An Extended Physical Map of the *TOX2* Locus of *Cochliobolus carbonum* Required for Biosynthesis of HC-Toxin

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Ahn, J.-H., Cheng, Y.-Q., and Walton, J. D. 2002. An extended physical map of the *TOX2* locus of *Cochliobolus carbonum* required for biosynthesis of HC-toxin. *Fungal Genetics and Biology* **35,** 31–38. In genetic crosses, HC-toxin production in the filamentous fungus *Cochliobolus carbonum* appears to be controlled by a single locus, *TOX2.* At the molecular level, *TOX2* is composed of at least seven duplicated and coregulated genes involved in HC-toxin biosynthesis, export, and regulation. All copies of four of the *TOX2* genes were previously mapped within a 540-kb stretch of DNA in strain SB111. Subsequently, an additional three *TOX2* genes, *TOXE*, *TOXF*, and *TOXG*, have been discovered. In this paper we have mapped all copies of the new genes, a total of seven, and show that except for one of the two copies of *TOXE*, which was previously shown to be on a chromosome of 0.7 Mb in strain SB111, they are all linked to the previously known *TOX2* genes within ~600 kb of each other on a chromosome of 3.5 Mb. We show here that this chromosome also contains at least one non-*TOX2* gene, *EXG2*, which encodes an exo-*β*1,3-glucanase. *EXG2* is still present in strains that have undergone spontaneous deletion of up to ~1.4 Mb of the 3.5-Mb chromosome. The results contribute to our understanding of the complex organization of the genes involved in HC-toxin biosynthesis and are consistent with the hypothesis that a reciprocal chromosomal translocation accounts for the pattern of distribution of the *TOX2* genes in different *C. carbonum* isolates. © 2002 Elsevier Science (USA)

Index Descriptors: cyclic peptide; gene cluster; host-selective toxin; plant pathogenesis.

Filamentous fungi produce a variety of secondary metabolites of medical, industrial, and ecological importance. Examples include penicillin, cyclosporin, ergot alkaloids, aflatoxins, and amatoxins. Ecologically, secondary metabolites are important virulence and specificity factors for many plant pathogenic fungi, especially those in the genera *Alternaria* and *Cochliobolus* (Kohmoto and Otani, 1991; Walton, 1996).

Many of the genes involved in fungal secondary metabolism share certain characteristic features, such as clustering, absence even from closely related nonproducing isolates, and association with chromosomal rearrangements or dispensable (supernumerary) chromosomes (Keller and Hohn, 1997; Walton, 2000). For example, 25 genes involved in sterigmatocystin biosynthesis are clustered within 60 kb in *Aspergillus nidulans* (Brown *et al.,* 1996), and 3 penicillin biosynthetic genes are clustered in *Penicillium chrysogenum* and *Aspergillus nidulans* (Diez *et al.,* 1990; MacCabe *et al.,* 1990). T-toxin production in *Cochliobolus heterostrophus* requires 2 genes that, because of a translocation, are genetically but not physically linked, and both genes are absent from closely related isolates that do

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not make T-toxin (Kodama *et al.,* 1999). Genes required for AK-toxin in *Alternaria alternata* Japanese pear pathotype are physically linked and absent from other species of *A. alternata* (Tanaka and Tsuge, 2000).

Other genes that control fungal virulence but that are not known to be associated with secondary metabolism have also been shown to have one or more of these characteristics. A set of genes required for virulence, including *PDA1* encoding pisatin demethylase, are clustered on a dispensable chromosome in *Nectria haematococca* MP VI (Han *et al.,* 2001; Covert, 1998). Some isolates of *Cladosporium fulvum* pathogenic on tomato containing *Cf9* lack the *avr9* gene (van Kan *et al.,* 1991). The telomere-localized avirulence gene *AVR-Pita* in *Magnaporthe grisea* is unstable, resulting in its complete absence in many virulent isolates (Orbach *et al.,* 2000). The gene for NIP1, a race-specific peptide elicitor from the barley pathogen *Rhynchosporium secalis,* is absent in most virulent isolates (Rohe *et al.,* 1995). A chromosome in *Colletotrichum gloeosporioides* affecting host range is dispensable and transferable (He *et al.,* 1998).

