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By

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ABSTRACT

MOLECULAR GENETICS OF VIRULENCE IN
COCHLIOBOLUS CARBONUM

By

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$Tox2^+$ isolates of the filamentous fungus *Cochliobolus carbonum* produce a cyclic tetrapeptide, HC-toxin, that is necessary for virulence on certain genotypes of maize. HC-toxin production is controlled by *TOX2* genes that are located on the supernumerary *TOX2* chromosome. In this work, the roles of horizontal gene transfer and *TOX2* chromosome instability in the evolution of the *C. carbonum* virulence were evaluated.

Four other filamentous fungi unrelated to *C. carbonum* produce cyclic peptides closely related to HC-toxin, raising the possibility that the genes involved in the synthesis of these compounds have moved between these fungi by horizontal gene transfer. In order to test this hypothesis, genomic DNA sequences encoding non-ribosomal peptide synthetases were amplified from different fungi by PCR. In addition, the genomic locus encoding a peptide synthetase from *Diheterospora chlamydosporia* was cloned, and a partial DNA sequence was obtained. The deduced amino acid sequences of the non-ribosomal peptide synthetases obtained from these fungi were found to be closely related to the *C. carbonum* HC-toxin synthetase (HTS), but the percent amino acid identity was lower than expected if these genes have been recently transferred horizontally.

A study of alterations in the *TOX2* chromosome of *C. carbonum* revealed exceptional meiotic instability of this chromosome. Of 200 progeny analyzed in crosses

between $Tox2^+$ and $Tox2^-$ isolates and between isolates in which the *TOX2* genes were on chromosomes of different sizes, eight (4%) had lost at least one copy of one of the *TOX2* genes. All of them still had at least one functional copy of each of the *TOX2* genes. The deletion strains were characterized with respect to virulence, HC-toxin production, *TOX2* gene expression, and size of the *TOX2* chromosome. Most deletions could be explained by simple chromosome breaks resulting in the loss of major contiguous portions (0.8 Mb to 1.4 Mb) of the 3.5-Mb *TOX2* chromosome. Most strains were still completely virulent, but two strains displayed a novel phenotype of reduced virulence (RV), characterized by lesions that expanded at a reduced rate and an inability to colonize plants systemically. Although the RV strains produced no detectable HC-toxin in culture, the RV phenotype was dependent on the presence of a functional copy of *HTS1*. We propose that the RV strains still make a low level of HC-toxin, at least *in planta*, and that the RV strains are missing unknown gene(s) that play a role in, but are not absolutely required for, HC-toxin production.

In addition, genomic and cDNA copies of *EXG1,* a gene encoding a novel exo-β-1,3-glucanase from *C. carbonum*, were isolated and sequenced. The deduced amino acid sequence of *EXG1* contains two imperfect copies of a 23-amino acid motif that is found in several other proteins that interact with polysaccharides, including two exo-β-1,3-glucanases, plant and bacterial polygalacturonases, PZA phage neck appendage protein, K1 phage endoneuraminidase, and bacterial mannuronan epimerase.

In a study of the function of the amino-acid activating domains of HTS, domain A was expressed separately in a $Tox2^-$ strain and found to activate L-Proline.

But you yourself should not be able

To tell defeats from victories of yours.

- Boris Pasternack

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INTRODUCTION

Interactions between plants and their microbial pathogens constitute an important area of study both because of the basic biological mechanisms involved and because plant diseases often pose a serious threat to agricultural productivity. Many plant-pathogen interactions are very complex, being a result of co-evolution directed by the reciprocal selection pressure that the pathogen and the host plant impose on each other. Pathogen specificity and plant resistance are the results of this co-evolutionary process.

The concepts of host specificity and host range describe the restriction of a particular pathogen to particular host plants. Both organisms, the pathogen and the plant host, contribute to their interaction and to the outcome of this interaction. Consequently, two genomes and two sets of biochemical and physiological traits are involved. The presence of two genomes adds to the challenge of dissecting the genetic elements controlling these interactions. If a particular pathogen can cause disease on a particular plant, that plant is considered to be susceptible to that pathogen, and the interaction is called "compatible". If no disease results, the interaction is called "incompatible". At the start of both types of interactions, one or a few plant cells are invaded by the pathogen. In an incompatible interaction, growth of the pathogen ceases without invading more than a few plant cells, but in a compatible interaction the pathogen continues to spread from the initial site of infection and causes disease symptoms (Walton, 1997).

All phytopathogenic microorganisms have a set of traits that allow them to colonize their host. These traits, or factors, are generally divided into basic and host-specific pathogenicity factors. Basic pathogenicity (or compatibility) factors are non-

specific; they can similarly affect a broad range of plants and allow a microorganism to penetrate and colonize host tissue and to use it as a source of nutrition. Without some minimum set of these factors, a microorganism is capable only of saprophytic growth and can not be a plant pathogen. Pathogen-secreted cell wall degrading enzymes are thought to be part of this attack "arsenal", helping the pathogen to penetrate the cuticle and epidermal cell walls of the host plant (Walton, 1994). Numerous studies of bacterial and fungal cell wall degrading enzymes have been aimed at determining which ones are important for pathogenicity. Of the many cell wall degrading enzymes studied, only three (one cutinase and two polygalacturonases) have so far been shown to have a role in host penetration and/or virulence. Introduction of a cutinase gene from *Nectria haematococca (Fusarium solani* f sp *pisi)* into the obligate wound pathogen *Mycosphaerella sp.* enabled this fungus to penetrate the intact cuticle of papaya fruits (Dickman et al., 1989). Recently, two polygalacturonases were shown to be required for full virulence of fungal plant pathogens: P2c contributes to invasion and colonization of cotton bolls by *Aspergillus flavus*, as determined by gene disruption and by introducing the gene into the strain that previously lacked it (Shieh et al., 1997) and BcPG1 contributes to the growth of *Botrytis cinerea* lesions on tomato leaves and on apple and tomato fruits, as determined by gene replacement experiment (ten Have et al., 1998).

