Parasexual fusion in Fusarium oxysporum; Biological and molecular characterization of avirulence recombinants

Teunissen, H.A.S.

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CHAPTER 3

The first genetic map of *Fusarium oxysporum* based on 
Foxy-AFLPs

Hedwic h A. S. Teunissen, Martijn Rep, Petra M. Houterman, Ben J. C. Cornelissen and Michel A. Haring

ABSTRACT

The first genetic map of the asexual fungus *Fusarium oxysporum* was constructed based on a population of 32 parsexual fusion products. Molecular markers were developed using a modified AFLP technique combining a Foxy-specific primer with standard adapter primers. The retroposon Foxy is abundantly present in the *F. oxysporum* genome and Foxy-AFLP fragments appeared to be polymorphic between strains Fol004 and Fol029 with a frequency of 43%. From the 102 Foxy markers obtained, 83 segregated in a 1:1 ratio. The remaining fragments showed a skewed segregation pattern in which the Fol004 derived Foxy fragments were overrepresented. Foxy markers were observed to be clustered suggesting that active Foxy elements may not travel far, or that preferred insertions sites may exist. Linkage analysis revealed 23 linkage groups. Physical linkage between segregating markers with a predicted genetic distance of 20 cM was confirmed, indicating that the genetic map is reliable.

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INTRODUCTION

*Fusarium oxysporum* Schlechtend.: Fr. f. sp. *lycopersici* (Sacc.) W.C. Snyder & H.N. Hansen is the causal agent of *Fusarium* wilt of tomato (*Lycopersicon esculentum*). The pathogen belongs to the *Fungi Imperfecti* since no sexual cycle has been observed. The various isolates of *F. oxysporum* f. sp. *lycopersici* are classified into physiological races and into vegetative compatibility groups (VCGs). Three physiological races (1, 2 and 3) are distinguished by their differential virulence on tomato cultivars containing different dominant resistance genes (Mes et al., 1999). Different races are proposed to contain avirulence genes that correspond to the resistance genes in the cultivars they are unable to infect (Flor, 1942 and 1971). In addition, four major VCGs (VCG0030-VCG0033) have been identified. Elias et al. (1993) indicated that isolates within each VCG are clonal derivatives of a common ancestor and that races arose independently within each VCG. Isolation and characterization of avirulence genes is important for understanding the race-specific resistance of tomato against *F. oxysporum* f. sp. *lycopersici* at the molecular level.

In order to genetically map avirulence genes in *F. oxysporum* f. sp. *lycopersici*, a mapping population was obtained. Two wild type strains originating from VCG0030 (and thus clonally related) were selected for parsexual crosses. Both strains are representatives of different physiological races: the race 1 isolate Fol004 contains avirulence gene *A1* (putative avirulence genotype *A1a2a3*) (Mes et al., 1999), whereas the race 3 isolate Fol029 contains *A3* (putative avirulence genotype *a1a2A3*). Fol004 and Fol029 were equipped with the phleomycin resistance gene (*ble*) and the hygromycin resistance gene (*hph*), respectively. Fusion products were generated by protoplast fusion and selected for the presence of both antibiotic resistance genes. In total 32 fusion products were obtained. Exchange of parental DNA, recombination and segregation of avirulence genes was observed in the fusion products (Chapter 2). From these observations we concluded that this population is suitable for mapping purposes.

For several plant pathogenic fungi and oomycetes, attempts to identify avirulence genes by a map based cloning strategy have been carried out. Crosses followed by molecular marker (RFLP, RAPD and AFLP) (bulked segregant) analysis and the generation of linkage maps were performed for e.g. *Uromyces appendilatus* (Martinez et al., 1996), *Leptosphaeria maculans* (Pongam et al., 1998), *Magnaporthe grisea* (Farman and Leong, 1998; Diao et al., 2000). *Phytophthora sojae* (Whisson et al., 1994), and *Phytophthora infestans* (Van der Lee et al., 2001). Until now, only the *Avr-Pita* gene from *Magnaporthe grisea* has been cloned and characterized using a map based cloning approach (Orbach et al., 2000).

