Fusarium oxysporum from iridaceous crops: analysis of genetic diversity and host specialisation
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A dispensable region from a 2.1-Mb chromosome of a race 1 isolate of *Fusarium oxysporum* f.sp. *iridacearum* harbours sequences correlated with pathogenicity to large-flowered gladiolus ‘Peter Pears’

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**ABSTRACT**

Race 1 of *Fusarium oxysporum* f.sp. *iridacearum* is pathogenic to the ‘large-flowered’ gladiolus cultivar ‘Peter Pears’, while the genetically closely related race 2n is not. Both races are pathogenic to the ‘small-flowered’ gladiolus ‘Nymph’. Fusion products obtained by fusion of protoplasts of hygromycin B and phleomycin resistant transformants of a race 1 and a race 2n isolate were subjected to phenotypic and genotypic analyses. Pathogenicity tests with ‘Peter Pears’ and ‘Nymph’ revealed that only some fusion products showed one of both parental phenotypes. Many fusion products showed an intermediate phenotype, being highly pathogenic to ‘Nymph’, but displaying reduced pathogenicity to ‘Peter Pears’ at variable levels. This indicated that the difference in pathogenicity to ‘Peter Pears’ is not determined by a single gene, but genetically more complex. AFLP- and RAPD-analysis yielded two groups of markers of which the absence was correlated with complete absence of pathogenicity to ‘Peter Pears’. Markers from both groups could be assigned to a 2.1-Mb chromosome of the race 1 parent. Southern analyses strongly suggested that homologous sequences of (part) of this chromosome are absent in the race 2n parental isolate, indicating that these markers are located on a dispensable region. A 1.6-Mb chromosome present in the race 1 parent, but absent in the race 2n parent, was also found to be correlated with pathogenicity to ‘Peter Pears’. Fusion products possessing both the 1.6-Mb chromosome and the marker groups located on the 2.1-Mb chromosome showed full pathogenicity to ‘Peter Pears’, those missing both were non-pathogenic to ‘Peter Pears’, and fusion products missing one of both showed reduced pathogenicity to variable extents. However, no molecular markers associated with this 1.6-Mb chromosome were found, hampering further analysis. One of the fusion products differed from its race 1 parent only by the absence of one of the two marker groups from the 2.1-Mb chromosome. Southern analyses and PFGE analysis indicated that loss of this marker group probably reflected the occurrence of a deletion of part of the 2.1-Mb chromosome, which resulted from a transloational event during the enforced fusion process. The fact that the concerning fusion product showed reduced pathogenicity to ‘Peter Pears’ suggested the presence of (a) putative pathogenicity gene(s) located on this dispensable region. Some but not all of the corresponding markers were shown to be physically closely linked. The minimum size of the marked region was calculated to be 120 kb.
**INTRODUCTION**

Race 1 of *Fusarium oxysporum* (Schlecht. : Fr.) f.sp. *iridacearum* (f.n.) was found highly pathogenic to the large-flowered gladiolus 'Peter Pears', while race 2 was non-pathogenic to this cultivar. Both races were pathogenic to small-flowered gladiolus 'Nymph' and several other iridaceous crops (chapter 2). Isolates G2 (race 1) and G6 (race 2n) were chosen to study the genetics underlying this distinct difference in pathogenicity to 'Peter Pears'. Since *F. oxysporum* is an imperfect fungus, classical genetic analysis of host specificity of physiological races, involving sexual crosses of isolates from different races, is not possible. Therefore, parasexual recombinants, enforced by fusing protoplasts of transgenic strains of both isolates, carrying two different dominant selection marker genes (resistances against the antibiotics hygromycin B or phleomycin; Punt and Van den Hondel, 1992) were used to study these genetics (chapter 4). Phenotypic and molecular analysis of parasexual progenies might enable the identification of markers linked to race phenotype, presuming that a single gene or a gene cluster determines this phenotype. Hybridisation of linked markers to chromosomes separated by pulsed field gel electrophoresis (PFGE; Birren and Lai, 1993) can subsequently be used to confirm physical linkage of molecular markers (Javerzat et al., 1993; Xu and Leslie, 1996) and to identify the chromosome on which the gene (cluster) determining (a)virulence to gladiolus 'Peter Pears' is located.

This chapter describes pathogenicity testing and a limited RAPD analysis of two parasexual progenies obtained by protoplast fusion, as well as an extensive AFLP analysis and PFGE analysis of a representative subset of fusion products from both populations. Results of these analyses will show if indeed a single gene(cluster) is responsible for the difference between G2 and G6 in their pathogenicity to 'Peter Pears' and can be assigned to a chromosome. Moreover, these analyses will give further insight in the nature and frequencies of molecular events occurring during the parasexual process.

**MATERIALS AND METHODS**

**Fungal material.** Wild type strains chosen for this study were G2 (race 1) and G6 (race 2n) of *F. oxysporum* f.sp. *iridacearum*. The transgenic parental strains G2p22 (p = phleomycin resistant) x G6h1 (h = hygromycin B resistant) and G2h12 x G6p8 were successfully used to produce double resistant protoplast fusion products (chapter 4). All strains were monosporid and kept as soil cultures at 4°C.

