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Chromosomal Organization of *TOX2*, a Complex Locus Controlling Host-Selective Toxin Biosynthesis in *Cochliobolus carbonum*

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Race 1 isolates of the filamentous fungus *Cochliobolus carbonum* are exceptionally virulent on certain genotypes of maize due to production of a cyclic tetrapeptide, HC-toxin. In crosses between toxin-producing ($Tox2^+$) and toxin-nonproducing ($Tox2^-$) isolates, toxin production segregates in a simple 1:1 pattern, suggesting the involvement of a single genetic locus, which has been named *TOX2*. Earlier work had shown that in isolate SB111, *TOX2* consists in part of two copies of a gene, *HTS1*, that encodes a 570-kD cyclic peptide synthetase and is lacking in $Tox2^-$ isolates. The genomic structure of *TOX2* and the relationship between the two copies of *HTS1* have now been clarified by using pulsed-field gel electrophoresis and physical mapping. In isolate SB111, both copies of *HTS1* are on the largest chromosome (3.5 Mb), which is not present in the related $Tox2^-$ strain SB114. Two other genes known or thought to be important for HC-toxin biosynthesis, *TOXA* and *TOXC*, are also on the same chromosome in multiple copies. Other independent $Tox2^+$ isolates also have two linked copies of *HTS1*, but in some isolates the size of the chromosome containing *HTS1* is 2.2 Mb. Evidence obtained with $Tox2^+$-unique and with random probes is consistent with a reciprocal translocation as the cause of the difference in the size of the *HTS1*-containing chromosome among the $Tox2^+$ isolates studied here. Physical mapping of the 3.5-Mb chromosome of SB111 that contains *HTS1* using rare-cutting restriction enzymes and engineered restriction sites was used to map the chromosomal location of the two copies of *HTS1* and the three copies of *TOXC*. The results indicate that *TOX2* is a complex locus that extends over more than 500 kb. The capacity to produce HC-toxin did not evolve by any single, simple mechanism.

INTRODUCTION

Several species of the plant pathogenic fungus *Cochliobolus* owe their virulence to the production of host-selective toxins, which are low molecular weight metabolites of diverse structures. Production of the characteristic toxins of *C. heterostrophus* race T, *C. carbonum* race 1, and *C. victoriae* appears to be under the control of single, different genetic loci because in each case toxin production segregates 1:1 with toxin nonproduction in crosses between toxin-producing (Tox^+) and toxin-nonproducing (Tox^-) isolates of these fungi (Nelson and Ullstrup, 1961; Scheffer et al., 1967; Lim and Hooker, 1971; Walton, 1990; Bronson, 1991). Until recently, the molecular nature of the *TOX* loci was unclear, but evidence is emerging that they are more complex than the earlier Mendelian genetic studies indicated. *TOX1* of *C. heterostrophus*, for example, is linked to a reciprocal translocation and appears to be large and complex (Bronson, 1991; Tzeng et al., 1992; Yoder et al., 1994).

Central to the biosynthesis of the host-selective toxin HC-toxin by race 1 ($Tox2^+$) isolates of *C. carbonum* is a 570-kD cyclic peptide synthetase called HC-toxin synthetase (HTS). HTS activates and thioesterifies three of the four amino acids (L-Pro, L-Ala, and D-Ala) in HC-toxin and epimerizes L-Ala and L-Pro (Walton, 1987; Walton and Holden, 1988). Because HTS has four amino acid–activating domains, HTS probably also activates the fourth amino acid in HC-toxin, 2-amino-9, 10-epoxy-8-oxodecanoic acid (Aeo) or an Aeo precursor (Scott-Craig et al., 1992). HTS is encoded by *HTS1*, which contains a 15.7-kb open reading frame (Scott-Craig et al., 1992). *HTS1*, as well as an additional 6 kb of flanking DNA, is present only in $Tox2^+$ isolates of *C. carbonum* and genetically cosegregates with HC-toxin production (Panaccione et al., 1992). The $Tox2^+$ isolate SB111 as well as six other independent $Tox2^+$ isolates examined have two functional copies of *HTS1*. When both copies (but not either copy alone) are mutated by targeted gene disruption, HTS activity, HC-toxin production, and pathogenicity are abolished (Panaccione et al., 1992).

Although *HTS1* is central to the biosynthesis of HC-toxin, its relation to the genetic locus *TOX2* remains unclear. One way to reconcile the molecular and Mendelian data is to

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propose that HTS catalyzes the complex synthesis of HC-toxin, and the two copies of *HTS1* are clustered, as are secondary metabolite genes in other fungi (e.g., Hohn et al., 1993). However, walking along the chromosome from *HTS1* in either the 3′ or the 5′ direction with overlapping genomic λ clones did not detect the other copy of *HTS1* within ~10 kb (further walking was hampered by repeated DNA; Panaccione et al., 1992), and screening of a cosmid library of SB111 failed to detect any cosmids containing more than a single copy of any part of *HTS1* (D.G. Panaccione, J.S. Scott-Craig, and J.D. Walton, unpublished results). These results indicate that the two copies of *HTS1* are not tightly clustered. Furthermore, several lines of evidence indicate that HTS by itself is not sufficient to catalyze the complete synthesis of HC-toxin. First, introduction of *HTS1* into a Tox2$^-$ background does not result in toxin production (Walton et al., 1994). Second, based on the deduced amino acid sequence of *HTS1*, HTS does not appear to be able to contribute to the synthesis of Aeo, which is a polyketide or fatty acid (Walton, 1990; Scott-Craig et al., 1992). Third, we have discovered a new Tox2$^+$-unique gene, *TOXC*, whose 6.5-kb open reading frame encodes a fatty acid synthase and is required for HC-toxin biosynthesis, most plausibly by catalyzing the synthesis of Aeo or its decanoic acid backbone (Walton et al., 1994; J.-H. Ahn and J.D. Walton, manuscript in preparation).

To resolve why HC-toxin production segregates as a single trait yet appears to involve multiple copies of multiple Tox2$^+$-unique genes, we used pulsed-field gel electrophoresis to analyze the genomic structure of *TOX2* and to map physically *HTS1* and the chromosome on which it lies. The results are relevant to the mechanism by which HC-toxin and hence the host range in *C. carbonum* has evolved.

RESULTS

Duplication of *HTS1* and Associated DNA

HC-toxin–producing isolates (Tox2$^+$) of *C. carbonum* have two functional copies of *HTS1* (Panaccione et al., 1992). Each copy of *HTS1* is part of a separate, contiguous 22-kb region that is found only in Tox2$^+$ isolates. The two copies of *HTS1* have identical restriction maps with every enzyme tested but can be distinguished from each other by restriction sites that lie outside the 22-kb Tox2$^+$-unique regions. In strain SB111, the two copies give different-sized fragments at the 3′ end of *HTS1* with many restriction enzymes, including BamHI, EcoRI, and SalI. BamHI, for example, produces fragments of 8.5 and 9 kb that hybridize with probe CC61 (Figure 1; Panaccione et al., 1992). Comparative restriction maps of the two copies of the 22-kb Tox2$^+$-unique region are shown in Figure 1. The maps diverge at the 3′ end within 1 kb of the end of the 22-kb Tox2$^+$-unique DNA (Figure 1). In contrast, a probe from the 5′ end of *HTS1* can distinguish copy 1 from copy 2 only if the DNA is cut with ApaI (of 22 six-base restriction enzymes tested) (Figure 1; Panaccione et al., 1992). When cut with ApaI and probed with CC48, genomic DNA of SB111 yields two bands of 18 and 30 kb (Figure 1).

