Transfer of a Supernumerary Chromosome Between Vegetatively Incompatible Biotypes of the Fungus *Colletotrichum gloeosporioides*

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ABSTRACT

Two biotypes (A and B) of *Colletotrichum gloeosporioides* infect the tropical legumes *Stylosanthes* spp. in Australia. These biotypes are asexual and vegetatively incompatible. However, field isolates of biotype B carrying a supernumerary 2-Mb chromosome, thought to originate from biotype A, have been reported previously. We tested the hypothesis that the 2-Mb chromosome could be transferred from biotype A to biotype B under laboratory conditions. Selectable marker genes conferring resistance to hygromycin and phleomycin were introduced into isolates of biotypes A and B, respectively. A transformant of biotype A, with the hygromycin resistance gene integrated on the 2-Mb chromosome, was cocultivated with phleomycin-resistant transformants of biotype B. Double antibiotic-resistant colonies were obtained from conidia of these mixed cultures at a frequency of approximately 10^{-7}. Molecular analysis using RFLPs, RAPDs, and electrophoretic karyotypes showed that these colonies contained the 2-Mb chromosome in a biotype B genetic background. In contrast, no double antibiotic colonies developed from conidia obtained from mixed cultures of phleomycin-resistant transformants of biotype B with biotype A transformants carrying the hygromycin resistance gene integrated in chromosomes >2 Mb in size. The results demonstrated that the 2-Mb chromosome was selectively transferred from biotype A to biotype B. The horizontal transfer of specific chromosomes across vegetative incompatibility barriers may explain the origin of supernumerary chromosomes in fungi.

MANY fungal species have been shown to contain variable electrophoretic karyotypes (Kistler and Miao 1992). Variable karyotypes have been found in both sexual (Zolan *et al.* 1994; Plummer and Howlett 1995) and asexual fungi (Kistler and Miao 1992) and this variation has even been demonstrated among supposedly clonal descendants (Masel *et al.* 1990; Talbot *et al.* 1993). Several mechanisms have been proposed to explain the generation of karyotype variation in fungi (Zolan 1995). These include partial or total chromosome deletion, reciprocal and nonreciprocal recombination between homologous chromosomes, and ectopic recombination between nonhomologous chromosomes. Karyotype variation may be of particular importance in the evolution of asexual fungi, which are potentially limited in their capacity to generate genotypic variation. For example, in *Candida albicans*, karyotype variation generated in culture has been associated with the acquisition of new physiological functions (Rustchenko *et al.* 1994).

Supernumerary chromosomes (Covert 1998) that are apparently dispensable for growth in standard culture media have been identified in several fungi (Tzeng *et al.* 1992; Masel *et al.* 1993, 1996; Covert *et al.* 1996). Supernumerary chromosomes have been identified by a lack of molecular hybridization to any chromosome in otherwise genetically similar genotypes within a fungal species (Masel *et al.* 1993; Covert 1998). The origin of supernumerary chromosomes in fungi is unknown, but might be explained by chromosome addition from a genetically distinct source (Masel *et al.* 1993). Evidence for chromosome addition may include (1) the identification of the donor of a supernumerary chromosome and (2) the experimental demonstration of the transfer of this chromosome into the recipient. So far, these two lines of experimental evidence for chromosome addition have not been obtained for any supernumerary chromosome in fungi.

Two biotypes (A and B) of *Colletotrichum gloeosporioides* causing anthracnose disease on the tropical forage legumes, Stylosanthes, have been recognized in Australia (Irwin and Cameron 1978; Manners *et al.* 1992). A fungal biotype has been defined as a subgroup within a species having a like genetic makeup (Maclean *et al.* 1993). Biotypes A and B have been shown to be genetically distinct (Manners *et al.* 1992; He *et al.* 1996), while isolates within each biotype appeared nearly monomorphic (Braithwaite *et al.* 1990; Manners *et al.* 1993; He *et al.* 1996). Sexuality has not been observed in the two biotypes (Ogle *et al.* 1986). In addition, field isolates of biotypes A and B have been shown to be vegetatively incompatible (Masel *et al.* 1996). All of these observations are consistent with the hypothesis that biotypes A

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and B are genetically isolated, clonal lines of *C. gloeosporioides* (Manners *et al.* 1992).

Considerable variation in the size and number of chromosomes in isolates of the two biotypes of *C. gloeosporioides* has been demonstrated (Masel *et al.* 1990, 1993). More recently, a supernumerary 2-Mb chromosome present in a biotype B isolate, termed Bx, was described (Masel *et al.* 1996). This chromosome either was absent from other biotype B isolates or was represented by a homologous supernumerary 1.2-Mb chromosome (Masel *et al.* 1996). The 1.2- and 2-Mb chromosomes appeared to be relatively recent additions to the biotype B genome because they lacked at least two dispersed repeat sequences that were both monomorphic in the biotype B population and present on all other resolved chromosomes (Masel *et al.* 1993, 1996). Using chromosome-specific markers, the 2-Mb chromosome in Bx was shown to be similar, if not identical to a 2-Mb chromosome present in biotype A isolates. No biotype A-like markers from other chromosomes were found in the Bx isolate, and the investigators (Masel *et al.* 1996) suggested that a form of "horizontal" transfer of the 2-Mb chromosome had recently occurred from biotype A to biotype B (Masel *et al.* 1996). In this article, we report on experiments designed to test the hypothesis that the 2-Mb chromosome can transfer from biotype A to biotype B during vegetative cocultivation under laboratory conditions.