**Pathogenicity test.** Corms of the two differential gladiolus cultivars 'Peter Pears' and 'Nymph' were inoculated with the different fusion products as described in chapter 2 with one modification. Because of the high number of fungal strains, corms were not dipped in a spore suspension prior to planting, but inoculated by pipetting 1 ml of the spore suspension on and around corms, which were already put in a planting hole, but not yet covered with peat soil. Plants were grown in a greenhouse at 25°C under strict quarantine conditions. Each
strain was tested on five independent plants of each cultivar. Replicates were randomised in blocks. Both populations were tested twice in two successive years. Plants inoculated with transgenic parental strains, wild types and water served as controls. After four weeks shoot length was measured and independently disease severity was assessed by visually scoring the amount of discoloured corn tissue on a scale from 0 to 5. Shoot length data were analysed by analysis of variance. Disease severity data were analysed by regression analysis after logit transformation. Strains that did not cause any symptoms were excluded from the regression analysis.

**RAPD analysis.** Random amplified polymorphic DNAs were generated using 10-mer oligonucleotide primers obtained from Operon technologies, Inc., Alameda, CA, USA as described in chapter 4. Primer codes and nucleotide sequences (5' → 3') of primers were: OPA-01 = CAGGCCCTTC and OPG-12 = CAGCTACGA.

**AFLP analysis.** A two step AFLP protocol as described in chapter 3 was used, but with some modifications in line with the final protocol published by Vos et al. (1995). After digestion and adapter ligation reaction mixtures (40 μl) were diluted to 500 μl with T0.1E (10 mM Tris.HCl (pH 8.0); 0.1 mM EDTA), stored at -20°C and used later as template for the preamplification reaction. The purification step with streptavidine beads, described in chapter 3, was omitted without noticeable changes in results. Preamplification reactions consisted of 20 cycles with annealing only at 56°C (selective AFLP reactions still consisted of 36 cycles with decreasing annealing temperature during the first 13 cycles) and preamplification products were diluted 10-fold with T0.1E (instead of 50-fold). The bottom strand of the EcoRI-adapter and both non-selective primers used were respectively three nucleotides shorter and one nucleotide longer at their 5'-ends than those given by Vos et al. (1995), as described in chapter 3.

The two parental wild type strains G2 and G6 were analysed with EcoRI-primers with the selective nucleotides +AT, +CG and +GA and +TA in combination with all sixteen possible Msel-primers with two selective nucleotides. Nine primer sets, yielding four to seven polymorphisms each, were selected for analysis of a subset of 25 fusion products, parental strains, and wild types. Since AFLP products of this set of samples, generated with a single primer set, were always analysed on the same gel it was easy to visually score presence or absence of AFLP fragments.

**Cluster analysis.** Linkage of AFLP and RAPD markers was analysed by cluster analysis of segregation profiles. For each marker the absence (0) or presence (1) in each individual of the fusion populations was recorded and the hence obtained two digit matrix was analysed with the help of a GENSTAT 5 (Payne et al., 1987) computer programme. Simplematching similarity coefficients (if \( x_i = x_j \), then 1, weight=1 and if \( x_i \neq x_j \), then 0, weight=1) were calculated for all possible pairs of markers using the FSIMILARITY directive, and from the similarity matrix a dendrogram was drawn using the HCLUSTER directive with average linkage. Results of this cluster analysis were used to group markers with similar segregation
profiles. Likewise, similarity of AFLP profiles of individual fusion products was analysed, leading to clustering of fusion products with similar AFLP profiles.

Cloning of RAPD- and AFLP-markers. RAPD markers separated by agarose gel electrophoresis were cut out and purified from the gel using the Geneclean II kit (Bio101 Inc., La Jolla, CA, USA) and finally dissolved in T0.1E. About 1 ng of DNA was reamplified using the original primer and PCR conditions (chapter 4) except that not 35 but 25 cycles were applied. A sample of the PCR product was analysed on agarose gel and a volume of the reaction mixture containing about 10 ng of reamplified marker DNA was ligated into vector pCR2.1 using the TA Cloning Kit (Invitrogen Corp., San Diego, CA, USA) and used to transform INVαF' Escherichia coli cells (One Shot Kit, Invitrogen). Plasmid DNA from transformed clones was isolated using standard techniques (Sambrook et al., 1989) and analysed by restriction analysis and by PCR using the original RAPD primer and amplification conditions.

Developed X-ray films containing AFLP autoradiograms were orientated in their original position on top of the corresponding gels (dried on absorbent filter paper). AFLP-marker bands were cut out through the X-ray film. Excised pieces of filter paper were transferred to micro tubes, soaked in 100 µl of water and incubated for at least 2 h at 4°C. A series of 0.1, 1 and 5 µl was used to reamplify the fragment using the original selective primer set and PCR conditions except that non-labelled primers were used (30 ng per reaction each). A sample of the PCR products was analysed on agarose gel and when enough product of approximately the expected size was produced a volume of the reaction product containing about 10 ng of marker DNA was ligated into vector pCR2.1 and used to transform E.coli cells as described for cloning of RAPD-markers. Plasmid DNA from several clones was used as template DNA in a selective AFLP reaction with the original primer set of which the EcoRI-primer was radioactively labelled. Reaction products were analysed on sequencing gels alongside samples of the original AFLP ladder to select the right clones.