The 5′ ApaI fragments and the 3′ BamHI fragments were assigned to copy 1 or copy 2 of *HTS1* by analysis of a particular fungal strain, 243-7, which is a progeny of SB111 and SB114. In crosses between SB111 and SB114, the progeny normally resemble one parent or the other in regard to *HTS1* and HC-toxin production; however, 243-7 is unusual in that it has only one copy of *HTS1* on a 2.1-Mb chromosome (see below). Re-

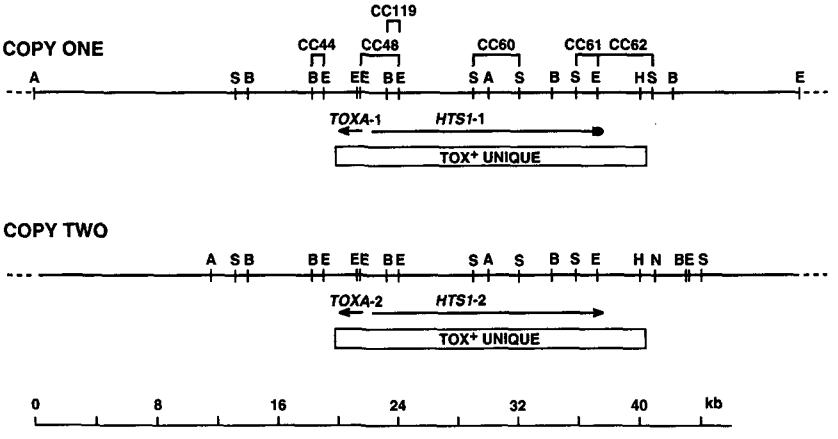

Figure 1. Restriction Maps of the Two Copies of *HTS1* and Flanking DNA.

HTS1-1 is copy one of *HTS1*, and *HTS1*-2 is copy two. The regions of contiguous DNA that are found only in Tox2$^+$ isolates are indicated. *TOXA* encodes a putative HC-toxin efflux pump. DNA fragments used as probes in this work are shown above the map of copy 1. The restriction maps of the two copies are identical between the SalI site at the 5′ end and the HindIII site at the 3′ end. A, ApaI; S, SalI; B, BamHI; E, EcoRI; H, HindIII; N, NotI. Additional SalI, BamHI, EcoRI, and HindIII sites to the left of CC44 are not shown. Additional ApaI sites to the right of CC60 are not shown.

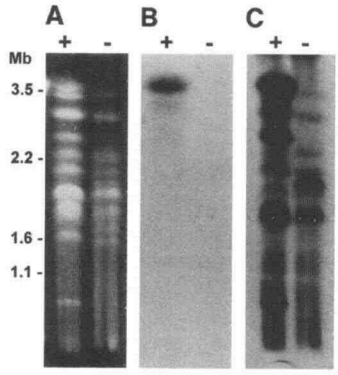

Figure 2. CHEF Separation of Chromosomes from the Tox2$^+$ Isolate SB111 (+) and the Related Tox2$^-$ Isolate SB114 (−).

CHEF conditions were 0.8% agarose, 50 V, with a 40-min switching interval for 72 hr, a 15- to 30-min switching interval for 72 hr, and a 10- to 20-min switching interval for 72 hr.

(A) Gel stained with ethidium bromide.

(B) Autoradiography of the same gel blotted and hybridized with probe CC61, representing *HTS1*.

(C) The same blot hybridized with probe CC44, representing repeated DNA that is adjacent to *HTS1* and common to Tox2$^+$ and Tox2$^-$ isolates.

Numbers at left indicate lengths in megabases.

striction enzyme mapping and contour-clamped homogeneous electric field (CHEF) electrophoresis indicated that in 243-7 the chromosome containing *HTS1* had undergone a break between the two copies of *HTS1*, resulting in the loss of one copy of *HTS1*. Strain 243-7 lacks both the 30-kb ApaI fragment that hybridizes with CC48 and the 8.5-kb BamHI fragment that hybridizes with CC61. On the basis of this strain, the 5′ ApaI and 3′ BamHI restriction polymorphisms were assigned to particular copies of *HTS1* as shown in Figure 1. The pathogenic phenotype of 243-7 is under investigation and will be reported elsewhere.

The restriction maps of copies 1 and 2 are identical between the 5′-most SalI site shown in Figure 1 and the 3′ end of *HTS1*. This region contains ~7 kb of DNA that has the same restriction map in both copies but is not Tox2$^+$ unique (Figure 1). At least some of this DNA is moderately repeated in both Tox2$^+$ and Tox2$^-$ isolates of *C. carbonum* and is found on most but not all chromosomes in both Tox2$^+$ and Tox2$^-$ isolates, as shown in Figure 2C. The sequence of CC44 indicates that it is related to the fungal transposable element Fot (Panaccione et al., 1996). Judging from relative hybridization intensity, CC44 appears to be particularly abundant on the same chromosome as *HTS1* (Figure 2).

Karyotypes of Tox2$^+$ and Tox2$^-$ Isolates of *C. carbonum*

Chromosomes of *C. carbonum* isolates SB111 (race 1, Tox2$^+$; produces HC-toxin) and SB114 (race 2, Tox2$^-$; does not produce HC-toxin) were analyzed by CHEF, as shown in Figure 2A. Although some chromosomes could not be separated due to similar mobilities, by varying the electrophoretic conditions it was possible to resolve 13 chromosomes in SB111. The total genome size of *C. carbonum* is estimated to be between 30 and 35 Mb. *C. heterostrophus* has been reported to have 15 chromosomes and 35 Mb of DNA (Tzeng et al., 1992). There are a large number of differences between SB111 and SB114. In particular, chromosomes of 0.7 and 3.5 Mb (the largest chromosome) in SB111 are lacking in SB114.

A probe from *HTS1* (CC61; see Figure 1) hybridized only with the 3.5-Mb, Tox2$^+$-unique chromosome, indicating that both copies of *HTS1* are on this chromosome. In 16 progeny of a cross between SB111 and SB114, all isolates rated as Tox2$^+$ on the basis of pathogenicity and production of HC-toxin have a 3.5-Mb chromosome containing *HTS1*, and all Tox2$^-$ progeny lack *HTS1* and a chromosome of 3.5 Mb (data not shown). The other chromosomes that can be resolved under our standard CHEF conditions and that are of different sizes in SB111 and SB114 (Figure 2) are distributed in the progeny, according to no particular pattern, which indicates that they assort independently of each other.

Chromosomal Differences in Independent Tox2$^+$ Isolates

Chromosomes of seven independent *C. carbonum* race 1 field isolates and SB111 were analyzed by CHEF, as shown in Figure 3A. No two isolates have exactly the same numbers and sizes of chromosomes. *HTS1* is located on a chromosome of either 3.5 (isolates SB111, 171, and 1101) or 2.2 Mb (isolates 141R, 151, 161, 181, and 1111) (Figure 3B). The strains in which *HTS1* is on a 2.2-Mb chromosome do not have a 3.5-Mb chromosome (Figure 3A). Not all chromosomes show a similar degree of variation in these strains; as shown in Figure 3C, all of them, as well as SB114, have a chromosome of 3.2 Mb that contains *XYL1* encoding endo-β1,4-xylanase (Apel et al., 1993).