Race 1 (Tox2$^+$) isolates of the maize pathogen *Cochliobolus* (*Helminthosporium*) *carbonum* race 1 produce the host-selective toxin, HC-toxin, of structure cyclo(D-Pro-L-Ala-D-Ala-L-Aeo), where Aeo stands for 2-amino-9,10-epoxi-8-oxodecanoic acid. Mendelian and molecular genetic analyses of the pathogen and the host indicate that HC-toxin is a critical virulence and specificity factor in the interaction between maize and *C. carbonum* (Walton, 1996).

In genetic crosses, production of HC-toxin appears to be controlled by a single locus, *TOX2* (Scheffer *et al.,* 1967). At the molecular level, *TOX2* is a complex locus composed of at least seven genes with an established or probable role in HC-toxin biosynthesis, export, and regulation. All of these genes are present in two or three copies in all naturally occurring toxin-producing (Tox2$^+$) isolates of *C. carbonum,* and all are absent from natural toxin nonproducing (Tox2$^-$) isolates. Unlike some of the copies of the ACR-toxin biosynthetic genes in *A. alternata* (Tanaka and Tsuge, 2000), all of the copies of the known *TOX2* genes appear to be functional. This conclusion is based on the fact that Tox2$^-$ strains have never been recovered by disrupting fewer than all of the copies of any particular *TOX2* gene. However, because not all copies of some of the *TOX2* genes have been disrupted in all combinations, it is possible that some of them are nonfunctional.

There are seven known genes within *TOX2*. *HTS1* encodes a 570-kDa nonribosomal peptide synthetase, *TOXA* encodes a putative HC-toxin efflux carrier, *TOXC* encodes a fatty acid synthase beta subunit, *TOXD* encodes a putative dehydrogenase (with no role yet established in HC-toxin biosynthesis), *TOXE* encodes a pathway-specific transcription factor, *TOXF* encodes a putative branched-chain amino acid transaminase, and *TOXG* encodes an alanine racemase (Panaccione *et al.,* 1992; Pitkin *et al.,* 1996; Ahn and Walton, 1996, 1997, 1998; Cheng *et al.,* 1999; Cheng and Walton, 2000).

In some isolates, including the laboratory strain SB111, all copies of *HTS1, TOXA, TOXC,* and *TOXD* are located within a 540-kb region on the largest (3.5-Mb) chromosome (Ahn and Walton, 1996, 1998; Canada and Dunkle, 1997). One copy of *TOXE* was later shown to be somewhere on this same chromosome, with the other copy on a chromosome of 0.7 Mb (Ahn and Walton, 1998). We call this particular pattern of *TOX2* gene distribution Type 1. In contrast, in other isolates, typified by isolate 151 and called Type 2, all of the *TOX2* genes, including both copies of *TOXE,* are on a chromosome of 2.2 Mb (Ahn and Walton, 1996, 1998; Canada and Dunkle, 1997).

The *TOX2* locus of *C. carbonum* is genetically unstable. Major deletions in the *TOX2* region occur in ~5% of random ascospore progeny (Pitkin *et al.,* 2000). Some of the deletion strains lack ~1.4 Mb of the 3.5-Mb chromosome yet show as their only phenotype a reduction in virulence. Thus, it appears that a large portion of the 3.5-Mb chromosome is dispensable (Pitkin *et al.,* 2000). It is unknown whether the entire chromosome is dispensable because all of the deletion strains retain at least ~1.9 Mb of the 3.5-Mb chromosome. Furthermore, there is evidence that this chromosome contains expressed single-copy genes common to Tox2$^+$ and Tox2$^-$ isolates that are not involved in toxin biosynthesis and might have housekeeping functions (Cheng *et al.,* 1999). Mapping studies with the *TOX2* genes and with other probes led to the hypothesis that the 3.5- and 0.7-Mb chromosomes (Type 1) are related to the 2.2-Mb chromosome (Type 2) by a reciprocal translocation (Ahn and Walton, 1996, 1998). The translocation partner of the 2.2-Mb chromosome in Type 2 isolates was postulated to be a chromosome of 2.0 Mb. To date, this chromosome has been found to contain only genes common to Tox2$^+$ and Tox2$^-$ isolates.