Southern analysis. Cloned RAPD and AFLP markers were labelled and used to probe Southern blots of EcoRI and HindIII digests to estimate their copy number, following protocols described in chapter 4. Cloned markers were digoxigenin-11-dUTP labelled by direct PCR-labelling using the original RAPD- or AFLP-primer set or [α3²P] dATP labelled by random primed labelling of purified inserts.

Rare cutter digests. DNA preparations from agarose embedded protoplasts identical to those used for PFGE of intact chromosomes (chapter 4) were used to prepare rare cutter digests. Agarose plugs were successively washed in 1 ml of TE (10 mM Tris.HCl (pH 8.0); 1 mM EDTA) and 1 ml of the appropriate digestion buffer for 30 min at 4°C, before they were digested at 37°C in 1 ml of fresh buffer containing 40 units of the restriction enzymes Ascl or Sfil (New England BioLabs, Inc., Beverly, MA, USA). After 4 hours again 40 units of restriction enzyme was added and samples were incubated for another 4 hours at 37°C. Subsequently plugs were washed in 1 ml of 0.5 x TBE (Sambrook et al., 1989) for 30 min at
4°C and put on gel (1% Seakem Gold agarose; FMC BioProducts, Rockland, ME, USA). Rare cutter digests were separated by PFGE on a CHEF DR-II system (Bio-Rad Laboratories Inc., Hercules, CA, USA) in 0.5 x TBE electrophoresis buffer at 5 V cm\(^{-1}\) at 15°C for 18 hours with switching intervals increasing from 1 to 25 s. As molecular size marker the Midrange PFG Marker I (New England BioLabs) with a size range of 15-300 kb was used.

**PFGE analysis of intact chromosomes.** Electrophoretic separation of chromosomes, staining, photographing and blotting was performed as described in chapter 4. To improve resolution of the smaller chromosomes gels were run at 10°C, 3 V cm\(^{-1}\) with switching intervals from 300-500 sec for 63 hours (3 day run). Hybridisation experiments with PFGE blots were carried out as for Southern blots of digested total DNA (chapter 4). To measure relative differences in intensities of chromosomal bands within a lane, photographs of PFGE-gels were digitalised and analysed using the computer programme BioImage advanced Quantifier 1-D Match (version 2.1.1; Bio-Image Systems Corp., Ann Arbor, MI, USA).

**Cloning of chromosomal fragments.** To obtain random clones from a specific chromosomal band, separated by PFGE, the band of interest was cut out of the gel. Excised pieces of gel were equilibrated in the appropriate restriction buffer for 8 hours. After 4 hours restriction buffer was changed. Twenty units of EcoRI and BamHI were added and samples were incubated overnight at 37°C. Again 20 units of both restriction enzymes were added and samples were incubated for another 8 hours. DNA was purified from the samples using the Geneclean II kit (Bio101 Inc., La Jolla, CA, USA) and digested in a small volume of TE buffer. Digest were ligated into the cloning vector pBluescript II SK+ (Stratagene, La Jolla, CA, USA) and transformed into *E.coli* INVαF' cells, following standard procedures (Sambrook et al., 1989). Clones containing non-repetitive sequences were selected by Southern analysis.

**RESULTS**

**Pathogenicity tests.** Pathogenicity of two populations of fusion products from isolate G2 (race 1) and isolate G6 (race 2n), both belonging to VCG 0340 of *F. oxysporum* f.sp. *iridacearum*, was tested on the two differential gladiolus cultivars ‘Peter Pears’ (susceptible to race 1, but resistant to race 2n) and ‘Nymph’ (susceptible to both races). The two populations were obtained by fusing two different pairs of parental strains, which showed chromosomal integration of selection markers in a ‘reciprocal’ fashion (chapter 4). In G2p22 x G6p8 vector DNA hybridised to chromosomal band III, while in G2h12 and G6h1 vector DNA hybridised to chromosomal band VII. Results of pathogenicity tests of fusion products from G2p22 x G6h1 (population A) and form G2h12 x G6p8 (population B) are presented in figure 1. Pathogenicity of transformed parental strains was not different from that of wild types G2 and G6. Phenotypes of fusion products, however could not simply be classified as either race 1 or race 2n, a result expected when race phenotype is determined by the presence
**Figure 1.** (On opposite page) Pathogenicity of fusion products and parental strains to the differential gladiolus cultivars ‘Peter Pears’ and ‘Nymph’. A, C and E: Population A: 40 fusion products of G2p22 and G6h1. B, D and F: Population B = 24 fusion products of G2h12 and G6p8. Fusion products are presented as triangles. Solid triangles represent the subset of fusion products subjected to AFLP- and PFGE-analysis. Wild types are presented as open circles, transformants as solid circles. Water controls are presented as diamonds. Predictions of disease severity scores as calculated by regression analysis were retransformed to the original scale (0-5). Corm rot of ‘Peter Pears’ plotted against corm rot of ‘Nymph’ are given in panels A and B. Data points below the dotted line showed significantly reduced severity of corm symptoms on ‘Peter Pears’ (P<0.05). Shoot length data are given in panels C and D. Data points above the dotted line had shoot lengths of ‘Peter Pears’ significantly (p<0.05) larger than the race 1 parents. Relationship between shoot length and corm rot data on ‘Peter Pears’ is presented in panels E and F. Controls, parental isolates and fusion products of special interest are labelled.