SB111 has two copies of *HTS1* (Panaccione et al., 1992), and both are on the 3.5-Mb chromosome (Figure 2B). As shown in Figures 3B and 4, other independent race 1 isolates, including those in which *HTS1* is not on a 3.5-Mb chromosome, also have two copies of *HTS1* on the same chromosome, judging by their hybridization patterns with CC61 and CC48 (Figure 1). All the isolates, as well as an additional four Tox2$^+$ field isolates (ARG, YUGX, ALN, and A2; data not shown), have an identical pattern of 8.5- and 9-kb BamHI fragments when probed with CC61 (Figure 4A). However, the copies of *HTS1* in these isolates have different patterns at their 5′ ends. All of the isolates have one copy of CC48 on a 30-kb ApaI fragment, but the second copy of CC48 is on a 13, 18, or 19-kb ApaI fragment (Figure 4B). Isolate 171 has three copies of CC48 but apparently only two copies of CC61 (Figure 4A). There is no correlation between the pattern of hybridization of CC48 and the size of the chromosome containing *HTS1* (Figure 3B). The

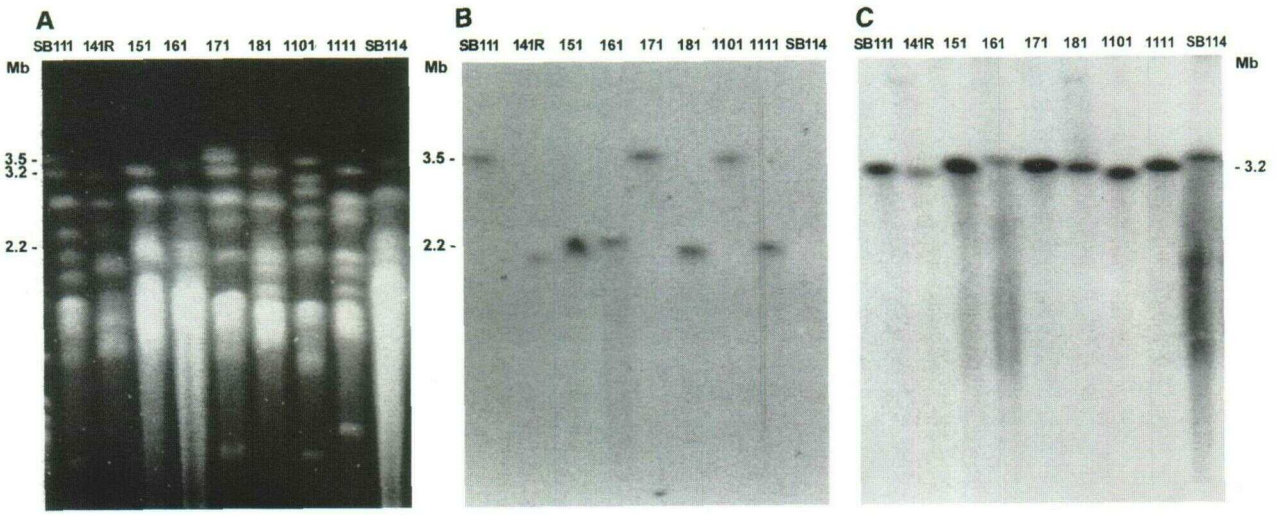

Figure 3. Electrophoretic Karyotype of Independent Tox2$^+$ Isolates of *C. carbonum*.

CHEF conditions are given in the legend to Figure 2.
(A) Gel stained with ethidium bromide.
(B) Autoradiography of gel blotted and hybridized with probe CC61.
(C) Same blot hybridized with *XYL1* encoding endo-β1,4-xylanase (Apel et al., 1993).
Numbers at left and right indicate lengths in megabases.

two copies of *HTS1* are on a 3.5-Mb chromosome in isolates SB111, 171, and 1101, yet CC48 shows a different pattern of hybridization to DNA cut with ApaI in each isolate (Figures 3B and 4B). Therefore, the source of the variation in *HTS1* chromosome size is probably different than that of the variation seen in the region that is 5′ to *TOXA*.

Single-Copy DNA Present on the Chromosome Containing *HTS1* Is Also Present in Tox2$^-$ Isolates

The complete lack of a homologous chromosome in Tox2$^-$ isolates could explain the genetic segregation of toxin production as a single locus (Nelson and Ullstrup, 1961; Scheffer et

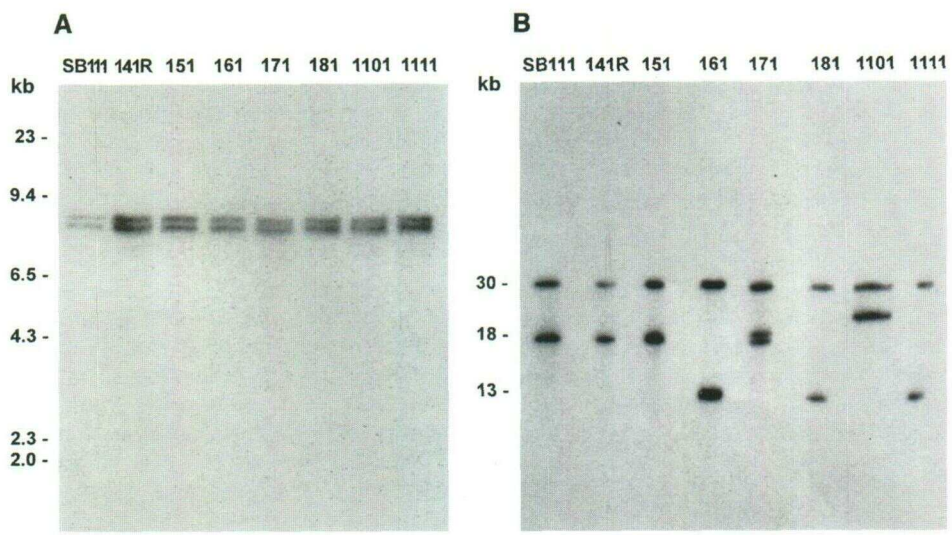

Figure 4. Duplication of *HTS1* in Independent Tox2$^+$ Isolates.

(A) DNA was cut with BamHI, separated by conventional electrophoresis, blotted, and hybridized with probe CC61 from the 3′ end of *HTS1*. Numbers at left indicate lengths of markers (λ DNA cut with HindIII) in kilobases.
(B) DNA was cut with ApaI, separated by CHEF (150 V with a 0.5- to 2.5-sec switching time for 20 hr), and hybridized with probe CC48 from the 5′ end of *HTS1*. Numbers at left indicate lengths of the hybridizing fragments in kilobases, using a λ DNA cut with XhoI, KpnI, or XbaI as markers.

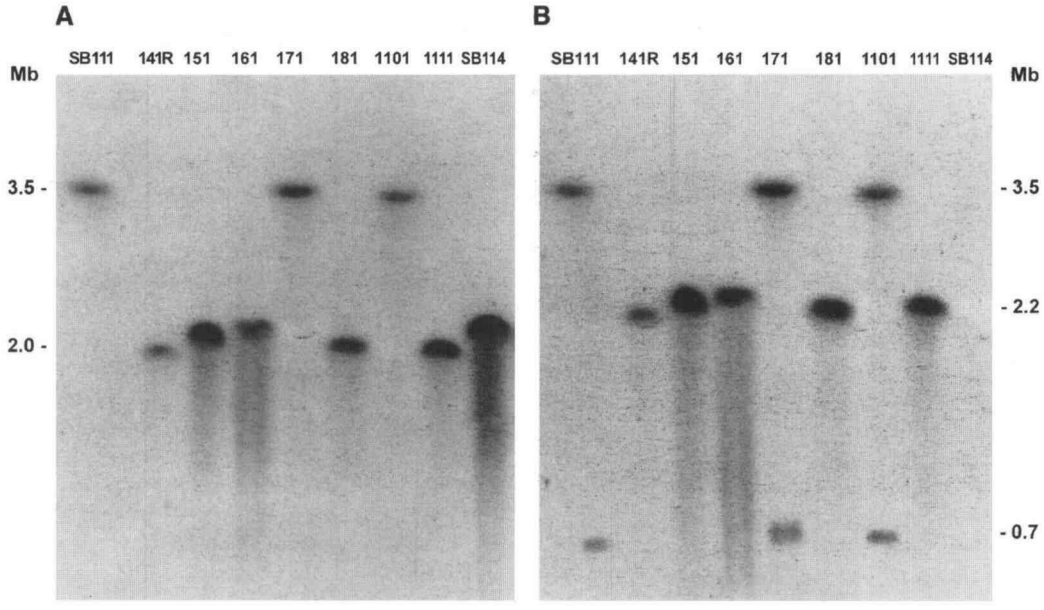

Figure 5. CHEF Separation of Chromosomes from Independent Tox2$^+$ Isolates and from Isolate SB114 (Tox2$^-$).