In contrast to the basic pathogenicity factors, host-specific, or host-selective, pathogenicity factors (e.g., host-selective toxins) are designed to break a defense of a particular host. Thus, they are positive determinants of host range and disease specificity. Plant species, varieties, cultivars or genotypes that are sensitive to a host-selective pathogenicity factor are susceptible to the producing pathogen, and the resulting plant-

microbe interaction is compatible (Walton, 1996). In contrast to host-selective toxins, host-selective elicitors are defined as pathogen-derived inducers of plant resistance leading to an incompatible interaction (Walton, 1997).

Although many plant pathogenic bacteria and fungi produce phytotoxins, most of these toxins are nonselective and could be considered to be basic pathogenicity factors. All known host-selective toxins are produced by fungi. Most of them are produced by fungi belonging to the genera *Cochliobolus* and *Alternaria*. Other well-studied fungi producing host-selective toxins are *Phyllosticta maydis* (PM-toxin) and *Periconia circinata* (peritoxin). All known host-selective toxins, except for Ptr-toxin from *Pyrenophora tritici-repentis*, are low molecular weight compounds with diverse structures. Ptr-toxin is a ribosomally synthesized polypeptide (Ballance et al., 1989; Walton, 1996).

Fungi, as a group, are the most economically important plant pathogens. They are also very diverse in their life cycles, morphology, and in the nature and complexity of their interactions with plants, ranging from obligate pathogens to saprophytes, which can cause opportunistic plant infections (Alexopoulos and Mims, 1979).

Cochliobolus carbonum Nelson (anamorph, *Helminthosporium carbonum* Ullstrup, synonym *Bipolaris zeicola* (G. L. Stout) Shoemaker) is a filamentous fungus belonging to the *Ascomycetes* (*Ascomycota*). It causes northern leaf spot and ear mold of maize (*Zea mays* L.) and was first discovered when it appeared suddenly on susceptible inbred cultivars of maize in the late 1930s (Ullstrup, 1941). $Tox2^{+}$ (race 1) isolates of this fungus produce a host-selective toxin known as HC-toxin, a cyclic tetrapeptide of the structure cyclo(D-prolyl-L-alanyl-D-alanyl-L-Aeo), where Aeo is 2-amino-9,10-epoxi-8-

oxodecanoic acid (Figure 1). This toxin is required for the pathogenicity of *C. carbonum* on susceptible cultivars of maize (Scheffer and Livingston, 1984). For the toxin to be active, the epoxide group and the carbonyl group of the Aeo component are required (Walton and Earle, 1983; Kim et al., 1987). Scheffer et al. (1967) showed that toxin production and virulence segregate 1:1 in the progeny of crosses between HC-toxin producing ($Tox2^+$ or race 1, virulent) and non-producing ($Tox2^-$ or race 2, non-virulent) isolates of *C. carbonum*. This indicated that a single Mendelian locus, termed *TOX2*, controls HC-toxin production.

Both race 1 and race 2 isolates of *C. carbonum* occur naturally in maize fields. Maize cultivars that are homozygous for the *hm1* allele are sensitive to HC-toxin and susceptible to race 1 isolates of *C. carbonum* (Nelson and Ullstrup, 1964; Meeley et al., 1992). If the inoculum is large enough, the whole plant dies in several days (Scheffer et al., 1967). If the maize cultivar harbors the dominant *Hm1* allele, it is resistant to race 1 isolates. Race 2 isolates of *C. carbonum* (non-toxin producing) cause only small, necrotic flecks on both susceptible (*hm1/hm1*) and resistant (*Hm1*/-) maize cultivars (Ullstrup, 1941; Scheffer et al., 1967). If HC-toxin is added to race 2 spores before inoculation, the fungus colonizes susceptible maize leaves with symptoms similar to those caused by race 1 (Comstock and Scheffer, 1973). *Hm1*, a dominant maize gene responsible for resistance to *C. carbonum*, was shown to encode HC-toxin reductase, an enzyme that inactivates HC-toxin by reducing the carbonyl group of the Aeo component (Meeley et al., 1992; Johal and Briggs, 1992).

The most important enzyme in HC-toxin biosynthesis is HC-toxin synthetase (HTS) (Walton, 1987; Walton and Holden, 1988). This enzyme catalyzes the L-proline,

