Polymorphic Chromosomes Bearing the *Tox2* Locus in *Cochliobolus carbonum* Behave as Homologs during Meiosis†

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The *HTS1* gene in the *Tox2* locus of the fungal pathogen *Cochliobolus carbonum* race 1 is required for synthesis of a host-selective phytotoxin and for increased virulence on susceptible genotypes of maize. The locus is present in race 1 isolates but absent from isolates of the other races, which do not produce the toxin. By pulsed-field gel electrophoresis and Southern analysis with *HTS1* sequences and chromosome-specific markers, the *HTS1* gene was detected on a 4-Mb chromosome in one group of isolates and on a 2.3-Mb chromosome in another group, which lacked the 4-Mb chromosome. A chromosome-specific marker from *C. heterostrophus* hybridized to a 2.3-Mb chromosome in non-toxin-producing isolates and in toxin-producing isolates, including those with a 4-Mb chromosome. A marker from *C. carbonum* hybridized to the 4-Mb chromosome, but in isolates lacking the 4-Mb chromosome, this marker hybridized to a smaller, 2.0-Mb chromosome. Thus, the *Tox2* locus is on different chromosomes in different groups of race 1 isolates. Single ascospore progeny from crosses between isolates having *HTS1* on different chromosomes were analyzed for toxin-producing ability, virulence, and the presence and chromosomal location of *HTS1*. All progeny produced HC toxin in culture, incited race 1-type lesions on susceptible maize genotypes, and contained *HTS1* sequences, as determined by PCR amplification with gene-specific primers. Analysis of the chromosomal complements of several progeny indicated that they all had only one *Tox2*-containing chromosome. Thus, despite their differences in size, these chromosomes behave as homologs during meiosis and may have arisen by a translocation.

Cochliobolus carbonum Nelson, a foliar pathogen of maize causing Helminthosporium leaf spot, is found in nature as its anamorph, *Helminthosporium carbonum* Ullstrup [syn. *Bipolaris zeicola* (Stout) Shoemaker]. The species is separated into races based on differences in pathogenicity and virulence on maize: race 0 is nonpathogenic (35); race 1 causes large, oval lesions on susceptible maize lines homozygous for the *hm1* allele (12, 29, 30); race 2 causes smaller necrotic lesions on most host genotypes (29, 30); race 3 causes linear lesions along the axis of the leaf (14), and some race 3 isolates are pathogenic on both maize and rice in Japan (27, 36). Race 1 isolates produce HC toxin, a cyclic tetrapeptide with the structure cyclo-(D-Pro-L-Ala-D-Ala-L-Aoe), where Aoe is 2-amino-8-oxo-9,10-epoxydecanoic acid (19, 32). The peptide is selectively toxic to susceptible (*hm1 hm1*) maize genotypes and, hence, is responsible for the increased virulence of race 1 isolates. Synthesis of this host-selective toxin requires HC toxin synthetase (HTS) (18), a large (ca. 574-kDa) peptide synthetase. This enzyme is encoded by the 15.7-kb *HTS1* gene within the *Tox2* locus, a single, complex locus containing two copies of *HTS1* (25). In crosses of race 1 with race 2 isolates, the ability to produce HC toxin cosegregates with increased virulence (15, 23).

The origin of *C. carbonum* is unknown. The pathogen appeared suddenly on susceptible inbred lines of maize and was named as a new species when first discovered and designated race 1, compared to a less-virulent race, race 2 (29, 30). In the absence of toxin-sensitive maize genotypes, the frequency of

race 1 isolates has declined to <1% of the total population of *C. carbonum*, presumably because of a decrease in parasitic fitness resulting from unnecessary toxin production (11). Race 2, the most prevalent race of *C. carbonum* (11), may have arisen as a result of the loss of toxin-producing ability. Because DNA sequences homologous to *Tox2* are not present in the genomes of non-toxin-producing races (18) and because members of the genus *Cochliobolus* frequently gain the ability to produce unique metabolites (22), it was proposed that HC toxin biosynthetic capacity was acquired by horizontal gene transfer (17, 31, 33). Although the molecular structure of the *Tox2* locus is consistent with a one-step acquisition of toxigenesis (31), recent evidence from analysis of the organization of *Tox2* (1) and from studies of other, unrelated fungal species that produce Aoe-containing cyclic peptides (16) suggests that the origin of race 1 was not by recent transfer of DNA.