MATERIALS AND METHODS

Fungal isolates: Isolate SR24 (termed A) of biotype A and isolates UQ62 (termed B3) and WRS36 (termed B4) of biotype B of *C. gloeosporioides* were used in these experiments. Isolate UQ396 (termed Bx; Masel *et al.* 1996) was included as a control in the molecular analysis. B3 and Bx contain the 1.2-Mb supernumerary chromosome, A and Bx contain a homologous 2-Mb chromosome, and B4 contains neither the 1.2-Mb nor the 2-Mb chromosome or any other homologue of these chromosomes. The isolates UQ38 and 22395 of *C. gloeosporioides*, which infect the tropical legumes *Centrosema pubescens* and *Aeschynomene falcata*, respectively, were also used in control experiments. All isolates have been described previously (Braithwaite *et al.* 1990; He *et al.* 1995, 1996; Masel *et al.* 1996). Recipes for oatmeal, V8, and potato-dextrose culture media were as described in Johnston and Booth (1983). All the fungal isolates, transformants, and recombinants described in this article have been deposited in the Colletotrichum culture collection at the University of Arkansas. Pathogenicity tests were conducted as described by He *et al.* (1995).

Fungal transformation: The plasmids pRCos1 (Crowhurst *et al.* 1992) and pAN8.1 (Farman and Oliver 1992) were used for transformation. The vector pRCos1 contains a hygromycin phosphotransferase (*hph*) gene that confers resistance to hygromycin B while pAN8.1 contains a phleomycin resistance gene (*phleo*). The plasmids pRCos1 and pAN8.1 were prepared from the cultures of *Escherichia coli* strain DH5α using a plasmid purification kit (QIAGEN Inc., Valencia, CA). The method for transformation of *C. gloeosporioides* was as described by Poplawski *et al.* (1997). All transformants were cultured from single spores to ensure genetic uniformity before further experimentation. Single spore isolations were conducted by plating diluted spore suspensions on water agar medium and incubating overnight at room temperature. Single germlings were then picked out with a dissection needle and transferred to fresh media.

DNA and chromosome manipulations: The methods for contour-clamped homogeneous electric field (CHEF) electrophoresis, the extraction of DNA, and Southern hybridization analysis were as described in Masel *et al.* (1990, 1993, 1996). PCR was used to assay for the presence of the *hph* and *phleo* genes in putative transformants using genomic DNA as a template for the PCR. A 664-bp fragment of the *hph* gene was amplified using the primers 5′-CTCGTGCTTTCAGCTTC GATGTAG-3′ and 5′-ATGAAGCTCGCCTCCGTAGGCCTC-3′. A 366-bp fragment was amplified from the *phleo* gene using the primers 5′-ATGGCCAAGTTGACCACTGCCGTT-3′ and 5′-ACGCACGTGAAGCACCGGCTCCTC-3′. Randomly amplified polymorphic DNA (RAPD) analysis was undertaken using the methods of Kazan *et al.* (1993) and employing commercial primers (Operon Technologies Inc., Alameda, CA).

Cocultivation of transformants of biotypes A and B: Spores of biotype A transformants with hygromycin resistance were mixed with an equal number of spores of biotype B transformants with phleomycin resistance and were spread on oatmeal agar plates (2×10^6/plate). After incubation for 5 days at room temperature, spores of the mixture were harvested from acervuli using an inoculation loop. The spores were suspended in sterile water and transferred onto plates of double antibiotic media (40 μg/ml hygromycin and 20 μg/ml phleomycin incorporated into V8 agar media) and incubated at room temperature. Each plate was inoculated with approximately 5×10^8 spores. Colonies growing on media containing both antibiotics were counted and some were removed and reisolated from single spores.

Staining of nuclei: Conidia produced on oatmeal agar plates were suspended in half-strength potato dextrose broth at a concentration of approximately 10^6 per ml and drops of the suspension were placed on glass slides for 4 hr at room temperature. The medium was washed from the slides with water and the spores that had attached to the slides were then washed for 30 min in 85% methanol and 15% acetic acid. The slides were dried, washed once in 95% ethanol and once in 70% ethanol, and then treated with 1 m HCl for 5 min at room temperature and subsequently treated with 1 m HCl at 60° for 10 min. The slides were washed in water, treated with Giemsa stain (0.07% Giemsa powder in 50 mm Na phosphate buffer, pH 6.9, 3% glycerol, 3% methanol) for 3 hr, and again rinsed in water. The stained nuclei in conidia (at least 10^3 per sample) were viewed by light microscopy.

RESULTS

The experimental strategy adopted first involved the identification of a transformant of biotype A with the *hph* gene located on the 2-Mb chromosome. This transformant, and other transformants with the *hph* gene on chromosomes larger than 2 Mb, were then cocultivated with a phleomycin-resistant transformant of biotype B. Subsequently, conidia from these mixed cultures were tested for resistance to both hygromycin and phleomycin. Finally, the genomes of double antibiotic-resistant progeny were characterized using a range of molecular markers.