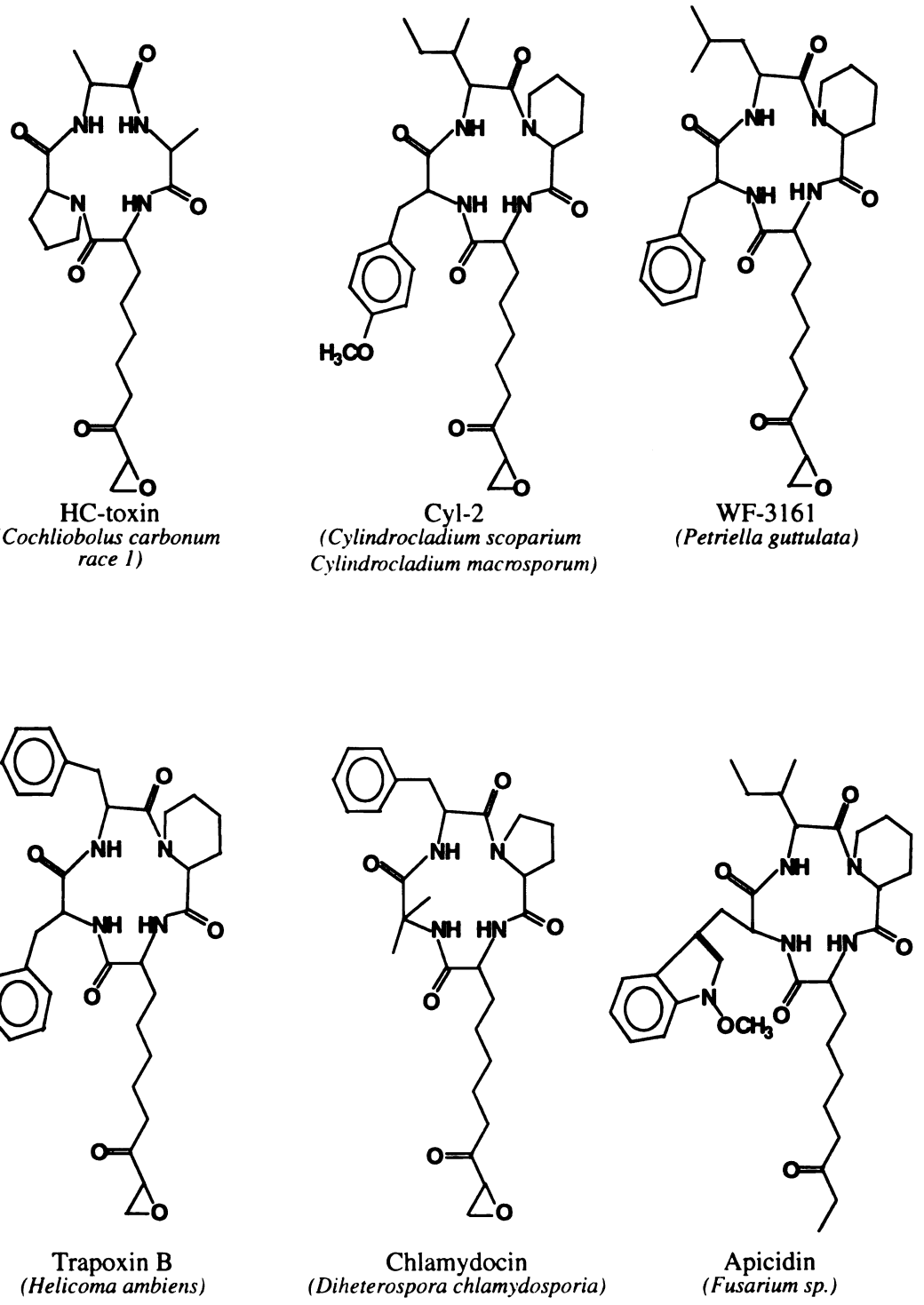

Figure 1. The family of cyclic tetrapeptides from filamentous fungi: Aeo-containing peptides and apicidin (after Walton et al., 1997; Darkin-Rattray et al., 1996).

D-alanine, and L-alanine dependent ATP/PP_i exchange and epimerizes L-proline to D-proline and L-alanine to D-alanine (Walton and Holden, 1988). HTS is encoded by a gene called *HTS1*, which consists of a single 15.7-kb open reading frame (Scott-Craig et al., 1992) and is present in two copies in most naturally occurring race 1 isolates of *C. carbonum* (Ahn and Walton, 1996). Both copies are functional, since disruption of both copies results in the loss of HTS enzyme activity, toxin production and virulence, while disruption of either copy alone results in a wild type phenotype (Panaccione et al., 1992).

In addition to *HTS1*, there are other genes involved in HC-toxin production. Adjacent to both copies of *HTS1*, and transcribed in the opposite direction, is *TOXA*, a gene predicted to encode a small molecule efflux pump. It has proven to be impossible to recover mutants with both copies of *TOXA* disrupted (Pitkin et al., 1996). Thus, *TOXA* may be essential for the protection of the fungus against HC-toxin, potentially by transporting the toxic compound out of the cell. Another gene, *TOXC*, encodes a protein that is highly similar to the β subunit of fatty acid synthetase and may be involved in the biosynthesis of Aeo. Different isolates of *C. carbonum* have two or three copies of *TOXC*. When all copies are disrupted, toxin production and virulence are lost (Ahn and Walton, 1997). Another gene unique to $Tox2^+$ strains, *TOXD*, has no known function and its disruption has no effect on toxin production or virulence (Ahn and Walton, 1996; Y. Cheng, unpublished data). *TOXE*, a gene encoding a DNA-binding protein, is required for expression of *TOXA*, *TOXC* and *TOXD*, but not *HTS1* (Ahn and Walton, 1998). TOXEp protein has been shown to bind the *TOXA* promoter (K. Pedley, unpublished data).

The genes that have been identified so far are probably not sufficient for HC-toxin

biosynthesis. There may be more genes involved in Aeo biosynthesis (e.g., α subunit of fatty acid synthetase interacting with *TOXC*, genes involved in the biosynthesis of the epoxide group, genes for the regulation of toxin production).

The complexity of HC-toxin biosynthesis and the number of genes involved raised the question of why toxin production appears to segregate genetically as a single Mendelian locus. This was addressed by physical mapping combined with pulsed field gel electrophoresis analysis (Ahn and Walton, 1996). All known HC-toxin biosynthetic genes are unique to $Tox2^{+}$ strains, being found only in isolates that produce HC-toxin and completely lacking in naturally occurring avirulent, non-toxin producing isolates. All known HC-toxin biosynthetic genes were found to reside on the same supernumerary chromosome, called the *TOX2* chromosome. In different $Tox2^{+}$ isolates, two partially homologous *TOX2* chromosomes, one 3.5 Mb and another 2.2 Mb in length, were found. The known *TOX2* genes were physically mapped on the 3.5-Mb chromosome in strain SB111 (Ahn and Walton, 1996). $Tox2^{-}$ isolates lack the chromosome of corresponding size, which can explain the Mendelian segregation of HC-toxin production. Since at least part of this chromosome is missing in $Tox2^{-}$ isolates, this part (called the *TOX2* module) is conditionally dispensable and is assumed to be essential only for HC-toxin production and thus for virulence on susceptible maize. Since *C. carbonum* competes well as a facultative saprophyte, the presence or absence of this *TOX2* module may be part of natural genetic diversity within this species. It is also possible that *TOX2* module has been acquired from some other species at the time prior to the first corn leaf spot disease outbreak. Isolates harboring the *TOX2* module may be favored when a compatible host is present, and in the absence of such a host, isolates without it may be favored because of a

decrease of parasitic fitness resulting from unnecessary toxin production. In fact, the frequency of $Tox2^+$ isolates in the absence of susceptible maize varieties in the field is less than 2% of the total population of C. *carbonum*, while in the presence of uniform plantings of susceptible maize $Tox2^+$ (race 1) isolates are frequent (ca. 50% of all the isolates from infected plants) and cause damage (Leonard, 1978).