In addition to conventional molecular markers, the use of AFLP markers based on mobile elements or repeated sequences has been shown to be particularly efficient in
mapping studies. Hamer and Givan (1990) were the first to map the *Magnaporthe grisea* SMO locus (spore morphology) using the dispersed repeated sequences called MGR. More recently, resistance against the fungal pathogen *Pyrenophora teres* was mapped in barley with the aid of retroposon-based molecular markers (Manninen et al., 2000). Since sexual recombination is lacking in *F. oxysporum*, the activity of transposable elements, sometimes leaving footprints or causing chromosome rearrangements and other genomic changes, has been suggested to be the major factor responsible for the genetic variation observed between different isolates (Daboussi, 1996). Mobile elements may also be involved in recombination events (Wickman et al., 1992; Britten, 1996). Several families of mobile elements have been characterized in isolates of *F. oxysporum* including transposons and retro(trans)posons (reviewed in: Daboussi and Langin, 1994; Daboussi, 1997; Hua-Van et al., 2000; Mes et al., 2000). Copies are present in numbers ranging from just a few elements to tens or hundreds per genome (Hua-Van et al., 2000). One of these mobile elements abundantly present in the *F. oxysporum* genome is Foxy, an active family of short interspersed nuclear elements (SINEs) (Mes et al., 2000). This 664 bp transposable element is flanked by a 12 bp target site duplication and contains sequence boxes homologous to binding sites for RNA polymerase III. Unique for Foxy are the tetranucleotide repeats found at the 5' end. Individual copies of Foxy show different primary structures of the tetra-nucleotide repeats (TATG), (TTTG) and (AAGG) as well as different numbers of repeats varying between three and seven (Mes et al., 2000). In contrast to transposing DNA elements, Foxy is mobilized via an RNA intermediate, which is converted into DNA by reverse transcription prior to reinsertion. This process results in an increase in the copy number of this element. Foxy is present on all chromosomes and is currently active. Hence it contributes to the genetic variation within *F. oxysporum* (Mes et al., 2000).

In this study, we describe the molecular analysis of 32 parasexual fusion products using a modified AFLP technique combining a Foxy-specific primer with standard adapter primers. This technique, designated Foxy-AFLP, resulted in an exceptionally high number of polymorphisms when compared to conventional molecular marker techniques. Most Foxy markers segregated in a 1:1 ratio in the population of fusion products. Based on 83 Foxy markers the first genetic map of *F. oxysporum* was constructed. The accuracy of the map was confirmed by physical linkage studies. Foxy-AFLP analysis in combination with the available Fol004 BAC library are strong tools in gene mapping and subsequent gene cloning in *F. oxysporum*.
MATERIAL & METHODS

Bacterial and Fungal isolates
E. coli strain DH5α was used for cloning and plasmid propagation purposes. Two F. oxysporum f. sp. lycopersici wild type isolates, Fol004 (race 1) and Fol029 (race 3), were used in this study and have been described by Mes et al. (1999). Four Fol004 phleomycin resistant transformants (A-D) and two Fol029 hygromycin resistant transformants (E and F) were obtained by transformation. Thirty-two doubly resistant fusion products were obtained by fusion experiments as described in Chapter 2. All strains were cultured from single spores and grown on potato dextrose agar (PDA: Difco Laboratories, Detroit, MI, USA) or Czapek Dox agar (CD: Oxoid). Strains were stored in Protect Bacterial Preservers (Technical Service Consultants LTD, Heywood Lancs, GB) at -70°C.

Genomic DNA isolation
F. oxysporum f. sp. lycopersici was grown in potato dextrose broth (PDB: Difco Laboratories, Detroit, MI, USA) 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 (1985).

Foxy-AFLP analysis
Amplified fragment length polymorphism analysis was based on the procedure described by Vos et al. (1995). Total genomic DNA (500 ng) was digested to completion by the restriction enzymes EcoRI and MseI (Gibco-BRL) for 3 hours at 37°C. Standard EcoRI and MseI adapters were ligated to the restriction fragments. Preamplifications were performed using EcoRI and MseI non-selective primer pairs (5'-GACTGCGTACCAATTC-3' and 5'-GATGAGTCCTGAGTAA-3', respectively). Preamplifications were diluted 50 times before selective amplification steps were carried out. Five different selective amplification reactions were performed using the 32P-end-labeled Foxy-specific primer AFLP1 (5'-GCTTCGTTACAGCCACCCAG-3') (Mes et al., 2000) in combination with the EcoRI-aspecific, 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, GB) using 1 x TBE as the anode-buffer and 1 x TBE: 0.25 M NaAc as the cathode-buffer at constant power (55 W) for approximately 3 hours. After electrophoresis gels were transferred to Whatman filter paper, vacuum dried at 80°C and exposed overnight to X-ray films (Kodak, Rochester, NY, USA).

Linkage analysis
Linkage analysis of 102 segregating markers in 32 fusion products was performed using MapDisto version 1.1b (http://www.mpl.ird.fr/~lorieux/mapdisto/mapdisto.html). To include a locus in a linkage group, a minimum logarithm of odds (LOD) threshold of 3.6 and a recombination fraction (r) of 0.20 were used. LOD score is defined as the log10 of the odds ratio that supports evidence for linkage between two markers versus non-linkage (Martinez et al., 1996). LOD 3.6 corresponds to a ~4000:1 odds ratio. Segregation values were calculated for each Foxy marker and a χ² test for goodness of fit was applied to test for significance of 1:1 segregation.

