Loss of Avirulence and Reduced Pathogenicity of a Gamma-Irradiated Mutant of *Fusarium oxysporum* f. sp. *lycopersici*

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ABSTRACT

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The tomato *Fusarium* resistance gene *I-2* confers resistance to *F. oxysporum* f. sp. *lycopersici* race 2, which expresses the corresponding avirulence gene *avrI-2*. To elucidate the molecular basis of this gene-for-gene interaction, we initiated a search for the *avrI-2* gene. Gamma irradiation mutagenesis, using ^{137}Cs, was performed to generate an *avrI-2* mutant of *F. oxysporum* f. sp. *lycopersici*. To this end, a race 2 isolate was first transformed with a phleomycine resistance gene and a GUS marker gene in order to distinguish mutants from contaminating isolates. A total of 21,712 mutagenized colonies was tested for loss of avirulence on *I-2*-containing tomato seedlings. One mutant was selected that showed the expected loss of avirulence but, surprisingly, also showed reduced pathogenicity toward susceptible tomato plants. DNA analysis was subsequently used to visualize genomic changes in the mutant. Southern analysis on contour-clamped homogeneous electrophoretic field blots demonstrated a translocation of a 3.75-Mb chromosome in the mutant. Random amplified polymorphic DNA and amplified fragment length polymorphism analysis identified at least nine polymorphisms between the wild-type and mutant isolates. Most of these polymorphisms appeared as extra fragments in the mutant and contained repetitive DNA sequences.

Fusarium oxysporum is a fungal pathogen infecting a wide range of host plants. Isolates of the species *F. oxysporum* are divided into at least 80 different formae speciales correlating with their host range (2). Within formae speciales, isolates are subdivided into races, depending on the cultivars they can infect successfully. We use the interaction between tomato and *F. oxysporum* f. sp. *lycopersici* as a model system to study wilting diseases (13,21,32). Recently, we gathered evidence for a gene-for-gene relationship (8) between *F. oxysporum* f. sp. *lycopersici* and tomato (21). Races of *F. oxysporum* f. sp. *lycopersici* lacking a functional avirulence gene fail to activate host defenses in time, resulting in successful colonization of the xylem vessels and disruption of the water-conduction system of the plant. Isolation of avirulence genes may give more insight into the early steps of race-specific defense 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 nucleotide binding site–leucine-rich repeat (NBS-LRR) class (7,23,32). While resistance genes share similar basic elements, no common features have been identified in the corresponding avirulence genes thus far (25). Therefore, cloning of avirulence genes based on structural similarity, as currently is performed for resistance genes (16), is not an option. Avirulence genes of plant-pathogenic bacteria have been cloned by complementation approaches (5). Fungal avirulence genes have been isolated by product-based cloning (10,28,40) and by map-based cloning (11,35,38). For *F. oxysporum*, shotgun complementation is not feasible because of the size of the genome and the low efficiency of transformation. Furthermore, avirulence genes are often expressed in planta only (10,39) and, therefore, attempts to isolate the avirulence gene product are complicated by the localization of the interaction, notably the xylem vessels. Map-based cloning is not possible because the life cycle of *F. oxysporum* does not include a sexual stage and, hence, classical genetic studies with the fungus cannot be carried out. The recently developed transposon tagging system for *F. oxysporum* f. sp. *lycopersici* (22) is also a promising method. Following the strategy to isolate avirulence genes from the flax rust fungus (37), we chose a random deletion mutagenesis approach to select for mutants that have lost avirulence. Subtraction techniques (18,34), RNA comparison analysis (17), and marker-based selection methods (42,44) can subsequently be used to trace the deletion and to clone the avirulence gene.

Here, we report on the efficiency of deletion mutagenesis using gamma irradiation to induce mutants of *F. oxysporum* f. sp. *lycopersici* and on the selection of a virulent mutant. As a next step toward the isolation of the avirulence gene, the mutant was subjected to random amplified polymorphic DNA (RAPD) and amplified fragment length polymorphism (AFLP) analysis to identify genomic changes.

MATERIALS AND METHODS

Fungal transformation. Two *F. oxysporum* f. sp. *lycopersici* isolates, race 2 isolate Fol007 and race 3 isolate Fol029 (21), were used in this study. Fol007 was used for fungal transformation to mark the isolate with phleomycine resistance and the GUS marker genes. Plasmid pAN8.1, containing the phleomycine resistance gene fused to the glyceraldehyde phosphate (*gpd*) promoter, was obtained from P. Punt, TNO, Zeist, the Netherlands (26). Plasmid pCF20, containing the *uidA* (GUS) gene controlled by the same *gpd* promoter, was obtained from G. Honee, Agricultural University, Wageningen, the Netherlands (27). Protoplast isolation and fungal transformation were essentially the same as described by Kistler and Benny (12), with some modifications. Isolates of *F. oxysporum* f. sp. *lycopersici* were grown in liquid potato dextrose broth (PDB) for 5 days at 25°C with continuous shaking. Washed conidia were used to inoculate an overnight culture by adding 5×10^8 conidia to