The size polymorphism of different variants of the *TOX2* chromosome and the hybridization pattern of different markers can be explained by a reciprocal translocation involving four chromosomal segments (Ahn and Walton, 1996). This explains two facts: at least some $Tox2^-$ isolates do contain a chromosome (2.0 Mb in size) that contains at least two single-copy sequences (markers G242 and Q) that are also present on the 3.5-Mb chromosome of SB111 (Ahn and Walton, 1996; Canada and Dunkle, 1997), and in some isolates the second copy of *TOXE* is located on the 0.7-Mb chromosome which is assumed to be involved in the translocation (Ahn and Walton, 1998).

The mode of action of HC-toxin is not clearly understood. It does not cause cell death. It is known to inhibit mammalian cell division (Walton et al., 1985) and to increase the uptake of certain ions, amino acids and nitrate (Yoder and Scheffer, 1973a, 1973b). It has been proposed that HC-toxin blocks the ability of a susceptible maize plant to develop a defense response to a pathogen attack, thus allowing the fungus to colonize the host plant tissue. It was shown that HC-toxin significantly inhibits histone deacetylase (Brosch et al., 1995). As histone deacetylase is considered to be involved in global gene regulation, this may be a way in which HC-toxin affects expression of plant defense genes.

Apart from the host-selective toxin HC-toxin, *C. carbonum,* like any other plant

pathogen, must produce and secrete a number of basic pathogenicity factors that allow colonization of susceptible host tissue. The ability to penetrate the plant tissue is very important in this respect. Germinating *C. carbonum* enters plants predominantly through the intact cuticle and epidermis, rather than using wounds or stomata (90% of fungal penetrations are at cell junctions and 10% at stomatal openings [Jennings and Ullstrup, 1957]). Based on the ultrastructural analysis of the initial penetration (Murray and Maxwell, 1975), and on the data on melanin-deficient mutants (which are deficient in hydrostatic pressure in the appressorium) (Kubo et al., 1989; Kubo et al., 1991; J. Pitkin, unpublished data), plant cell wall dissolution is thought to be more important than mechanical pressure for the *C. carbonum* penetration. Therefore, cell wall-degrading enzymes must play an important role in *C. carbonum* pathogenicity. To date, 17 genes encoding various cell wall degrading enzymes from *C. carbonum* have been cloned and null mutants have been created for most of them via transformation-mediated gene disruption (reviewed by Scott-Craig et al., 1998b). None of the genes known so far was found to be essential for virulence. This may be due in part to their redundancy, since most cell wall-degrading enzymes exist as multiple isoenzymes, encoded by different genes. Studies of the mutants deficient in several of these genes (multiple gene disruptions) are in progress and may reveal sets of enzymes at least one of which is required to be present in order for the fungus to be virulent.

The mechanisms by which plant pathogenic fungi evolve and change the host range and virulence are poorly understood. In this study, molecular mechanisms of *C. carbonum* virulence and pathogenicity were investigated. The role of horizontal gene transfer and supernumerary chromosome (*TOX2* chromosome) instability in the evolution

of the *C. carbonum* virulence were evaluated. In order to assess the possible role of horizontal gene transfer in the acquisition of non-ribosomal peptide synthetase-encoding genes by *C. carbonum* and several other fungal species, the corresponding deduced amino acid sequences were compared, and found to be not similar enough to support the horizontal gene transfer hypothesis. A study of alterations in the *TOX2* chromosome revealed exceptional meiotic and mitotic instability of this chromosome, and it was shown that alterations of this chromosome can result in a novel phenotype of reduced virulence. In addition, *EXG1*, the gene encoding β-1,3-exoglucanases from *C. carbonum*, was cloned and found to be a member of a novel glucanase family.

Chapter 1

IDENTIFICATION OF PEPTIDE SYNTHETASE-ENCODING GENES FROM FILAMENTOUS FUNGI PRODUCING HOST-SELECTIVE PHYTOTOXINS OR THEIR ANALOGS

Abstract

Part of this chapter was published as Nikolskaya et el., 1995.

Race 1 ($Tox2^+$) isolates of *Cochliobolus carbonum* produce a cyclic tetrapeptide, HC-toxin, that is necessary for their virulence on certain genotypes of maize. The synthesis of HC-toxin is catalyzed by a 570-kDa, multifunctional enzyme, HC-toxin synthetase (HTS). The gene encoding HTS (*HTS1*) is absent from other races of *C. carbonum* and from other species of *Cochliobolus*. Four other unrelated filamentous fungi produce cyclic peptides closely related to HC-toxin, raising the possibility that the corresponding non-ribosomal peptide synthetase-encoding genes have moved between these fungi by horizontal gene transfer. Degenerate PCR primers were designed based on several highly conserved amino acid motifs common to known non-ribosomal peptide synthetase domains and used to amplify genomic sequences from different fungi. PCR products representing non-ribosomal peptide synthetase genes from *Diheterospora chlamydosporia*, which produces the HC-toxin analog chlamydocin, and *Cylindrocladium macrosporum*, which produces the analog Cyl-2, were cloned and analyzed. The genomic locus encoding the corresponding putative peptide synthetase from *D. chlamydosporia* was cloned and mapped, and partial sequence was obtained. All putative non-ribosomal

peptide synthetase amino acid sequences obtained from these fungi were found to be closely related to HTS, but the percent amino acid identity was not consistent with a very recent horizontal movement of these genes.

Introduction

Cyclic peptides (and some linear peptides) are synthesized by a class of enzymes known as non-ribosomal peptide synthetases (reviewed by Kleinkauf and von Döhren, 1996; Cane et al., 1998). Multifunctional peptide synthetases, those that catalyze activation of more than one amino acid, are organized into synthetase units (ca. 1000 - 1500 amino acids in length), one for each amino acid substrate. Each unit contains conserved motifs known or believed to be involved in adenylate formation, ATP binding, peptide bond formation, and sometimes epimerization and cyclization (Gocht and Marahiel, 1994; Kleinkauf and von Döhren, 1996). The most highly conserved part of each unit (ca. 600 amino acids), the amino acid activating domain, performs aminoacyl adenylation and thioester binding.

