A Near-Isogenic *Fusarium oxysporum* f. sp. *lycopersici* Strain with a Novel Combination of Avirulence Characteristics

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ABSTRACT

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A novel *Fusarium oxysporum* f. sp. *lycopersici* strain (F1-27) was obtained from protoplast fusions between race 1 Fol004 (putative avirulence genotype *A1a2a3*) and race 2 Fol007 (*a1A2A3*). Bioassays using different tomato cultivars revealed new virulence characteristics for F1-27 that were mitotically stable. The corresponding avirulence

genotype for F1-27 was assigned *a1A2a3*. Despite their distinction in avirulence genotype, molecular analysis revealed that parent Fol007 and F1-27 were near-isogenic strains. The electrophoretic karyotype of F1-27 was identical to that observed for Fol007. *Foxy*-amplified fragment length polymorphism (AFLP) marker analysis showed that all Fol007-specific bands were present in F1-27. In addition, 11 new F1-27-specific *Foxy* insertions were identified. Segregation of both virulence and these new *Foxy*-AFLP markers was observed in a backcross between F1-27 and its parent Fol007. One marker was found to cosegregate with the *a3* allele. The nature of the genetic change in this strain is discussed.

Fusarium oxysporum Schlechtend.:Fr. is a soilborne fungal pathogen able to infect over 100 plant species. Individual isolates, however, have the ability to cause disease in a limited number of plant species. Based on their host range, isolates of *F. oxysporum* are grouped into more than 80 formae speciales (2). *F. oxysporum* f. sp. *lycopersici* (Sacc.) W.C. Snyder & H.N. Hansen is the causal agent of Fusarium wilt of tomato and its host range is restricted to *Lycopersicon* spp. Three different races (1, 2, and 3) are distinguished by their differential virulence on tomato cultivars containing different dominant resistance genes (16). Based on the existence of monogenic dominant resistance traits, a gene-for-gene relationship between *F. oxysporum* f. sp. *lycopersici* and tomato is generally assumed. Fungal races contain dominant avirulence genes that correspond to dominant resistance genes in the cultivars they are unable to infect (7,8). Identification of the avirulence genes involved in race-specific resistance is essential to understand the interaction between *F. oxysporum* f. sp. *lycopersici* and tomato on a molecular level.

To identify avirulence genes, different approaches can be envisaged. Avirulence genes of pathogenic bacteria were cloned by complementation (6). For *F. oxysporum*, however, a complementation strategy is not feasible because of the large size of the genome (~50 Mb) and the low transformation efficiency (14). A product-based cloning strategy has been successful in cloning avirulence genes of the fungal pathogens *Cladosporium fulvum* (9,11,32) and *Rhynchosporium secalis* (24). Attempts to isolate avirulence gene products of *F. oxysporum* f. sp. *lycopersici* from xylem sap were not successful, but new (induced) pathogenesis-related proteins were identified in this way (22). Recently, the *Avr2* gene of *C. fulvum* was cloned by employing a functional

screen based on the hypersensitive response (HR)-inducing activity of the encoded protein (12,27). Such an approach does not seem feasible for *F. oxysporum* because an HR has never been observed macroscopically. Transposon tagging might be successful because many transposable elements have been identified in *F. oxysporum* (5), but this approach has yet to yield useful results. Random deletion mutagenesis using gamma irradiation to generate an *F. oxysporum* f. sp. *lycopersici* *Avr1-2* mutant appeared to be very inefficient, and no DNA markers were found that would allow mapping of the *Avr* gene (17). Genetic mapping of avirulence genes (31) and subsequent map-based cloning has been successful in *Magnaporthe grisea* (18,30).

Because a sexual stage has never been observed in *F. oxysporum*, parasexual crosses between race 1 and race 3 isolates were performed to obtain avirulence recombinants (28,29). In addition to new combinations of avirulence genes, molecular analysis confirmed exchange of parental DNA and mitotic recombination. A modified amplified fragment length polymorphism (AFLP) technique based on the *F. oxysporum* retroposon *Foxy* revealed high polymorphism rates between genetically closely related strains (15,28). *Foxy*-AFLP analysis of the parental strains and the progeny revealed an approximately equal contribution of both parents and random segregation of *Foxy*-specific markers in the progeny. Based on *Foxy* markers, a first mitotic linkage map of *F. oxysporum* was constructed (28). From these findings, we can conclude that genetic analysis is feasible in the asexual *F. oxysporum* and, therefore, a map-based cloning approach could be used to map avirulence genes.