Cloning of AFLP markers
All race 1-specific Foxy fragments belonging either to linkage group 7 and 11 or to group V were eluted from radioactive gels in water. The DNA solution (5-10 μl) was then PCR amplified using the same primer pairs that generated the AFLP fragment. The PCR products were cloned into pGEM-T (Promega). Sequencing was done by the di-deoxy chain termination method (Sanger et al., 1977) using a LI-COR IR2 system (LI-COR Inc). Marker-specific primers were designed on the unique flanking region upstream of the Foxy-specific 5' terminal tetranucleotide repeats (Table 1). To verify that the correct marker was cloned, PCR amplification on genomic DNA of parents and fusion products using the unique, marker-specific primer in combination with the Foxy-specific
primer was performed. If 100% correlation between PCR and Foxy-AFLP was observed we concluded that the fragment corresponded to the desired marker.

Table 1: Marker-specific PCR primers.

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</tr>
<tr>
<td>A5</td>
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Screening of a BAC library

A BAC library of Fol004 was constructed using the plasmid pBelOBA11 (Shizuya et al., 1992) containing the gpdA driven hygromycin B resistance gene (hph) from pAN7.1 (Punt and Van den Hondel, 1992). Partial digestion of fungal genomic DNA was performed using 0.5 U HindIII (MBI Fermentas, Vilnius, Lithuania) for 30 minutes at 37°C. Three different partially digested DNA pools (50-100 kb, 100-150 kb and 150-200 kb) were isolated from gel, ligated into pBelOBA11-hph and transformed to E. coli. Insert containing colonies were selected by blue/white screening. 6144 Individual colonies were collected in 16 microtiter plates each containing 384 wells and stored as glycerol stock at -70°C. The BAC library was organized in a three-dimensional manner: every plate was pooled separately (16 pools), all 24 columns were pooled per four plates (4 x 24 = 96 pools) and all 16 rows were pooled per four plates (4 x 16 = 64 pools) resulting in 176 pools. From all 176 pools DNA was isolated. Screening of the BAC library was performed by PCR on the DNA pools using the unique, marker-specific primer in combination with the Foxy-specific primer (Table 1). PCR-positive pools identified possible coordinates of positive BAC clones. Due to the three-dimensional organization, a maximum of X' coordinates can be found for X positive BACs. Since columns and rows were pooled per four plates, the obtained number of possible coordinates was much lower than the maximum number of possible coordinates. BACs from all coordinates were screened individually by PCR to identify the clones that contain the marker of interest.

Hybridization of CHEF blots with BAC specific probes

Contour-clamped homogeneous electric field analysis was performed as described in Chapter 2 with some modifications. Chromosomes were separated in a 14-days run in 1% Seakem Gold
agarose (PFGE agarose, BMA, Rockland, ME USA). Blotting of CHEF gels onto Hybond N’ membrane (Amersham Pharmacia Biotech, GB) was performed as described in Sambrook et al. (1989), using 0.4M NaOH as transfer buffer. CHEF blots were rinsed in 5x SSC, wrapped in Saran wrap (Dow) and stored at 4°C.

DNA of all positive BAC clones was extracted from 500 ml E. coli culture according to the Qiagen protocol for midi preparations of plasmid DNA. BAC DNA was digested with either HindIII, XhoI or with both enzymes together (MBI Fermentas, Vilnius, Lithuania). BAC fragments were separated on a 1% agarose gel overnight at 30 V. DNA fragments between 500-1200 bp for at least one BAC per marker were isolated and extracted from the agarose using the QIAEXII kit (Qiagen). These BAC restriction fragments were α32P dATP-labeled by the Deca Label DNA Labeling System (MBI Fermentas, Vilnius, Lithuania) and hybridized on CHEF blots containing Fol004 and Fol029 chromosomes. Probes were also hybridized on blots containing the HindIII digestion profiles of all BACs to confirm that they contained unique sequences.

RESULTS

Foxy-AFLP analysis of closely related isolates

In a previous study we obtained a population of 32 fusion products from parasexual crosses between the clonally related isolates Fol004 and Fol029 (Chapter 2). The close genetic relationship between these two isolates does not allow molecular analysis of the fusion products using RFLP, RAPD or AFLP markers. To identify a sufficient number of molecular markers, we developed a modified AFLP technique based on Foxy, as suggested by Mes et al. (2000). AFLP amplifications were carried out on EcoRI/MseI amplicons of both wild type strains. One radioactively labeled Foxy-specific primer was used in combination with standard MseI adapter primers plus one selective nucleotide (MseI+A, MseI+T, MseI+C and MseI+G) or with the standard, non-selective EcoRI adapter primer (EcoRI-Asp) in five separate amplification reactions. Amplified fragments were analyzed on polyacrylamide gels as for standard AFLP analyses (Fig. 1). Using standard electrophoretic conditions, separating fragments between approximately 90-850 bp, 126 Foxy-specific bands were identified for Fol004, whereas 114 Foxy-specific bands were identified for Fol029. This indicates that at least as many copies of the SINE retroposon are present in both isolates. Of these fragments, 69 were shared by both isolates. It should be noted that some strongly labeled bands might consist of several fragments that segregate individually. Therefore, the number of Foxy-specific polymorphic bands is an underestimation. In total, 102 Foxy insertions (43%) appeared to be polymorphic between Fol004 and Fol029 (Fig. 1). This high percentage of polymorphic bands is an exceptional increase when compared to standard AFLP (~2% polymorphic bands), making the Foxy-AFLP method an excellent tool for segregation analysis.
**Figure 1:** Foxy-AFLP analysis of the two wild type strains Fo1004 and Fo1029. AFLP amplification was performed using the radiolabeled Foxy-specific primer in combination with MseI adapter primer, extended with one selective nucleotide (Mse-A, Mse-T, Mse-C and Mse-G) or with the standard, non-selective EcoRI adapter primer (Eco-Asp). Fragment sizes are indicated in basepairs (bp).
Segregation analysis of fusion products using *Foxy-AFLP*