HC-toxin (Figure 1, p. 5) is a cyclic tetrapeptide produced by the filamentous fungus *Cochliobolus carbonum*. It is a critical pathogenicity determinant in the interaction between *C. carbonum* and its host, maize, *Zea mays* (Panaccione et al., 1992). A non-ribosomal peptide synthetase of 570 kDa called HTS, encoded by *HTS1* (Scott-Craig et al., 1992), catalyses activation of L-Pro, D-Ala and L-Ala, and epimerizes L-Pro and L-Ala, and is presumed to activate the fourth amino acid, 2-amino-9,10-epoxi-8-oxodecanoic acid (Aeo) or an Aeo precursor and to polymerize and cyclize the peptide. HTS contains four conserved domains, each approximately 600 amino acids long, which

are similar to each other and to conserved amino-acid activating domains from other non-ribosomal peptide synthetases. The protein sequences between domains are approximately 1000, 550 and 550 amino acids long. Each of the four domains is believed to activate one of the four amino acids, L-Pro, D-Ala, L-Ala and Aeo.

Naturally occurring isolates that do not produce HC-toxin completely lack the locus encoding HTS, which raises the question of the origin of HC-toxin production in *C. carbonum*. Non-toxin producing isolates might have lost *HTS1* or it might have been acquired by the toxin-producing strains by horizontal gene transfer from another organism. Four other unrelated filamentous fungi produce cyclic tetrapeptides structurally similar to HC-toxin (Figure 1; Table 1). To explore the possibility that *C. carbonum* might have acquired the capacity to synthesize HC-toxin by horizontal gene transfer of *HTS1* from one of these other fungi, we isolated the corresponding non-ribosomal peptide synthetase genes from these species and compared their sequences.

Prior to this study, the strategies used to isolate fungal non-ribosomal peptide synthetase genes have involved cross-hybridization with bacterial non-ribosomal peptide synthetase genes (Smith et al., 1990) or purification of the non-ribosomal peptide synthetases to raise antibodies to screen expression libraries (Haese et al., 1993) or to obtain amino acid sequences to be used for designing oligodeoxyribonucleotide probes (Scott-Craig et al., 1992). While this work was in progress, another PCR strategy to isolate non-ribosomal peptide synthetase from bacteria was described (Turgay and Marahiel, 1994), and since the publication of the results described in this chapter (Nikolskaya et al., 1995), numerous other peptide synthetase genes from different sources have been cloned (e.g., Schauwecker et al., 1998; Konz et al., 1997; Annis and Panaccione, 1997; Van

Table 1. Fungal species used in this study and peptide toxins that they produce.

Fungus[a]	Cyclopeptide toxin[b]	Ecological niche	Reference
C. carbonum SB111 (ATCC 90305)	HC-toxin cyclo(D-Pro-L-Ala-D-Ala-L-Aeo)	parasitizes maize	Walton, 1990
C. carbonum SB114	none known	non-pathogenic isolate, related to SB111	Walton, 1990
D. chlamydosporia (ATCC 36384)	chlamydocin cyclo(D-Pro-L-Phe-Aib-L-Aeo)	parasitizes rotifers and nematodes	Barron, 1985; Bursnall and Tribe, 1974
P. guttulata	WF-3161 cyclo(L-Pip-L-Leu-D-Phe-L-Aeo)	soil saprophyte	Closse and Huguenin, 1974; Umehara et al., 1983
Cyl. macrosporum (ATCC 34395)	Cyl-2 cyclo(L-Pip-L-Ile-D-O-MetTyr-L-Aeo)	parasitizes various higher plants	Hirota et al., 1973; Pirone, 1970
H. ambiens	trapoxin cyclo(D-Pro-L-Phe-L-Phe-L-Aeo)	saprophyte	Goos, 1986; Itazaki et al., 1990
H. olivaceum (ATCC 60292)	none known	saprophyte	Goos, 1986
H. monilipes (ATCC 22623)	none known	saprophyte	Goos, 1986
H. palmigenum (ATCC 60295)	none known	saprophyte	Goos, 1986
C. victoriae 1146A	victorin	parasitizes oats	Walton, 1990
C. heterostrophus C4	none known	parasitizes maize, produces T-toxin	Yoder, 1988
C. heterostrophus C3	none known	near-isogenic to C4, does not produce T-toxin	Yoder, 1988

(Table 1 - continued)

[a] Isolates of *D. chlamydosporia, Cyl. macrosporum, H. olivaceum, H. monilipes* and *H. palmigenum* were obtained from ATCC. *H. ambiens* RF-1023 was a kind gift of Shionogi and Co., Japan; *P.guttulata* of Fujisawa Pharmaceutical Co., Japan; *C. victoriae* of Dr. R.P.Scheffer, MSU; and *C. heterostrophus* of Dr. O.C. Yoder, Cornell University.
[b] Toxin production by the strains of *D.chlamydosporia, Cyl. macrosporum, P. guttulata* and *H. ambiens* was confirmed for all species except for *P. guttulata* from which WF-3161 could not be detected (Figure 2).

Wageningen et al., 1998). Here we describe a method to isolate fungal non-ribosomal peptide synthetase genes using PCR. PCR primers based on highly conserved non-ribosomal peptide synthetase motifs were used to isolate nucleotide sequences encoding non-ribosomal peptide synthetase domains from several different fungi.

