with EcoRI; Lane 3, Fol007pg digested with HindIII; Lane 4, Fol007avr digested with HindIII.

DISCUSSION

According the gene-for-gene hypothesis, expression of race-specific resistance responses depends on the presence of both a pathogen avirulence gene and the corresponding R-gene in the plant (Flor, 1971). For many interactions the existence of a pathogen avirulence gene and a corresponding R gene in the plant has been proven genetically. Nowadays more than 15 resistance genes have been isolated and characterized (Ellis and Jones, 1998), and in some cases corresponding avirulence genes as well. For the F. oxysporum f.sp. lycopersici-tomato interaction a gene-for-gene relationship is generally assumed. Indeed, monogenic, dominant resistance traits against the three known races of this tomato pathogen have been described. One of these traits, the I-2 gene, conferring resistance to race 2 isolates has been cloned recently (Ori et al., 1997; Simons et al., 1998). The gene product shares many features with the proteins encoded by other R genes thought to be involved in gene-for-gene relationships. However, due to the imperfect character of the fungus, the existence of F. oxysporum f.sp. lycopersici avr-genes has not been proven yet. Indirect evidence for their existence includes the
presence of all avirulent genotypes of avirulence 1 and 2 within the natural population (Mes et al., 1999, chapter 2). The *F. oxysporum* f.sp. *lycopersici* mutant described here shows the change from avirulence to virulence. Whatever the nature of this change it proofs the capacity of *F. oxysporum* f.sp. *lycopersici* race 2 to gain virulence for a plant containing the 1-2 gene. This is striking evidence for avirulence genes to play an important role in the interaction between *F. oxysporum* f.sp. *lycopersici* and tomato.

The mutant identified in our gamma irradiation screen was changed in both avirulence and pathogenicity. This could imply that (I) deletions were induced affecting both pathogenicity and avirulence, (II) a common factor was deleted involved in signalling, regulation or secretion of both pathogenicity and avirulence factors, or (III) pathogenicity and avirulence are encoded by the same genetic trait. The first explanation can not be excluded completely. Testing the effect of gamma-irradiation we found that more than 40% of the nitrate reductase mutants had one or more deletions that could be made visible by Southern analysis. One out of these 24 mutants had a deletion of more than 4 kb, containing the entire nitrate reductase gene. The second option can not be excluded either. Many avirulence and pathogenicity genes are induced *in planta*. This suggests that the fungus senses the plant. Fungal receptors and down stream signals will have a major role in activation of these genes. When the expression of fungal pathogenicity and avirulence genes is regulated by common factors, mutations in genes encoding such factors will knock out proper function of both. If Fol007avr has a mutation in a common signalling factor it would be interesting to clone the gene encoding this factor since such a gene could lead us to the identification of pathogenicity and avirulence factors. The third explanation seems to be most likely to explain a change in both pathogenicity and avirulence: pathogenicity and avirulence are determined by the same factor. For many bacterial avirulence genes it has been found that they are involved in fitness or pathogenicity (virulence) of the pathogen (Dangl, 1995; Leach and White, 1996; Vivian and Gibbon, 1997). The same seems to hold true for fungal avirulence genes: NIP1, the host specific toxin of *Rynchosporum secalis* which is necessary for symptom development (Rohe et al., 1995) and ECP2 of *Cladosporium fulvum* which is required for full virulence of the fungus on tomato, induces a hypersensitive response-based resistance (Lauge et al., 1998). These results show the thin line between pathogenicity and avirulence. The *F. oxysporum* f.sp. *lycopersici* race 3 isolate that infects the same set of tomato lines as the mutant Fol007avr, is very pathogenic. Compared to race 1 and 2 isolates from the same genetic group (VCG0030), race 3 is even more pathogenic on susceptible plants like C32. This could imply that single mutations in a pathogenicity / avirulence gene could result in a more effective pathogenicity factor able to circumvent plant recognition. Disruption of such a gene could result in a non or less pathogenic mutant. Fol026 (Table 1), an isolate originating from Australia, gave comparable plant weight reduction as Fol007avr (unpublished results). This isolate was classified as race 3 because it could break 1-2 resistance. In our inoculation experiments Fol026 was not as pathogenic as three other race 3
isolates showing a pathogenicity comparable to Fol029. The low pathogenicity of Fol026 may be explained by its low spore production compared to the other race 3 isolates.

One factor determining both pathogenicity and avirulence could explain the low frequency at which we have found avirulent mutants. Using the nitrate reductase gene as target sequence we found a deletion mutation frequency of one out of every 2600 surviving conidia. Taken into account the low number of mutants tested in this pilot experiment and the relative large size of the nitrate reductase gene, we expected to find more mutants than one out of 21,712 mutants tested for loss of avirulence. When pathogenicity and avirulence are encoded by the same gene, a mutation would affect both traits. Only in case the mutation knocks out avirulence completely but affects pathogenicity only slightly or not at all, avirulence mutants can be selected. Obviously, such mutants will be found at frequencies lower than can be expected for a single trait encoding gene. Likewise, when the avirulence trait is not present as a single gene or when it is located within a small locus, an avirulence mutant will never be found or at very low frequencies only.

Whatever the genetic basis of pathogenicity and avirulence, it will be interesting to see which factor of *F. oxysporum* f.sp. *lycopersici* is responsible for the observed phenotype of the mutant. DNA analysis have revealed genomic alterations in the mutant. By CHEF analysis and hybridization experiments using chromosome specific probes, a translocation in the mutant could be identified. The conclusion that we are dealing with a chromosome translocation rather than a loss of a chromosome is supported by the low frequency at which polymorphisms are detected by RAPD and AFLP analysis. Loss of a 3.75 Mb chromosome is equal to the loss of approximately 7.5% of the genome. Both RAPD analysis and AFLP analysis revealed only 0.1% of polymorphisms, which were not even fragments absent in the mutant. This chromosomal translocation might have been induced by gamma irradiation. However, spontaneously chromosomal translocations have been observed in fungi as well (Zolan, 1995). In this context it is noteworthy that all isolates tested in the CHEF analysis are from linear clonal origin but that they all exhibited other chromosomal profiles.

The RAPD polymorphism and AFLP polymorphisms identified in this study are important. They show that detectable mutations have been induced in the mutant. Detailed study of the RAPD polymorphism will be straightforward because it is present as a polymorphic single copy fragment. The AFLP polymorphisms, however, are very puzzling. They were generated by the same selective Eco-primer and all appeared as extra fragments in the mutant whereas absence of fragment are more expected for a deletion mutagenesis. Moreover they all seem to be of repetitive origin what will complicate detailed analysis. It might be that these polymorphisms have been generated by a retroposon which have been duplicated and inserted at new sites. Further characterization of these polymorphisms will unravel the genomic alteration in the mutant and hopefully will lead us to the cloning of the avirulence gene of *F. oxysporum* f.sp. *lycopersici*. 
LITERATURE CITED