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40 ml of PDB. After 15 h of growth, mycelium was collected by centrifugation and washed with 1.2 M $MgSO_4$ (pH 5.8). Digestion of cell walls was performed with Glucanex (25 mg/ml; Novo Nordisk Ferment AG, Neumalt, Switzerland) 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, CA). Four volumes of 1 M sorbitol solution was added, and protoplasts were collected by centrifugation. Protoplasts were washed once in sorbitol, counted, and diluted to 2×10^8 protoplasts (pps) per ml. For transformation, 10 µg of plasmid was added to 200 µl of protoplasts. During transformation, DNase inhibitor aurintricarboxylic acid was added to a final concentration of 10 mM (26). Subsequently, polyethylene glycol (PEG) 6000 (60% in 10 mM Tris-HCl, pH 7.5, and 50 mM $CaCl_2$) was added to the protoplast/DNA mixture. After incubation for 30 min, 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) osmostabilized with 0.5 M $MgSO_4$ (30) containing 100 µg of phleomycine per ml (Cayla, Toulouse, France).

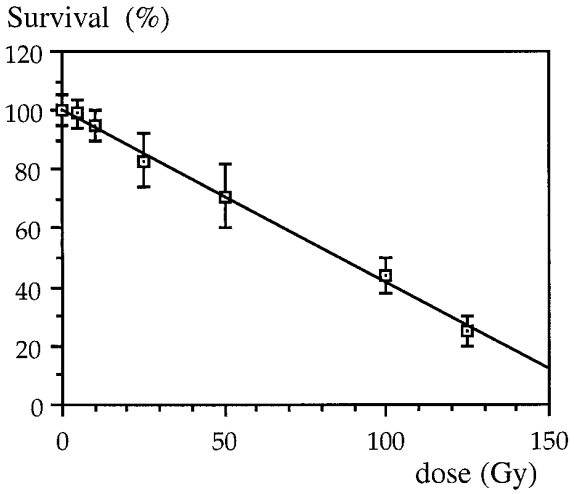

Fig. 1. Dose response of conidia of *Fusarium oxysporum* f. sp. *lycopersici* exposed to ^{137}Cs irradiation. Dose is expressed in grays (Gy). $R^2 = 0.996$.

Irradiation mutagenesis. Conidia scraped from *F. oxysporum* f. sp. *lycopersici* cultures grown for 2 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 ^{137}Cs-unit H 622 at different dosage levels (0.9 Gy per min) and then incubated at room temperature for several days until colonies grew from the surviving conidia.

Irradiation efficiency was analyzed by testing for chlorate-resistant mutants, of which some have a mutation at the nitrate reductase gene. Conidia were plated on CDA supplemented with 5% chlorate before and during gamma irradiation. Chlorate-resistant colonies were transferred to CDA to confirm the nitrate nonutilizing deficiency, visible as thin, growing mycelium. Mutants with mutations in the nitrate reductase gene were selected by media complementation; only mutants whose morphology is restored by CDA media supplemented with NO_2 (0.5 g/liter) or hypoxanthine (0.2 g/liter) have a mutation at the nitrate reductase locus (4).

DNA isolation and DNA gel blot analysis of the nitrate reductase mutants were performed as reported previously (20). A 4-kilobase (kb) *Hin*dIII fragment of plasmid pNE24 (obtained from M.-J. Daboussi, University Paris-Sud, Orsay, France), containing the complete nitrate reductase gene (6) was used as a probe.

For large-scale selection of a virulent mutant, conidia were plated on PDA (500 conidia per plate), irradiated, and grown for 2 days

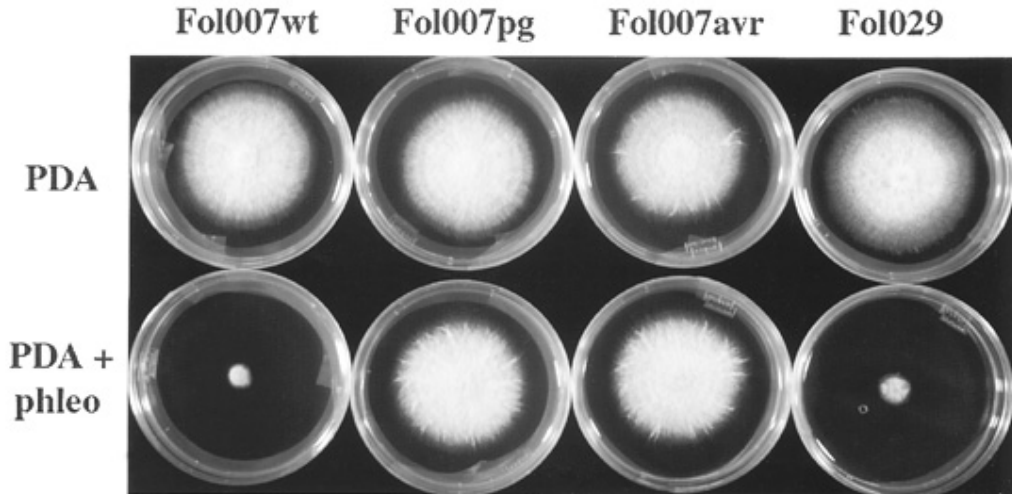

Fig. 2. Growth of *Fusarium oxysporum* f. sp. *lycopersici* isolates on potato dextrose agar plates with or without the addition of phleomycine. Fol029 is one of the four race 3 isolates tested, which all showed to be susceptible to phleomycine.