or absence of a single gene(cluster). Within both populations, fusion products displaying intermediate phenotypes were found. Their pathogenicity to both cultivars was apparent, but disease severity on ‘Peter Pears’ was significantly reduced compared to the race 1 parental strain, while their pathogenicity to ‘Nymph’ was comparable to that of the parental strains. Of the 40 fusion products from G2p22 x G6h1, 13 showed a phenotype similar to the race 1 parent. Another 11 showed significantly (p<0.05) less reduction in shoot length compared to race 1, while their corm rot scores were comparable to race 1. Fifteen isolates also caused corm rot symptoms on ‘Peter Pears’, but these symptoms were significantly (P<0.05) less severe than those caused by their race 1 parent G2p22. One fusion product (A18) of this population showed no symptoms on ‘Peter Pears’. Of the 24 fusion products from the G2h12xG6p8 cross, only one showed a race 1 phenotype. All other fusion products showed significantly (P<0.05) less severe symptoms on ‘Peter Pears’. One of these (B6) only showed weak symptoms on few corms of ‘Peter Pears’. Another fusion products (B19) showed no symptoms at all on ‘Peter Pears’.

**RAPD analysis.** The two RAPD primers OPA-01 and OPG-12 were selected on the basis of their ability to discriminate between race 1 isolates and isolates belonging to race 2n or other races within VCG 0340 of *F. oxysporum* f.sp. *iridacearum* (Mes et al., 1994a and De Haan et al. 2000). Both primers yield a RAPD-fragment present in G2, but absent in G6. The marker obtained with primer A01 was designated RAPD1 and had an estimated size of 1125 base pairs, while marker RAPD2 obtained with primer G12 was 600 base pairs in size. All fusion products of both populations were analysed for the presence of these two RAPD markers. Marker RAPD2 was found in all fusion products except in A18 (from G2p22 x G6h1) and B19 (from G2h12 x G6p8), two fusion products showing a true race 2n phenotypes (figure 1). Marker RAPD1 was also found to be absent in these two fusion products. In all other fusion products, except B7, this marker was present. Fusion product B7 was highly pathogenic to ‘Nymph’, but showed slight corm rot symptoms on ‘Peter Pears’ (figure 1).
CHAPTER 5

Population A (G2p22 x G6h1)

Population B (G2h12 x G6p8)
**Southern analysis.** Both RAPD marker fragments were cloned. Neither contained *Hind*III recognition sites. Hybridisation to Southern blots of *Hind*III digests of total DNA revealed the presence of two copies of both fragments in wild type isolate G2 and its transformant, but neither fragment did hybridise to DNA of G6 or its transformants (figure 2). This proved that both RAPD markers are the consequence of the complete absence of their template sequences in G6. The three fusion products which showed absence of one or both markers, and two randomly selected fusion products from both populations which were positive for both RAPD markers, were analysed in the same Southern (figure 2). The two fusion products (A18 and B19) that missed both RAPD markers showed hybridisation with neither of both fragments like the G6 parental strains. Fusion product B7, which was positive for marker RAPD2 but negative for RAPD1 in the PCR, contained one of the two copies of the RAPD2 fragment, while both copies the RAPD1 fragment were missing.

**AFLP analysis.** From both fusion populations a representative subset of 25 (presented by solid triangles in figure 1) strains was subjected to AFLP-analysis with nine different primer sets. This subset included the three fusion products showing absence of one or both RAPD markers. To facilitate comparison between marker data and pathogenic phenotypes, pathogenicity of each strain to 'Peter Pears' as presented in figure 1, are also given in figure 3. Disease severity scores on this cultivar are presented as bars at the top of this figure.

In total 528 AFLP fragments were scored, of which 29 were present in G2, but absent in G6 and 10 were present in G6, but absent in G2. Presence or absence of these AFLP markers and of both RAPD markers within the representative subset of fusion products and parental strains is presented in figure 3.

Markers with identical segregation patterns were assigned to the same marker group (figure 3, second column). To visualise similarity between marker groups, they were assorted according to the result of cluster analysis. Likewise, fungal strains with similar marker profiles as revealed by cluster analysis were assorted.

Six of the G2-AFLP-markers (31, 33, 47, 49, 53 and 63) showed the same segregation profile as RAPD1 and were designated marker group 1. Marker group 2 consisted of nine G2-AFLP-markers (1, 3, 7, 13, 19, 62, 51 and 54) and RAPD2. This group differed from marker group 1 only in its presence in fusion product B7. Marker group 3 (G2-AFLP-markers 14, 15, 22, 23, 24, 35, 42, 67 and 69) was found to be present in all fusion products. G2-AFLP-markers 65 and 66 (marker group 4) co-segregated with marker group 3 in all cases except one. The remaining G2-AFLP-markers (16, 18 and 21) each showed distinct segregation patterns. Within the set of G6-AFLP-markers seven marker groups (8 to 14; figure 3) were recognised. G6-AFLP-markers 12 and 17 both showed unique segregation patterns. Segregation patterns of marker groups 10 and 11 were similar as were the profiles of marker groups 12, 13 and 14. Besides rearrangement of G2 and G6 markers, also presence of new AFLP fragments and absence of AFLP fragments present in both parents were observed in 18 of the 25 fusion products analysed (data not shown). These observations indicate the frequent occurrence of molecular rearrangements during the fusion process.
Figure 2. Southern analysis of parental isolates and a selection of fusion products. HindIII digest of total DNA was probed with cloned marker fragments RAPD1 (panel A) and RAPD2 (panel B). M = molecular size marker (lambda HindIII+EcoRI).