Determination of the chromosomal location of the *Tox2* locus may provide clues relevant to the origin of races in *C. carbonum* and the source of genetic variation in other pathogenic fungi. In the present study, we examined the *Tox2*-containing chromosome in several field isolates of *C. carbonum* to characterize the diversity within race 1, which has been demonstrated by previous studies to be a relatively uniform population (8, 26, 34, 35). If race 1 is a clonal lineage, the *Tox2* locus would be expected to be located on the same chromosome in all isolates of race 1. A recent report by Ahn and Walton (1) indicated that the two copies of *HTS1* are always on the same chromosome, but the size of that chromosome varied among isolates.

Our results confirm that *Tox2* is on chromosomes of different sizes in different race 1 isolates of *C. carbonum*. Chromosomes of corresponding sizes were distinguished by hybridization with chromosome-specific markers, indicating that the *Tox2* locus occupies different positions in the genomes of different isolates. To determine whether these polymorphic *Tox2*-

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TABLE 1. Isolates of *C. carbonum* used in this study, including our laboratory designation, race, the person who isolated or provided the isolate, origin, mating type, and chromosome bearing the *Tox2* locus (from results presented herein)

Isolate	Race	Source	Geographic origin	Mating type	Chromosome bearing <i>Tox2</i> locus (Mb)
SB111	1	S. P. Briggs	Iowa	MAT-2	4
CC111 ^a	1	K. J. Leonard	N.C.	MAT-1	4
CC141	1	D. R. Smith	Ill.	MAT-1	2.3
CC151	1	R. P. Scheffler	Mich.	MAT-2	2.3
CC161	1	M. L. Carson	N.C.	MAT-2	2.3
CC171	1	M. L. Carson	Mich.	MAT-2	4
CC181	1	M. L. Carson	N.C.	MAT-2	2.3
CC1101	1	R. R. Nelson	Argentina	MAT-2	4
CC1211	1	S. P. Briggs	Miss.	MAT-1	2.3
CC1311	1	L. D. Dunkle	Ind.	MAT-1	2.3
CC221	2	K. J. Leonard	N.C.	MAT-2	None
CC3131	3	L. D. Dunkle	Ind.	MAT-1	None
HMO ^b	O	L. D. Dunkle	Ind.		None

^a Albino mutant (10).

^b *C. heterostrophus* race O.

containing chromosomes are homologs, sexually compatible isolates with *Tox2* on chromosomes of different sizes were crossed, and the progeny were analyzed for segregation of *HTS1* sequences, expression of the race 1 disease phenotype, and production of HC toxin in culture. If these *Tox2*-containing chromosomes, despite their size differences, are functional homologs, we would predict that all of the progeny would receive one or the other *Tox2*-containing chromosome. If the two chromosomes assort independently during meiosis, we would expect approximately 25% of the progeny to receive both of the *Tox2* chromosomes, 50% to receive one or the other of those chromosomes, and 25% to receive neither and, therefore, lack the ability to produce HC toxin and cause large, race 1-type lesions on maize leaves. A loss of *Tox2* or the *Tox2*-containing chromosome as a result of such crosses might explain the evolution of the non-toxin-producing races of *C. carbonum*.

MATERIALS AND METHODS

Fungal isolates. Cultures of the *C. carbonum* isolates used in this study (Table 1) were grown on potato dextrose agar (PDA) (Difco) at 24°C. Conidia from 2-week-old cultures were harvested in sterile 30% glycerol and stored at -80°C.

Isolation of genomic DNA. Genomic DNA was isolated from mycelium produced by conidia (10⁴ conidia/ml) incubated on a rotary shaker (200 rpm) in modified Fries medium (20) at 24°C for 2 days. Mycelium was filtered from the medium onto sterile cheesecloth, rinsed with cold, sterile water, lyophilized for 15 min, and frozen at -80°C. The mycelium was ground to a fine powder in liquid nitrogen, extracted in lysis buffer, and then extracted with phenol-chloroform-isoamyl alcohol (25:24:1), and the DNA was precipitated with ethanol (2). For some preparations, precipitation with 3 M NaCl was used to remove polysaccharides from the DNA (5). Genomic DNA (25 µg) from each isolate was digested with *ApaI*, *EcoRI*, *PstI*, or *XmnI*. The digestion products were separated by electrophoresis in a 0.8% agarose gel in 0.5× TBE (45 mM Tris-borate, 0.1 mM EDTA [pH 8.0]) and transferred to Nytran filters (Schleicher & Schuell) for Southern analysis as described below.