In this study, we describe the identification and characterization of an *F. oxysporum* strain, F1-27, with a novel combination of avirulence genes. Although F1-27 was obtained from a protoplast fusion experiment between race 1 isolate Fol004 and race 2 isolate Fol007, no Fol004 parental contribution to the F1-27 genome could be demonstrated. Karyotype analysis revealed a chromosome pattern for F1-27 that is identical to its Fol007 parent. Furthermore, *Foxy*-AFLP analysis shows nearly identical band patterns for F1-27 and Fol007. Therefore, they can be considered

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near-isogenic. The identification of these isogenic strains in combination with the *Foxy*-AFLP markers provides a unique opportunity to map avirulence gene 3.

MATERIALS AND METHODS

Fungal isolates. The two wild-type isolates of *F. oxysporum* f. sp. *lycopersici* used in this study, Fol004 (putative avirulence genotype *Ala2a3*) and Fol007 (putative avirulence genotype *a1A2A3*), belong to the same vegetative compatibility group (VCG0030) and have been described previously by Mes et al. (16). Strain F1-27 originated from a parasexual cross between Fol004 and Fol007. Its observed phenotype was stable. F1-27 was used in parasexual backcross experiments with three different Fol007 transformants (T-1, T-2, and T-3) resulting in 14 backcross progeny (BC1-1, BC2-1 to BC2-4, and BC3-1 to BC3-9). All strains were cultured from single spores and stored in Protect Bacterial Preservers (Technical Service Consultants, Heywood Lancs, UK) at -70°C .

Fungal transformation. To allow selection, fungal strains Fol007 and Fol004 were transformed with plasmid pAN7.1 containing the hygromycin B resistance gene (*hph*) from *Escherichia coli* or plasmid pAN8.1 containing the phleomycin resistance gene (*ble*) from *Streptoalloteichus hindustanus*. Expression of both resistance marker genes was regulated by the *gpdA* promoter from *Aspergillus nidulans* and the *A. nidulans trpC* terminator (20). Protoplast preparation and transformation were performed as described by Teunissen et al. (29). Protoplasts transformed with pAN7.1 or pAN8.1 were plated on Czapek-Dox agar (CDA; Oxoid, Hampshire, UK) plates osmostabilized with 1 M sorbitol and 100 μg of hygromycin (Sigma-Aldrich Chemie, Steinheim, Germany) per ml or 100 μg of phleomycin (Cayla, Toulouse, France) per ml, respectively.

Parasexual (back)crosses. Parasexual (back)crosses by means of protoplast fusion were performed as described by Teunissen et al. (29). Fast growing colonies appearing after 5 to 14 days on CDA plates osmostabilized with 1 M sorbitol containing 100 μg of hygromycin per ml or 100 μg of phleomycin per ml (CDA) were transferred to fresh CDA plates. To obtain stable fusion products or backcross products (BCs), colonies were grown on nonselective potato dextrose agar (Difco Laboratories, Detroit) for at least 14 days. Spores were collected from these agar plates and plated on CDA plates. A single colony was cultured for further experiments.

Plant material. Four plant lines were used in this study, each with a unique resistance gene against *F. oxysporum* f. sp. *lycopersici*. This included two near-isogenic lines of tomato cv. Money-maker, viz. C32 and GCR161, which were previously described by Kroon and Elgersma (10). C32 served as the susceptible control line because it has no resistance genes against *F. oxysporum* f. sp. *lycopersici*. GCR161 contains the resistance gene *I* of Missouri Accession 160 of *Lycopersicon pimpinellifolium* (PI79532) (3) against *A1* but is susceptible to isolates containing *A2* or *A3*. Plant line OT364, homozygous for the *I-2* locus of accession PI26915, was described by Mes et al. (16) and is resistant against isolates with *A2*. The fourth plant line, E779, provided by J. W. Scott (University of Florida, Gainesville), is resistant to isolates containing *A3* but is susceptible to isolates without *A3*. Plant line E779 contains the *I-3* resistance gene without the *I-1* locus introgressed from the *L. pennellii* accession LA716 (26).

Bioassays. Eleven-day-old seedlings, 10 to 20 per fungal strain, were inoculated with a standard root-dip inoculation method (16,34). Spore suspensions were prepared 1 day before inoculation and stored overnight at 4°C . The final concentration of the inoculum was $\geq 1 \times 10^7$ spores per ml. Seedlings were potted in individual containers and randomly distributed. Greenhouse conditions were as described by Teunissen et al. (29). After 3 weeks, the weight of the plant part above the cotyledons was determined.