Chromosomes were separated by CHEF (conditions are as given in the legend to Figure 2), and the gel was blotted and hybridized.
(A) Blot hybridized with probe G242 from *C. heterostrophus*.
(B) The same blot hybridized with probe CC62.
Numbers at left and right indicate lengths in megabases.

al., 1967). In this case, the entire 3.5-Mb chromosome of SB111 could be considered *TOX2*. However, because SB111 has at least 44 kb of DNA that is unique to Tox2$^+$ isolates, a chromosome in Tox2$^-$ isolates that is homologous to the 3.5-Mb chromosome might be present but smaller. A series of restriction fragment length polymorphism probes that had been used to map the *C. heterostrophus* genome (Tzeng et al., 1992) was used to screen for DNA sequences that are present on the 3.5-Mb *HTS1* chromosome in SB111 and also present in the Tox2$^-$ isolate SB114. Of 40 probes tested, two hybridized with the 3.5-Mb chromosome containing *HTS1*. One of these, G213, is present as four or five copies in *C. carbonum* SB111 on different chromosomes; the other, G242, is present as a single copy. Figure 5A shows that G242 hybridized with the 3.5-Mb chromosome in isolates that have CC61 (representing *HTS1*) on a chromosome of that size, and in other isolates, G242 hybridized to a 2.0-Mb chromosome. G242 also hybridized with a chromosome of 2.0 Mb in SB114 (Tox2$^-$), but it is not known if this chromosome is the same as the 2.0-Mb chromosome in the Tox2$^+$ isolates. The actual similarity of the DNA represented by G242 in SB111 and SB114 is indicated in Figure 6 by the similarity of the hybridization patterns of G242 to SB111 and SB114 DNA cut with several restriction enzymes. Therefore, although SB114 does not have a chromosome of 3.5 Mb, it is not lacking this entire amount of DNA. Because this conclusion is based on a single probe, it is not known how extensive the homology is between the 3.5-Mb chromosome of SB111 and the 2.0-Mb chromosome of SB114. G242 is also located

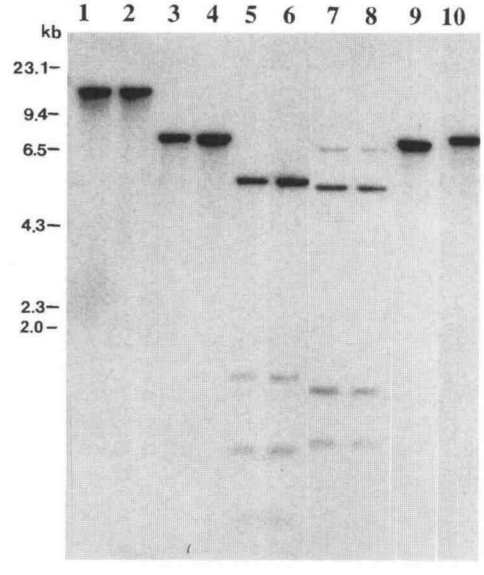

Figure 6. Restriction Mapping of Tox2$^+$ and Tox2$^-$ Isolates with Probe G242.

DNA from isolate SB111 (lanes 1, 3, 5, 7, and 9) and SB114 (lanes 2, 4, 6, 8, and 10) was cut with EcoRI (lanes 1 and 2), HindIII (lanes 3 and 4), SalI (lanes 5 and 6), XbaI (lanes 7 and 8), or XhoI (lanes 9 and 10), separated by conventional electrophoresis, blotted, and hybridized with probe G242. Numbers at left indicate lengths of markers (λ DNA cut with HindIII) in kilobases.

on a chromosome of 2.0 Mb in six other Tox2$^-$ field isolates (data not shown).

Tentative Evidence for a Translocation Associated with *TOX2* among Tox2$^+$ Isolates

Probe G242 hybridized with the 3.5-Mb chromosome in those Tox2$^+$ isolates that have *HTS1* on this chromosome and with a 2.0-Mb chromosome in those Tox2$^+$ isolates that have *HTS1* on a 2.2-Mb chromosome (Figures 3B and 5A). CC62 represents a sequence that is Tox2$^+$ unique and present in four to six copies per genome (Panaccione et al., 1992). Two of the copies of CC62 are immediately 3′ of the two copies of *HTS1* (Figure 1). CC62 hybridized with a chromosome of the same size as CC61 and CC48, representing *HTS1*, in all Tox2$^+$ isolates examined (Figure 5B). In addition, CC62 hybridized with a 0.7-Mb chromosome in those isolates that have *HTS1* on a 3.5-Mb chromosome (Figure 5B). In other Tox2$^+$ isolates, CC62 hybridized only with the 2.2-Mb chromosome containing *HTS1* (Figure 5B).

A plausible explanation for these hybridization patterns is that a reciprocal translocation has occurred in the evolution of Tox2$^+$ isolates of *C. carbonum*. The pattern typified by SB111 could give rise to the pattern typified by isolate 151 by breakage of the 3.5-Mb chromosome (containing the two copies of *HTS1*, at least two copies of CC62, and G242) into two fragments of 1.5 (containing the two copies of *HTS1* and associated copies of CC62) and 2.0 Mb (containing G242), with the 1.5-Mb fragment then joining with the 0.7-Mb fragment (containing the other copies of CC62). This would produce new chromosomes of 2.2 (containing both copies of *HTS1* and all copies of CC62) and 2.0 Mb (containing G242), as seen in isolates 141R, 151, 161, 181, and 1111. Because fungal translocations tend to be reciprocal (Zolan, 1995), a piece of the 0.7-Mb chromosome containing one of its telomeres likely translocates at the same time to the broken end of the 2.0-Mb fragment containing G242.

Physical Mapping of *TOX2*

Since the cloning and characterization of *HTS1* (Panaccione et al., 1992; Scott-Craig et al., 1992), three new genes with a possible role in HC-toxin biosynthesis have been found. All are unique to toxin-producing isolates and in SB111 are on the same 3.5-Mb chromosome as *HTS1*. *TOXA*, encoding a putative HC-toxin efflux pump, is present in two copies, one copy being immediately 5′ of each copy of *HTS1* (Figure 1; Walton et al., 1994; Pitkin et al., 1996). *TOXC*, encoding a fatty acid synthase, is present in three copies in SB111. Gene disruption experiments indicate that *TOXC* is required for HC-toxin production, probably for the synthesis of Aeo (Walton et al., 1994; J.-H. Ahn and J.D. Walton, manuscript in preparation). *TOXD* (GenBank accession number X92391), present in SB111 in three copies, does not yet have any demonstrated role in HC-toxin biosynthesis and was used in this study solely as a mapping probe.

TOX2 was first mapped using rare-cutting restriction enzymes and endogenous sites. As shown in Table 1, the 8-bp recognition enzymes NotI, SfiI, PmeI, and AscI each generated two bands that hybridized with probe CC61 (Figure 1) and therefore cut at least once between the two copies of *HTS1*. PacI, however, generated a single band of 1 Mb containing both copies of *HTS1* (Table 1). PacI is a particularly useful mapping enzyme, because there is a PacI site ~20 kb in the 3′ direction of *HTS1*-2, as determined by mapping a genomic cosmid (pCos1) that begins near the 5′ end of *HTS1*-2 and extends in the 3′ direction for ~40 kb. The sizes of restriction fragments with each of the 8-bp recognition restriction enzymes that hybridized with a probe from *TOXC* are also shown in Table 1. Two copies of *TOXC* are on a 1-Mb PacI fragment and another is on a 20-kb PacI fragment. NotI, AscI, and PmeI generated three bands that hybridized with *TOXC*, whereas SfiI generated only two, of 240 and 280 kb. Therefore, two of the three copies of *TOXC* are within 280 kb of each other.