TABLE 1. Mean plant fresh weight 3 weeks after inoculation of different tomato lines infected by the wild type of Fol007 (Fol007wt), the phleomycine and GUS transformant of Fol007 (Fol007pg), the mutant of Fol007 (Fol007avr), and race 3 isolate Fol029

		Tomato lines						
Race	Isolate	C32 –[y]	GCR161 *I*	90E402F *I-1*	OT364 *I-2*	90E341F *I-2*	C295 *I, I-2*	90E218F *I-2, I-3*
2	Fol007wt	0.1 ± 0.2 c[z]	1.1 ± 1.4 c	0.9 ± 0.8 c	9.6 ± 2.1 b	11.4 ± 1.3 a	10.2 ± 1.9 a	11.9 ± 2.0 a
2	Fol007pg	1.3 ± 1.5 c	0.9 ± 1.7 c	2.2 ± 2.6 c	9.3 ± 1.2 b	8.4 ± 1.2 c	8.7 ± 0.7 ab	8.4 ± 3.1 b
?	Fol007avr	3.9 ± 2.6 b	6.4 ± 4.5 b	7.8 ± 4.0 b	11.4 ± 2.9 ab	3.8 ± 1.5 d	7.0 ± 3.7 b	12.0 ± 2.3 a
3	Fol029	0.1 ± 0.2 c	0.0 ± 0.0 c	0.1 ± 0.1 d	0.6 ± 1.0 c	0.1 ± 0.1 e	0.1 ± 0.1 c	11.8 ± 1.7 a
H_2O	Control	9.8 ± 1.5 a	10.5 ± 1.7 a	10.2 ± 2.3 a	11.6 ± 2.7 a	9.9 ± 2.0 b	9.3 ± 3.5 a	9.4 ± 2.1 b

[y] Indicates the *Fusarium* resistance loci (*I, I-1, I-2*, and *I-3*) that is present in the respective tomato lines.
[z] Vertical row values followed by the same letter do not differ significantly according to an *F* test ($P = 95\%$).

at room temperature. Colonies were transferred to tubes containing 3 ml of liquid Czapek-Dox (CD) and grown for 4 days at 25°C with continuous motion. The whole mixture of mycelia and conidia was used to inoculate seedlings (7 days old) of tomato line C295. Inoculated plants were grown in the greenhouse at 25°C for 3 weeks and then examined for Fusarium wilt symptoms. Infected plants were used for reisolation of the mutant causing the symptoms. Single-spore cultures of the reisolate were tested the same way as described above to distinguish real virulent mutants from false positives.

Plant material. The previously described near-isogenic lines of tomato cv. Moneymaker, C32, GCR161, and C295 (21), were used. C32 is susceptible to races 1 and 2; GCR161 contains the resistance to race 1; C295 (also known as Mobox; H. Laterrot, INRA, Versailles, France) is resistant to both races 1 and 2. Three tomato lines were provided by J. W. Scott (University of Florida, Gainesville): (i) 90E341F, contains the race 2 resistance of the *I-2* locus of PI126915 (33) without the linked resistance against race 1; (ii) 90E402F, contains the race 1 resistance (*I-1*) obtained from LA716 (31); and (iii) 90E218F, contains the *I-3* locus of LA716 giving resistance against races 2 and 3. KeyGene (Wageningen, the Netherlands) provided a transgenic line (KG324) of susceptible tomato line KG52201 transformed with cosmid B22 containing the *I-2* gene (32). The original transformant was selfed and a homozygous T2 line selected.

Plant infection. Seedlings (12 days old) were infected by a standard root-dip inoculation (21). All inoculation experiments were performed in 10 replicates that were randomly arranged in equal blocks, each block containing all treatments once. After 3 weeks, the degree of vascular browning was estimated and the weight of the plant above the cotyledons was determined. Data were statistically analyzed with analysis of variance.

Contour-clamped homogeneous electrophoretic field (CHEF) analysis. 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 STE containing 1.2% InCert agarose (FMC BioProducts, Rockland, ME) and mounted in mold chambers. Plugs were incubated in NDS (0.1 M Tris, 0.5 M EDTA, and 1% laurylsarcosyl, pH 9.5) containing 2 mg of pronaseE per ml at 50°C overnight and washed three times in 50 mM EDTA (pH 8.0). 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 BioProducts) at 4°C using switch times between 20 to 80 min at 1.5 V/cm. Running buffer (0.5× Tris-borate-EDTA) was refreshed every 2 days. Gels were stained with ethidium bromide and destained with water. CHEF gel blot analysis was basically as described by Sambrook et al. (29).