Plant weight data were statistically analyzed by analysis of variance (StatViewSE + Graphics, version 1.03; StatView, Berkeley, CA).

Genomic DNA isolation and polymerase chain reaction analysis. *F. oxysporum* f. sp. *lycopersici* was grown in potato dextrose broth (Oxoid), without shaking, at room temperature in the dark for approximately 4 weeks. The mycelial layer was collected by filtration and freeze-dried overnight. Genomic DNA was extracted as described by Raeder and Broda (21).

Amplification of both the *hph* gene and the *ble* gene was performed using primers specific for the *gpdA* promoter (5'-TTTCC-CACATTCATCGCAG-3') and the *trpC* terminator (5'-CACCATT-TGTCTCAACTCCG-3'). The *hph*-specific polymerase chain reaction (PCR) product was 1,190 bp, whereas for the *ble* gene, a 583-bp fragment was amplified.

Strain F1-27 was maintained on agar medium alternating with or without selection pressure during the interval between its isolation and the subsequent PCR tests for the presence of the marker genes (described below).

Contour-clamped homogeneous electric field analysis. Contour-clamped homogeneous electric field (CHEF) analysis was performed according to Teunissen et al. (29). Blotting of CHEF gels onto Hybond N⁺ membrane (Amersham Pharmacia Biotech, Uppsala, Sweden) was performed as described in Sambrook et al. (25), using 0.4 M NaOH as transfer buffer. Hybridization was performed using an *hph*- or *ble*-specific DNA fragment $\alpha^{32}\text{P}$ dATP-labeled by the Deca Label DNA Labeling System (MBI Fermentas, Vilnius, Lithuania). Hybridization was performed in 0.5 M phosphate buffer, pH 7.2, containing 7% sodium dodecyl sulfate (SDS), 1% bovine serum albumin, and 1 mM EDTA. The blot was incubated overnight at 65°C and subsequently washed with 2 $\times$, 1 $\times$, and 0.5 $\times$ SSC (1 $\times$ SSC is 0.15 M NaCl plus 0.015 M sodium citrate) with 0.1% SDS for 15 min each at 65°C .

Foxy-AFLP analysis. AFLP analysis was based on the procedure described by Vos et al. (33). Total genomic DNA (500 ng) was digested to completion by restriction enzymes *EcoRI* and *MseI* (Gibco-BRL, Bethesda, MD) for 3 h at 37°C . Standard *EcoRI* and *MseI* adapters were ligated to the restriction fragments. Preamplifications were performed using *EcoRI* and *MseI* nonselective primer pairs (5'-GACTGCGTACCAATTC-3' and 5'-GAT-GAGTCCTGAGTAA-3', respectively). Preamplifications were diluted 50 times before selective amplification steps were carried out. Four different selective amplification reactions were performed using the ^{32}P -end-labeled *Foxy*-specific primer AFLP1 (5'-GCTTCGTTACAGCCACCCAG-3') (15) in combination with the *MseI*+A, *MseI*+T, *MseI*+C, and *MseI*+G primer, respectively. Amplified fragments were separated on a 6% acrylamide gel (Sequa-Gel 6, National Diagnostics, UK) using 1 $\times$ Tris-borate-EDTA (TBE) as the anode-buffer and 0.25 M NaAc as the cathode-buffer at constant power (55 W) for approximately 3 h. After electrophoresis, gels were transferred to Whatman filter paper, vacuum dried at 80°C , and exposed overnight to X-ray films (Kodak, Rochester, NY).

RESULTS

Identification of a strain with new (a)virulence characteristics. Protoplast fusions between race 1 isolate Fol004 (putative avirulence genotype *Ala2a3*) and race 2 isolate Fol007 (putative avirulence genotype *a1A2A3*) were performed as described by Teunissen et al. (29). To select for parasexual progeny, Fol004 was transformed with the phleomycin resistance gene (*ble*) to obtain a resistant strain (designated Fol004-*ble*), whereas Fol007 was marked with the hygromycin resistance gene (*hph*) to obtain a resistant strain (designated Fol007-*hph*). In total, 84 primary colonies grew on the selective medium, of which 57 were stable, doubly resistant fusion products after single-spore cultivation and repeated subculturing. Colony morphology and growth on

media containing antibiotics was normal compared with their transgenic parents.