Preparation of *HTS1* probe, chromosome-specific probes, and genomic library. A 966-bp DNA fragment within the *HTS1* open reading frame was amplified by PCR as described previously (8), cloned into the pCRII vector (Invitrogen Corporation), and used as a probe for *HTS1*. Single-copy restriction fragment length polymorphism markers that mapped to specific chromosomes of *Cochliobolus heterostrophus* (28) were provided by C. Bronson (Iowa State University). For use as chromosome probes, plasmid DNAs were purified on a CsCl gradient and digested with *HindIII*, and the insert DNAs were radiolabeled with [α -³²P]dATP by random priming (6, 7). Genomic libraries were prepared by digesting DNA with *BamHI* and cloning it into the λ ZAP vector (Stratagene). Potential chromosome markers were selected from these libraries by hybridiza-

tion with randomly labeled DNA extracted from the 4-Mb chromosome purified by contour-clamped homogeneous electric field (CHEF) gel electrophoresis.

Preparation of chromosomes. Conidia from 2-week-old cultures were suspended in sterile distilled water containing 0.05% Tween 20. They were concentrated by centrifugation, suspended in 100 ml of modified Fries liquid medium at 10⁴ conidia/ml, and incubated in a rotary shaker (200 rpm) at 24°C for 5 to 6 h until germ tubes with a high nucleus/cell ratio had developed. The germinated conidia were collected on Nitex gauze (20-µm pores) (Tetko, Inc.), resuspended in filter-sterilized enzyme solution (2 mg of Novozyme [Interspec Products, Inc.] per ml, 2 mg of Driselase [Sigma Chemical Co.] per ml, 10 µg of β -glucuronidase [Sigma Chemical Co.] per ml in 0.7 M NaCl), and incubated for approximately 1 h. The protoplasts were then filtered through three layers of Nitex gauze, concentrated by centrifugation at 2,000 rpm, and separated from debris and conidia by placement of a 1.2 M sorbitol solution beneath the protoplast suspension (37). Protoplasts were suspended at 10⁸/ml in 0.8% low-melting-point agarose (50°C) and allowed to solidify on ice as agarose plugs. Agarose-protoplast plugs were treated with proteinase and detergent and stored in 0.5 M Na₂EDTA as described by Schwartz and Cantor (24).

CHEF gel electrophoresis. Chromosomal DNAs were loaded into wells as agarose plugs (24) and separated in 0.9% agarose gels at 13.5°C in 0.5× TBE with the CHEF-DR11 (Bio-Rad Laboratories) under the following conditions: 1.8 V/cm with a switch interval ramp from 700 to 1,100 s for the first 30 h and 1.7 V/cm with a switch interval ramp of 1,100 to 1,700 s for the next 80 h. Gels were stained in a solution of 0.8 µg of ethidium bromide/ml for 1 h, destained in water, and photographed with UV illumination. The sizes of chromosomes were determined by comparison with chromosome size standards from *Schizosaccharomyces pombe* and *Candida albicans* (Bio-Rad).

Southern analysis. The gels were treated for 5 min with 0.25 M HCl and for 30 min in 1.5 M NaCl-0.5 N NaOH. Chromosomal DNAs or DNA fragments from genomic digests were transferred by capillary action onto Nytran filters for 24 h in 20× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate). DNA was cross-linked to the membrane by illumination with UV light before hybridization. All blots were prehybridized in 1 ml of hybridization solution (5× SSC, 5× Denhardt's solution, 5 mM Na₂HPO₄, 0.1% sodium dodecyl sulfate, 10 µg of sheared calf thymus DNA per ml/cm² of membrane at 65°C before denatured probe was added to the hybridization bag. Hybridizations were performed for 20 h at 65°C for the *HTS1* probe or at 60°C for the chromosome-specific probes. Blots were washed twice in 2× SSC at 60°C for 15 min and once in 0.1× SSC at 60°C for 10 min. In some experiments, the probe was stripped from blots with 0.5 N NaOH for 30 min, followed by neutralization in 0.5 M Tris (pH 8.0) containing 10 mM Na₂EDTA. The blots were rinsed with 0.1× SSC containing 0.1% sodium dodecyl sulfate before additional hybridization analysis.