Tagging the 2-Mb chromosome with an antibiotic resistance gene: The biotype A isolate was transformed with pRCos1 and 50 hygromycin-resistant transformants

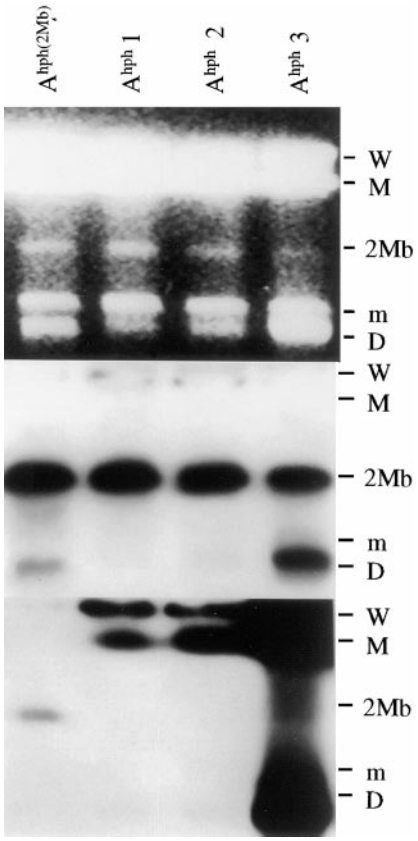

Figure 1.—Identification of a transformant of biotype A of *C. gloeosporioides* with the *hph* gene integrated into the 2-Mb chromosome. Protoplasts of biotype A were transformed with the vector pRCos1 and the independent transformants A^{hph} 1–3 and $A^{hph(2Mb)}$ were analyzed by CHEF gel electrophoresis using conditions described by Masel *et al.* (1996) and stained with ethidium bromide (top). This resolved the 2-Mb chromosome (2 Mb) while other chromosomes were either retained in the loading well (W) or compressed in a high molecular weight band (M) >3 Mb in size or in a low molecular weight band <1 Mb (m). Degraded DNA (D) was also present at the electrophoretic front. A Southern blot of the CHEF gel was hybridized to the cosmid Cos60 that is specific to the 2 Mb chromosome (middle) and to the pRCos1 vector (bottom).

were subsequently analyzed using CHEF gel analysis, Southern blotting, and hybridization to a pRCos1 probe (Figure 1). Most transformants carried the vector sequences only in the large and poorly resolved chromosomes, >3 Mb in size (Figure 1). All transformants of biotype A contained the 2-Mb chromosome (Figure 1) but only one transformant carrying *hph* on this chromosome was identified (Figure 1). No vector sequences were detected on any other chromosome in this transformant. The *hph* gene was stably maintained for at least 10 subcultures, each from a single conidium, and this transformant was termed $A^{hph(2Mb)}$. Nine transformants that carried vector sequences on larger chromosomes were arbitrarily chosen and these were termed A^{hph}1–9. Single transformants of B3 and B4 that carried pAN8.1 integrated into chromosomes >5 Mb were also selected and were termed $B3^{phleo}$ and $B4^{phleo}$, respectively. The conidia of the transformants of biotypes A and B were inspected for their nuclear content by Giemsa staining and all conidia viewed were mononuclear.

Isolation of double antibiotic-resistant recombinants: Spores of $A^{hph(2Mb)}$ and spores of either $B3^{phleo}$ or $B4^{phleo}$ were cocultivated on media without antibiotics for five days. Microscopic inspection of these plates did not reveal the presence of perithecia or any other evidence of sexual structures. Conidia from acervuli produced on these mixed cultures were transferred to growth medium containing both hygromycin and phleomycin. The growth of colonies was observed after 5 days and the frequency of recovery of double antibiotic-resistant recombinants determined on three separate occasions with a total of 13 experimental plates. The number of colonies that grew on the double antibiotic media ranged from 53 to 125 per plate. The total number of double antibiotic-resistant colonies isolated from 6.5×10^9 conidia tested in the 13 plates was 1001. The mean numbers of colonies recovered from 5×10^8 conidia per plate were 71 and 94 when $B3^{phleo}$ and $B4^{phleo}$, respectively, were used as parents in the cocultivations. Therefore approximately 10^{-7} conidia were hph^R and $phleo^R$. Ten double antibiotic-resistant colonies, five from the mixed cultures of $A^{hph(2Mb)}$ with $B3^{phleo}$ and five from the mixed cultures of $A^{hph(2Mb)}$ with $B4^{phleo}$, were subcultured, purified from a single conidium, and used for further analysis. Giemsa staining and light microscopy of approximately 10^3 conidia again revealed that each conidium contained only one single nucleus. These results indicated that genetic exchange between $A^{hph(2Mb)}$ and $B3^{phleo}$ and $B4^{phleo}$ probably had occurred.