To determine the presence of avirulence genes *A1*, *A2*, and *A3*, the 57 fusion products and their parental strains were tested for virulence on four plant lines harboring different resistance genes. A universally susceptible cultivar, C32, was used to check for loss of virulence. Cultivar GCR161, containing resistance gene *I*, was used to determine the presence of *A1*. Fungal isolates harboring *A1* are avirulent on this plant line, whereas isolates without *A1* are virulent. Line OT364 (containing *I-2*) and line E779 (*I-3*) were used to check for the presence of *A2* and *A3*, respectively. Bioassays were carried out for Fol004 and Fol004-*ble*, Fol007 and Fol007-*hph*, and the 57 fusion products. The virulence of both Fol004-*ble* and Fol007-*hph* was comparable to the virulence observed for the wild-type isolates from which they were derived (data not shown). Although random exchange of virulence characteristics was expected in the progeny, 56 of 57 fusion products behaved like the Fol007 parent (data not shown). These strains were not further analyzed. Only one fusion product (F1-27) showed virulence behavior that differed from both parents (Fig. 1). Inoculation of susceptible line C32 revealed that all three strains were virulent, although F1-27 caused less severe disease symptoms than Fol004 and Fol007. As expected, Fol004 was virulent on E779 (*I-3*) plants and Fol007 was avirulent on E779 (*I-3*) plants. In contrast to Fol007, strain F1-27 was virulent on E779 and caused more severe disease symptoms than Fol004. Quantification of disease symptoms confirmed these observations (Fig. 2). Although Fol004 (and Fol007) caused a more severe reduction in plant weight than F1-27 on C32 (Fig. 2, panel C32), the latter strain completely destroyed E779 (Fig. 2, panel E779). Infection of GCR161 (*I*) showed that Fol004 was avirulent, whereas Fol007 was virulent on this plant line, as expected. Strain F1-27 was found to be virulent as well. Isolate Fol004 was virulent on OT364 (*I-2*) plants, whereas Fol007 and F1-27 were not. Again, F1-27 behaved as Fol007. Therefore, with F1-27, we identified an *F. oxysporum* f. sp. *lycopersici* strain with new virulence properties and a putative *a1A2a3* genotype. These new virulence properties were stable because the results shown in Figure 2 could be reproduced in four independent bioassays carried out over a period of 12 months. Also, reisolation of strain F1-27 from plant material did not alter its virulence behavior (data not shown).

In addition to altered virulence, an additional phenotypic characteristic was observed, discriminating F1-27 from either parent when grown on potato dextrose medium (Fig. 3). Fol004 mycelium is pink on this medium and Fol007 mycelium shows pink/purple pigmentation, whereas F1-27 cultures are white. Because the changes in avirulence and mycelium color in strain F1-27 might be explained by chromosomal rearrangements, we conducted molecular analysis.

Molecular analysis of F1-27. Strain F1-27 was initially isolated as resistant to both hygromycin and phleomycin. PCR amplification from genomic DNA isolated 6 months after the actual fusion experiment revealed the presence of both markers genes in F1-27. However, only the *hph*-specific band was amplified using genomic DNA that was isolated in a later stage (12 months after fusion), indicating that the *ble* gene had been lost (Fig. 4A). To investigate the presence of chromosomes of different parental origin in F1-27, electrophoretic karyotyping was performed. Chromosomes of Fol004-*ble*, F1-27, and Fol007-*hph* were separated by CHEF analysis (Fig. 4B). The karyotypes of both Fol004-*ble* and Fol007-*hph* were identical to the wild types (data not shown). The F1-27 karyotype appeared to be identical to the Fol007 karyotype. Hybridizing the CHEF blot using *ble*- and *hph*-specific probes revealed the presence of phleomycin resistance gene in Fol004-*ble* on chromosome band 9 and the hygromycin marker in Fol007-*hph* on chromosome band 1. The presence of only the hygromycin marker confirmed that the phleomycin marker was lost in F1-27 (Fig. 4C).