RAPD and AFLP analysis. RAPD analysis was performed as described previously (21) using primers obtained from kits A, B, C, F, and K from Operon Technologies, Inc. (Alameda, CA).

AFLP analysis was performed according to Vos et al. (42), with some modifications. Standard adapters were ligated to *Eco*RI- and

TABLE 2. Mean plant fresh weight 3 weeks after inoculation of transgenic lines and control tomato lines with strains of *Fusarium oxysporum* f. sp. *lycopersici*

		Tomato lines			
Race	Isolate	KG52201 –[y]	GCR161 *I* locus	KG324 cosmid B22	C295 *I-2* locus
2	Fol007wt	0.0 ± 0.1 b[z]	1.1 ± 1.8 c	7.7 ± 1.4 a	10.3 ± 1.5 a
2	Fol007pg	0.6 ± 2.5 b	1.8 ± 3.0 c	7.7 ± 1.4 a	10.4 ± 1.4 a
?	Fol007avr	1.6 ± 1.9 b	4.5 ± 3.8 b	5.5 ± 2.1 b	7.3 ± 3.8 b
3	Fol029	0.3 ± 0.8 b	0.1 ± 0.2 c	0.1 ± 0.2 c	0.1 ± 0.2 c
H_2O	Control	10.1 ± 1.3 a	10.8 ± 1.4 a	8.0 ± 1.5 a	11.4 ± 1.7 a

[y] Indicates the *Fusarium* resistance loci (*I* and *I-2*) or the *I-2* resistance gene containing cosmid B22 that is present in the respective tomato lines.
[z] Vertical row values followed by the same letter do not differ significantly according to an *F* test (*P* = 95%).

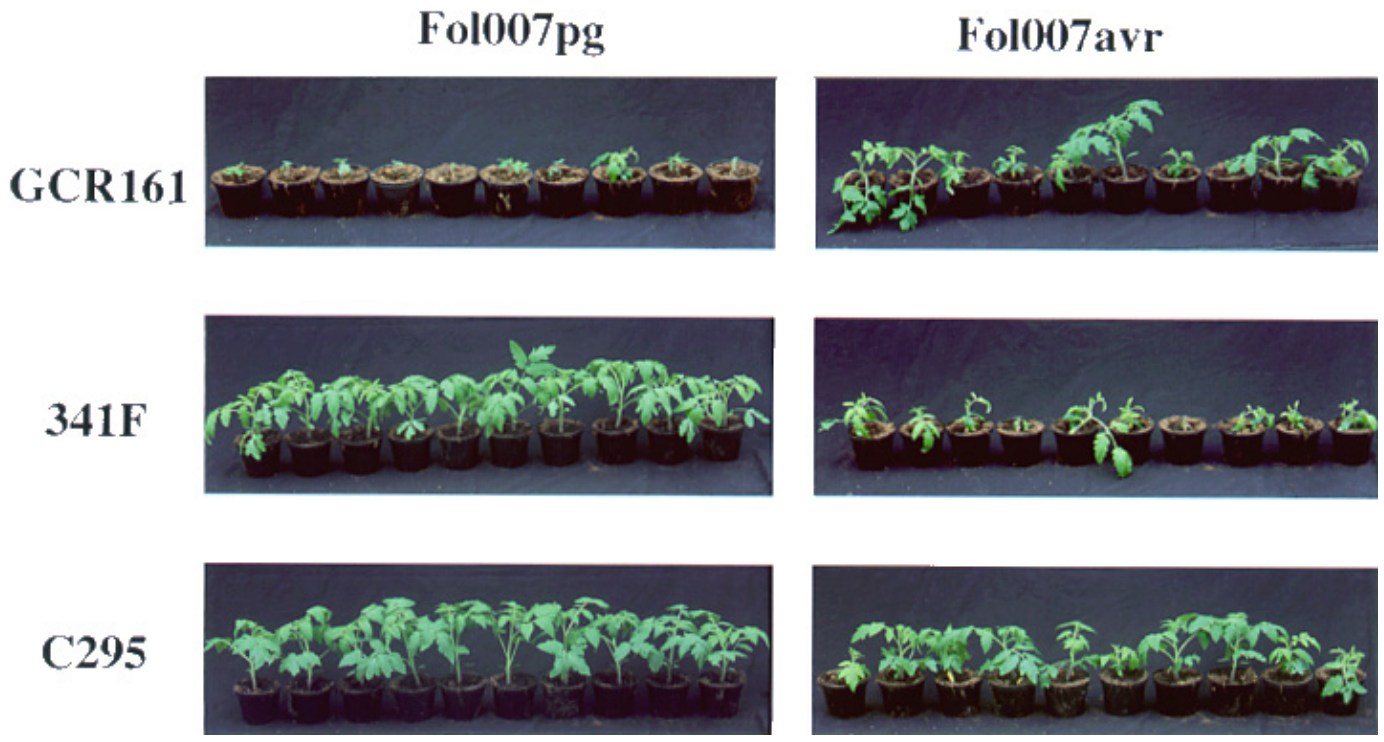

Fig. 3. Plant growth 3 weeks after infection with *Fusarium oxysporum* f. sp. *lycopersici* isolates Fol007pg and Fol007avr.