The transformants A^{hph}1–9 were cocultivated with each of $B3^{phleo}$ and $B4^{phleo}$ to determine whether genetic exchange between biotypes occurred when the *hph* gene was located on chromosomes other than the 2-Mb chromosome in the biotype A parent. Three independent cocultivation experiments were conducted on three occasions, each using fresh parent plates. On each occasion, at least three plates each containing 5×10^8 conidia from the mixed cultures were tested for the production of colonies resistant to the double antibiotics. In total, 1.3×10^{10} conidia were tested in experiments using A^{hph}1–9 as parents. However, all these experiments failed to produce any colonies that grew on media with both antibiotics. These observations were consistent with the results of Poplawski *et al.* (1997), who also failed to obtain recombinants with five randomly selected transformants of biotype A as a parent in similar cocultivation experiments with a phleomycin-resistant biotype B parent.

A control experiment was conducted to test whether transformation interfered with the ability of biotype A to recombine with any other biotype of *C. gloeosporioides.* The transformants A^{hph} 1 and A^{hph} 3 were each cocultivated with phleomycin-resistant transformants of iso-

lates UQ38 and 22395. These two fungal isolates infect the host species *C. pubescens* and *A. falcata*, respectively, and appear to be vegetatively compatible with biotype A. In these experiments, 50–200 double antibiotic-resistant colonies were obtained from each cocultivation after $\sim 5 \times 10^8$ conidia were plated. This experiment indicated that transformation of biotype A to hygromycin resistance did not impair vegetative recombination with all *C. gloeosporioides*.

Putative recombinants contain both selectable marker genes: Two putative recombinants, termed $B3^{2Mb}$ and $B4^{2Mb}$, were selected arbitrarily from the $A^{hph(2Mb)}+B3^{phleo}$ and $A^{hph(2Mb)}+B4^{phleo}$ pairings, respectively. PCR analysis was undertaken using primers specific for the *hph* and *phleo* genes to test whether these putative recombinants contained both selectable marker genes. Bands of approximately 664 bp and 336 bp in size were amplified from the genomic DNA of $B3^{2Mb}$ and $B4^{2Mb}$ using primer pairs specific for the *hph* and *phleo* genes (data not shown). This demonstrated that $B3^{2Mb}$ and $B4^{2Mb}$ were true recombinants.

The recombinants carry a 2-Mb chromosome derived from biotype A: To determine whether the intact 2-Mb chromosome from the biotype A parent $A^{hph(2Mb)}$ was present in the $B3^{2Mb}$ and $B4^{2Mb}$ recombinants, CHEF gel electrophoresis and Southern hybridization with DNA probes specific to the 2-Mb chromosome were undertaken. The results (Figure 2) indicated that both $B3^{2Mb}$ and $B4^{2Mb}$ contained a 2-Mb chromosome that was absent in the $B3^{phleo}$ and $B4^{phleo}$ parental genotypes.

Restriction fragment length polymorphism (RFLP) analysis was undertaken, using DNA probes specific to the 1.2- and 2-Mb chromosomes, to test whether the 2-Mb chromosomes in the recombinants $B3^{2Mb}$ and $B4^{2Mb}$ were identical to those of the parent $A^{hph(2Mb)}$ (Masel *et al.* 1993, 1996). The RFLP patterns obtained for $B4^{2Mb}$ were identical to those of $A^{hph(2Mb)}$, while RFLP patterns of $B3^{2Mb}$ comprised a combination of the bands observed in the analysis of $B3^{phleo}$ and $A^{hph(2Mb)}$ (Figure 3). The RFLP banding patterns revealed by the chromosome-specific DNA probes with DNA of $B3^{2Mb}$ were identical to those obtained using the field isolate Bx that contains both 1.2- and 2-Mb chromosomes (Figure 3). All of the 10 chromosome-specific RFLP probes of Masel *et al.* (1993) and one chromosome-specific cosmid clone (He *et al.* 1995) gave similar results. These results suggest that the intact 2-Mb chromosome of biotype A has been incorporated into the biotype B genome.

The background genome of the recombinants $B3^{2Mb}$ and $B4^{2Mb}$ is like that of biotype B: Previous research has demonstrated that the differences in the karyotypes of biotypes A and B were particularly well resolved for chromosomes in the 200–600-kb size range (Masel *et al.* 1990). Examination of the chromosomes in this size range in the parents and recombinants failed to detect chromosomes specific to $A^{hph(2Mb)}$ in the recombinants $B3^{2Mb}$ and $B4^{2Mb}$ (Figure 4). In contrast, the recombinants were identical in their chromosome complement to their respective biotype B parents $B3^{phleo}$ and $B4^{phleo}$ (Figure 4).

