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

Joong-Hoon Ahn and Jonathan D. Walton
Department of Energy-Plant Research Laboratory, Michigan State University, East Lansing, Michigan 48824

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
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 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 Sall. 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 Apal (of 22 six-base restriction enzymes tested) (Figure 1; Panaccione et al., 1992). When cut with Apal and probed with CC48, genomic DNA of SB111 yields two bands of 18 and 30 kb (Figure 1).

The 5' Apal 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-
Chromosomes of C. carbonum; isolates SB111 (race 1, Tox2 carbonum 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 Mb (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-β-1,4-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 Apal fragment, but the second copy of CC48 is on a 13, 18, or 19-kb Apal 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 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.

The restriction maps of copies 1 and 2 are identical between the two copies of HTS1, resulting in the loss of one copy of HTS1. Strain 243-7 lacks both the 30-kb Apal fragment that hybridizes with CC48 and the 8.5-kb BamHI fragment that hybridizes with CC61. On the basis of this strain, the 5' Apal 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 Sall 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.

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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 Apal 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 TOX4.

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 al., 1993).
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 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.

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 both copies of HTS7 and all copies of HTS7) and 2.0 Mb (containing G242), with the 1.5-Mb fragment then joining with the 0.7-Mb fragment (containing G242), as seen in chromosomes of 2.2 Mb (containing both copies of HTS7 and all copies of HTS7) and 2.0 Mb (containing G242), 2.0 Mb (containing CC61), 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 HTS7 and all copies of HTS7) 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. TOX2, 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 TOX2 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, Pmel, and AciI each generated two bands that hybridized with probe CC61 (Figure 1) and therefore cut at least once between the two copies of HTS1. Pacl, however, generated a single band of 1 Mb containing both copies of HTS1 (Table 1). Pacl is a particularly useful mapping enzyme, because there is a Pacl site ~20 kb in the 3' direction of HTS1-2, as determined by mapping a genomic cosmid (pCost) 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 Pacl fragment and another is on a 20-kb Pacl fragment. NotI, AciI, and Pmel 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 Pacl 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 (Apal 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 Pacl 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 Pacl site was inserted into HTS1-2, digestion with Pacl resulted in fragments of 280, 200, and 120 kb.

| Table 1. Restriction Fragment Lengths in Strains SB111 and 243-7 |
|-------------------|------------------|------------------|------------------|
| Strain            | Probea           | CC61 (kb)        | TOXC (kb)        |
|                   |                  |                  |                  |
|                   | Pacl             | 1000             | 280              | 20; 1000          | 20; 280           |
|                   | Notl             | 40; 350          | 40               | 20; 60            | 20               |
|                   | SfiI             | 120; 280         | 280              | 240; 280          | 280              |
|                   | AciI             | 80; 200          | 200              | 35; 170; 200      | 35; 200          |
|                   | Pmel             | 35; 270          | 35               | 220; 270; 350     | 200; 350         |
|                   |                  |                  |                  |                  |
| SB111             |                  |                  |                  |                  |
|                   |                  |                  |                  |
|                   |                  |                  |                  |

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<td>35</td>
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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.
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 Pacl 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 Pacl site, then strain T570-1, with a Pacl site in H7S7-1, reprobed with TOXA, confirmed the orientation of H7S7-1 and contains a Pacl 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 TOAXA-1, TOAXC-1, TOXD-1, and TOXD-3 and has the remaining genes (HTS1-2, TOAXA-2, TOAXC-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 Pacl 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

![Figure 7. Mapping of HTS1 and TOXC with Engineered Restriction Sites.](image)

Chromosomal DNA from wild-type SB111, spontaneous chromosomal mutant 243-7, and transformants containing new Pacl sites at the indicated locations was digested with Pacl and fractionated by CHEF on a 1% agarose gel at 170 V with a 2- to 5-s switching time for 22 hr and then a 60- to 120-s switching time for 24 hr and blotted. (A) Blot probed with CC61. (B) The same blot probed with TOXA. Lanes 1 contain SB111 (untransformed); lanes 2, transformant T569-3 with a new Pacl site at HTS1-2; lanes 3, transformant T569-2 with a new Pacl site at TOXC-1; lanes 4, strain 243-7 (untransformed); lanes 5, transformant T570-2 with a new Pacl site at HTS1-1; lanes 6, transformant T570-1 with a new Pacl 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 Pacl sites. Numbers at left and right indicate lengths in kilobases.

![Table 2. Restriction Fragment Lengths in Strains with Engineered Pacl Sites](image)

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<td>T569-1</td>
<td>TOAXC-1</td>
<td>1000</td>
<td>&lt;200; 1000</td>
<td></td>
</tr>
<tr>
<td>T569-2</td>
<td>TOAXC-2</td>
<td>80; 920</td>
<td>20; 80; 920</td>
<td></td>
</tr>
<tr>
<td>T569-3</td>
<td>TOAXC-3</td>
<td>520</td>
<td>20; 480; 520</td>
<td></td>
</tr>
<tr>
<td>T506-1</td>
<td>TOXO-1</td>
<td>950</td>
<td>20; 950</td>
<td></td>
</tr>
<tr>
<td>T506-2</td>
<td>TOXO-2</td>
<td>410</td>
<td>20; 410; 590</td>
<td></td>
</tr>
<tr>
<td>T506-3</td>
<td>TOXO-3</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

- a The gel was blotted and probed with either CC61 (from the 3' end of HTS1) or a 1-kb internal fragment of the TOX1 gene. Integration at HTS1 was at the 5' end of the gene.
- b A Pacl restriction site was transformed into the indicated gene, and DNA from the transformants was isolated, cut with Pacl, and fractionated by CHEF.
- c Size was too small to determine accurately.
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 Pacl 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 Pacl 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
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. hetrostrrophus (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. hetrostrrophus, 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. hetrostrrophus, 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. hetrostrrophus 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, 1990). Isolates APG, 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 homogeneous field (CHEF) electrophoresis (CHEF-DR II; Bio-Rad) was performed in 0.5 x TBE buffer (1 x TBE is 45 mM Tris, 45 mM boric acid, 1.5 mM EDTA, pH 8.0) with a field strength of 5 V/cm and a pulse ramp ranging from 0 to 120 seconds over 20 hours at 14°C.
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 x SSPE (1 x SSPE is 150 mM NaCl, 10 mM NaH₂PO₄, 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; Stratagene). The membranes were prehybridized at 65°C for 1 hr in 5 x 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-32P-dCTP by random priming and used at a concentration of 10⁶ to 10⁷ dpm/μL. After hybridization, the membranes were washed twice in 2 x SSPE plus 0.1% SDS for 10 min at 21°C and twice in 0.1 x 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 (CTTAAATTAAGG) (New England Biolabs) to the unique SmaI site of pCCII9 (Turgeon et al., 1987). The vector for inserting PacI sites into TOXD, pAJ20, was a 0.7-kb SalI-SmaI fragment from the 5’ end of TOXD (GenBank accession number X92391) along with the PacI linker into the SmaI site. The vector for insertion of PacI sites into TOXD, pAJ19, was a 1-kb HindIII-HindIII fragment from the middle of TOXO 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, pAJ19, 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 lac 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 1 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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TOX2 of Cochliobolus carbonum


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