To refine the restriction map of *TOX2*, targeted transformation was used to introduce PacI sites into each of the copies of *HTS1*, *TOXC*, and *TOXD*. The location of the transforming plasmid in each case was determined by conventional agarose gel electrophoresis based on diagnostic enzyme digestions that could distinguish the different copies of each of the genes (ApaI fragments of 30 and 18 kb for *HTS1*-1 and *HTS1*-2, respectively; XhoI fragments of 14, 10, and 9 kb for *TOXC*-1, *TOXC*-2, and *TOXC*-3, respectively; and BamHI fragments of 15, 9, and 8 kb for *TOXD*-1, *TOXD*-2, and *TOXD*-3, respectively). As shown in Figure 7A, integration of a new PacI site into the 5′ end of *HTS1*-1 caused the 1-Mb band that hybridized with CC61 to disappear and one new band of 300 kb to appear. When the PacI site was inserted into *HTS1*-2, digestion with PacI resulted

Table 1. Restriction Fragment Lengths in Strains SB111 and 243-7

	Probe[a]			
	CC61		*TOXC*	
	Strain			
Enzyme[b]	SB111 (kb)	243-7 (kb)	SB111 (kb)	243-7 (kb)
PacI	1000	280	20; 1000	20; 280
NotI	40; 350	40	20[c]; 60	20[c]
SfiI	120; 280	280	240; 280	280
AscI	80; 200	200	35; 170; 200	35; 200
PmeI	35; 270	35	220; 270; 350	200; 350

[a] Probe CC61 is from the 3′ end of *HTS1*; the *TOXC* probe is an internal 1-kb portion of the *TOXC* gene.
[b] Genomic DNA was cut with the indicated enzyme, fractionated by CHEF, and analyzed by blotting and probing with the indicated probe.
[c] Based on hybridization intensity, this band is a doublet.

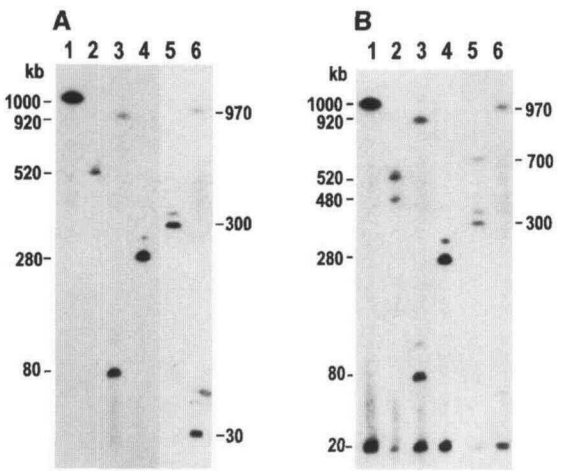

Figure 7. Mapping of *HTS1* and *TOXC* with Engineered Restriction Sites.

Chromosomal DNA from wild-type SB111, spontaneous chromosomal mutant 243-7, and transformants containing new PacI sites at the indicated locations was digested with PacI and fractionated by CHEF on a 1% agarose gel at 170 V with a 2- to 5-sec switching time for 22 hr and then a 60- to 120-sec switching time for 24 hr and blotted.

(A) Blot probed with CC61.

(B) The same blot probed with *TOXC*.

Lanes 1 contain SB111 (untransformed); lanes 2, transformant T569-3 with a new PacI site at *TOXC*-3; lanes 3, transformant T569-2 with a new PacI site at *TOXC*-2; lanes 4, strain 243-7 (untransformed); lanes 5, transformant T570-2 with a new PacI site at *HTS1*-1; lanes 6, transformant T570-1 with a new PacI site at *HTS1*-2. The fainter hybridized bands whose lengths are not indicated in lanes 4 to 6 in **(A)** and lanes 3 to 6 in **(B)** are consistent with partial cutting at endogenous PacI sites. Numbers at left and right indicate lengths in kilobases.

in two bands of 970 and 30 kb (Figure 7A). Therefore, the distance between the two copies of *HTS1* is 270 kb. The pattern of hybridization is consistent only with a tandem orientation of the two copies of *HTS1* (i.e., they are transcribed in the same direction). If the two copies of *HTS1* were transcribed in opposite directions, then strain T570-1, with a PacI site in *HTS1*-1, would give an additional hybridizing band of 700 kb with CC61, which it does not (Figure 7A, lane 5). Stripping the blot and reprobing with *TOXA*, which is on the other side of the inserted PacI site from CC61 (see Figure 1), gave a pattern that confirmed the orientation of *HTS1*-1 and *HTS1*-2; *TOXA* hybridized with two fragments of 300 and 700 kb in T570-1 and with one fragment of 970 kb in T570-2 (data not shown).

A similar strategy was used to map the location and transcriptional orientation of each copy of *TOXC*. The *TOXC* probe (Figure 7B and Table 2) extends over the site of integration of the PacI site and therefore hybridized with fragments from either side of the PacI site. As shown in Figure 7B, two copies of *TOXC* are on the same 1-Mb fragment as the two copies of *HTS1*, whereas the third copy (*TOXC*-1) is on a separate 20-kb PacI fragment. The locations of the three copies of *TOXD* in strain SB111 were also determined. The restriction fragment sizes generated with engineered PacI sites are summarized in Table 2. The transcriptional directions of *TOXC*-2 and *TOXC*-3 were determined by probing the transformed strains listed in Table 2 with *TOXC* probes from either side of the introduced PacI site. The orientation of *TOXC*-1 was determined by chromosome walking and sequencing: a genomic λ clone containing *TOXC*-1 that overlaps pCos1, which extends into *HTS1*-2 and contains a PacI site, was analyzed in detail by restriction mapping and sequencing.

Strain 243-7, described above, was used to test and extend the map predicted by the above results. In addition to *HTS1*-1, strain 243-7 is missing *TOXA*-1, *TOXC*-3, *TOXD*-1, and *TOXD*-3 and has the remaining genes (*HTS1*-2, *TOXA*-2, *TOXC*-1, *TOXC*-2, and *TOXD*-2) on a 2.1-Mb instead of a 3.5-Mb chromosome (Figure 8). As shown in Table 1, the PacI fragment that contains both copies of *HTS1*, which is 1 Mb in SB111, is only 280 kb in strain 243-7. All of the map distances are consistent with 243-7 having undergone a single, simple chromosomal break between *HTS1*-1 and *HTS1*-2 at ~20 kb from *HTS1*-1 (Table 1).

Because the *HTS1*-containing chromosome in 243-7 is 1.4 Mb smaller than the *TOX2* chromosome in SB111, it can be deduced that in SB111, *HTS1*-2 is 1.9 Mb from one end of the chromosome and 1.6 Mb from the other end, and that *HTS1*-1 is 1.3 and 2.2 Mb from the ends of the chromosome. To test these locations, the *cos* site of λ was introduced by targeted transformation into *HTS1*-1, and DNA from the transformant was cut with λ terminase (Wang and Wu, 1993). As shown in Figure 8, λ terminase does not cut the native *TOX2*

Table 2. Restriction Fragment Lengths in Strains with Engineered PacI Sites

Transformant[b]	Integration Site	Probe[a] CC61 (kb)	Probe[a] *TOXC* (kb)
Wild type	None	1000	20; 1000
T570-1	*HTS1*-1	300	20; 300; 700
T570-2	*HTS1*-2	30; 970	20; 970
T569-1	*TOXC*-1	1000	<20[c]; 1000
T569-2	*TOXC*-2	80; 920	20; 80; 920
T569-3	*TOXC*-3	520	20; 480; 520
T506-1	*TOXD*-1	350	20; 350; 650
T506-2	*TOXD*-2	50; 950	20; 950
T506-3	*TOXD*-3	410	20; 410; 590

[a] The gel was blotted and probed with either CC61 (from the 3′ end of *HTS1*) or a 1-kb internal fragment of the *TOXC* gene. Integration at *HTS1* was at the 5′ end of the gene.