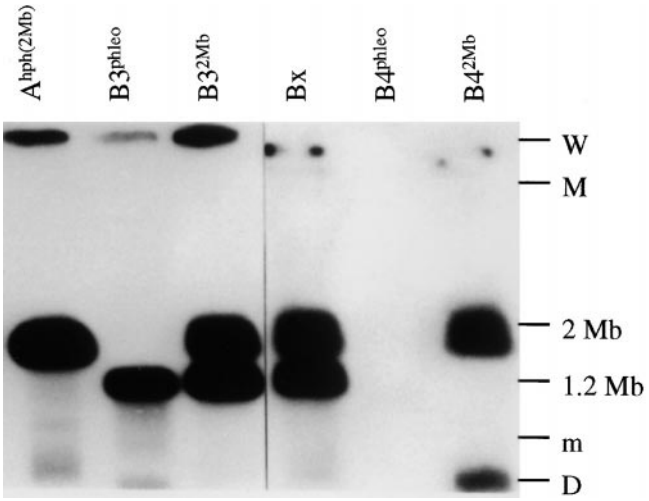

Figure 2.—Recombinants $B3^{2Mb}$ and $B4^{2Mb}$ contain a 2-Mb chromosome homologous to the 2-Mb chromosome of biotype A. Chromosomes of the parent biotype A transformant ($A^{hph(2Mb)}$), parent biotype B transformants ($B3^{phleo}$ and $B4^{phleo}$), and recombinant progeny ($B3^{2Mb}$ and $B4^{2Mb}$) were separated by CHEF gel electrophoresis using conditions of Masel *et al.* (1996) to resolve the 1.2- and 2-Mb chromosomes. The partial recombinant field isolate Bx (Masel *et al.* 1996) that contains both 1.2- and 2-Mb chromosomes was included as control. A Southern blot of the CHEF gel was hybridized to a DNA probe specific to the 1.2- and 2-Mb homologous chromosomes. W refers to well, M to unresolved large chromosomes, m to unresolved small chromosomes (200–600 kb), and D to degraded DNA. The positions of the 1.2- and 2-Mb chromosomes are also marked.

RFLP analysis was undertaken to test whether chromosomes of $A^{hph(2Mb)}$ other than those of the 2-Mb chromosome were present in the recombinants $B3^{2Mb}$ and $B4^{2Mb}$. The RFLP analysis utilized a ribosomal DNA probe and 10 arbitrary low-copy cDNA probes located on chromosomes >3 Mb in size (Masel *et al.* 1996). Previous results have shown that the rDNA of biotypes A and B differs in an *Xba*I-generated RFLP (Braithwaite *et al.* 1990). When the rDNA probe was used, the banding patterns of $B3^{2Mb}$ and $B4^{2Mb}$ were identical to those of the biotype B parental isolates $B3^{phleo}$ and $B4^{phleo}$, and distinct from those of the biotype A parent $A^{hph(2Mb)}$ (Figure 5). When the 10 cDNAs were used as probes, $B3^{2Mb}$ and $B4^{2Mb}$ had hybridization patterns identical to the biotype B parents (*e.g.*, Figure 5) and were distinct from the biotype A parent. These results indicated that $B3^{2Mb}$ and $B4^{2Mb}$ were not heterokaryons or hybrids but partial recombinants comprising mainly a biotype B-like genome. Eight other monoconidial putative recombinant progenies, four from pairings of $A^{hph(2Mb)}+B3^{phleo}$ and four from pairings of $A^{hph(2Mb)}+B4^{phleo}$, were also analyzed for RFLPs using five of the arbitrary cDNA clones used for RFLP analysis. The banding patterns of