Previous tests for race-specificity of the 32 fusion products by infection of different plant lines revealed an unexpected skewed segregation for avirulence gene 1 (*A1*) (Chapter 2). To investigate whether this skewed segregation is unique for the avirulence locus or occurs genome-wide, *Foxy-AFLP* analysis was performed on the 32 fusion products and their parental strains (A-F). Figure 2 shows the *Foxy-AFLP* patterns resulting from the *Foxy-Mse1+G* selective amplification reaction on the amplicons of the parental strains A-F and the fusion products. A skewed segregation was observed for some markers. For example, the *Foxy* fragment denoted with arrow 1 in Figure 2, is present in the race 1 parental strains A-D and in all fusion products, but is absent in the race 3 parents E and F. However, segregation of most race 1- and race 3-specific *Foxy* markers conformed to a 1:1 ratio as exemplified by the fragments denoted with arrows 2 and 3 in Figure 2. These markers are present in approximately half of the fusion products, suggesting that parental *Foxy* fragments have been randomly distributed during parasexual fusion. From the 102 markers, 83 segregated in a 1:1 ratio (probability (*P*) > 0.05) whereas 19 markers showed a significant segregation distortion (*P* < 0.05). For the *Foxy-AFLP* patterns of these 19 biased markers, Fol004-specific *Foxy* fragments were overrepresented and Fol029-specific *Foxy* markers were always underrepresented. Since the vast majority of the *Foxy* markers is randomly distributed we conclude that skewed segregation as observed for the avirulence locus is the exception rather than the rule.

*Foxy-AFLP* bands present in both parental strains but missing in a fusion product were observed at low frequencies. Two fusion products (FP1-3 and FP1-4) were identified missing one *Foxy* insertion, and one fusion product (FP6-6) missed four *Foxy* insertions. One of the missing *Foxy* bands in FP6-6 is shown in Figure 2 (arrow 4). The loss of *Foxy* fragments might be explained by the occurrence of deletions. Indeed, karyotype analysis already indicated that a deletion has occurred in FP6-6 (Chapter 2). Newly appearing *Foxy* bands, probably resulting from transposition of the SINE retroposon, were observed in the transformants used as parents for parasexual crosses as well as in some fusion products. Parent B contained one extra *Foxy* band, parent D contained two extra bands and both parents E and F contained three extra *Foxy* insertions. The extra *Foxy* band present in parent D (Fig. 2; arrow 5) is also present in the two fusion products FP3-1 and FP3-2 derived from a cross using this race 1 parental strain. New *Foxy* insertions were also identified in eight fusion products. Fusion products FP4-2, FP5-4, FP 6-2, FP6-4 (Fig. 2; arrow 6) and FP7-1 all contained one extra *Foxy*-specific fragment, whereas FP1-1, FP2-1 and FP5-2 contained two extra *Foxy* insertions. These new *Foxy* bands are easily identified and do not interfere with linkage analysis.
Figure 2: Foxy-AFLP analysis of the race 1 parental strains (A-D), the race 3 parental strains (E and F) and 32 fusion products (numbered 1-1 to 7-1) using the Foxy-specific primer in combination with the Msel-G primer. For all strains the avirulence genotype is presented as previously determined (Chapter 2). \( A1 = Avr1; \) \( A3 = Avr3; \) \# = undefined.

The genetic contribution of the parents to each fusion product was estimated by counting the race 1 or race 3 derived Foxy bands of 102 polymorphic bands in total. Figure 3 shows that most fusion products contain an approximately equal contribution of each parent, though with some notable exceptions (FP1-1, FP1-2 and FP6-8). The average race 1 contribution is \~60\% instead of the expected 50\%, which can be attributed to skewed segregation of a subset of markers (see below). The number of polymorphic Foxy insertions found in the race 1 Fol004 and race 3 Fol029 isolates was 57 and 45, respectively. A diploid fusion product should thus contain 102 Foxy insertions. Figure 3 shows that none of the fusion products is fully diploid. In fact, in most fusion products the number of Foxy markers varied between 45 and 57. One fusion product (FP6-2) contained fewer polymorphic Foxy bands than the race 3 parent. This may be the result of a deletion but can also be explained by recombination events. Seven fusion products (FP2-2, FP2-5, FP2-6, FP4-1, FP5-4, FP6-5 and FP6-7) contained more Foxy bands than the race 1 parent,
suggesting that these fusion products might be partially diploid. However, recombination events can also result in more *Foxy*-specific fragments than originally observed in the parental strains. Thus, although partial diploidy cannot be rigorously excluded, our results suggest that the extent of partial diploidy is limited if occurring at all.