Production and analysis of sexual crosses. Sexual crosses were produced on sterile, senescent corn leaves on the surface of modified Sach's medium as described by Nelson (13). Mycelial plugs (5 mm in diameter) were removed from 2-week-old PDA cultures of compatible mating types and placed on the agar surface on opposite sides of the leaf. After incubation in the dark at 21°C for 21 days, ascospores were isolated from pseudothecia and germinated on complete agar medium (37). Pieces of agar with individual ascospores were transferred to PDA.

All ascospore progeny were screened for the presence of *HTS1* sequences by PCR amplification with *HTS1*-specific primers (8) to amplify a 600-bp sequence near the 5' end and an upstream 406-bp sequence which spans the translation start site of *HTS1* (8). This second reaction served to confirm the presence of the entire *HTS1* open reading frame.

After 14 days of growth in modified Fries medium, all progeny of the cross CC141 × SB111 were analyzed for their ability to produce HC toxin. The toxin in 0.4 ml of medium was partitioned into an equal volume of chloroform. The chloroform was evaporated, the residue containing the toxin was suspended in 20% acetonitrile, and duplicate samples were analyzed by high-performance liquid chromatography (4). The concentration of HC toxin was determined by integration of peak areas relative to those of purified toxin standards.

Each single-ascospore progeny from the cross CC141 × SB111 was analyzed for race phenotype by inoculation of the susceptible maize inbred Pr (*hm1 hm1*). Conidia from 2-week-old cultures were suspended in 0.05% Tween 20 at 3 × 10⁴ spores/ml and sprayed onto leaves of 3-week-old seedlings. The seedlings then were placed in a dew chamber and removed after 20 h to the greenhouse bench (4). Symptom development and lesion size on plants inoculated with each of the progeny or the parents were observed daily to establish the time required for expression of the definitive race 1 disease phenotype.

RESULTS

Genomic Southern blots. Genomic DNA from several isolates was digested with *ApaI*, *EcoRI*, *PstI*, or *XmnI*, and the digestion products were hybridized with the 966-bp *HTS1* probe to detect potential variation within the *HTS1* gene and adjacent sequences within the *Tox2* locus. The *HTS1* probe hybridized to the fragments of the same sizes in restriction digests of DNA from all race 1 isolates, indicating that recog-

FIG. 1. CHEF gel showing separation of chromosomes from isolates of *C. carbonum*. The electrophoretic conditions resolved chromosomes in the range of 1 to 4 Mb. This range includes those chromosomes that contain the *HTS1* gene. The isolate designations are listed above each lane. On the right, chromosomes from *Schizosaccharomyces pombe* and *Candida albicans* were included as size markers, and the sizes of the chromosomes are indicated in megabases in the left margin.

nitition sequences for several recognition enzymes are conserved within the *HTS1* gene and up to 20 kb 3' of the *HTS1* probe. As expected, no hybridization of this marker was detected with digested DNA from isolates of non-toxin-producing races.

Chromosomal polymorphisms. Electrophoretic karyotypes obtained from several *C. carbonum* isolates revealed differences in the sizes and numbers of chromosomal bands in many of the isolates (Fig. 1). The most notable difference was the presence of a chromosome with a size of approximately 4 Mb in isolates CC111, SB111, CC171, and CC1101 which was absent from the other isolates. Not all of the chromosomes were fully resolved by the electrophoretic conditions, because the parameters were optimized for separating the chromosomes containing *Tox2*. The possible comigration of some chromosome bands in these electrophoretic karyotypes is suggested by the intensity and thickness of some of the bands (Fig. 1). Some of the differences among isolates may be due to variation in the sizes of homologous chromosomes.