No molecular markers showed linkage to one of the selection markers (presented at the bottom of figure 3). Such markers would be present in all fusion products from the same crossing and, considering the reciprocal integration of selection markers in both parental sets, absent in all fusion products from the other crossing. Since many fusion products showed significantly reduced pathogenicity to ‘Peter Pears’ (figure 1 and top of figure 3), the correlation between molecular markers and race phenotype was not obvious. The absence of marker groups 1 and 2 in the two fusion products showing a true race 2n phenotype, however suggested a correlation between the presence of these groups and pathogenicity to ‘Peter Pears’.

**PFGE analysis.** All wild types, parental strains and fusion products subjected to AFLP analysis were also characterised by pulsed field gel electrophoresis (PFGE). Gels containing representative strains, displaying all observed chromosomal polymorphisms, are presented in figure 4.
**Figure 3.** Overview of molecular analyses of wild type isolates G2 (race 1) and G6 (race 2n) of *F. oxysporum f.sp. iridacearum*, transgenic parental strains (G2h12, G2p22, G6h1 and G6p8) and a representative subset of 25 fusion products. AFLP- and RAPD- markers with identical segregation patterns are assigned group numbers (second column). Markers are assorted according to mutual similarity as revealed by cluster analysis. Likewise fungal strains are assorted on the basis of similarity in marker profiles. Dark grey = marker is present; White = marker is absent; Middle grey = missing data. At the top of the figure black bars represent pathogenicity to ‘Peter Pears’. Presence of the 1.6-Mb chromosome X (top) and selection markers (bottom) is also given.
Electrophoretic karyotypes of wild type isolates G2 and G6 showed some striking differences, especially for the smaller chromosomes. Isolate G2 showed the presence of a 1.6-Mb chromosomal band (nr. X), which is absent in G6. Presence of this chromosome within the subset of fusion products was scored and is included in figure 3 (at top of the figure). All fusion products showing the presence of chromosome X (except fusion product B7) showed full pathogenicity to ‘Peter Pears’. No chromosome-specific probe was found after testing several random cloned restriction fragments obtained from isolated DNA of chromosomal band X. All cloned fragments were of a multicopy nature and present in both G2 and G6 (data not shown).

Chromosomal band IX (2.1 Mb) of G2 showed an intensity which was higher than that of chromosomal band X. Intensities of both bands, measured by computerised image analysis, yielded a 2:1 ratio. Presuming that chromosomal band X consist of a single chromosome it can be concluded that chromosomal band IX of G2 contained two unresolved chromosomes. Also after applying electrophoresis conditions with improved resolution at the concerning size range, these two chromosomes could not be resolved (figure 4C). Chromosomal band IX of G6 also appeared to consist of two chromosomes (figure 4A and 4B), but in contrast with chromosomal band IX of G2 these chromosomes could be resolved completely (figure 4C). The larger of these two chromosomes (IX-a) was of the same size as chromosomal band IX of G2 (2.1 Mb), while the smaller one (IX-b) had an estimated size of 2.0 Mb.

Transformants showed electrophoretic karyotypes similar to those of wild types except for G2h12, which missed chromosomal band VI (3.3 Mb), but had an extra band of 2.9 Mb (figure 4B). The putative deletion of 0.4 Mb, causing this polymorphism, did apparently not effect viability or pathogenicity of this transformant (figure 1). Of all fusion products from G2h12 xG6p8 analysed by PFGE, four inherited the 3.3 Mb band, presumably from G6p8, eight inherited the 2.9 Mb band from G2h12 and one (fusion product 174-6) seemed to have inherited neither of both (figure 4B).

Some fusion products showed chromosomes with non-parental sizes. Fusion products A11 (figure 4A) as well as A3, A28 and B18 (not shown) showed the presence of a chromosomal band of approximately 2.5 Mb, which was absent in the parental strains. Fusion products A10, A15 (figure 4A), B24 (figure 4B), A2 and A10 (not shown) appeared to have extra bands at the position of band IX (2.1 Mb). Running conditions with higher resolution in this size range, revealed two separate bands of 2.1 Mb and 2.0 Mb (A15 shown in figure 4C), as in the parental strain G6h1, but with a ratio of band intensities of 3:1 instead of 1:1. This might be the result by the occurrence of aneuploidy due to inheritance of the two (unresolved) 2.1-Mb chromosomes from G2p22 as well as the 2.1-Mb and 2.0 MB chromosomes of G6h1.

Fusion product B7 displayed a unique novel electrophoretic karyotype of the smaller chromosomes. Three bands of 1.6 Mb, 1.7 Mb and 2.1 Mb, with an intensity ratio of 1:1:1, were resolved (figure 4C).

Chromosomal localisation of markers. Marker groups 1 and 2 were the only marker groups that showed a possible correlation with pathogenicity. Both groups are distinguished only by
their differential presence in fusion product B7. Whether this implies that both groups should be considered different linkage groups or not, was tested by hybridisation of cloned markers from both groups to PFGE blots. The two RAPD markers were considered most suitable for this purpose, because of their relative large molecular size compared to AFLP markers.