![Graph showing the number of *Foxy* markers derived from the race 1 Fol004 parent (black) and from the race 3 Fol029 parent (grey) in parasyexual fusion products.](image)

**Figure 3:** The number of *Foxy* markers derived from the race 1 Fol004 parent (black) and from the race 3 Fol029 parent (grey) in parasyexual fusion products. Upper horizontal line represents the number of polymorphic bands in Fol004; lower horizontal line represents the number of polymorphic bands in Fol029.

When the *Foxy*-AFLP pattern of the various markers was investigated throughout the fusion products, linkage was observed. In Figure 4 some examples of linked *Foxy* markers are shown. The segregation pattern of the two race 3 derived markers A15 and G14 are nearly identical except for bands in two fusion products indicated by the arrows (Fig. 4A). These differences in segregation of *Foxy* markers suggests that recombination has occurred between A15 and G14. Linkage between the race 1 derived markers G11 and G12 is 100%, since their *Foxy*-AFLP pattern is identical (Fig. 4B). Compared to G11 and G12, the *Foxy*-AFLP pattern of the race 3 derived marker C4 is complementary. When a G11/G12-specific *Foxy* band is present in parents and fusion products, this band is absent in C4 (and vice versa). However, one exception was observed. No *Foxy* fragment is present in fusion product FP6-6 for either the G11/G12 or C4 marker, indicating that recombination or deletion has taken place.
In conclusion, from the 102 bands found to be polymorphic between the two wild type strains Fol004 and Fol029, the vast majority (83) in the population of 32 fusion products segregated in a Mendelian manner (1:1) and can therefore be used for linkage analysis.

**Linkage analysis - the first genetic map of *Fusarium oxysporum***

Linkage analysis was performed based on the polymorphic Foxy-specific AFLP markers using MapDisto version 1.1b (Lorieux). To determine the optimal parameters for generation of a linkage map, several combinations of minimum logarithm of odds (LOD) thresholds (2.9-3.7) and maximum recombination fraction (r) values (0.20-0.50) were applied to the data set. Any LOD value set between 3.0 and 3.6 did not influence the results. A genetic map of *Fusarium oxysporum* was generated using LOD 3.6 and r = 0.20. In total, 23 linkage groups were identified (Fig. 5A). Some linkage groups contain only race 1 derived markers (linkage groups 2, 10 and 14) and others only consist of race 3 derived markers (linkage groups 3, 9, 16 and 17). Most linkage groups, however, contain markers derived from both parents. A remarkable observation is that Foxy markers cluster together. Clusters were defined as groups of two or more markers that completely cosegregated. Of the 83 markers, 62 were found to be clustered on 16 positions. In addition, six linkage groups were observed to contain just one marker (18-23). Five groups (I-V) including 19 markers showed a significant segregation distortion (Fig. 5B). They did not segregate in a 1:1 ratio and were therefore excluded from linkage analysis. The distribution patterns of these markers revealed an overrepresentation of Fol004 derived Foxy fragments whereas Fol029 derived Foxy markers were always underrepresented. The 19 skewed
markers were divided into five groups based on similarity of their distribution in the population of fusion products.

**Figure 5**: Genetic map of *F. oxysporum* f. sp. lycopersici. Linkage analysis was performed with LOD 3.6 and \( r = 0.2 \) using the MapDisto program. A: 23 Linkage groups were identified, based on the segregation of 83 Foxy-AFLP markers. B: 19 Foxy markers showing skewed segregation were organized in 5 groups (I-V) according to their segregation pattern. The markers were named after the particular standard adapter primer from which they were obtained (A=Mse+A; C=Mse+C; G=Mse+G; T=Mse+T; E=Eco-Asp) and numbered according to their gel position (from high to low molecular weight). Genetic linkage was confirmed using JoinMap (Stam, 1993). The only difference between the two programs was the order of markers in linkage group 4. T20 was mapped at 7 cM from A3b using JoinMap.