[b] A PacI restriction site was transformed into the indicated gene, and DNA from the transformants was isolated, cut with PacI, and fractionated by CHEF.

[c] Size was too small to determine accurately.

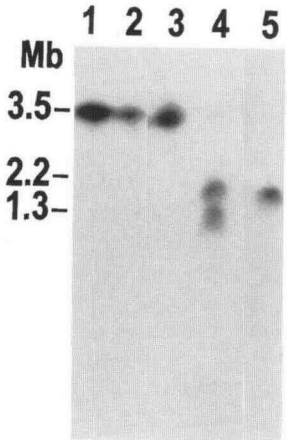

Figure 8. Mapping of the 3.5-Mb Chromosome of SB111 with an Engineered *cos* Site.

The fungus was transformed by homologous recombination at CC60 within *HTS1*-1 with a plasmid containing the *cos* site of λ. DNA was separated by CHEF (conditions are as given in the legend to Figure 2). The gel was blotted and probed with CC60. Lane 1 contains SB111; lane 2, SB111 cut with λ terminase; lane 3, transformant T571 containing *cos* at *HTS1*-1 (uncut); lane 4, transformant T571 cut with λ terminase; and lane 5, strain 243-7 (untransformed and uncut). Numbers at left indicate lengths in megabases.

chromosome of SB111 but does cut the chromosome into two fragments of 1.3 and 2.2 Mb after the introduction of the *cos* site into *HTS1*-1. This confirms the chromosomal locations of *HTS1*-1 and *HTS1*-2 determined by analysis of 243-7.

The maps of the *TOX2* chromosome of SB111 and 243-7 are summarized in Figure 9. G242 is deduced to lie to the right of the PacI site that is 20 kb to the right of *HTS1*-2 (Figure 9B), on the basis that it hybridized with the 2.1-Mb chromosome of 243-7 and did not hybridize with a 1-Mb PacI fragment in SB111. Figure 9A is an enlargement of the portion of the 3.5-Mb chromosome that contains *HTS1*, *TOXA*, *TOXC*, and *TOXD*. Figure 9C is a map of the *HTS1*-containing chromosome of strain 243-7.

DISCUSSION

Earlier Mendelian genetic studies indicated that a single gene, *TOX2*, controls HC-toxin production (Nelson and Ullstrup, 1961; Scheffer et al., 1967). However, in molecular terms, *TOX2* is not a single, simple genetic locus but rather a complex locus extending over >540 kb and containing multiple copies of multiple genes.

From this and earlier studies, the reasons HC-toxin production segregates as a single trait despite the size and complexity of *TOX2* are emerging. One is simply that, as shown here, all of the genes required for HC-toxin production are linked. Therefore, without analyzing large numbers of progeny, toxin production appears to segregate 1:1 in crosses between $Tox2^+$ and $Tox2^-$ isolates. The second is that $Tox2^-$ isolates lack homologous DNA necessary for crossing over within and perhaps between the genes of *TOX2*. All of the genes studied here (*HTS1*, *TOXA*, *TOXC*, and *TOXD*) are found only in $Tox2^+$ isolates. At least some of the DNA found between the copies of *HTS1* is

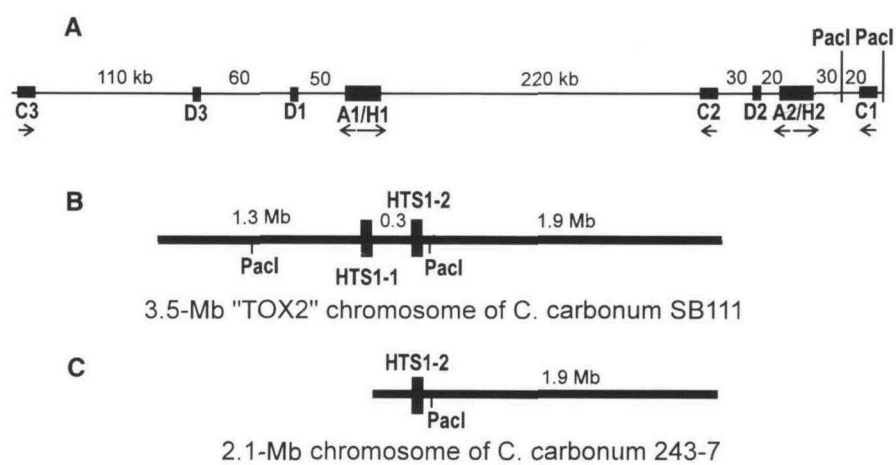

Figure 9. Physical Maps of the *HTS1*-Containing Chromosomes of SB111 and 243-7.

(A) Detailed map of the *HTS1* region of the 3.5-Mb chromosome of SB111. Distances are in kilobases. A1/H1 indicates *TOXA*-1 plus adjacent *HTS1*-1, and A2/H2 indicates *TOXA*-2 plus adjacent *HTS1*-2. C1, C2, and C3 are *TOXC*-1, *TOXC*-2, and *TOXC*-3, respectively. D1, D2, and D3 are *TOXD*-1, *TOXD*-2, and *TOXD*-3, respectively. Arrows indicate the directions of transcription of *HTS1*-1, *HTS1*-2, *TOXC*-1, *TOXC*-2, and *TOXC*-3. The black boxes that indicate gene locations are not to scale.

(B) Map of the entire 3.5-Mb *HTS1*-containing chromosome of SB111. Lines connect the corresponding sites in **(A)** and **(B)**. Distances are indicated in megabases.

(C) Deduced map of the entire *HTS1*-containing chromosome of 243-7. Distances are in megabases.

repetitive and is also found in Tox2$^-$ isolates, but whether there is sufficient homology to allow chromosome pairing and crossing over during meiosis is not known. Third, some mechanism appears to suppress crossing over within the *TOX2* complex. If the relationship between map distance and physical distance is the same in *C. carbonum* as it is in *C. heterostrophus* (23 kb per centimorgan; Tzeng et al., 1992), recombination between the two copies of *HTS1* should occur in ~12% of the progeny of a cross between two Tox2$^+$ isolates. However, the actual measured recombination in a cross between two strains with the identical haplotype of *TOX2* genes is <1% (Pitkin et al., 1996), indicating that crossing over in this region is, in fact, suppressed.

C. carbonum is like many other plant pathogenic fungi in that it has a high degree of heterogeneity in the sizes and numbers of its chromosomes (Mills and McCluskey, 1990; Talbot et al., 1991; Kistler and Miao, 1992; Andrew et al., 1993; Masel et al., 1993; Xu et al., 1995; Zolan, 1995). In particular, *C. carbonum* also has chromosomal variation associated with the pathogenicity gene *TOX2*. Translocations are a common cause of chromosome-length polymorphisms in filamentous fungi (Kistler and Miao, 1992; Zolan, 1995), and this is a plausible explanation for the variation seen in the sizes of the *HTS1*-containing chromosome among the *TOX2* isolates studied here. The mechanism driving high chromosomal recombination rates in fungi is not known, but the involvement of repetitive DNA has been suggested (Kistler and Miao, 1992; Zolan, 1995). It may therefore be significant that *HTS1* is flanked on both sides by regions of repetitive DNA related to transposable elements and that the 3.5-Mb chromosome containing *HTS1* is particularly rich in these sequences.

A chromosomal translocation is genetically tightly linked to the *TOX1* locus of *C. heterostrophus*, although it is not clear what significance this has for the structure of *TOX1* and the biosynthesis of T-toxin (Tzeng et al., 1992; Yoder et al., 1994). The putative translocation associated with *TOX2* within Tox2$^+$ isolates appears to have no effect on pathogenicity because all of the Tox2$^+$ isolates studied here are indistinguishable in their ability to produce HC-toxin and to cause disease.