Results and discussion

PCR strategy

As potential sources of non-ribosomal peptide synthetase genes several taxonomically unrelated species of filamentous fungi were examined (Table 1) that produce Aeo-containing cyclic tetrapeptides. We also used several other species from the genus *Helicoma* that are not known to produce any cyclic peptides. Data on the non-ribosomal peptide synthetase sequences obtained by D. Panaccione (Nikolskaya et al., 1995) from *Cochliobolus victoriae* using the same strategy are used in Figures 3 and 5 for the purpose of comparison. *C. victoriae* is an oat pathogen that produces the cyclic pentapeptide victorin. Victorin is not a member of the Aoe-containing cyclic tetrapeptide family. Although they are in the same genus, *C. victoriae* does not produce HC-toxin and *C. carbonum* does not produce victorin (Walton et al., 1995).

Preparations made from C*yl. macrosporum, P. guttulata, D. chlamydosporia and H. ambiens* culture filtrates were analyzed by TLC for the presence of the epoxide groups. Epoxide group-containing compounds were detected in C*yl. macrosporum* and *D. chlamydosporia*-produced culture filtrates, trace amounts were detected in a *H. ambiens* preparation (Figure 2).

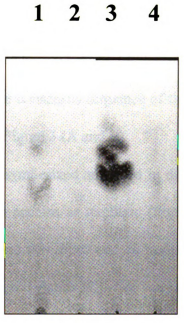

Figure 2. TLC analysis of epoxide-containing toxin production by representatives of the fungal species used in this study. Toxins were purified from 24-day culture filtrates, fractions were lyophilized, resuspended in methanol and loaded onto silica gel TLC plates. Epoxide-containing compounds were detected by spraying plates with the epoxide indicator 4-(*p*-nitrobenzyl)-pyridine (*p*-NBP) and are visible as bright spots. Lanes: 1, *Cyl. macrosporum*; 2, *P. guttulata*; 3, *D. chlamydosporia*; 4, *H. ambiens*.

Degenerate oligodeoxyribonucleotide PCR primers were designed based on highly conserved amino acid motifs in the non-ribosomal peptide synthetase genes for which sequence information was available (Turgay et al., 1992). Codon bias was used in the design of some of the primers based on codon usage in *HTS1* in order to reduce degeneracy (Figure 3B). The sequences of the oligodeoxyribonucleotides and their relative positions within the consensus sequence of the conserved domains of HTS from *C. carbonum* are shown in Figure 3 (A and B).

Four primers were synthesized and used in different combinations for PCR with the genomic DNA of each species as template (Figure 3C). All species tested except *Petriella guttulata, Helicoma monilipes* and *H. palmigenum* yielded at least some PCR products of the predicted sizes (Figure 3C). We were unable to detect WF-3161 (Table 1) in our culture of *P. guttulata*, which may explain the negative PCR results for this species. These PCR fragments were isolated from gels and used as template for a second PCR reaction (reamplification) with the same primers in order to increase the amount of material for cloning. Figure 4 shows reamplified PCR products from four PCR reactions in which *Cyl. macrosporum* DNA was used as template.

Analysis of putative non-ribosomal peptide synthetase gene fragments

Reamplified PCR fragments were isolated from gels and subcloned into the plasmid pUC18. A number of independent plasmid clones were obtained from each size-class of the PCR products and partial nucleotide sequences were determined: 18 clones from *D. chlamydosporia* yielded seven different sequences, six from *Cyl. macrosporum* yielded three, 12 from *H. ambiens* yielded eight, and three from *H.*

A.

```
AI--WDG--S Y-EL---S-- LA--L---G- -------V-- -FEKS--AVV -M-A--KAGG 60
-FVP-DP--P --RL--II-- --A---L--- -----L---- ---------- K-I------- 120
                                    GKPKG
---------- ---------- -----AY-LF TSGSTG-PKG -V--H----- ---------- 180
--G-----R- LQF-SY-FD- SI-DIF--L- -GGCLCIP-E E-R---NL-- -------N-- 240
                                                YGP TE
-LTPS----L -P---P---- -L---GE--- -S----W--- ---L-N-YGP TE------A- 300
                                                    RGYLN
---------- --S-IG---- ---WV--P-- ---LVP-GA- GEL-IE---L AR-YL--P-R 360
                           YKTG DL
T---F----- WL-------- ----R-Y-TG DLVRY--DG- L---GRKD-- Q-K--GQR-E 420
LGE-E----- ----DP---- --V-L----- ---R------ -------A-L --G------- 480
---------- ------L--- ---------- ---LP--MVP ---------L P---SGKLDR 540
---R-----L ---------- T--------- ---------- -LR--W---- ---------- 600
L--------- -F---GG-SI -A--L----- R--G--L-V- DIF                    643
```

B.

Primer number	amino acid sequence	nucleotide sequence	Length	Degeneracy
78	YGPTE	5'-TCTAGATAYGGNCCNACNGA	14mer	96-fold
79	YKTGDL	5'-TCTAGAARRTCNCCNGTYTTRTA	17mer	256-fold
114	GKPKG	5'-TATCTAGAGGNAARCCNAARGG	14mer	64-fold
115	RGYLN	5'-TATCTAGARTTNAGRTADCCHCG	15mer	144-fold

C.

Primer combination	Predicted size (bp)	Resulting size (bp) *Dc*	*Cm*	*Ha*	*Ho*	*Hm*	*Hp*	*Pg*	*Cv*
78 + 79	300	300	300	np	np	np	np	np	300
114 + 115	600	nd	600	np	800	np	np	np	nd
78 + 115	180	nd	180	700	500	np	np	np	nd
114 + 79	720	nd	700	700	650	np	np	np	nd

Figure 3. PCR strategy to amplify peptide synthetase sequences from several fungal species. **(A)** Consensus sequence of the four amino acid-activating domains of HTS encoded by *HTS1* (accession # M98024). Consensus amino acids are indicated in normal type when three of the four amino acids in the four HTS domains are identical and in bold type when all four are identical. Sequences on which the PCR primers were based are shown above the consensus sequence and highlighted. When there was no consensus between HTS domains, amino acids for the primers were chosen based on other available non-ribosomal peptide synthetase sequences (Turgay et al., 1992). **(B)** Design of the PCR primers. The underlined linker sequence was added to each primer to facilitate cloning. In the nucleotide sequence, R represents G or A; Y represents C or T; H represents A, C or T; D represents G, A or T; N represents A, C, G or T. **(C)** PCR results with various primer combinations. *Dc, D. chlamydosporia; Cm, Cyl. macrosporum; Ha, H. ambiens; Ho, H. olivaceum; Hm, H. monilipes; Hp, H. palmigenum; Pg, P. guttulata; Cv, C. victoriae;* nd, not done; np, no product generated by PCR.