*Mse*I-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 three or four selective nucleotides.

RESULTS

Marking the wild-type isolate. Wild-type race 2 isolate Fol007 (Fol007wt) of *F. oxysporum* f. sp. *lycopersici* is avirulent on plants containing the *I-2* gene and pathogenic on plants lacking this race-specific resistance trait. Circumstantial evidence suggests that an avirulence gene is present in this isolate corresponding to the *I-2* resistance gene (21). To distinguish Fol007wt mutants lacking a functional *avrI-2* gene from naturally occurring race 3 isolates that can infect *I-2* plants, the wild-type race 2 isolate was marked with a phleomycine resistance gene and the β-glucuronidase (GUS) marker gene. The latter was introduced by cotransformation with the former. Transformants that were both phleomycine resistant and GUS positive (9) were single-spored and characterized by Southern 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, designated Fol007pg, was used in further studies.

Induction of mutants. A gamma irradiation approach was followed to create virulent mutants. Conidia spores of Fol007wt were irradiated for different periods of time and allowed to germinate on PDA plates. Emerging colonies were 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 to achieve approximately 20% survival.

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 by selection for chlorate resistance and subsequent media complementation analysis (4). Nitrate reductase mutants were found at a frequency of 1 per 2,600 surviving conidia, approximately 140 times higher than spontaneous mutations. Twenty-four mutants were characterized by Southern analysis. Nine mutants (37.5%) showed an altered hybridization pattern compared with wild-type isolates (data not shown), suggesting a deletion in or close by the nitrate reductase gene. One mutant did not hybridize at all, 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 analysis.

In a large-scale experiment to produce and select virulent mutants, conidia of Fol007pg were irradiated (130 Gy) and allowed to grow. To eliminate 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 colonies showed affected growth or spore formation. Wild-type growing cultures were individually used to inoculate seedlings of C295 tomato. Efforts to analyze pools of mutants were unsuccessful, probably due to the induction of resistance by avirulent isolates in the mixture. A total of 21,712 colonies that survived the gamma irradiation and selection on minimal medium was tested for virulence on tomato seedlings. In 37 cases, plants showed Fusarium wilt symptoms. From these diseased plants, the pathogen was reisolated, purified, and retested on plants. Only 1 of the 37 reisolates repeatedly showed Fusarium disease symptoms on C295 tomato and seemed affected in a function required for full expression of resistance brought about by the *I-2* gene. This isolate was designated Fol007avr.

Biological characterization of the mutant. On phleomycine-containing agar plates, Fol007avr showed the same radial growth and colony morphology as Fol007pg, whereas none of the race 3 isolates in our collection 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

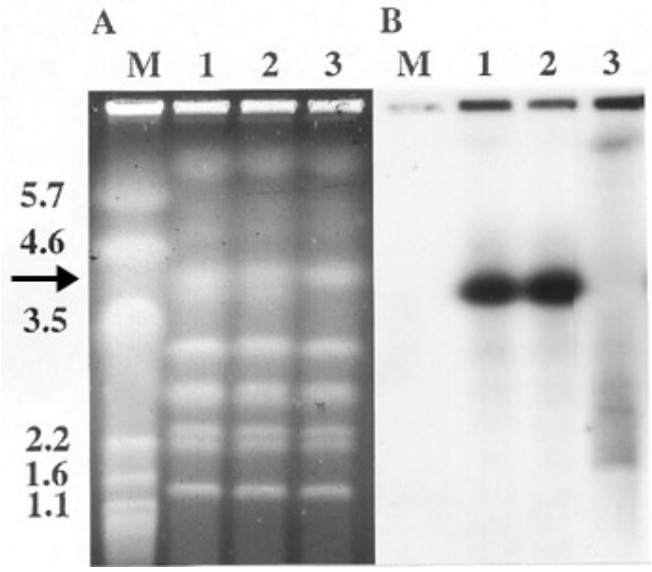

Fig. 4. Contour-clamped homogeneous electrophoretic field analysis of isolates of *Fusarium oxysporum* f. sp. *lycopersici*. **A,** Gel-stained with ethidium bromide. **B,** Gel-blotted and hybridized with a chromosome-specific probe. Lane M, marker (Mb); lane 1, Fol007wt; lane 2, Fol007pg; and lane 3, Fol007avr.