Because no visible changes in karyotype were detectable, a more sensitive molecular technique was applied, *Foxy*-AFLP (15, 28). Figure 5 shows the separated *Foxy*-specific fragments resulting from four selective amplifications. In total, 285 *Foxy* bands were observed (162 for Fol007 and 123 for Fol004) of which 107 were polymorphic between Fol004 and Fol007. Because standard AFLP analysis between these two closely related isolates only revealed $\approx 2\%$ polymorphisms (15), the polymorphism rate (38%) increased substantially when *Foxy*-AFLP was applied. Although the *Foxy*-AFLP pattern for F1-27 was very similar to the pattern observed for Fol007, 13 reproducible polymorphisms were detected between Fol007 and F1-27 (Fig. 5, arrows). Eleven of these markers represented new insertions of the *Foxy* element present in F1-27 but absent in both Fol007 and

Fig. 1. A to C, Bioassays assign a novel avirulence phenotype to strain F1-27. Wilting phenotype of tomato lines C32 (susceptible) and E779 (*I-3*) 3 weeks after infection with different *Fusarium oxysporum* f. sp. *lycopersici* strains. **A,** Infection with strain Fol004 (*A1a2a3*). **B,** Infection with strain F1-27. **C,** Infection with strain Fol007 (*a1A2A3*).

Fig. 2. Quantification of disease symptoms of tomato lines carrying individual *Fusarium* resistance genes. Average plant weight 3 weeks after inoculation with strain Fol004, F1-27, and Fol007. Fifteen seedlings of each differential plant line (C32 [susceptible], GCR161 [resistant only to race 1], OT364 [resistant only to race 2], and E779 [resistant only to race 3]) were inoculated with the respective fungal strain. White bars represent the average weight of mock-inoculated control plants. Black bars represent the average weight of race 1 strain Fol004-inoculated plants. Hatched bars represent the average weight of F1-27-inoculated plants. Gray bars represent the average weight of race 2 strain Fol007-inoculated plants. Different lowercase letters above each of the bars indicate significant differences between the values according to analysis of variance (StatViewSE + Graphics, version 1.03; StatView, Berkeley, CA).

Fol004. Two *Foxy* markers (Fig. 5, asterisk) were present in Fol007 but absent in Fol004 and F1-27, suggesting that the fusion product shares this region with Fol004.

In summary, molecular analysis revealed identical karyotypes for F1-27 and Fol007-*hph*. In addition, *Foxy*-AFLP showed nearly identical band patterns for these two strains; from the 162 *Foxy* bands present in Fol007, 160 are also present in F1-27. Eleven new *Foxy* insertions were identified in F1-27 due to recent activation of the retroposon. Because F1-27 can be considered nearly isogenic to Fol007, varying only in mycelium color, avirulence, the presence of 11 new *Foxy* markers, and the absence of two *Foxy* markers, we started a backcross analysis to map the altered loci.

Segregation analysis in a backcross population. Strain F1-27 was backcrossed with Fol007 transformants marked with *ble* to investigate linkage of the polymorphic bands to one of the new traits of F1-27. Three different transformants (T-1, T-2, and T-3), each with the *ble* marker on a different chromosome, were used for backcrosses. These backcrosses resulted in 14 BCs (BC1-1, BC2-1 to BC2-4, and BC3-1 to BC3-9). For these 14 BCs, the presence of the *ble* and *hph* genes, mycelium color, avirulence, and segregation of the 13 *Foxy* markers was determined (Table 1). PCR amplification using *hph*- and *ble*-specific primers revealed that *hph* was present in all BCs, but *ble* was only present in BC1-1, BC2-4, BC3-1, and BC3-9, confirming that these four strains contain DNA from both parents. Furthermore, these four BCs were the only strains with pink/purple (Pp)-colored mycelium and an A3 phenotype (Table 1). Mycelium was white and A3 was absent (*a3*) in the other BCs, like in F1-27.

Investigation of the *Foxy* markers showed different segregation patterns. Some BCs shared identical marker patterns (BC2-1, BC2-2, and BC2-3; BC3-3 and BC3-6; BC3-1 and BC3-9; and BC3-2, BC3-4, BC3-5, and BC3-8), suggesting a clonal origin. However, unique, new *Foxy* bands were observed for all these BCs (Table 1). These new *Foxy* insertions resulted from *Foxy* transposition events that must have occurred during the backcross process because they were absent in the parental strains (T-1, T-2, T-3, and F1-27). Counting parent-specific *Foxy* fragments revealed enormous variation in parental contribution to the BCs. Products in which the *ble* marker had been lost had a Fol007-*ble* parental contribution varying from zero markers in BC3-4, BC3-5, and BC3-8 to seven markers (54%) in BC2-1, BC2-2, and BC2-3. The parental Fol007-*ble* contribution in the doubly resistant BCs varied from 6 markers (46%) in BC3-1 and BC3-9 to 13 markers (100%) in BC1-1. The extent of parental DNA exchange differed in each backcross experiment. In the single BC1 product, the Fol007-*ble* (T-1) parent was dominant, whereas in the nine BC3 products, the Fol007-*hph* (F1-27) contribution seemed to be dominating (Table 1). The segregation patterns of *Foxy* markers observed for the four progeny strains of BC2 suggested an equal contribution of parental strains T-2 and F1-27 (Table 1).