Since the original mapping studies (Ahn and Walton, 1996), three additional *TOX2* genes (*TOXE, TOXF,* and *TOXG*) have been characterized. *TOXE* is present in two copies in all Tox2$^+$ isolates, and in isolates with a 3.5-Mb *TOX2* chromosome one copy was shown to be somewhere on this chromosome (Ahn and Walton, 1998). *TOXF* and *TOXG* are tightly and divergently clustered, their tran-

scriptional start sites being separated by 195 bp. Most Tox2^{+} isolates contain three copies of the *TOXF/G* cluster (Cheng *et al.,* 1999; Cheng and Walton, 2000). Because *TOXF* and *TOXG* were identified by their presence on the same bacterial artificial chromosome (BAC) as one copy of *TOXD,* at least one copy of *TOXF/G* must be physically linked to the other *TOX2* genes (Cheng *et al.,* 1999), but otherwise their locations have not been established.

To understand the structure of the *TOX2* locus more completely, we have now physically mapped *TOXE, TOXF,* and *TOXG* relative to the other *TOX2* genes in isolate SB111 of *C. carbonum.* The results shed light on the evolutionary history of this complex locus and support the hypothesis that a reciprocal translocation has affected the chromosomal distribution of the *TOX2* genes since the origin of HC-toxin biosynthesis in *C. carbonum.*

MATERIALS AND METHODS

C. carbonum strain SB111 (race 1; Tox2^{+}; ATCC 90305) was stored as a spore suspension in 25% (v/v) glycerol at −80°C and routinely grown on V8-juice agar plates. Wild-type *C. carbonum* Tox2^{+} isolates such as 151 were obtained from L. Dunkle, Purdue University (Ahn and Walton, 1996; Canada and Dunkle, 1997). Deletion strains are described in Pitkin *et al.* (2000).

pCB1004 (Carroll *et al.,* 1994) containing the gene encoding hygromycin phosphotransferase driven by the *trpC* promoter of *A. nidulans* was digested with *Sma*I, dephosphorylated, and ligated with a *Pac*I linker (New England Biolabs). The resulting plasmid was digested with *Pst*I and ligated with a 2.9-kb *Pst*I fragment containing fragments of *TOXG* and *TOXF* (Cheng *et al.,* 1999). This plasmid (AJ62) was linearized with *Bbr*PI before transformation. Protoplast isolation and fungal transformation were done as described (Scott-Craig *et al.,* 1990; Apel *et al.,* 1993). Transformants were selected for their ability to grow in the presence of hygromycin (100 μg/ml) and purified to nuclear homogenity by two rounds of single-spore isolation.

C. carbonum genomic DNA was isolated as described by Pitkin *et al.* (1996). The genomic lambda library has been described (Panaccione *et al.,* 1992). The BAC library, described by Cheng *et al.* (1999), was transferred from 96-well microtiter plates to nylon membranes (ZetaProbe; Bio-Rad) and screened with a 2.9-kb *Pst*I fragment containing *TOXF/G.* The *TOXE* fragment used as probe was a 1-kb *Eco*RI/*Eco*RI fragment (Ahn and Walton, 1998).

DNA blotting and hybridization followed standard protocols (Sambrook *et al.,* 1989).

The alkaline lysis method (Sambrook *et al.,* 1989) was used to isolate BAC DNA. BAC DNA was digested with *Asc*I and fractionated by pulsed-field gel electrophoresis (PFGE) using a CHEF II system (Bio-Rad). Conditions were 1% agarose, 170 V, with a 0.5- to 2-s switching interval over 22 h.