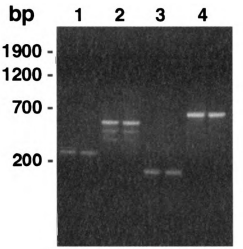

Figure 4. Analysis of the products of the second round of PCR (reamplification) of *Cyl. macrosporum* DNA by gel electrophoresis. Lanes: 1, primer combination 78+79; 2, primer combination 114+115; 3, primer combination 78+115; 4, primer combination 114+79.

olivaceum yielded three. Based on the presence of characteristic non-ribosomal peptide synthetase amino acid motifs other than those used to design the PCR primers, one sequence from *D. chlamydosporia* and three from *Cyl. macrosporum* encoded portions of non-ribosomal peptide synthetase domains. Complete sequences of these clones were obtained and their predicted amino acid sequences are shown in Figure 5. Three *C. victoriae* sequences obtained by D. Panaccione are also shown in Figure 5. No PCR products obtained from *Cyl. macrosporum* with primer combinations 78+115 and 114+79 were sequenced. None of the PCR products from *H. ambiens* and *H. olivaceum* contained non-ribosomal peptide synthetase -like sequences.

Comparison of the predicted amino acid sequences of the PCR products from *C. carbonum*, *D. chlamydosporia* and *Cyl. macrosporum* (Figure 5) indicates that these products are clearly related. When compared against the GenBank DNA database, the highest BLAST (Altschul et al., 1993; Altschul et al., 1997) scores for all of the PCR products were to domain B of HTS, followed by other domains of HTS and then domains from other non-ribosomal peptide synthetases. We conclude that degenerate oligodeoxyribonucleotide PCR primers based on conserved non-ribosomal peptide synthetase motifs can be used to identify non-ribosomal peptide synthetase-like sequences in a variety of fungal species. Indeed, since the publication of this work, several researchers have requested and used our primers to clone non-ribosomal peptide synthetase genes.

The significance of the fact that the deduced amino acid sequences of the PCR products always showed more similarity to domain B of HTS than to the other domains of HTS is not clear. Percent amino acid identity as determined by BESTFIT (Program

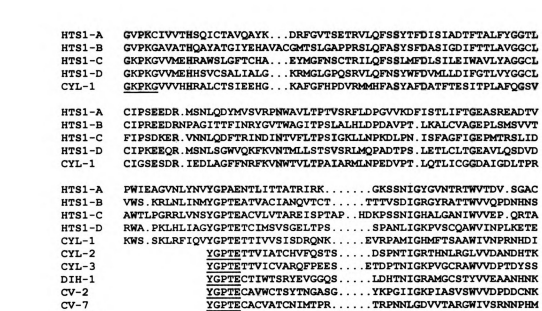

Figure 5. Alignment of the predicted amino acid sequences of the PCR products containing conserved peptide synthetase sequences with the corresponding sequences from the functional domains of HTS. Peptide synthetase sequences are as follows: one amplified from *D. chlamydosporia* (DIH-1), three from *Cyl. macrosporum* (CYL-1, CYL-2 and CYL-3), three from *C. victoriae* (CV-2, CV-7, CV-19) (sequenced by D. Panaccione, see Nikolskaya et al., 1995), and corresponding conserved sequences from the four functional domains of HTS (HTS1-A through D). Conserved amino acids are highlighted, and amino acids that were used to design the PCR primers are underlined. Alignments were done using PILEUP (Program Manual for the Wisconsin Package, 1994). The corresponding nucleotide sequences have been deposited in GenBank with accession numbers L42329 (CYL-1), L42330 (CYL-2), L42331 (CYL-3), L42332 (DIH-1), L42312 (CV-19), L42313 (CV-7), L42314 (CV-2).

Manual for the Wisconsin Package, 1994) was also slightly higher between domain B of HTS and all PCR products. However, the percent amino acid identity between the different PCR products and the different domains of HTS ranged from 34 to 48%, which is only somewhat higher than, for example, the percent identity between the corresponding fragments of the four domains of HTS itself (33 to 44%) and between the domains of HTS and other non-ribosomal peptide synthetases of bacterial and fungal origin (28 to 34%) (Scott-Craig et al., 1992).

Since the PCR primers were based on the amino acid sequence of HTS, the products most likely represent the sequences in *D. chlamydosporia* and *Cyl. macrosporum* that are the most closely related to HTS. However, we do not know if the PCR products from *D.chlamydosporia* and *Cyl. macrosporum* (Figure 5) are in fact parts of the genes for the putative "chlamydocin synthetase" and "Cyl-2 synthetase", respectively, because these sequences might be involved in the synthesis of other cyclic peptides or might be nonfunctional. If these sequences do encode "chlamydocin synthetase" and "Cyl-2 synthetase", then based on their low degree of similarity to *HTS1* it seems unlikely that they derived from each other by horizontal gene transfer in the recent evolutionary past.

When used as probes at high stringency, PCR products from the fungi producing Aeo-containing tetrapeptides hybridized to genomic DNA of the species from which they were derived but not to that of the other species listed in Table 1 (results not shown). Likewise, *HTS1* does not cross-hybridize to DNA from the other fungi (results not shown). Thus, among the fungi studied these genes are unique to their corresponding species.