Fig. 3. Mycelium color of strains Fol004, F1-27, and Fol007 grown on potato dextrose agar medium.

Segregation patterns of the markers throughout the BCs suggested different groups of linked markers: first group: P, Pp, A3, and absence of *MseT3*; second group: absence of *MseA3*, *MseT1*, and *MseG1*; third group: *MseA1* and *MseA2*; and fourth group: *MseT2*, *MseC1*, *MseC2*, *MseG2*, and *MseG3*. Three markers (*H*, *MseG4*, and *MseC3*) were unlinked, and marker *MseT3* was found to cosegregate with the absence of avirulence gene 3 (*a3*) and also with the absence of the *ble* gene and mycelium color (Table 1, shown in gray).

Fig. 4. A, Polymerase chain reaction (PCR) amplification using primers specific for the plasmid backbone of pAN7.1 (containing *hph*) and pAN8.1 (containing *ble*). PCR was performed on pAN8.1 DNA, pAN7.1 DNA, F1-27-6m genomic DNA (isolated 6 months after the actual fusion experiment), and F1-27-12m genomic DNA (isolated 12 months after the fusion experiment). B, Electrophoretic karyotyping of parental strains Fol004-*ble* and Fol007-*hph* and fusion product F1-27. Contour-clamped homogeneous electric field gel stained with ethidium bromide. C, Blot of this gel hybridized with a probe specific for the plasmid backbone of pAN7.1 and pAN8.1.

Fig. 5. *Foxy*-amplified fragment length polymorphism (AFLP) analysis of race 1 strain Fol004 (1), F1-27 (M), and race 2 strain Fol007 (2). AFLP amplification was performed using the radiolabeled *Foxy*-specific primer in combination with *MseI* adapter primer extended with one selective nucleotide (*MseA*, *MseT*, *MseC*, and *MseG*). Arrows indicate new insertions of the *Foxy* element in F1-27. Arrows with asterisk indicate a *Foxy* fragment present in strain Fol007 but absent in Fol004 and F1-27. *Foxy* markers are named after the particular *Mse* primer used to generate them and numbered according to their gel position (from high to low molecular weight). Reference fragment sizes are indicated in basepairs (bp).

DISCUSSION

Pathogenicity studies using four tomato lines, each with a unique resistance gene against *F. oxysporum* f. sp. *lycopersici*, revealed that strain F1-27 exhibited a pattern of behavior that matched putative genotype *ala2a3*. This new combination of avirulence genes appeared to be unique and had never been observed in our wild-type strain collection of isolates from all over the world. Although F1-27 is virulent on plants without any resistance genes (C32), the virulence on this plant line is significantly reduced when compared with parental strains Fol004 and Fol007 (Figs. 1 and 2). Reduction of virulence on C32 plants was also seen in a gamma-irradiated *avr1-2* mutant of *F. oxysporum* f. sp. *lycopersici* that had lost avirulence gene 2 (*ala2A3*) (16). The reduced disease symptoms might be the result of the loss of avirulence, because avirulence factors have been suggested to have functions in avirulence as well as in virulence (11,35). Surprisingly, virulence of F1-27 was not generally reduced because it appeared to be more virulent on plant line E779 than that of Fol004 and Fol007 (Figs. 1 and 2). This observation underscores the influence of the tomato line and its genetic background on the outcome of bioassays. Although resistance genes are apparently present in these lines, other plant and fungal factors determine the strength of the biological response.