Restriction enzyme digestion of agarose-embedded chromosomal DNA was performed as described with some modifications (Ahn and Walton, 1996). The plugs were soaked in restriction enzyme buffer and 20 units of restriction enzyme in Eppendorf tubes, stored overnight on ice, and then incubated at the recommended temperature for 4 h. Conditions for fractionation of large restriction fragments by pulsed-field gel electrophoresis were as previously described (Ahn and Walton, 1996).

RESULTS

Mapping of TOXE, TOXF, and TOXG

The location of the copy of *TOXE* that is on the 3.5-Mb *TOX2* chromosome in strain SB111 was determined by chromosome walking. A 40-kb cosmid insert (called cos2), which includes part of *HTS1* copy 2 and flanking genomic DNA beyond the 3′ end, was overlapped with a genomic lambda clone insert (called λE) that contained an endogenous *Pac*I site and *TOXE.* The distance from the end of copy 2 of *HTS1* to *TOXE* was estimated to be ~20 kb based on restriction mapping of cos2 and λE.

The lambda clone was mapped with restriction enzymes, and a 2.0-kb *Bam*HI fragment containing the *Pac*I site was used to probe a blot of *Pac*I-digested genomic DNA from *C. carbonum* SB111. The *Bam*HI fragment hybridized to two fragments of 1 Mb and 20 kb, which are the same sizes as the fragments to which the three copies of *TOXC* hybridize, and we assume that they are, in fact, the same fragments (Ahn and Walton, 1996). This result indicated that the 20-kb *Pac*I fragment containing copy one of *TOXC* (*TOXC*-1) abuts the 1-Mb *Pac*I fragment that contains the two other copies of *TOXC.*

In *C. carbonum* isolate SB111, the three copies of *TOXF/G* are present on restriction fragments of different sizes when cut with *Apa*I, *Bgl*I, *Bst*XI, *Kpn*I, or *Pvu*I. *TOXF/G*-1, *TOXF/G*-2, and *TOXF/G*-3 refer to the copies found on *Apa*I fragments of 18, 16, and 9.6 kb, respectively (Cheng and Walton, 2000). To determine the chro-
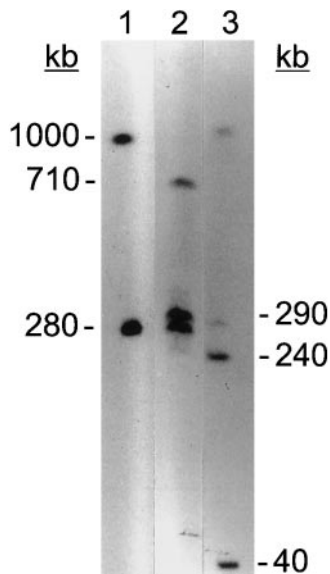
FIG. 1. Mapping of *TOXF/G* by introduction of *Pac*I sites. Chromosomal DNA was digested with *Pac*I, fractionated by PFGE, blotted, and probed with a *TOXG* cDNA. Lane 1, *C. carbonum* wild type (SB111); lane 2, SB111 transformed with a *Pac*I site into copy 1 of *TOXF/G*; lane 3, SB111 transformed with a *Pac*I site into copy 3 of *TOXF/G*.

mosomal location of these three copies, a DNA blot of intact chromosomes from isolate SB111 was probed with a *TOXG* cDNA. All copies of *TOXG* and *TOXF* were located on the 3.5-Mb chromosome in those isolates that have *HTS1* on this chromosome, and all copies were on a 2.2-Mb chromosome in those isolates that have the *TOX2* genes on a chromosome of this size (data not shown).

To obtain a physical map of the three copies of *TOXG* and *TOXF* on the 3.5-Mb chromosome, chromosomal DNA of strain SB111 was digested with *Pac*I, the fragments were separated by PFGE, and the gel was blotted and probed with a *TOXG* cDNA. *TOXG* hybridized to fragments of 1 Mb and 280 kb (Fig. 1, lane 1). The 1-Mb *Pac*I fragment was assumed to be the same fragment that contains two copies of *HTS1, TOXA,* and *TOXC* and three copies of *TOXD* (Ahn and Walton, 1996). The 280-kb *Pac*I fragment that hybridizes to *TOXG* was not the same size as any of the *Pac*I fragments that hybridize with any of the other *TOX2* genes.