Both RAPD fragments hybridised to chromosomal band IX of G2 on PFGE blots and showed no hybridisation to G6. This means that of each fragment both copies are located on one of the two chromosomes composing this band, or that both chromosomes contain one copy each. Hybridisation experiments with PFGE blots demonstrated that in all fusion products of the subset, except A18, B19 and B7, both RAPD fragments also hybridised to the 2.1-Mb chromosomal band. In all these cases this chromosomal band existed of at least two non-resolved chromosomes. RAPD1 did not hybridise to any chromosome of the three exceptional fusion products. This was expected since in a Southern analysis of HindIII digested total DNA also no hybridisation signal was observed (figure 2). RAPD2 did also not hybridise to any chromosome of A18 or B19 as already seen in the Southern analysis shown in figure 2. In fusion product B7, however, one copy of the RAPD2 sequence is still present (figure 2). On PFGE blots the RAPD2 clone was observed to hybridise to one of the larger chromosomes (>2.5 Mb), which were not resolved after a 3-day-run, indicating the occurrence of a translocational event during the fusion process.

Chromosomal band IX of G2 was excised from a PFGE gel. From this DNA a non-repetitive 1.1 kb EcoRI-BamHI fragment designated G2-IX-1 was cloned, which hybridised both to G2 and G6 DNA. When used as a probe on PFGE blots, this fragment did hybridise to chromosomal band IX of G2 and to the 2.1-Mb chromosomal band of G6 (IX-a). Hybridisation results obtained with both RAPD fragments and clone G2-IX-1 could be explained by the assumption that one of the two chromosomes (IX-a) composing chromosomal band IX of G2 is homologous to chromosome IX-a of G6 and both hybridise to G2-IX-1, while the other 2.1-Mb chromosome of G2 (IX-b) contains both copies of both cloned RAPD fragments. The conclusion that both RAPD markers are located on the same chromosome implies that markers group 1 and 2 (figure 3) should be considered one linkage group, corresponding to chromosome IX-b of G2.

Further analysis of marker group 1. Absence of marker group 1 was the only difference observed between marker profiles of fusion product B7 and G2 (figure 3). The strongly reduced pathogenicity to 'Peter Pears' of B7 might be correlated with the absence of this marker group. Of the RAPD1 sequence two copies were found present in the G2 genome, while both copies were absent in G6 and B7 (figure 2). AFLP-markers belonging to marker group 1 were also cloned. Cloned fragments were probed to Southern blots of EcoR1 digests of total DNA from G2, G6 and of fusion product B7. AFLP33, AFLP47 and AFLP63 each showed a single hybridising band in G2 but did not hybridise to G6 or B7. From these results we concluded that these AFLPs are a consequence of the complete absence of their sequences in G6 and B7, as was the case for RAPD1. AFLP53 showed a more complex hybridisation pattern. In G2 three bands were found, of which one was missing in B7 and two were missing in G6, indicating that the observed PCR-product in the AFLP analysis was
probably the result from the copy present in G2 but absent in G6 and B7. This means that also AFLP53 is probably due to a deletion. Signals obtained with AFLP31 and AFLP49 were too weak, probably due to their small molecular sizes (< 100 bp including adapter sequences).

![Image of electrophoretic karyotypes](image)

**Figure 4.** Analysis of electrophoretic karyotypes of fusion products. Panels A and B: 10 day run; 4 °C; 1.5 V.cm⁻¹; switching intervals from 1200 to 4800 sec. Chromosomal bands resolved were numbered from large to small using Roman numbers indicated at the left. Panel C: 3-day run; 3 V.cm⁻¹; switching intervals from 300 to 500 secs. When previously unresolved bands (panel A and B) were resolved, suffixes of letters were added to the roman number. Positions of size markers are indicated at the right (mega base pairs). Within a lane numbers presented next to bands indicate their relative intensity.
Since all group 1 markers analysed were found to be the result of deletions, they might be located on a single unbroken region present on chromosome IX-b of G2 but absent in G6 and hence physically linked. To investigate physical linkage, cloned fragments of AFLP33, -47, -53 and -63, as well as the two cloned RAPD fragments, were probed to blots from rare cutter digests of G2 obtained with \textit{Ascl} and \textit{SfiI}. Restriction fragments ranging in size from 20 to 400 kb were separated by PFGE. RAPD2 was also used as a probe in this analysis, since fusion product B7 was missing one copy of this marker (figure 2), which therefore could \textit{de facto} be considered a RFLP-marker belonging to marker group 1. Hybridisation patterns with Southern blots of rare cutter digest showed that some, but not all, markers of group 1 are physically closely linked. In both digests the AFLP63 fragment hybridised to the same two rare cutter fragments as the RAPD1 fragment. Estimated sizes of these restriction fragments were 78 and 85 kb (\textit{Ascl} digest) and 63 and 80 kb (\textit{SfiI} digest). The AFLP33 fragment hybridised to a 60 kb \textit{Ascl} fragment and a 20 kb \textit{SfiI} fragment, which were of the same size as those present in Southern blots probed with the AFLP53 fragment. The AFLP53 fragment hybridised to more than one rare cutter fragment, but these probably correspond to the two other copies already observed in the Southern analysis of the \textit{EcoRI} digest. The remaining two marker fragments RAPD2 and AFLP47 hybridised to rare cutter fragment of sizes different from those obtained with any of the other cloned marker fragments. Although close physical linkage of RAPD1 and AFLP63 was evident and AFLP33 is possibly closely linked to AFLP53, not all members of marker group 1 were clustered. The minimum size of the region spanned by the markers used in the rare cutter Southern analyses was calculated for each rare cutter enzyme by totalling the sizes of all except the two largest independent hybridising restriction fragments. This yielded values of 90 kb and 120 kb for \textit{Ascl} and \textit{SfiI} respectively, which means that the two distal marker fragments are separated by at least 120 kb.