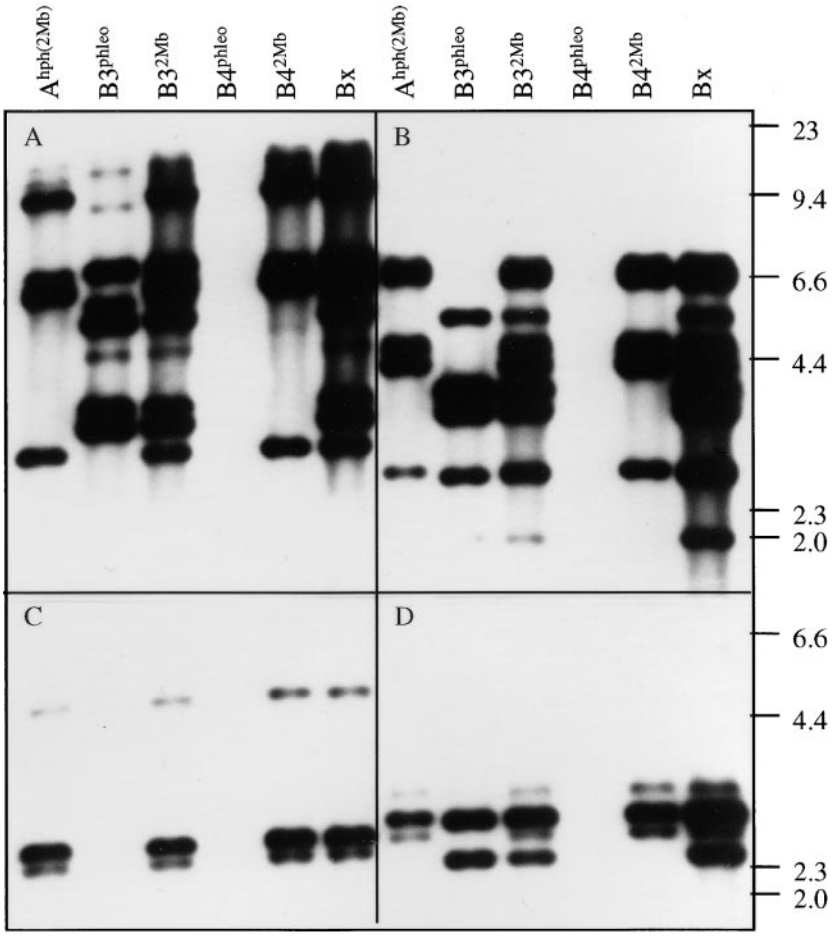

Figure 3.—RFLP analysis of *Eco*RI (A and C) and *Hin*dIII (B and D) restriction digests of total DNA from the recombinants $B3^{2Mb}$, $B4^{2Mb}$, and their parents, $B3^{phleo}$, $B4^{phleo}$, and $A^{hph(2Mb)}$. The field isolate Bx was included as a control. Hybridization probes 8 (A and B) and 4 (C and D) specific to the 1.2- and 2-Mb supernumerary chromosomes (Masel *et al.*1993) were used. The sizes of *Hin*dIII cut λ DNA markers are indicated in kilobases.

these eight recombinants were also identical to their respective biotype B parents $B3^{phleo}$ and $B4^{phleo}$ and were distinct from those of $A^{hph(2Mb)}$. These results suggested that most of the progeny from these pairings contained a biotype B genome with the 2-Mb chromosome of biotype A.

The genomes of $B3^{2Mb}$, $B4^{2Mb}$, and their respective parental isolates were also analyzed by RAPD markers to scan the genomes more widely for the possible presence of biotype A markers in the partial recombinants (*e.g.*, Figure 6). A total of eight primers revealed 56 bands that distinguished the biotype A parent $A^{hph(2Mb)}$ and the biotype B parents $B3^{phleo}$ and $B4^{phleo}$. The primers were selected on an arbitrary basis. $B3^{phleo}$ and $B4^{phleo}$

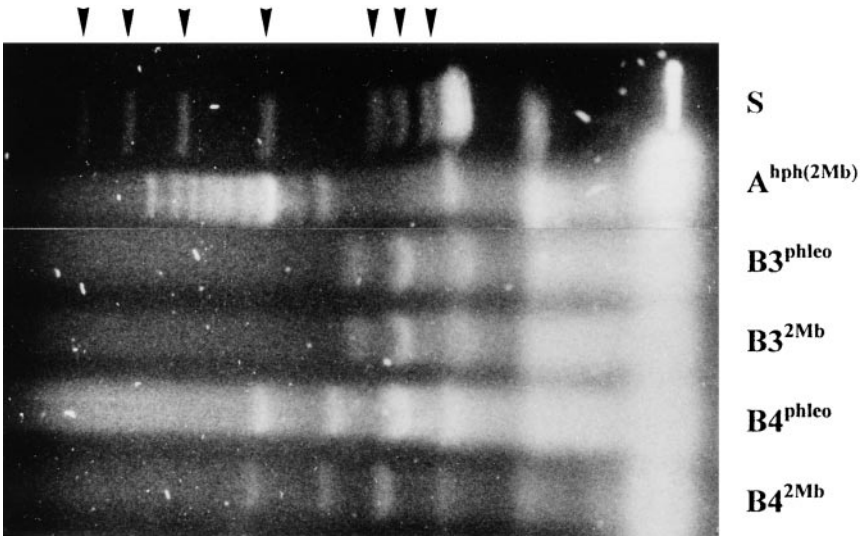

Figure 4.—Electrophoretic karyotypes of the recombinants $B3^{2Mb}$, $B4^{2Mb}$, and their vegetative parents, $B3^{phleo}$, $B4^{phleo}$, and $A^{hph(2Mb)}$. Chromosomes in the size range 200–600 kb were resolved by CHEF electrophoresis using the condition 1 of Masel *et al.* (1990). Lane S contains chromosomes of *Saccharomyces cerevisiae* as size markers. The sizes of the resolved markers (arrowed) were 225, 295, 375, 450, 555, 610, and 680 kb from bottom to top. Chromosomes >700 kb were not resolved under these running conditions.