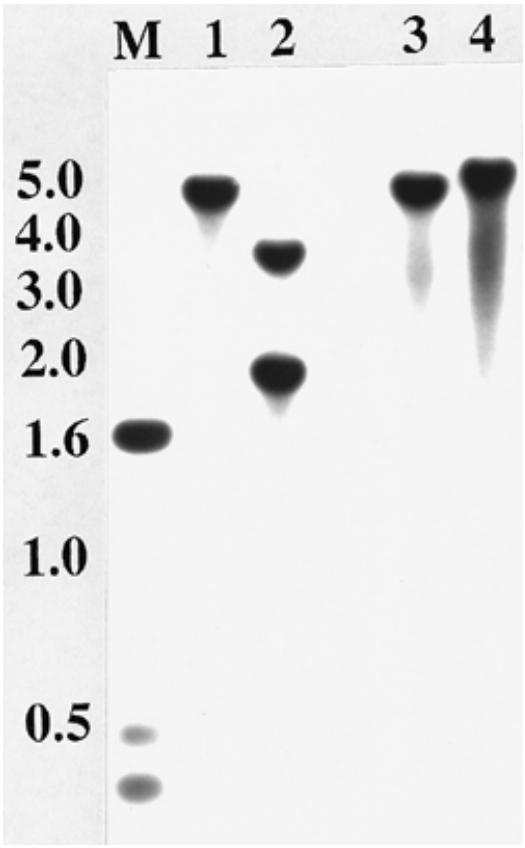

Fig. 5. Polymorphism between Fol007pg and Fol007avr detected by Southern blot analysis using $A14_{1300}$ as a probe. Lane M, marker; lane 1, Fol007pg digested with *Eco*RI; lane 2, Fol007avr digested with *Eco*RI; lane 3, Fol007pg digested with *Hin*dIII; and lane 4, Fol007avr digested with *Hin*dIII.

and comparable to Fol029, a race 3 isolate of *F. oxysporum* f. sp. *lycopersici*. All these observations suggest that the primary metabolism of the mutant was not affected and that no genes responsible for growth and spore production were mutated.

To further characterize Fol007avr, a range of tomato lines containing various race-specific resistance genes against *F. oxysporum* f. sp. *lycopersici* was inoculated. Experiments were repeated at least five times, and the results were all comparable. The outcome of one experiment is shown in Table 1. Tomato lines susceptible to race 2 (C32, GCR161, and 90E402F) are heavily infected by Fol007wt and Fol007pg, resulting in a significant loss of plant weight. Fol007avr infects these lines as well, although less than the isolate it originates from. This suggests that the mutant is affected in its pathogenicity. Tomato lines containing the *I-2* locus (90E341F and C295) or *I-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 the plants infected with Fol007pg differed significantly from the mean weight of the water control plants. However, this weight reduction was not found consistently (Table 2). In contrast, both *I-2*-containing lines, 90E341F and C295, were infected by Fol007avr; all 10 infected plants of 90E341F and 7 out of 10 infected C295 plants showed Fusarium wilt symptoms (Fig. 3). In addition, weight reduction was significant compared with that of Fol007wt and Fol007pg plants. This result confirms the suggestion that Fol007avr is impaired in facilitating expression of resistance brought about by the *I-2* gene. Resistance of the *I-3* locus (line 90E218F) was not broken by any of the isolates or by the mutant, indicating that functional expression of *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.

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 2). As expected, strains Fol007wt, Fol007pg, and Fol007avr could infect line KG52201 and control line GCR161. And again, Fol007avr showed a reduced pathogenicity. After inoculation with Fol007wt or Fol007pg, no Fusarium wilt disease symptoms were found in control line C295 or in line KG324, a KG52201 derivative transgenic for the *I-2* gene (32). 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 support the hypothesis that Fol007avr is affected in its pathogenicity and is able to break the *I-2*-dependent resistance.

Karyotyping of the isolates. Gamma irradiation may cause large chromosomal alterations (1,19). To examine the virulent mutant for major DNA rearrangements, CHEF analysis was carried out. Mutant Fol007avr (Fig. 4A, lane 3) was compared with the original isolate Fol007wt (lane 1) and Fol007pg (lane 2). 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 show an identical karyotype, but Fol007avr misses a chromosome of 3.75 Mb (Fig. 4A, lane 3 arrow). 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 3.75-Mb chromosome and these were used in Southern analysis. In Southern analysis of digested genomic DNA, none of the probes revealed 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 (Fig. 4B, lane 1) and Fol007pg (lane 2), the probe hybridized to the chromosome of 3.75 Mb, confirming the chromosome specificity of the cloned fragment. However, in Fol007avr (Fig. 4B, lane 3), hybridization was found to the largest chromosome only, suggesting a translocation of the 3.75-Mb chromosome to the largest chromosome.