It has been estimated, based on comparisons of total genome size in near-isogenic strains of *C. heterostrophus*, that isolates of this fungus that make T-toxin have ~1 Mb more DNA than isolates that do not (Tzeng et al. 1992). Whether this is true for all isolates of *C. heterostrophus* is not known. We have found that a lower limit of the amount of Tox2$^+$-unique DNA associated with *TOX2* is to date ~110 kb of DNA that is present in SB111 (Tox2$^+$) on the 3.5-Mb chromosome and absent in SB114 (Tox2$^-$). This includes all of the copies of *HTS1*, *TOXA*, *TOXC*, and *TOXD* as well as their flanking DNA. An upper limit of the amount of Tox2$^+$-unique DNA can be estimated at 1.5 Mb, because this is the difference in size between the chromosomes in SB111 and SB114 that hybridize to probe G242.

The molecular structure of *TOX2* is quite different than expected from its Mendelian behavior. In this regard, *TOX2* resembles several other genetic loci in plant pathogenic fungi whose Mendelian behavior belied their molecular structure (e.g., Lu et al., 1994; Van Etten et al., 1994; Yoder et al., 1994). Mechanisms by which genetically complex traits can appear to be controlled by single, simple loci in fungi include suppression of recombination, absence of homologous DNA in one of the parents in a cross, multifunctional enzymes, and gene clustering. *TOX2* contains elements of all of these.

The evolutionary origin of new races of toxin-producing plant pathogenic fungi has been a major issue for many years (Yoder et al., 1994). In regard to the origin of HC-toxin production, horizontal gene transfer originally appeared plausible for two reasons. One is the complete absence of *HTS1* and *TOXA* in Tox2$^-$ strains of *C. carbonum* (Panaccione et al., 1992). The other is the existence of Aeo-containing cyclic tetrapeptides in four other fungi that are not closely related to *C. carbonum* (Walton et al., 1995). On this basis, we tested the hypothesis that *HTS1* might have arisen in *C. carbonum* in the evolutionarily recent past by horizontal gene transfer from one of the other fungi that make Aeo-containing peptides (Nikolskaya et al., 1995). However, it now appears on several grounds that if horizontal gene transfer did contribute to the ability of *C. carbonum* to make HC-toxin, and hence to the origin of race 1, it was not by the recent transfer of a single, discrete piece of DNA. First, at least one other gene in addition to *HTS1*, *TOXC*, is required for HC-toxin production (J.-H. Ahn and J.D. Walton, manuscript in preparation). Second, the presence of two copies of *HTS1* and *TOXA* that are 270 kb apart in most if not all Tox2$^+$ isolates of *C. carbonum* argues that duplication and chromosomal rearrangements occurred subsequent to any putative horizontal gene transfer event. Third, if *TOXC* originally came with *HTS1* and *TOXA* by horizontal gene transfer, there have since been additional duplication and chromosomal rearrangement events that have resulted in three copies of *TOXC* being dispersed over 540 kb of DNA.

METHODS

Fungal Strains

All fungi were stored as spore suspensions in glycerol at −80°C. *Cochliobolus carbonum* SB111 (race 1, Tox2$^+$; ATCC 90305) and SB114 (race 2, Tox2$^-$) have been described previously (Walton, 1987). Field isolates 141R, 151, 161, 171, 181, 1101, and 1111 were obtained from L.D. Dunkle (Purdue University, West Lafayette, IN; Jones and Dunkle, 1993). Isolates ARG, YUGX, YUGY, and A2 were collected in the 1960s by the late R.R. Nelson (Pennsylvania State University, University Park) and were obtained from R.P. Scheffer (Michigan State University, East Lansing).

Pulsed-Field Gel Electrophoresis Conditions

Intact chromosomal DNA was prepared as described by Orbach et al. (1988). Chromosomal-grade agarose (Bio-Rad) was used. Contour-clamped homogenous field (CHEF) electrophoresis (CHEF-DR II; Bio-Rad) was performed in 0.5 × TBE buffer (1 × TBE is 45 mM Tris,

45 mM boric acid, 1 mM EDTA, pH 7.6) at 14°C. Voltage, switching intervals, and total running times are indicated in Figures 2, 4, and 7. The gels were stained with ethidium bromide for 30 min and destained in distilled water for 30 min. Sizes of chromosomes and chromosome fragments were calculated using yeast chromosome markers (Bio-Rad) and lambda concatemers (New England Biolabs, Beverly, MA).

For transfer and hybridization, CHEF gels were soaked in 0.25 M HCl for 30 min and then in 0.5 M NaOH plus 1.5 M NaCl for 30 min. DNA was transferred to Zetaprobe membrane (Bio-Rad) by capillary action with 0.4 M NaOH for 48 hr. The membranes were rinsed in 2 × SSPE (1 × SSPE is 150 mM NaCl, 10 mM $NaHP_2O_4$, 1 mM EDTA, adjusted to pH 7.4 with NaOH) and cross-linked with 120 μJ/cm² UV light using a UV Stratalinker (model 1800; Strategene). The membranes were prehybridized at 65°C for 1 hr in 5 × SSPE, 7% SDS, 0.5% nonfat dry milk, and 0.1 mg/mL denatured salmon sperm DNA, and hybridized in the same solution for 15 hr at 65°C. Probes were labeled with α-^{32}P-dCTP by random priming and used at a concentration of 10^5 to 10^6 dpm/mL. After hybridization, the membranes were washed twice in 2 × SSPE plus 0.1% SDS for 10 min at 21°C and twice in 0.1 × SSPE plus 0.1% SDS for 30 min at 65°C.

Construction of Mapping Plasmids and Fungal Transformation

The vector pAJ21, used for inserting PacI sites into the 5′ end of *HTS1*, was constructed by ligating a phosphorylated PacI linker (CCTTAATTAAGG) (New England Biolabs) to the unique SmaI site of pCC119 (see Figure 1; Panaccione et al., 1992). The vector for inserting PacI sites into *TOXC*, pAJ19, was a 1-kb HindIII-HindIII fragment from the middle of *TOXC* subcloned into the HindIII site of pHYG1 (Sposato et al., 1995) along with the PacI linker into the SmaI site. The vector for insertion of PacI sites into *TOXD*, pAJ20, was a 0.7-kb SalI-SmaI fragment from the 5′ end of *TOXD* (GenBank accession number X92391) subcloned into pHYG2 along with the PacI linker into the SmaI site. pHYG2 is pSP72 (Promega) containing the *C. heterostrophus* P1 promoter driving the *hph* gene (Turgeon et al., 1987). The vector for introduction of the λ *cos* site into *HTS1*-1 was constructed by subcloning the 2.1-kb BamHI-BamHI fragment from pCC60 (see Figure 1; Panaccione et al., 1992) into the unique BamHI site of pCosHyg1 (Turgeon et al., 1987). Fungal transformation was performed as described by Panaccione et al. (1992).

Restriction Enzyme Digestion of Agarose-Embedded Chromosomal DNA

Agarose plugs containing DNA (1 to 2 μg) were immersed in 1 mL of TE (10 mM Tris plus 1 mM EDTA, pH 8.0) and chilled on ice for 30 min. TE was replaced with the appropriate restriction endonuclease buffer, and the agarose plugs were again put on ice for 30 min. The restriction endonuclease buffer was replaced, and 30 units of restriction endonuclease (New England BioLabs) or 30 units of λ terminase (PanVera, Madison, WI) was added; the plugs were incubated overnight at the recommended temperature. After digestion, enzyme and buffer were removed by aspiration, and the plugs were washed with TE and loaded onto the CHEF gel.

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REFERENCES

Andrew, M.M., Irwin, J.A.G., and Manners, J.M. (1993). DNA addition or deletion is associated with a major karyotype polymorphism in the fungal phytopathogen *Colletotrichum gleosporioides*. Mol. Gen. Genet. **237,** 73–80.