**From genetic linkage to physical linkage**

The map shown in Figure 5A is based on LOD 3.6. Lowering the LOD score to 2.9 resulted in fusion of linkage groups. Groups 2 and 4, groups 9 and 23 and groups 7 and 11 were joined together using LOD 2.9 (Fig. 6). To investigate whether the genetic linkage at LOD 2.9 represents the actual situation, physical linkage was studied by means of hybridization experiments. In addition, physical linkage between skewed markers was investigated. For these purposes we focused on two groups: firstly, linkage groups 7 and 11
were chosen as representatives of non-skewed groups. Markers in these groups were derived from both the race 1 and the race 3 parent and all segregated in a 1:1 ratio. Secondly, group V was chosen for physical linkage analysis as this group contains both avirulence genes. We were able to clone race 1-specific Foxy bands from linkage group 11 (C20 and A10) and linkage group 7 (G21 and C14) as well as from group V (C19, C9, G17, A1 and A5) into the pGEM vector (Table 1). Subsequently, the Foxy fragments were sequenced.

Since Foxy represents a family of SINEs that can be divided into families based on the 5' terminal tetranucleotide repeats, these specific repetitive sequences were investigated for all sequenced Foxy fragments (Fig. 7). Compared to the three families described for Fol007 by Mes et al. (2000), only members of the TTTG and TATG families were characterized for Fol004 and Fol029. However, two new variants were observed: markers A1 and C19 contain a (TTTG)4 tetranucleotide that is repeated four times whereas in marker C20 this motif is repeated two and four times divided by sequence TTTTTG (Fig. 7). The sequence upstream of the tetranucleotide repeats is unique for each marker and did not suggest any sequence specificity for Foxy integration. Primers were designed on these unique flanking sequences upstream of the Foxy specific 5' terminal tetranucleotide repeats (Table 1). PCR amplification steps using the unique primer in combination with the Foxy-specific primer were performed on genomic DNA of the parents and the fusion products. Only when the amplification pattern was identical to the pattern known from the AFLP analysis for the particular marker, we were convinced to have cloned the correct Foxy-specific polymorphic fragment. Primers designed on the unique flanking region of markers A10, C19, G17 and A1 were not specific since fragments were amplified in both race 1 and race 3 parental strains (data not shown). PCR
with primers specific for C20, G21 (group 11), C14 (group 7), C9 and A5 (group V), showed the expected segregation pattern. For all five markers, the unique flanking fragment upstream of the Foxy-specific 5' terminal tetranucleotide repeats was too small to

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<th>A1</th>
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Figure 7: Alignment of the Foxy-specific 5' terminal tetranucleotide repeats of 10 Foxy markers derived from either Fo004 or Fo029 (this study) and 9 polymorphic AFLP fragments derived from Fo007 (Mes et al., 2000). The Foxy-specific primer sequence is shown in grey. Sequences upstream of the tetranucleotide repeats are unique for each marker (only four nucleotides shown).

be used directly as a probe on CHEF blots. To obtain larger DNA fragments, a Fo004 BAC library was screened by PCR. For the C20 marker one positive BAC clone was found while for G21 and C14 five positive BAC clones were identified. Screening of the BAC library with the skewed markers resulted in two positive BAC clones for A5 and one positive BAC clone for C9. Digestion of all positive BAC clones with HindIII revealed similar patterns for BACs derived from the same marker, whereas no overlap was observed with BACs containing other markers (Fig. 8A). Randomly chosen BAC fragments (1.2 - 0.5 kb) from digestions using either HindIII or XhoI, were gel-purified and used as a probe on a CHEF blot containing chromosomes of the wild type strains Fo004 and Fo029. Between 2 and 8 different BAC fragments per marker were used for subsequent hybridization experiments. All fragments tested were single copy sequences and fragments derived from different positive BAC clones for one particular marker showed identical hybridization patterns (results not shown). Figure 6B shows hybridization patterns for one
representative probe per marker. Based on genetic linkage with LOD 2.9, these markers were expected to be physically linked. This is clearly not the case for linkage groups 7 and 11 since C20 is located on a different chromosome as G21 and C14. However, markers G21 and C14 with a genetic distance of 20 cM are located on chromosome 3 and 23 in Fol004 and Fol029, respectively. Marker C20, and therefore linkage group 11, was mapped to chromosome 11 in Fol004 and chromosome 27 in Fol029. We therefore assume that
genetic linkage with LOD 3.6 is in accordance with physical linkage. For the skewed group V, the A5 marker was mapped to a different chromosome compared to C9. The C9 marker was observed to be race 1-specific on chromosome 10, whereas A5 was present in both Fol004 and Fol029 on different chromosomes (chr. 8 and chr. 27, respectively). From these results we conclude that the markers C9 and A5 that show a highly similar (skewed) distribution pattern over the progeny are not physically linked, indicating the importance of taking 1:1 segregation into account. However, physical linkage of other markers in this biased group (G17 and A1, C9 and C19, A5 and Avr-1) cannot be excluded.

We have shown that genetic analysis is a functional approach in the asexual *F. oxysporum* f. sp. *lycopersici*, resulting in the first linkage map. Further, we have shown that the *Foxy*-AFLP technique is an efficient and strong tool for segregation analysis. *Foxy*-AFLP markers linked to any gene of interest can be exploited to screen a BAC library. Positive BAC clones can be used in complementation studies, subsequently leading to gene cloning.