The new combination of avirulence genes in F1-27 could result from exchange of parental DNA during the parasexual fusion. Contribution of the *a3* allele by the race 1 Fol004 parent would then explain the *a3* phenotype for F1-27. However, several observations indicated that rearrangements in the A3 locus of the race 2 Fol007 parent are the cause of the *a3* phenotype in F1-27. Firstly, the phleomycin resistance gene from the Fol004-*ble* parent was lost in F1-27 (Fig. 4A). The loss of the *ble* gene in F1-27 is probably due to the nondisjunctional haploidization process, which takes place during the parasexual cycle and might take several generations (29). Despite this loss, F1-27 is able to grow on media containing phleomycin. The basis for phleomycin tolerance is unknown but this phenomenon has been observed in other fusion products that lack the resistance gene (H. A. S. Teunissen, Y. Boer, J. Verkooijen, B. J. C. Cornelissen, and M. A. Haring, *unpublished*

data). Secondly, the electrophoretic karyotype of F1-27 was identical to its Fol007-*hph* parent, indicating that no large-scale exchange of parental DNA had occurred (Fig. 4B). Thirdly, the *Foxy*-AFLP pattern of F1-27 was nearly identical to the *Foxy*-specific band pattern found for the Fol007 parent (Fig. 5). Only 2 of the 162 bands present in Fol007 were absent in F1-27. These two bands also were absent in Fol004 and might therefore be an indication of the presence of Fol004 parental DNA. Nevertheless, deletion events can also explain the absence of these *Foxy* fragments. Stronger evidence for exchange of parental DNA would be the presence of Fol004-specific *Foxy* bands in F1-27 and absence of these bands in Fol007. However, such bands have not been observed. Therefore, no direct evidence was found for Fol004 parental contribution to the F1-27 genome. Finally, the white mycelium color observed for F1-27 completely cosegregated with the *a3* phenotype but was different from the color of the Fol004 parent (Fig. 3). The mycelium color locus might have been affected by chromosome breakage due to exchange of the *a3* allele. However, the *a3* phenotype of F1-27 is strikingly different from that observed for Fol004, suggesting that contribution of the *a3* allele by the Fol004 parent is improbable. Therefore, the altered phenotypic characteristics observed for F1-27 might be explained by the occurrence of rearrangements without visible consequences for the karyotype but affecting the A3 locus and mycelium color.

Eleven polymorphic *Foxy*-AFLP bands were new insertions of the retroposon in F1-27. New insertions of *Foxy* previously were shown to occur in low frequencies ($\approx 1\%$) in transformants and in fusion products resulting from parasexual crosses between isolates Fol004 and Fol029 (29). New *Foxy* insertions in BCs were also shown to occur in low frequencies; the highest number of new *Foxy* insertions ($\approx 2\%$) was observed in BC2-3 (Table 1). The number of new bands caused by *Foxy* activity is intriguingly high in F1-27 and comparable to the situation described for the gamma-irradiated *Avr1-2* mutant of *F. oxysporum* f. sp. *lycopersici* in which nine new *Foxy*-AFLP polymorphisms were identified (17). Genomic stress is known to activate transposable elements (13). DNA damage (4), irradiation with gamma rays (15), and exposure to chlorate (1) have been identified as factors that are able to

TABLE 1. Segregation patterns of markers in a population from three different backcrosses between F1-27 and Fol007parents T-1, T-2, and T-3^a

	Fol007 (P)			Fusion products														Fol007 (H)
	T-1	T-2	T-3	BC1-1	BC2-1	BC2-2	BC2-3	BC2-4	BC3-3	BC3-6	BC3-1	BC3-9	BC3-7	BC3-2	BC3-4	BC3-5	BC3-8	F1-27
Hygromycin	–	–	–	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H
Phleomycin	P	P	P	P	–	–	–	P	–	–	P	P	–	–	–	–	–	–
Pigment ^b	Pp	Pp	Pp	Pp	W	W	W	Pp	W	W	Pp	Pp	W	W	W	W	W	W
Genotype ^c	A3	A3	A3	A3	<i>a3</i>	<i>a3</i>	<i>a3</i>	A3	<i>a3</i>	<i>a3</i>	A3	A3	<i>a3</i>	<i>a3</i>	<i>a3</i>	<i>a3</i>	<i>a3</i>	<i>a3</i>
<i>Foxy</i> -markers ^d																		
<i>MseT3</i>	–	–	–	–	+	+	+	–	+	+	–	–	+	+	+	+	+	+
<i>MseA3</i>	–	–	–	–	+	+	+	+	+	+	–	–	+	+	+	+	+	+
<i>MseT1</i>	+	+	+	+	–	–	–	–	–	–	+	+	–	–	–	–	–	–
<i>MseG1</i>	+	+	+	+	–	–	–	–	–	–	+	+	–	–	–	–	–	–
<i>MseG4</i>	–	–	–	–	+	+	+	+	+	+	–	–	+	+	+	+	+	+
<i>MseA1</i>	–	–	–	–	–	–	–	–	–	–	–	–	+	+	+	+	+	+
<i>MseA2</i>	–	–	–	–	–	–	–	–	–	–	–	–	+	+	+	+	+	+
<i>MseT2</i>	–	–	–	–	–	–	–	–	–	–	+	+	+	+	+	+	+	+
<i>MseC1</i>	–	–	–	–	–	–	–	–	–	–	+	+	+	+	+	+	+	+
<i>MseC2</i>	–	–	–	–	–	–	–	–	–	–	+	+	+	+	+	+	+	+
<i>MseG2</i>	–	–	–	–	–	–	–	–	–	–	+	+	+	+	+	+	+	+
<i>MseG3</i>	–	–	–	–	–	–	–	–	–	–	+	+	+	+	+	+	+	+
<i>MseC3</i>	–	–	–	–	+	+	+	–	+	+	+	+	+	+	+	+	+	+
No. of new unique bands	–	–	–	0	2	1	3	0	1	2	1	0	0	2	1	0	1	–