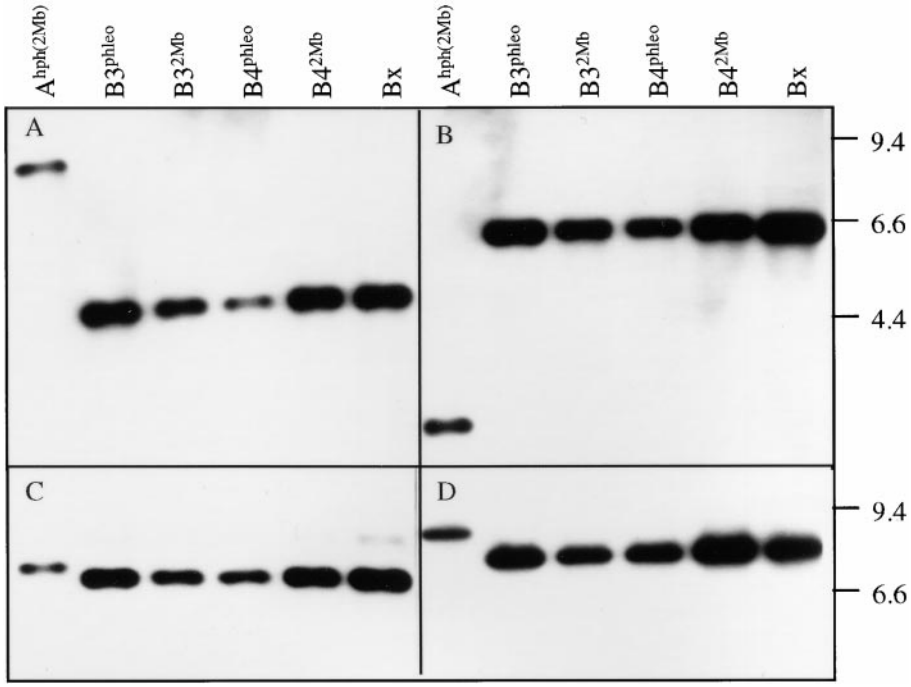

Figure 5.—RFLP analysis of (A) *Eco*RI, (B and C) *Hin*dIII and (D) *Xba*I restriction digests of total DNA from the recombinants $B3^{2Mb}$, $B4^{2Mb}$, and their vegetative parents, $B3^{phleo}$, $B4^{phleo}$, and $A^{hph(2Mb)}$. The field isolate Bx was included as another biotype B background genotype (Masel *et al.* 1996). The hybridization probes used included low-copy cDNAs (A, B, and C) from *C. gloeosporioides* (Masel *et al.* 1996) and a rDNA clone (D) from *A. nidulans* (Lockington *et al.* 1982). The probes detect sequences on the large unresolved chromosomes of *C. gloeosporioides* (position M in Figure 1). The sizes of *Hin*dIII cut λ DNA markers in kilobases are indicated.

were monomorphic in this analysis (Figure 6). A total of 21 RAPD bands amplified from DNA of $A^{hph(2Mb)}$ were absent from the PCR products generated from $B3^{phleo}$ and $B4^{phleo}$. None of the 21 biotype A-specific RAPD bands were detected in the PCR products generated from $B3^{2Mb}$ and $B4^{2Mb}$. In contrast, all of the 35 bands generated from the biotype B parents and absent from the biotype A parent were detected in $B3^{2Mb}$ and $B4^{2Mb}$.

Stability of 2-Mb chromosome in recombinants: To test whether the 2-Mb chromosome was mitotically stable in the absence of antibiotic selection in the recombinants, the 10 independent double antibiotic-resistant putative recombinants (5 from the $A^{hph(2Mb)}+B3^{phleo}$ pairings and 5 from the $A^{hph(2Mb)}+B4^{phleo}$ pairings) were grown from a single conidium on oatmeal agar without antibiotic for 10 subcultures. Following the 10 subcultures, dilutions of ~100 conidia per plate for each of the recombinant progeny tested were incubated on medium containing both phleomycin (20 μg/ml) and hygromycin (40 μg/ml) and the germination frequency compared microscopically to similar samples on medium lacking antibiotics. All of the plated conidia germinated on both media. These results suggested that the 2-Mb chromosome was not highly mitotically unstable in the absence of antibiotic selection.

Pathogenicity of recombinants: Biotype A isolates are virulent on *Stylosanthes scabra* and cause limited lesions while isolates of biotype B are specifically virulent on *S. guianensis* and produce a blight-like symptom (Irwin and Cameron 1978). Pathogenicity tests were carried

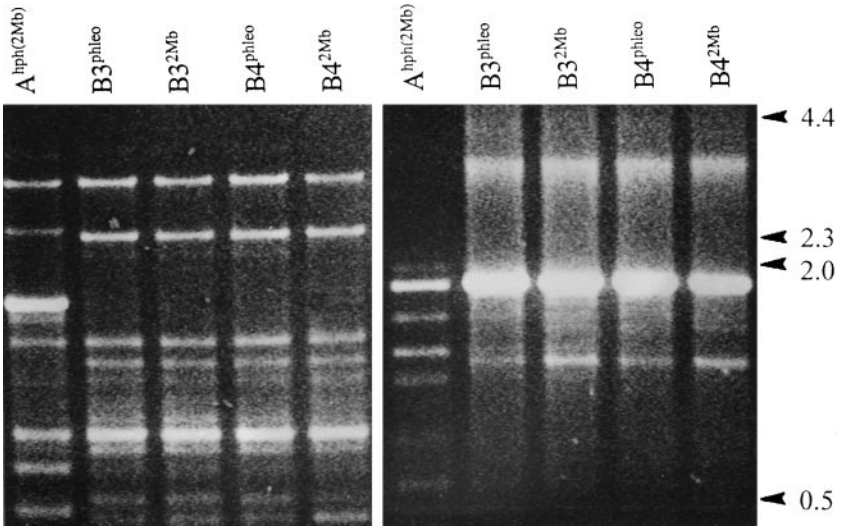

Figure 6.—Examples of the RAPD analysis of the recombinants $B3^{2Mb}$, $B4^{2Mb}$, and their parents, $B3^{phleo}$, $B4^{phleo}$, and $A^{hph(2Mb)}$. Banding patterns obtained with the primers AB4 (left) and AB8 (right) of Operon Technologies Inc. (Alameda, CA) are shown. The molecular weight markers are in kilobases.

out to assess the host specificity of the two recombinants $B3^{2Mb}$ and $B4^{2Mb}$. The parent $A^{hph(2Mb)}$ was pathogenic on *S. scabra* cv. Seca. The parent $B3^{phleo}$ and the recombinant $B3^{2Mb}$ were pathogenic on *S. guianensis* cv. Graham. The parent $B4^{phleo}$ and the recombinant $B4^{2Mb}$ were pathogenic on *S. guianensis* cv. Cook. No symptoms were observed on *S. scabra* cv. Seca inoculated with $B3^{2Mb}$, $B4^{2Mb}$, $B3^{phleo}$, and $B4^{phleo}$. It would appear from this analysis that the genes from biotype A that condition pathogenicity on *S. scabra* were not all present in the recombinants $B3^{2Mb}$ and $B4^{2Mb}$.