**DISCUSSION**

Pathogenicity tests of fusion products of complementary antibiotic resistant transformants of isolates G2 (race 1) and G6 (race 2n), both belonging to VCG 0340 of \textit{Fusarium oxysporum} f.sp. \textit{iridacearum}, on the two differential gladiolus cultivars 'Peter Pears' (susceptible to race 1) and 'Nymph' (susceptible to both race 1 and race 2n) did not result in a clear-cut separation of the progeny population in the two parental phenotypes. Many fusants were found to be pathogenic to both cultivars, but caused less severe corm rot symptoms on 'Peter Pears' than G2 parental strains (figure 1). This result can be explained by assuming that pathogenicity of isolate G2 to 'Peters Pears' is not simply determined by the presence or absence of a single gene, but has a more complicated genetic basis. Alternatively, pathogenicity to 'Peter Pears' is determined by the presence or absence of a single gene, but reduced pathogenicity to 'Peter Pears' is the results of defects of pathogenicity genes present in both parents, causing lower aggressiveness of these isolates. A reduction of disease
severity on 'Nymph' similar to that of 'Peter Pears', however, was not observed (figure 1). But, since 'Peter Pears' and 'Nymph' are genetically not closely related (Ohri and Khoshoo, 1983) also their levels of horizontal resistance (Parlevliet and Zadoks, 1977) might differ. Presuming a lower level of horizontal resistance of 'Nymph', reduction in aggressiveness might not have led to reduced symptoms on this cultivar considering the high infection pressure applied in our tests. Since fusion products from the two race 1 parental strains G2h12 and G2p22 did not differ from their parents in their pathogenicity to both cultivar (data not shown), we have no indications that defects in mutual pathogenicity factors might occur during parasexual recombination. However, this observation does not rule out that such defects occur when transformants from different isolates (in casu G2 and G6) are crossed.

Two RAPD markers, selected for their ability to discriminate between race 1 isolates and isolates belonging to other races within VCG 0340, were both absent only in fusion products A18 and B19. The fact that these two belonged to the only three fusion products out of a total of 64, which did not show any corn rot symptoms on 'Peter Pears' suggested a correlation between absence of both markers and lack of pathogenicity to 'Peter Pears'. Both RAPD markers were shown to be linked to two large groups of AFLP markers, all present in G2 but absent in G6 (figure 3). Hybridisation to PFGE blots indicated that both RAPD markers, and hence both marker groups, are situated on a 2.1-Mb chromosome of G2, designated IX-b.

Although these two large marker groups 1 and 2 were absent in two fusion products which did not show any symptoms on corms of 'Peter Pears', their presence was not correlated with (full) pathogenicity to this cultivar. Many of the fusion products with both marker groups present did show pathogenicity to 'Peter Pears' albeit at reduced levels. This indicated that genes elsewhere on the genome are also required for full pathogenicity to 'Peter Pears'. Putative race 1 specific genes linked to marker groups 1 and 2 and located on chromosome IX-b of G2 should therefore be considered essential but not sufficient to express a race 1 phenotype.

The finding that fusion product B7 only differed from the G2 parent in the absence of marker group 1, while its pathogenicity to 'Peter Pears' was strongly reduced (figure 3), supported the idea that this marker group might be linked to genes involved in pathogenicity to 'Peter Pears'.

PFGE analysis revealed a possible role of chromosome X, a 1.6 Mb chromosome present in G2 which but absent in G6, in pathogenicity to 'Peter Pears'. In all fusion products which did have marker groups 1 and 2, but showed reduced pathogenicity to 'Peter Pears', this chromosome X was missing (figures 3 and 4). The fact that absence of chromosome X did apparently not effect the viability of fusion products indicates that this chromosome might be considered a "dispensable" chromosome, harbouring genes involved in pathogenicity. Other plant pathogenic filamentous fungi have been reported to possess minichromosomes, which are non-essential for survival of the organism (Miao et al., 1991; Tzeng et al., 1992; Masel et al., 1993). Such minichromosomes are referred to as "supernumary", "dispensable" or "B" chromosomes. In Nectria haematococca (anamorph: Fusarium solani) mating population VI, genes involved in pathogenicity are located on a 1.6 Mb dispensable chromosome (Van Etten, 1994).
Cochliobolus carbonum (Ahn and Walton, 1996) genes involved in the synthesis of the host specific HC-toxin are located on a large (>0.5 Mb) dispensable portion of a chromosome. Although PFGE analysis strongly suggested that chromosome X is of a dispensable nature, molecular evidence for the presence of dispensable sequences on this chromosome is lacking, since G2-AFLP-markers co-segregating with chromosome X were not found (figure 3). When chromosome X contains mostly repetitive DNA and few (dispensable) genes, a feature thought to be common to B chromosomes (Kistler and Miao, 1992), this could explain the lack of AFLP markers on this chromosome. The finding that no chromosome specific clones could be obtained from chromosome X is in line with this idea. The variation in electrophoretic karyotypes observed within collections of field isolates (Boehm et al., 1994) and after protoplast fusion (figure 4), however, illustrates the flexibility in chromosome architecture of *F. oxysporum*. The absence of the 1.6-Mb chromosomal band in G6 or one of the fusion products does therefore not necessarily imply the absence of homologues of sequences present on this chromosome. They might be (partly) present in one of the larger chromosomes. The complete lack of markers on chromosome X hampers further analysis of this chromosome.