Hybridization with chromosome-specific probes. The *HTS1* gene was expected to be on the same chromosome in isolates of race 1 if these isolates are clonal descendants of a common progenitor. To examine the diversity among *C. carbonum* isolates of race 1, the 966-bp *HTS1* probe was hybridized by chromosomal Southern blotting. The probe hybridized to different-sized chromosomes in different isolates (Fig. 2). In isolates CC111, SB111, CC171, and CC1101, the probe hybridized with a chromosome with a size of approximately 4 Mb. In other race 1 isolates (CC141, CC151, CC161, CC181, CC1211, and CC1311), the *HTS1* probe hybridized with a smaller chromosome with a size of approximately 2.3 Mb. Each of the race 1 isolates that had the 4-Mb chromosome also had a 2.3-Mb chromosome; in those isolates, only the 4-Mb chromosome hybridized with the *HTS1* probe. There was no relationship between geographic origin and the size of the chromosome bearing *HTS1* (Table 1). As expected, in numerous repeated experiments, the *HTS1* probe did not hybridize to any chro-

mosomes in karyotypes of non-toxin-producing isolates CC221 (race 2) and CC3131 (race 3). These non-toxin-producing races had a 2.3-Mb chromosome but lacked the 4-Mb chromosome (Fig. 1).

Chromosome-specific probes from the related species *C. heterostrophus* (28) were used to distinguish chromosomes of *C. carbonum* on chromosomal Southern blots. Of the 45 markers analyzed, 35 hybridized to chromosomes of *C. carbonum* (data not shown). A *C. heterostrophus* isolate (HMO) was included in each analysis as a positive control, and the markers hybridized with a chromosome that, in most cases, is nearly the same size as the hybridizing *C. carbonum* chromosome. None of the markers hybridized in a pattern corresponding to that obtained with the *HTS1* marker. The size of the hybridizing chromosome usually varied less than 0.2 Mb among isolates. The variation was never as large as 2 Mb as with the *HTS1* marker. A probe prepared from the clone G214 hybridized to a 2.3-Mb chromosome in all *C. carbonum* isolates tested, except SB111 (Fig. 3, right panel), including isolates of race 2 (CC221) and race 3 (CC3131). Thus, as in *C. heterostrophus*, this marker is chromosome specific in *C. carbonum*. Clone B71, a marker that is on the same *C. heterostrophus* chromosome as G214 (28), also hybridized with a 2.3-Mb chromosome in the *C. carbonum* isolates and hybridized with a slightly larger chromosome in SB111 (data not shown). These results suggested that the 2.3-Mb chromosome and the 4-Mb chromosome are distinct, even though the *Tox2* locus is contained on one or the other of them.

Genomic libraries were constructed from isolates CC111 and CC1101 and were screened to obtain a marker for the 4-Mb chromosome. One marker, designated Q2, was found that hybridized with the 4-Mb chromosome in all four isolates previously found to have *Tox2* on a 4-Mb chromosome (CC111, SB111, CC171, and CC1101) (data not shown). In the other isolates that did not have a 4-Mb chromosome (CC141, CC151, CC161, CC181, CC1211, CC1311, and CC221), this marker hybridized with a 2.0-Mb chromosome, not with a 2.3-Mb chromosome. These results indicate that the 2.0- and 2.3-Mb chromosomes have sequences in common with that of the 4-Mb chromosome.

FIG. 2. Chromosomal Southern blot of *C. carbonum* isolates. The *HTS1* probe hybridized with a 4-Mb chromosome in isolates CC111, SB111, CC171, and CC1101 and with a 2.3-Mb chromosome in isolates CC141, CC151, CC161, CC181, CC1211, and CC1311. The *HTS1* probe did not hybridize with chromosomes of the race 2 (CC221) or race 3 (CC3131) isolates.

FIG. 3. Chromosomal Southern blot hybridized with the *HTS1* probe (left) showing with the 4-Mb chromosome in isolates CC111, SB111, and CC1101 and with a 2.3-Mb chromosome in isolates CC181 and CC1311. In a separate blot, the chromosome-specific probe G214 (right) hybridized to the 2.3-Mb chromosome in all isolates of *C. carbonum* except SB111 and with an isolate of *C. heterostrophus* (HMO) as a positive control. The lanes are labeled with the isolate designations. Arrowheads in right-hand lane indicate the positions of the 4- and 2.3-Mb chromosomes, respectively.