DISCUSSION

The results presented herein demonstrate limited genetic recombination between biotypes A and B of *C. gloeosporioides* that infect the legumes Stylosanthes in Australia. The genetic recombination detected between the biotypes in these experiments was restricted to the transfer of a 2-Mb chromosome of biotype A to biotype B during vegetative growth. This chromosome transfer occurred without any obvious sexual interaction. The results indicate that the transfer of the 2-Mb chromosome from biotype A to biotype B is very selective. For example, double antibiotic-resistant recombinants (*e.g.*, $B3^{2Mb}$ and $B4^{2Mb}$) were readily obtained when the biotype A parent with the *hph* selectable marker gene located on the 2-Mb chromosome ($A^{hph(2Mb)}$) was used in interbiotype cocultivation experiments, but no recombinants were isolated when biotype A parents with the *hph* gene located on other chromosomes (A^{hph} 1–9) were tested. Therefore, the results indicate that a horizontal transfer of the 2-Mb chromosome occurred via a nonsexual pathway between otherwise genetically incompatible genotypes of this fungal plant pathogen. Supernumerary chromosomes are common in fungi (Covert 1998) and the horizontal transfer of chromosomes across incompatibility barriers provides a possible explanation for their origin.

The mechanism of transfer of the 2-Mb chromosome between the biotypes of *C. gloeosporioides* was not determined. Vegetative incompatibility between fungi has been reported to result in either the lysis or the retardation of growth of heterokaryotic cells (Begueret *et al.* 1994; Saupe *et al.* 1996). The 2-Mb chromosome may be transferred by either of two processes during a transient or slow-growing stage of heterokaryosis. First, nuclear fusion may occur, followed by loss of almost all of the biotype A genome, leaving the 2-Mb chromosome in a predominantly biotype B genome. This mechanism would conform to a parasexual cycle with an extreme nonrandom assortment of chromosomes following karyogamy. There is evidence for a nonrandom assortment of chromosomes in the generation of aneuploid and addition lines in *Aspergillus nidulans* via the parasexual cycle (Bos 1996). Alternatively, a mechanism to transfer the 2-Mb chromosome between nuclei may exist in an unstable interbiotype heterokaryon. The ability to directly introduce transformation vectors and yeast artificial chromosome vectors into fungi indicates that DNA molecules can traverse the nuclear envelope in fungal cells. One might expect that such a mechanism would favor small chromosomes and genetic elements. We have previously demonstrated the transfer of a small (10 kb) autonomously replicating vector between biotypes at similar frequency to that observed here for the 2-Mb chromosome in vegetative pairing experiments (Poplawski *et al.* 1997). In the present study, the 2-Mb chromosome was the smallest chromosome tagged with the *hph* gene, so comparisons of the efficiency of transfer of the 2-Mb chromosome with smaller chromosomes were not made.

Vegetative incompatibility in fungi is conditioned by the interaction of alleles at *het* loci in the interacting genotypes (Glass and Kuldau 1992; Leslie 1993). Although fungi are believed to carry multiple *het* loci, it is possible that some chromosomes may lack these loci. These compatible chromosomes may transfer between nuclei in transient heterokaryons. Cells containing nuclei with compatible foreign chromosomes may in turn reproduce and escape the incompatibility processes programmed by complementary *het* alleles that normally would prevent the persistence of complete hybrids or heterokaryons. This process potentially provides a powerful mechanism of gene transfer between fungal genotypes in nature and would be comparable to plasmid transfer between bacterial species via conjugation.

The demonstration of selective transfer of the 2-Mb chromosome from biotype A into a predominantly biotype B genetic background is consistent with previous observations of partial recombinants isolated from infected plants of *S. guianensis* in the field. Isolates of biotype B, termed Bx, were obtained in northern Australia in 1992 and the genome of these isolates resembled that of $B3^{2Mb}$ described in this article (Figures 2, 3, and 5). At present, the functions encoded on the 2-Mb chromosome are unknown. In the work described herein, antibiotic resistance genes located on the chromosome were used to select recombinants in the laboratory. It is not certain whether the 2-Mb chromosome derived from biotype A confers any selective advantage to the Bx genotype in the field (Masel *et al.* 1996). The plant inoculation tests on the recombinants indicated that genes determining pathogenicity on *S. scabra*, one host of biotype A grown widely in northern Australia, were not carried on the 2-Mb chromosome. Future studies of genes expressed on the 2-Mb chromosome may help identify its function.

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