To map the copies of *TOXF/G* more precisely on the 3.5-Mb chromosome, a *Pac*I site was introduced into each copy of *TOXG* by homologous transformation. Twelve transformants were isolated and analyzed by DNA blotting. The location of the transforming plasmid was determined by conventional agarose gel electrophoresis after

digesion of genomic DNA with *Apa*I, which can distinguish the different copies of *TOXF/G.* Chromosomal DNA from transformants in which the plasmid containing the *Pac*I site had integrated into different copies of *TOXG* was digested with *Pac*I, fractionated by PFGE, blotted, and probed with a *TOXG* cDNA. When a *Pac*I site was inserted into copy one of *TOXF/G* (*TOXF/G*-1), subsequent digestion with *Pac*I resulted in disappearance of the 1-Mb hybridizing band and appearance of two new bands of 290 and 710 kb (Fig. 1, lane 2). On the same blot, *TOXE* hybridized to a 290-kb band, presumed to be the same one (data not shown). Therefore, *TOXF/G*-1 is 290 kb away from an endogenous *Pac*I site, which places it ~20 kb from the end of *HTS1*-1 in the direction of *HTS1*-2 (Fig. 2). Insertion of a *Pac*I site into *TOXF/G*-2 and subsequent digestion of the transformant DNA with *Pac*I resulted in the generation of *TOXG*-hybridizing fragments of 370 and 630 kb, and *TOXE* hybridized to a 370-kb fragment, presumed to be the same one (data not shown). This places *TOXF/G*-2 between *TOXD*-1 and *TOXD*-3 (Fig. 2).

The location of *TOXF/G*-3 was determined by chromosome walking using the lambda and BAC genomic libraries. The BAC library was screened with a *TOXC* cDNA, and a 65-kb BAC insert, 24F11, that contains copy 1 of *TOXC* on a 20-kb *Pac*I fragment, was found. Restriction analysis of this BAC indicated that its two *Pac*I sites were on *Bam*HI fragments of 4.0 and 2.0 kb. The 2.0-kb *Bam*HI fragment is the same one that is present in λE (see above) and thus contains the *Pac*I site that is closest to *TOXE*. The *Pac*I site on the 4.0-kb *Bam*HI fragment represents a *Pac*I site on the other side of *TOXE* away from copy 2 of *HTS1.* This 4.0-kb *Bam*HI fragment hybridized to fragments of 20 and 280 kb on a blot of total chromosomal DNA digested with *Pac*I. The 20-kb fragment is assumed to correspond to the *Pac*I fragment on which *TOXC*-1 is located, and the 280-kb fragment is assumed to be the same *Pac*I fragment to which the third copy of *TOXF/G* hybridized. In summary, the order of *Pac*I fragments across the *TOX2* region is 1 Mb, 20 kb, and 280 kb from left to right (Fig. 2).

The *Pac*I/*Bam*HI fragment from the 4.0-kb *Bam*HI fragment that hybridized only with the 280-kb *Pac*I fragment was cloned and named pPC/B. The location of copy 3 of *TOXF/G* (*TOXF/G*-3) was determined by introducing a *Pac*I site into this copy. Digestion of the transformant with *Pac*I and probing of the blot with a *TOXG* cDNA resulted in hybridization to two fragments of 40 and 240 kb and to a fragment of 1 Mb representing copies 1 and 2 of *TOXF/G* (Fig. 1, lane 3). When stripped and reprobed with pPC/B, only the 40-kb band hybridized. These results
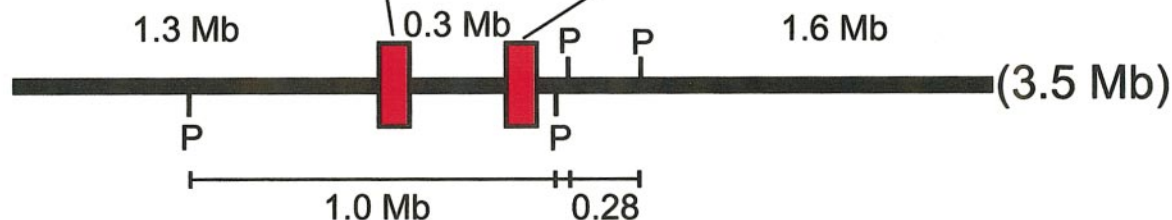
A *TOX2* region