RAPD and AFLP analysis. RAPD analysis was performed to detect polymorphisms between Fol007pg and Fol007avr. One hundred primers were tested, generating more than 1,000 fragments reflecting almost 2% of the genome. All amplified fragments, except for one, were found in both Fol007pg and Fol007avr. Only primer OPA-14 reproducibly gave a polymorphism between Fol007pg and Fol007avr: a fragment of approximately 1,300 base pairs (bp) ($A14_{1300}$) present in Fol007pg was not found in Fol007avr; recip-

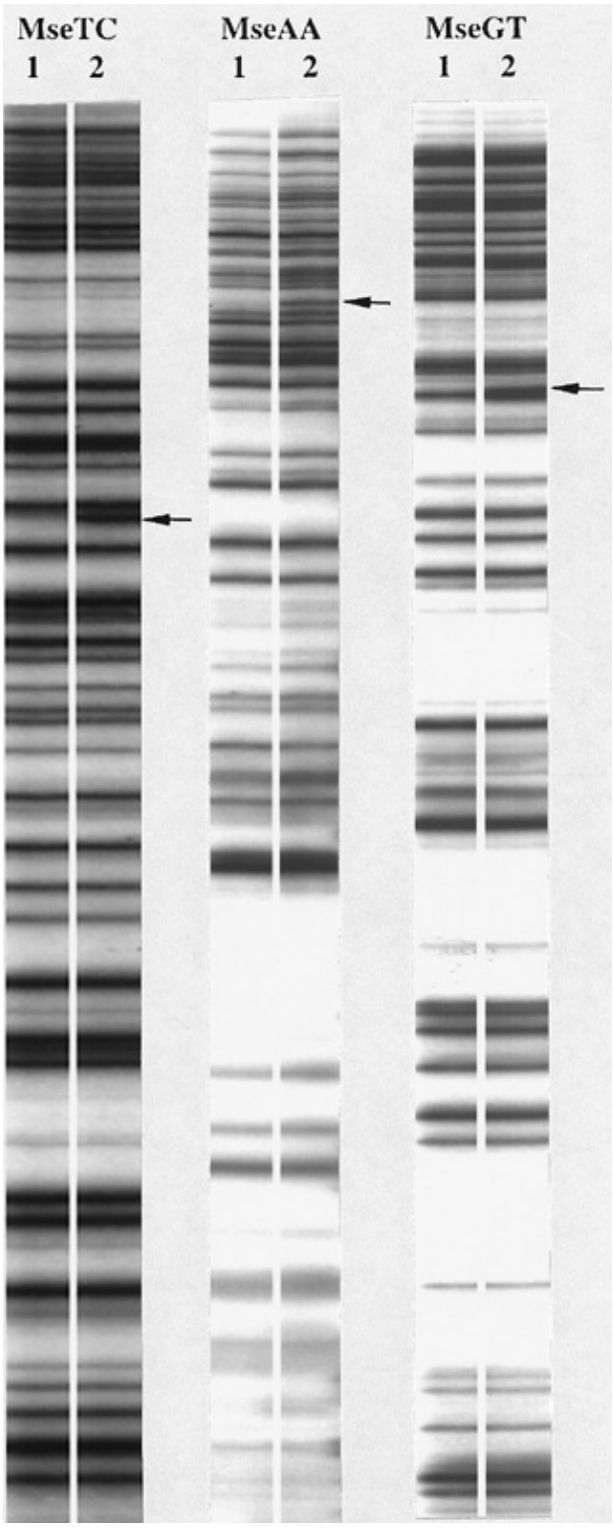

Fig. 6. Amplified fragment length polymorphism pattern comparison between Fol007pg and the mutant Fol007avr. Lanes 1, Fol007wt; and lanes 2, Fol007avr. Arrows indicate polymorphisms p6 (*Eco*TG × *Mse*TC), p12 (*Eco*TG × *Mse*AA), and p11 (*Eco*TG × *Mse*GT).

rocally, a fragment of 2,000 bp ($A14_{2000}$) 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 *Eco*RI or *Hin*dIII, was probed with either the $A14_{1300}$ fragment or the $A14_{2000}$ fragment. The latter probe hybridized with numerous bands in both Fol007pg and Fol007avr, suggesting that it contained a repetitive sequence (data not shown). In contrast, hybridization with $A14_{1300}$ 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 *Eco* × *Mse* primer combinations with a total of three selective nucleotides were used in the amplification reactions. Approximately 125 bands were generated per primer combination, and it was estimated that approximately 5% of the genome was covered. When a potential polymorphism was found, the reaction was repeated in four amplification mixtures with primers containing an additional selective nucleotide. In this way, nine reproducible AFLP polymorphisms (p3, p4, p5, p6, p11, p12, p13, p14, and p15) with variable lengths were identified that all were generated by the same *Eco*TG primer and appeared as extra fragments in the mutant Fol007avr. Figure 6 shows the AFLP polymorphisms p6, p11, and p12, which can be generated by *Eco*TG in combination with *Mse*TC, *Mse*GT, and *Mse*AA, respectively. Southern 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. However, it suggests that, like the RAPD $A14_{2000}$, the AFLP polymorphisms all contain repetitive sequences.