^a Gray shading highlights linkage between marker *MseT3* and *a3*.

^b Mycelium color during in vitro growth. Pp = purple/pink; W = white.

^c Presence (A3) or absence (*a3*) of avirulence gene 3 as assigned according to (a)virulence behavior on E779 plants containing the *I-3* resistance gene.

^d Presence (+) or absence (–) of a polymorphic *Foxy*-amplified fragment length polymorphism fragment.

activate transposition. Transposable elements also were found to be activated during protoplast isolation (19). Since protoplast generation is necessary for both polyethylene glycol-mediated transformation and fusion experiments, *Foxy* might have been activated. Thus, loss of a functional *A3* gene in F1-27 could have resulted from insertion of a *Foxy* element.

To investigate which of the 11 extra *Foxy* insertions might be responsible for loss of avirulence, backcross experiments were performed. Three different Fol007-*ble* parents were backcrossed with the *hph* containing F1-27. Only 4 (BC1-1, BC2-4, BC3-1, and BC3-9) of the 14 BCs contained both the *hph* and *ble* marker genes and therefore should contain genetic material from both parents. The absence of the *ble* gene, the white mycelium color, and the absence of *A3* (*a3*) in the other BCs suggested that no exchange of parental DNA had occurred. However, *Foxy*-AFLP markers indeed segregated in the 14 BCs, indicating that exchange of parental DNA did occur in these BCs. The extent of parental DNA exchange in the BCs differs in each backcross experiment. The genetic background of the parental strains used in the three backcrosses is identical (Fol007) and therefore should not influence the extent of this parent's contribution. Chromosomal position of the *ble* marker gene in the three different Fol007-*ble* parents (T-1, T-2, and T-3) could be possible factors influencing the extent of parental exchange. Although different parents were used, the presence of the *ble* marker gene in the BCs seemed to automatically account for the presence of pink/purple mycelium, *A3*, and the absence of *MseT3*. A possible influence of the *ble* marker on segregation of avirulence has been suggested previously for parasexual crosses between Fol004-*ble* and Fol029-*hph* (29). From 31 fusion products containing both the *hph* and *ble* markers, 30 fusion products harbored *AI*. In addition, Roebroeck (23) observed a correlation between the virulence phenotype of fusion products and the virulence phenotype of their *ble* parent. This tendency was also observed for fusion products obtained from the reciprocal cross, although less extreme (23). Therefore, the phleomycin resistance gene and/or gene product might play a role in segregation of parental DNA.

Because the novel *Foxy* fragments in F1-27 segregated as four independent clusters (although a limited number of BCs were analyzed), this suggested that the genome of F1-27 has been altered at several positions. Interestingly, marker *MseT3* was found to cosegregate with the absence of *A3* (*a3*) and white mycelium color. Loss of a functional *A3* gene could then be explained by *Foxy* insertion of *MseT3* in this locus. Also, markers *MseA3*, *MseT1*, and *MseG1* seem closely linked to *A3* and mycelium color. More BCs need to be analyzed in order to confirm the correlation between one of these markers and the altered loci. Cloning of the *MseA3*, *MseT1*, *MseG1*, and *MseT3* *Foxy* fragments and sequencing of their flanking regions will allow us to screen a Fol007-specific Bacterial Artificial Chromosome (BAC) library. Positive BAC clones can subsequently be used in complementation studies and might lead us to the *A3* locus.

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