B whole chromosome

FIG. 2. Map of the *TOX2* locus of *C. carbonum* SB111 (revised from Ahn and Walton, 1996). A/H, the *TOXA/HTS1* cluster; C, *TOXC;* D, *TOXD;* E, *TOXE;* F/G, the *TOXF/TOXG* cluster. Directions of transcription are indicated where known. (A) Local region spanning ~600 kb; distances are in kb. (B) Map of the entire 3.5-Mb chromosome of SB111; distances are in Mb. P stands for native *Pac*I sites (not all sites on the chromosome are shown). The copies of *HTS1* in (A) and (B) are connected by lines. The boxes representing the genes are not drawn to scale.

indicate that *TOXF/G*-3 is located ~40 kb from the end of the 20-kb *Pac*I fragment containing *TOXC*-1.

The deduced absolute and relative locations of all copies of all known *TOX2* genes in isolate SB111, with the exception of one copy of *TOXE,* is shown in Fig. 2. The other copy of *TOXE* was earlier shown to be on a 0.7-Mb chromosome in SB111 and has not been further mapped (Ahn and Walton, 1998). The accuracy of this map (Fig. 2) is limited by the resolution of PFGE and by the assumption that hybridization of two probes to restriction fragments of the same size means that the fragments are the same DNA.

Is the 3.5-Mb TOX2 Chromosome Dispensable?

Because the *TOX2* genes are present only in Tox2$^+$ isolates and because engineered Tox2$^-$ mutants are fully viable, the genes necessary for HC-toxin biosynthesis, which to date total >90 kb of DNA, are clearly dispensable. It was earlier shown by the analysis of strains with spontaneous deletions of the *TOX2* genes in isolate SB111 that ~1.4 Mb of the 3.5-Mb *TOX2* chromosome of SB111 is dispensable (Pitkin *et al.,* 2000). This 1.4-Mb region

maps to the left-hand end of the 3.5-Mb chromosome as drawn in Fig. 2. However, the right-hand end of the *TOX2* chromosome is still present in all of the deletion strains described by Pitkin *et al.* (2000), and therefore this region of the 3.5-Mb chromosome might contain essential and dispensable genes. The 3.5-Mb chromosome of SB111 does, in fact, contain at least one non-*TOX2* gene, *EXG2*, which encodes an exo-β1,3-glucanase (GenBank Accession No. AF229446; Kim et al., 2001)(Fig. 3). In Type 2 isolates, in which all of the *TOX2* genes are on a 2.2-Mb chromosome, *EXG2* is on a 2.0-Mb chromosome (data not shown). *EXG2* is on a chromosome of 2.0 Mb in Tox2$^-$ isolates also (data not shown). Because *EXG2* is still present in all of the *TOX2* deletion strains (Fig. 3) it is on the right-hand end of the 3.5-Mb chromosome (Fig. 2). Insofar as *EXG2* can be considered a housekeeping gene (although it is not a *TOX2* gene and is present in all tested *C. carbonum* isolates, it is dispensable for growth and virulence; Kim *et al.,* 2001), the 3.5-Mb chromosome is thus a composite of housekeeping and dispensable genes.