DISCUSSION

According to the gene-for-gene hypothesis, a race-specific resistance response depends on the presence of both a pathogen avirulence gene and a corresponding resistance gene in the plant (8). For many interactions, the existence of a pathogen avirulence gene and a corresponding resistance gene in the plant has been proven genetically. So far, more than 20 resistance genes have been isolated and characterized (7) and, in some cases, the corresponding avirulence genes as well (3,24,36,43). For the interaction between tomato and *F. oxysporum* f. sp. *lycopersici*, a gene-for-gene relationship is generally assumed because monogenic, dominant resistance traits against the three known races have been described. One of these traits, the *I-2* gene, conferring resistance to race 2 isolates, has been cloned recently (23,32). The gene product shares many features with the proteins encoded by other resistance genes thought to be involved in gene-for-gene relationships. To study the early signaling steps in the *I-2*-dependent resistance pathway, we aimed at isolating the *avrI-2* gene. Until now, there was only indirect evidence for the existence of avirulence genes in *F. oxysporum* f. sp. *lycopersici* (21). Because of a lack of alternative methods, we chose the random deletion mutagenesis approach to select for mutants that have lost avirulence. Tracing the induced deletion, which would be much easier than tracing point mutations induced by most commonly used mutagens, could eventually lead to the cloning of the avirulence gene. The mutant of *F. oxysporum* f. sp. *lycopersici* described here shows the change from avirulence to virulence. The results demonstrate the capacity of *F. oxysporum* f. sp. *lycopersici* race 2 to gain virulence to a plant containing the *I-2* gene.

The mutant identified in our gamma irradiation screen was changed in both avirulence and pathogenicity. This could imply that (i) different deletions were induced, affecting both pathogenicity and avirulence; (ii) a common factor involved in signaling, regulation, or secretion of both pathogenicity and avirulence factors was deleted; or (iii) pathogenicity and avirulence are encoded by the same gene. The first explanation cannot 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 detected by Southern analysis. One out of these twenty-four mutants had a deletion of more than 4 kb, removing the entire nitrate reductase gene. The second option cannot be excluded either. Many avirulence and pathogenicity genes are induced in planta. When the expression of fungal pathogenicity and avirulence genes is regulated by common factors, mutations in genes will knock out proper function of both. The change in both pathogenicity and avirulence is probably explained best by the third option: pathogenicity and avirulence are determined by the same factor. For many bacterial avirulence genes, it has been found that they are involved in the fitness or pathogenicity (virulence) of the pathogen (5,15,41). The same seems to hold true for some fungal avirulence genes; NIP1, the host-specific toxin of *Rhynchosporium secalis*, which is necessary for symptom development (28), and ECP2 of *Cladosporium fulvum*, which is required for full virulence of the fungus on tomato, induces a hypersensitive response-based resistance (14). Most *F. oxysporum* f. sp. *lycopersici* race 3 isolates within our collection, which infect the same set of tomato lines as the mutant Fol007avr, are highly pathogenic. In many experiments, these race 3 isolates were significantly more pathogenic compared with race 1 and race 2 isolates from the same vegetative compatibility group (VCG0030) (data not shown). This could imply that single mutations in a pathogenicity/avirulence gene result in a more effective pathogenicity factor able to circumvent plant recognition. Disruption of such a gene could result in a less or non-pathogenic mutant.

Regardless of the genetic basis of pathogenicity and avirulence, it will be interesting to determine the factor of *F. oxysporum* f. sp. *lycopersici* responsible for the observed phenotype of the mutant. DNA analysis has revealed genomic alterations in the mutant. By CHEF blot analysis with chromosome-specific probes, a translocation has been identified in the mutant. Based on the low frequency at which polymorphisms are detected, we conclude that a chromosome translocation has occurred rather than a loss of a chromosome. Loss of a 3.75-Mb chromosome is equal to the loss of approximately 7.5% of the genome, whereas less than 0.1% of polymorphisms were detected, which were not even fragments absent in the mutant. This chromosomal translocation might have been induced by gamma irradiation, although spontaneous chromosomal translocations have been observed in fungi as well (45).

Although undetectable point mutations can still be responsible for the phenotypic change of the mutant, as demonstrated by Southern analysis on the irradiation-induced nitrate nonutilizing mutants, it is encouraging that polymorphisms have been identified. A 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. Deletion mutagenesis is expected to result in the loss of fragments. Instead, extra fragments were found in the mutant, all generated by the same selective *Eco* primer. Moreover, they all seem to contain repetitive sequences. It might be that these polymorphisms have been generated by a mobile element that has been duplicated and inserted at new sites. Currently, we are exploring the possibility of using a parasexual cycle for *F. oxysporum* via protoplasts fusions to link one of these polymorphisms to pathogenicity and avirulence genes.

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