**DISCUSSION**

The high abundance and widespread distribution of the *Foxy* element in the *F. oxysporum* genome was used to develop a modified AFLP technique, using the *Foxy* sequence in combination with standard AFLP adapter sequences: *Foxy*-AFLP. The *F. oxysporum* f. sp. *lycopersici* Fol007 genome was estimated to contain at least 160 *Foxy* elements (Mes et al., 2000). In the different strains used in this study, the number of visible *Foxy*-AFLP bands is lower (126 for Fol004 and 114 for Fol029) (Fig. 1). Most probably these numbers underestimate the actual number of *Foxy* insertions for several reasons. Large DNA fragments could be missed because of amplification failure. In addition, polymorphic and non-polymorphic fragments may be of equal length and will therefore not be separated under the electrophoretic conditions used. Hybridization experiments on CHEF blots revealed the presence of *Foxy* sequences on every single chromosome from several different isolates belonging to VCG0030 (Mes et al., 2000). The same result was obtained for Fol004 and Fol029 (data not shown), indicating that *Foxy* is distributed throughout the genome.

Modified AFLP techniques have previously only been described based on (retro)transposable elements derived from plants e.g. *PDR1*, *Tps12* and *Tps19* (the *Tyl-copia* group) of *Pisum* (Ellis et al., 1998; Pearce et al., 2000), the *BARE-1* of Barley (Waugh et al., 1997; Kalender et al., 1999), the *dTphl* family of *petunia* (Van den Broeck et al., 1998) and the MITE family *Heartbreaker* of *Maize* (Casa et al., 2000). The purpose of using a transposable element was to increase the number of polymorphisms above that
revealed by conventional molecular markers (RFLP, RAPD and AFLP). The proportion of polymorphic bands detected by BARE-1-AFLP (26%) was higher than the polymorphism rates observed with standard AFLP (9.2-19.3%) (Waugh et al., 1997). Comparable results were obtained for maize as the level of polymorphism detected by Heartbreaker-AFLP (60%) was higher than standard AFLP (26-40%) but comparable to RFLP (50-80%) (Casa et al., 2000). The high level of polymorphism of Foxy between Fol004 and Fol029 (43%), is exceptional when compared to the ~2% of polymorphic bands determined by standard AFLP analysis between these closely related isolates (Mes et al., 2000), indicating that Foxy has been active in the recent past. Foxy is not the only repetitive element in F. oxysporum (Daboussi and Langin, 1994; Hua-Van et al., 2000) and the retroposon-based AFLP approach would be applicable to any class of homogeneous sequence. However, AFLP analysis based on the DNA transposon Fotl (Daboussi et al., 1992) showed less than 5% polymorphic Fotl fragments between closely related strains (Mes et al., 2000). The high rate of Foxy activity may provide an explanation for the variability with respect to electrophoretic karyotype, virulence and avirulence found between strains of F. oxysporum. We showed that Foxy was activated during transformation and fusion processes. Genomic stress is known to activate transposable elements (McClintock, 1984). DNA damage (Bradshaw and McEntee, 1989), irradiation with gamma rays (Mes et al., 2000) and exposure to chlorate (Anaya and Roncero, 1996) have been identified as factors that are able to activate transposition. Transposable elements were also found to be activated during protoplast isolation (Pearce et al., 1996). Since protoplast generation is necessary for both transformation and fusion experiments, Foxy might hence be activated.

Foxy insertions were observed to be clustered, with some clusters only containing markers derived from either the race 1 parent or the race 3 parent. The existence of these clusters could be explained by nearby transposition of just one active Foxy marker. Most clusters, however, contain complementary markers derived from both parents and might be explained by nearby transposition of two allelic active Foxy markers. Alternatively, these clusters might represent hot spots of integration, which different active Foxy elements prefer as integration site. Since the estimated genome size for F. oxysporum f. sp. lycopersici is approximately 50 Mb (Migheli et al. 1993; Zolan, 1995) and 102 Foxy markers are taken into account, one Foxy marker is predicted to occur every 500 kb in case they are randomly distributed. However, considering the clustering of 62 Foxy fragments at 16 positions, one Foxy marker is expected to occur every 900 kb. Local clustering of retrotransposable elements was suggested for BARE-1 elements in barley (Kalendar et al., 1999) and for class II transposons from F. oxysporum (Hua-Van et al., 2000). Clusters of transposable elements have been found around centromeric and telomeric regions in Magnaporthe grisea (Valent and Chumley, 1994) and in Neurospora crassa (Cambareri et
al., 1998). Avirulence genes from *M. grisea* and *Phytophthora infestans* have been observed to cluster near telomeric sequences as well (Dioch et al., 2000; Orbach et al., 2000; Van der Lee et al., 2001). The clustering of *Foxy*’s may appear to limit its usefulness in mapping avirulence genes. However, when avirulence genes and clusters of *Foxy* or other transposable elements of *F. oxysporum* are also localized near centromeric or telomeric regions, *Foxy*-AFLP and modified AFLP techniques in general may provide useful markers for mapping avirulence genes.