The deduced chromosomal distribution of all copies of all *TOX2* genes and the U-repeat, G242, and *EXG2* in Type 1 and Type 2 Tox2$^+$ isolates of *C. carbonum* are shown schematically in Fig. 4. The U-repeat and G242 are
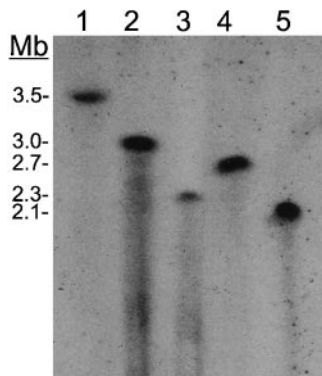
FIG. 3. Sizes of the chromosomes containing *EXG2* (Kim *et al.,* 2001) in strains of SB111 with spontaneous deletions in the 3.5-Mb *TOX2* chromosome. Lane 1, *C. carbonum* SB111; lane 2, strain 164R10; lane 3, strain 512-3; lane 4, strain 543-48; lane 5, 243-7 (Pitkin *et al.,* 2000).

probes that were previously mapped to the chromosomal level (Ahn *et al.,* 1996). The "U-repeat" is a sequence present in subcloned fragment CC62, which is adjacent to the 3′ end of *HTS1* (Panaccione *et al.,* 1992). The U-repeat is present in multiple copies in Tox2⁺ isolates (see Fig. 1 of Panaccione *et al.,* 1992; J.-H. Ahn and J. D. Walton, unpublished results). The U-repeat is absent from Tox2⁻ isolates and is found flanking both copies of *HTS1,* all three copies of *TOXC,* and both copies of *TOXE* in isolate SB111 (Ahn and Walton, 1997, 1998). The U-repeat is on chromosomes of 3.5 and 0.7 Mb in Type 1 isolates and on a chromosome of 2.2 Mb in Type 2 isolates (Ahn and Walton, 1996). G242 is a mapping probe from *C. heterostrophus* (Tzeng *et al.,* 1992). It was previously shown to be present as a single copy in both Tox2⁺ and Tox2⁻ isolates and to be present on a 3.5-Mb chromosome in Type 1 isolates and on a 2.0-Mb chromosome in Type 2 isolates (Ahn and Walton, 1996).

DISCUSSION

All copies of the *TOX2* genes had previously been mapped to a region of ~500 kb on a 3.5-Mb chromosome in isolate SB111 of *C. carbonum* (Ahn and Walton, 1996, 1998). Here we show that, with one exception, all copies of three new genes (*TOXE, TOXF,* and *TOXG*) are also part of the same extended gene cluster. The exception is one of the copies of *TOXE,* which had previously been shown to be on a different chromosome in SB111 (Ahn and Walton, 1998). However, in other isolates both copies of *TOXE* are

also linked to the other *TOX2* genes on the same 2.2-Mb chromosome (Ahn and Walton, 1998). Collectively, these results are consistent with the original genetic studies showing that the trait of HC-toxin biosynthesis appears to be controlled by a single Mendelian locus (Scheffer *et al.,* 1967).

The physical organization of the genes for HC-toxin biosynthesis have characteristics that are different from those of other fungal secondary metabolite pathways. Some of the genes are tightly clustered (e.g., *HTS1* with *TOXA* and *TOXF* with *TOXG*) like other fungal secondary metabolite genes, but altogether all copies of the known genes of *TOX2* are dispersed over more than 600 kb. There is evidence that some of the genes involved in other fungal secondary metabolite pathways are also dispersed, e.g., *TRI101,* which encodes an *O*-acetyltransferase involved in trichothecene biosynthesis in *Fusarium* species, and at least one of the aflatoxin biosynthetic genes (Kimura *et al.,* 1998; McCormick *et al.,* 1999; Woloshuk and Prieto, 1998). The *TOX2* genes are also unusual among fungal secondary metabolite genes in being duplicated or triplicated. The penicillin biosynthetic gene cluster is present in 5–14 copies in some strains of *Penicillium chrysogenum,* but this is probably a result of intensive artificial selection for strain improvement (Fierro *et al.,* 1995). One consequence of the duplication of the *TOX2* genes is that the trait of HC-toxin production would be predicted to be quite refractory to loss by mutation. On the other hand, this genetic stability appears to be counteracted, at least in isolates such as SB111, by the tendency of the 3.5-Mb *TOX2* chromosome to undergo major deletions (Pitkin *et al.,* 2000).