Apel, P.C., Panaccione, D.G., Holden, F.R., and Walton, J.D. (1993). Cloning and targeted gene disruption of *XYL1*, a β-xylanase gene from the maize pathogen *Cochliobolus carbonum*. Mol. Plant-Microbe Interact. **6,** 467–473.

Bronson, C.R. (1991). The genetics of phytotoxin production by plant pathogenic fungi. Experientia **47,** 771–776.

Hohn, T.M., McCormick, S.P., and Desjardins, A.E. (1993). Evidence for a gene cluster involving tricothecene-pathway biosynthetic genes in *Fusarium sporotrichioides*. Curr. Genet. **24,** 291–295.

Jones, M.G., and Dunkle, L.D. (1993). Analysis of *Cochliobolus carbonum* races by PCR amplification with arbitrary and gene-specific primers. Phytopathology **83,** 366–370.

Kistler, H.C., and Miao, V. (1992). New modes of genetic change in filamentous fungi. Annu. Rev. Phytopathol. **30,** 131–152.

Lim, S.M., and Hooker, A.L. (1971). Southern corn leaf blight: Genetic control of pathogenicity and toxin production in race T and race O of *Cochliobolus heterostrophus*. Genetics **69,** 115–117.

Lu, S., Lyngholm, L., Yang, G., Bronson, C., Yoder, O.C., and Turgeon, B.G. (1994). Tagged mutations at the *Tox1* locus of *Cochliobolus heterostrophus* by restriction enzyme–mediated integration. Proc. Natl. Acad. Sci. USA **91,** 12649–12653.

Masel, A.M., Irwin, J.A.G., and Manners, J.M. (1993). DNA addition or deletion is associated with a major karyotype polymorphism in the fungal phytopathogen *Colletotrichum gloeosporioides*. Mol. Gen. Genet. **237,** 73–80.

Mills, D., and McClusky, K. (1990). Electrophoretic karyotypes of fungi: The new cytology. Mol. Plant-Microbe Interact. **3,** 351–357.

Nelson, R.R., and Ullstrup, A.J. (1961). The inheritance of pathogenicity in *Cochliobolus carbonum*. Phytopathology **51,** 1–2.

Nokolskaya, A.N., Panaccione, D.G., and Walton, J.D. (1995). Identification of peptide synthetase–encoding genes from filamentous fungi producing host-selective phytotoxins or analogs. Gene **165,** 207–211.

Orbach, M.J., Vollrath, D., Davis, R.W., and Yanofsky, C. (1988). An electrophoretic karyotype of *Neurospora crassa*. Mol. Cell. Biol. **8,** 1469–1473.

Panaccione, D.G., Scott-Craig, J.S., Pocard, J.-A., and Walton, J.D. (1992). A cyclic peptide synthetase gene required for pathogenicity of the fungus *Cochliobolus carbonum* on maize. Proc. Natl. Acad. Sci. USA **89,** 6590–6594.

Panaccione, D.G., Pitkin, J.W., Walton, J.D., and Annis, S.L. (1996). Transposon-like sequences at the *TOX2* locus of the plant-pathogenic fungus *Cochliobolus carbonum*. Gene, in press.

Pitkin, J.W., Panaccione, D.G., and Walton, J.D. (1996). A putative HC-toxin efflux pump encoded by the *TOXA* gene of the plant pathogenic fungus *Cochliobolus carbonum*. Microbiology, in press.

Scheffer, R.P., Nelson, R.R., and Ullstrup, A.J. (1967). Inheritance of toxin production and pathogenicity in *Cochliobolus carbonum* and *Cochliobolus victoriae*. Phytopathology **57,** 1288–1289.

Scott-Craig, J.S., Panaccione, D.G., Pocard, J.-A., and Walton, J.D. (1992). The cyclic peptide synthetase catalyzing HC-toxin production in the filamentous fungus *Cochliobolus carbonum* is encoded by a 15.7-kilobase open reading frame. J. Biol. Chem. **267,** 26044–26049.

Sposato, P., Ahn, J.-H., and Walton, J.D. (1995). Characterization and disruption of a gene in the maize pathogen *Cochliobolus carbonum* encoding a cellulase lacking a cellulose binding domain and a hinge region. Mol. Plant-Microbe Interact. **8,** 602–609.

Talbot, N.J., Oliver, R.P., and Codington, A. (1991). Pulsed field gel electrophoresis reveals chromosome length differences between strains of *Cladosporium fulvum* (syn. *Fulvia fulva*). Mol. Gen. Genet. **229,** 267–272.

Turgeon, B.G., Garber, R.C., and Yoder, O.C. (1987). Development of a fungal transformation system based on selection of sequences with promoter activity. Mol. Cell. Biol. **7,** 3297–3305.

Tzeng, T.-W., Lyngholm, L.K., Ford, C.F., and Bronson, C.R. (1992). A restriction fragment length polymorphism map and electrophoretic karyotype of the fungal maize pathogen *Cochliobolus heterostrophus*. Genetics **130,** 81–96.

Van Etten, H., Soby, S., Wasmann, C., and McCluskey, K. (1994). Pathogenicity genes in fungi. In Advances in Molecular Genetics of Plant–Microbe Interactions, Vol. 3, M.J. Daniels, J.A. Downie, and A.E. Osbourn, eds (Dordrecht, The Netherlands: Kluwer Academic Publishers), pp. 163–170.

Walton, J.D. (1987). Two enzymes involved in biosynthesis of the host-selective phytotoxin HC-toxin. Proc. Natl. Acad. Sci. USA **84,** 8444–8447.

Walton, J.D. (1990). Peptide phytotoxins from plant pathogenic fungi. In Biochemistry of Peptide Antibiotics, H. Kleinkauf and H. von Döhren, eds (Berlin: De Gruyter), pp. 179–203.

Walton, J.D., and Holden, F.R. (1988). Properties of two enzymes involved in biosynthesis of the fungal pathogenicity factor HC-toxin. Mol. Plant-Microbe Interact. **1,** 128–134.

Walton, J.D., Ahn, J.-H., Akimitsu, K., Pitkin, J.W., and Ransom, R. (1994). Leaf spot disease of maize: Chemistry, biochemistry, and molecular biology of a host-selective cyclic peptide. In Advances in Molecular Genetics of Plant–Microbe Interactions, Vol. 3, M.J. Daniels, J.A. Downie, and A.E. Osbourn, eds (Dordrecht, The Netherlands: Kluwer Academic Publishers), pp. 231–237.

Walton, J.D., Bronson, C.R., Panaccione, D.G., Braun, E.J., and Akimitsu, K. (1995). *Cochliobolus*. In Pathogenesis and Host Specificity in Plant Diseases, Vol. 2, K. Kohmoto, U.S. Singh, and R.P. Singh, eds (Oxford, UK: Elsevier), pp. 65–81.

Wang, Y., and Wu, R. (1993). A new method for specific cleavage of megabase-size chromosomal DNA by λ-terminase. Nucleic Acids Res. **21,** 2143–2147.

Xu, J.-R., Yan, K., Dickman, M.B., and Leslie, J.F. (1995). Electrophoretic karyotypes distinguish the biological species of *Gibberella fujikuroi* (*Fusarium* section Liseola). Mol. Plant-Microbe Interact. **8,** 74–84.

Yoder, O.C., Yang, G., Rose, M.S., Lu, S.W., and Turgeon, B.G. (1994). Complex genetic control of polyketide toxin production by *Cochliobolus heterostrophus*. In Advances in Molecular Genetics of Plant–Microbe Interactions, Vol. 3, M.J. Daniels, J.A. Downie, and A.E. Osbourn, eds (Dordrecht, The Netherlands: Kluwer Academic Publishers), pp. 223–230.

Zolan, M. (1995). Chromosome-length polymorphisms in fungi. Microbiol. Rev. **59,** 686–698.