Marker groups 1 and 2 (figure 3) provided a better starting point for further work aiming at cloning genes involved in pathogenicity to 'Peter Pears'. Hybridisation experiments revealed that both marker groups are linked and located on one of the two unresolved 2.1-Mb chromosomes composing chromosomal band IX of G2 (designated chromosome IX-b). Hybridisation experiments indicated the absence of homologous sequences in G6. Assuming that the ratio (15/528) between the number of AFLP markers in group 1 and 2 and the total number of scored AFLP fragments is indicative for the proportion of the genome covered by these markers, they would represent a region of 1.4 Mb, but it can not be ruled out that they span the entire chromosome or that they are clustered on a much smaller region. Segregation of marker group 1 and marker group 2 was only observed in fusion product B7 (figure 3). Lack of hybridisation of most of the cloned marker fragments from group 1 to DNA of fusion product B7 can be explained by the occurrence of a deletion in chromosome IX-b. This might be the result of chromosomal breakage and healing (Zolan, 1995). In that case, the novel 1.7-Mb chromosome present in fusion product B7 (figure 4C) would be the remnant of chromosome IX-b. The cloned RAPD2 fragment, of which one of the two copies is still present in B7 (figure 3), however, did not hybridise to this 1.7-Mb chromosome of B7. This means that the loss of marker group 1 is the result of a more complex translocation event (Zolan, 1995) and the 0.4 Mb difference between the IX-b chromosome of G2 and the novel 1.7-Mb chromosome of B7 is not indicative for the size of the region harbouring the group 1 markers.

Also in other fusion products novel electrophoretic karyotypes were observed (figure 4), indicating that chromosomal rearrangements occur frequently during the enforced fusion process. The finding of many *de novo* AFLP markers in most of the fusion products further illustrated the frequent occurrence of genomic rearrangements (e.g. translocations or transpositions).
The fact that (besides the presence of the phleomycin resistance selection marker) absence of marker group 1 was the only detected difference in marker profile between fusion product B7 and its G2h12 parent, while pathogenicity to 'Peter Pears' of this fusion product was strongly reduced (figure 1), supported the hypothesis that markers of group 1 are probably linked to (a) gene(s) involved in pathogenicity to 'Peter Pears'. Southern analysis of rare cutter digests of G2, using cloned marker fragments of group 1 as probes showed that, although some of the markers are physically closely linked, the total region spanned by this marker group is at least 120 kb in size. Nevertheless cloning of the gene(s) of interest might be feasible. When the cloned marker fragments from group 1 are used to screen a cosmid bank of isolate G2, selected cosmid clones containing a substantial part of the marked region can be obtained. These cosmids clones can subsequently be analysed for the presence of transcribed sequences by hybridisation with cDNA from infected tissue. Sequence analysis, followed by data bank screening, might enable the identification of genes putatively involved in pathogenicity to 'Peter Pears'.

The data presented in this chapter led to the working hypothesis that chromosome IX-b of G2 contains dispensable sequences harbouring (a) gene(s) with an essential role in the pathogenicity of race 1 of *F. oxysporum* f.sp. *iridacearum* to 'Peter Pears'. It should however be emphasised that the presented data only provide circumstantial evidence. The working hypothesis assumes that virulence and not avirulence is the dominant factor determining the difference in pathogenicity between G2 (race 1) and G6 (race 2n). Although we found no evidence for the existence of determinants of avirulence, it can not be ruled out that they exist. Putative virulence gene(s) located on chromosome IX-b are not the only determinants of pathogenicity to 'Peter Pears'. Genes present on chromosome X are probably also involved. Furthermore, the large group of fusion products with intermediate levels of pathogenicity to 'Peter Pears' showed significant variation in pathogenicity levels which can not be explained by absence or presence of marker group 1 and 2 and chromosome X.

Due to the frequent occurrence of complicating genomic rearrangements, analysis of enforced parasexual recombinants has shown to be not an effective experimental tool to unravel the unexpectedly complex genetics underlying the difference in pathogenicity to 'Peter Pears' between isolate G2 (race 1) and G6 (race 2). Nevertheless, a dispensable region on chromosome IX-b of isolate G2 was identified which contained (a) gene(s) putatively involved in pathogenicity of this race 1 isolate to 'Peter Pears'. Cloning and functional analysis of these genes will be necessary to prove that they are indeed required for full pathogenicity of isolate G2 to 'Peter Pears'.

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