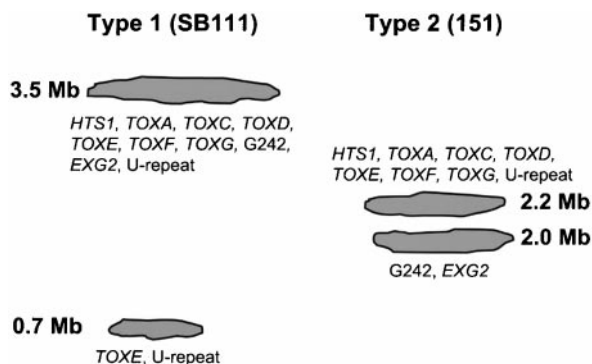The results in this paper are consistent with the hypothesis (Ahn and Walton, 1996) that the Type 1 (3.5 Mb) and

FIG. 4. Graphic summary of the distribution of known DNA sequences among the chromosomes in two types of Tox2⁺ isolates of *C. carbonum* (Ahn and Walton, 1996; Canada and Dunkle, 1997).

Type 2 (2.2 Mb) *TOX2* chromosomes are related to each other by a reciprocal translocation (Ahn and Walton, 1996) (Fig. 4). Additional strong support for the relatedness of these two chromosomes is the fact that they behave as homologs during meiosis (Canada and Dunkle, 1997).

With regard to whether Type 1 evolved from Type 2 or vice versa, it is significant that all of the copies of all of the *TOX2* genes are on a single chromosome of 2.2 Mb in Type 2 isolates, whereas the *TOX2* genes are found on chromosomes of two sizes, 3.5 and 0.7 Mb, in Type 1 isolates. Furthermore, in Type 2 isolates no *TOX2* genes and all of the DNA sequences common to Tox2$^+$ and Tox2$^-$ isolates (i.e., G242 and *EXG2*) are found on a chromosome of 2.0 Mb, whereas in Type 1 isolates the 3.5-Mb chromosome contains both *TOX2* and non-*TOX2* genes. Because *known* mechanisms of genomic rearrangements in fungi (e.g., translocation, unequal crossing over, etc.) are more likely to lead over time to greater gene dispersal rather than to greater gene linkage, Type 2 is probably ancestral to Type 1.

A relatively recent origin for the *TOX2* genes is suggested by the fact that all natural Tox2$^-$ isolates, and other species of *Cochliobolus,* completely lack the genes for HC-toxin biosynthesis (Panaccione *et al.,* 1992). Because the genes are clustered, it would have been possible for the trait of HC-toxin biosynthesis to have moved between fungi by a process such as horizontal gene transfer of a subchromosomal fragment of DNA (Rosewich and Kistler, 2000; Walton, 2000). Based on our current knowledge, the smallest contiguous fragment of DNA that could have resulted in transfer of the trait of HC-toxin biosynthesis is 100 kb, which is the size of the region surrounding copy 2 of *HTS1* containing at least one copy of each of the known *TOX2* genes (Fig. 2). The minimum amount of DNA for complete HC-toxin production might be larger, however, because not all of the steps of HC-toxin biosynthesis are accounted for by the known genes; e.g., the fatty acid synthase β subunit (the product of *TOXC*) requires an unknown interacting α subunit (Ahn and Walton, 1997). If one assumes that the *TOX2* genes arrived in the genome of *C. carbonum* as a contiguous single-copy gene cluster, the present distribution could have arisen from a combination of gene duplication and translocation (Nikolskaya *et al.,* 1995; Walton, 1996). Because the *TOX2* genes are duplicated or triplicated in both Type 1 and Type 2 isolates (Ahn and Walton, 1996, 1997; Cheng *et al.,* 2000), the generation of the extra copies probably occurred before the translocation. Thus, the chain of events leading to the present genomic organization of *TOX2* in Type 1 isolates

of *C. carbonum* is proposed to be horizontal transfer, duplication, and translocation.

ACKNOWLEDGMENTS

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