Sexual crosses and analysis of ascospore progeny. To determine whether the two *Tox2*-containing chromosomes are homologs or assort independently, we crossed isolates that had *HTS1* on a 4-Mb chromosome with those that had *HTS1* on a 2.3-Mb chromosome. Among the 12 crosses made, viable ascospore progeny were isolated only from the following: CC111 $\times$ CC181, CC1211 $\times$ CC171, CC1311 $\times$ CC171, and CC141 $\times$ SB111. Other crosses resulted in the production of pseudothecia, but few of those yielded asci with viable ascospores. Forty-three ascospore progeny from cross CC141 $\times$ SB111 and 22 progeny from the cross CC111 $\times$ CC181 were analyzed for race 1 characteristics.

DNA from all ascospore progeny was screened for the presence of *HTS1* sequences. *HTS1*-specific primers were used in PCRs to amplify a 600-bp fragment from DNA of the progeny of two different crosses. A 600-bp fragment was amplified and detected in DNA of every progeny isolate (data not shown). In addition, primers were used to amplify an upstream 406-bp *HTS1* sequence from all progeny in a different set of PCRs (data not shown). Thus, all progeny contained *HTS1*.

All sporulating progeny of the cross CC141 $\times$ SB111 produced a race 1 phenotype when inoculated onto maize seedlings susceptible to *C. carbonum* race 1. However, some isolates (23%) developed the large necrotic lesions characteristic of a race 1 disease phenotype only after a longer time (4 to 6 days) following inoculation compared to the remaining (77%) progeny and the parents (2 to 3 days).

All of the progeny from the cross CC141 $\times$ SB111 produced HC toxin in culture, although the amounts varied. Most of the progeny (65%) produced large amounts of HC toxin, comparable to amounts produced by the parents (>60 $\mu\text{g/ml}$). The remainder of the progeny produced small (<15 $\mu\text{g/ml}$) or intermediate (16 to 60 $\mu\text{g/ml}$) amounts of toxin. No correlation was observed between the amount of toxin produced in culture and the virulence of the isolates described above. Disease symptoms developed as rapidly among the progeny producing small amounts of toxin as among the progeny producing large amounts of toxin, and delayed symptom development was ob-

served for some of the progeny producing large amounts of toxin as well as those producing small amounts.

Chromosomes of several of the CC141 $\times$ SB111 progeny were separated by CHEF gel electrophoresis (Fig. 4). Some of the progeny isolates had nonparental chromosomal bands in their karyotypes. Southern analysis with the *HTS1* probe indicated that each isolate had only one *Tox2*-containing chromosome (Fig. 4). One isolate (isolate 14) had a *Tox2*-containing chromosome that was slightly larger than the 2.3-Mb chromosome of the CC141 parent. All of the other progeny analyzed had *Tox2*-containing chromosomes that were either 2.3 or 4 Mb, the same sizes as those in the parental strains. None of the progeny lacked a *Tox2*-containing chromosome, and none had two such chromosomes. Evidence from the analysis of the progeny indicated that the chromosomal position of *Tox2* does not directly influence virulence or the amount of HC toxin produced.

FIG. 4. CHEF gel showing separation of chromosomes from selected progeny of the cross CC141 $\times$ SB111 (top). Chromosomal Southern analysis of the CHEF gel hybridized with the *HTS1* probe. All of the progeny have a single *Tox2*-containing chromosome. Arrowheads in the right-hand lane indicate the positions of the 4- and 2.3-Mb chromosomes, respectively.