Dispersed and repeated DNA sequences have been useful in segregation analysis and mapping as demonstrated for MGR sequences in *M. grisea* (Hamer and Givan, 1990) and *BARE-l* in barley (Manninen et al., 2000). Similarly, *Foxy* sequences can be applied for genetic analysis and mapping purposes. Although *F. oxysporum* is an asexual fungus, we showed that exchange of parental DNA and recombination of phenotypic markers like avirulence occurred in parасexual crosses (Chapter 2). In addition, *Foxy*-AFLP revealed random exchange of most polymorphic *Foxy* fragments in asexual fusion products (Fig. 2). The extent of partial diploidy, if occurring at all, is limited (Fig. 3). The total number of *Foxy* markers in race 1 parents (57) was higher than the number of *Foxy* markers in the race 3 parental strains (45). Despite correction for this higher number of race 1 markers, the race 1 contribution in the fusion products was 60%, which is 10% higher than expected. This was caused by preferred inheritance of several race 1 derived markers and against several race 3 derived markers. Such significant segregation distortion was found for 19 markers which is only a minority of the total number of 102 *Foxy* markers. Skewed segregation, as also seen for the inheritance of avirulence gene 1 (*A I*), is therefore exception rather than rule.

Segregation distortion is commonly detected when mapping molecular markers in plants (Zamir and Tadmore, 1986; Guo et al., 1991; Jenczewski et al., 1997; Casa et al., 2000). Further, in the oomycete *Phytophthora infestans*, non-Mendelian segregation of mating type loci was observed (Judelson et al., 1995). Aberrant segregation of genetic markers has also been reported in fungal species. For example, in *Gibberella fujikuroi* and *Neurospora tetrasperma*, spore killer loci (Xu and Leslie, 1996; Raju and Perkins, 1991) and in *Leptosphaeria maculans*, AFLP markers (33%) (Pongam et al., 1998) have been shown to segregate abnormally. Skewed segregation of RFLPs and RAPDs with preferential maternal transmission was observed in wide crosses of the bean rust fungus, *Uromyces appendiculatus* (Martinez et al., 1996). Since the crosses between race 1 Fol004 and race 3 Fol029 are not naturally occurring but were forced, like the wide crosses described by Martinez et al. (1996), the race 1 parent might have a higher fitness than the race 3 parent and could therefore be genetically dominant (Madhosingh, 1994). Hybridization experiments using random probes revealed the presence of race 1-specific
sequences in all fusion products (Chapter 2). These particular sequences may be preferentially inherited due to the presence of an essential gene or because homologous sequences are absent in the race 3 parent. Five groups of markers (I-V) did not segregate in a 1:1 ratio and showed preferential race 1 inheritance. Markers from the skewed group V appeared not to be physically linked. Physical linkage of markers from the other four skewed groups is therefore doubtful. Disregarding the 19 markers that showed skewed segregation, 83 markers showing a 1:1 segregation pattern were used to generate the first genetic map of *Fusarium oxysporum*. In total, 23 linkage groups were identified. The exact number of chromosomes in the parents used in this study is not known but varies among different isolates of *F. oxysporum f. sp. lycopersici* between 10-15 chromosomes. We assume therefore that the 23 linkage groups do not correspond to the number of chromosomes. For the genetic map of *Gibberella fujikuroi* mating population A, which is the sexual stage of *Fusarium moniliforme*, 12 linkage groups were identified (Xu and Leslie, 1996). In addition, expanded genetic mapping analysis in *Gibberella moniliformis* (*Fusarium verticillioides*) resulted in 636 markers distributed across 12 chromosomes (Jurgenson et al., 2002). To improve the accuracy of the linkage map of *Fusarium oxysporum f. sp. lycopersici*, the population size should be increased. To improve the coverage of this genetic map, other transposable elements as well as new Foxy family members should be exploited for retroposon-based AFLP analysis in order to increase the number of markers.

In conclusion, Foxy-AFLP was demonstrated to be a powerful technique in the genetic analysis of clonally related isolates of *F. oxysporum* since the level of polymorphisms appeared to be much higher than that revealed with other molecular marker approaches. Since Foxy is presumably present in almost all *Fusarium* species (Mes et al., 2000), Foxy-AFLP is useful for mapping and phylogenetic studies within or between any species of this genus. The retroposon-based AFLP approach is not restricted to Foxy or *Fusarium* species; it is applicable to any class of homogeneous sequence in any species. The availability of a genetic map in combination with a BAC library provides us unique tools for gene mapping in *Fusarium oxysporum f. sp. lycopersici*.

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