DISCUSSION

Hybridization of chromosomal DNA with the *HTS1* probe indicated that the *Tox2* locus is on two chromosomes of different sizes in different isolates. Chromosome-specific markers confirmed that the two *Tox2*-containing chromosomes are different. Although all of the race 1 isolates in our collection had a *Tox2*-containing chromosome with a size of either 2.3 or 4 Mb, the possibility cannot be eliminated that the *Tox2* locus is located on still other chromosomes in isolates that were not analyzed. As expected (18), the coding sequence within the *HTS1* gene is highly conserved; all of our race 1 isolates had restriction fragments of a uniform size that hybridized to an *HTS1* probe, regardless of the size of the *Tox2*-containing chromosome in those isolates. Although slight variation in chromosome sizes was demonstrated with the chromosome-specific markers from *C. heterostrophus*, a size difference comparable to that of the two *Tox2*-containing chromosomes was never observed with those markers (data not shown). The only marker other than *HTS1* that showed a substantial size polymorphism was Q2, which hybridized with the *Tox2*-containing 4-Mb chromosome in one group of isolates and with a non-*Tox2*-containing 2.0-Mb chromosome in the other. Thus, the 4-Mb chromosome shares DNA sequences (Q2) with a 2.0-Mb chromosome, which is present in isolates that do not contain a 4-Mb chromosome. Also, the 4-Mb and the 2.3-Mb chromosomes share the *Tox2* sequences but do not share chromosome-specific markers from *C. heterostrophus* (G214 and B71) and *C. carbonum* (Q2).

These results have important implications concerning the origin and variability of *C. carbonum*. The difference in *Tox2*-containing chromosomes in different isolates indicates that the population of race 1 is not homogeneous as predicted from previous studies (8, 26, 34, 35). Indeed, chromosome-length polymorphisms are known to result from sexual reproduction (9, 21, 38, 39). However, the teleomorphic stage of *C. carbonum* has never been found in nature, perhaps in part because a single gene conditioning the inability to form pseudothecia is common in populations of *C. carbonum* (3, 11). Although evidence for genetic exchange and gene flow in the pathogen population is equivocal, Welz and Leonard (35) concluded from their studies that individuals within a specific race reproduce sexually but that sexual reproduction does not occur between different races. When race 1 was the predominant race in nature, it is possible that race 1 individuals reproduced sexually, generating chromosomal polymorphisms, including the location of *Tox2*. In our crosses between two race 1 isolates, we observed the generation of a *Tox2*-containing chromosome of a different size (nonparental) in one isolate (isolate 14). Although we did not analyze this isolate further, it may have resulted from rare recombination events within the *Tox2* locus. According to estimates based on the size and organization of the locus (1), recombination should occur in approximately 12% of the progeny of a cross between two race 1 isolates.

The chromosomal polymorphisms observed in our analyses indicate that genomic rearrangements are common in *C. carbonum*. These genomic rearrangements may include translocations, recombination between repeated sequences such as those that flank the *Tox2* locus, or generation of two smaller chromosomes (e.g., the 2.0-Mb chromosome, which retained the 4-Mb marker, and the 2.3-Mb chromosome, which retained *HTS1*) from the 4-Mb chromosome. To investigate the relationship between the two *Tox2*-containing chromosomes, it was important to determine whether they behave as distinct linkage groups and assort independently in sexual crosses. Isolates with a 4-Mb, *Tox2*-containing chromosome were crossed to com-

patible isolates with *Tox2* on a 2.3-Mb chromosome. Analysis of the progeny of the cross (CC141 × SB111) indicated that all of the progeny produced HC toxin, contained *HTS1* sequences, and produced race 1 type lesions on susceptible leaves. None of the progeny analyzed lacked a *Tox2*-containing chromosome. The failure to detect such progeny may have been due to lethality resulting from the loss of both chromosomes. If so, we would predict that a corresponding proportion of the progeny would receive both *Tox2*-containing chromosomes. However, we found by chromosomal Southern analyses that the progeny received only one functional *Tox2*-containing chromosome, indicating that the two chromosomes did not assort independently.

The most straightforward explanation of the results of progeny analysis is that the *Tox2* loci in different chromosomal positions behave as homologous loci during meiosis, although the chromosomes differ in other DNA sequences. These results are predicted if the two *Tox2*-containing chromosomes with different sizes arose as the result of a translocation(s). The progeny of the crosses either result from pairing between chromatids that are not entirely homologous or from mutual exclusion of the different chromosomal regions. This hypothesis is consistent with the observations of Ahn and Walton (1), who concluded that a reciprocal translocation is responsible for the differences in size of the *Tox2*-containing chromosomes. Likewise, the *Tox1* locus in *C. heterostrophus*, which determines the ability of this pathogen to produce HmT toxin, maps near a translocation breakpoint (28). Therefore, translocations or other similar genomic rearrangements may represent an important mechanism by which new pathogens or new races with altered virulence arise.

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