In contrast to the PCR products from *D. chlamydosporia* and *Cyl. macrosporum*, PCR products obtained from *C. victoriae* by D. Panaccione (Figures 3 and 5) and by J. Pitkin (not shown), hybridized not only to *C. victoriae* DNA but also to that of *C. carbonum* and *C. heterostrophus*, although the degree of their similarity to HTS was within the same range (CV-2, -7 and -19 are 43 to 48% identical to domain B of HTS). If any of them represent the putative "victorin synthetase", then the gene for victorin synthetase is unlike *HTS1* in being present even in species and isolates of *Cochliobolus* that do not produce victorin. J. Pitkin has shown by targeted gene disruption that two of the *C. victoriae* products are not parts of the victorin synthetase (J. Pitkin, unpublished data). Alternatively, these sequences from *C. victoriae* might be nonfunctional or represent one or more non-ribosomal peptide synthetase genes involved in the biosynthesis of an unknown peptide common to all three species of *Cochliobolus*.

Cloning and mapping of the non-ribosomal peptide synthetase gene locus from D. chlamydosporia

In order to clone non-ribosomal peptide synthetase gene genes from *D. chlamydosporia* we constructed a genomic DNA library of this species in phage λEMBL3 and screened it with the corresponding cloned PCR products. Three overlapping lambda clones containing a genomic locus with a putative non-ribosomal peptide synthetase gene gene from *D. chlamydosporia* were isolated (Figure 6). The locus was mapped by restriction analysis of these lambda clones and plasmid subclones (Figure 6). The map was confirmed by comparison to restriction enzyme-digested genomic DNA on Southern blots probed with various regions of the locus (data not shown). Positions of the four

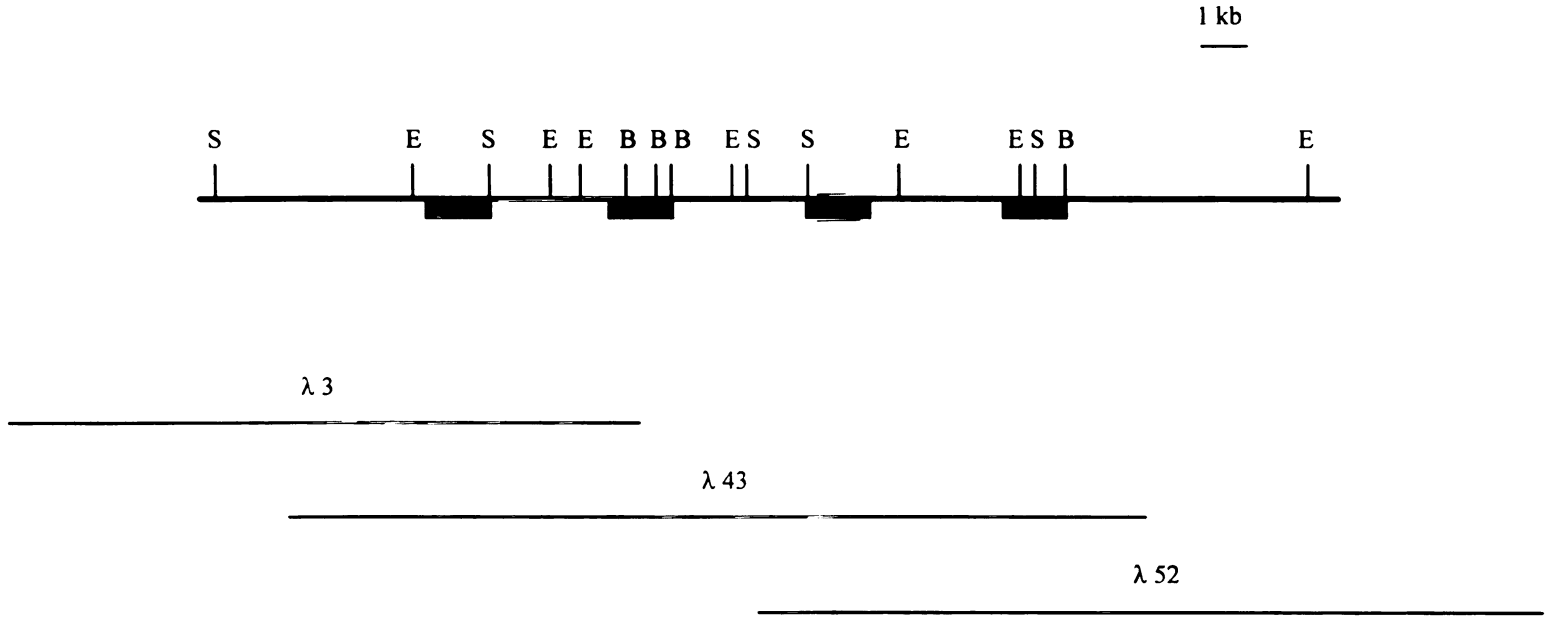

Figure 6. Restriction map of *D. chlamydosporia* locus that contains the putative chlamydocin synthetase gene. Conserved domains are shown as black boxes. S, *Sal*I; E, *Eco*RI; B, *Bam*HI.

conserved domains (Figure 6) were determined by sequencing parts of the locus and comparing the sequence to the consensus sequence of the known non-ribosomal peptide synthetase gene domains. Two of the domains were sequenced completely on one strand. Predicted amino acid sequences of domains A and B were compared to protein sequence databases. They showed significant similarity to various peptide synthetases. One of the best matches was to the sequences of HTS, surpassed only by the sequences of a putative peptide synthetase from the entomopathogenic fungus *Metarhizium anisopliae* (Bailey et al., 1996). Alignment of the sequences of *D. chlamydosporia* domains A and B and four domains from HTS is shown in Figure 7.

Thioester binding core motifs, located in the C-terminal part of the peptide synthetase domains, are SGGDSISAM in domain A and LGGDSLTAM in domain B (Figure 7). They are in a reasonably good agreement with the consensus sequence LGGXSIXAI deduced from other known non-ribosomal peptide synthetases (Stachelhaus et al., 1995; Kleinkauf and von Döhren, 1996; Cane et al., 1998). The conserved motifs involved in adenylate formation and ATP binding are also present in both domains (Figure 7). They show some deviations from the previously reported consensus sequences SGSTGXPKG, YGXTE, GELXIXGXXVAR, RLYRTGDL, and LXXYMVP (Kleinkauf and von Döhren, 1996) deduced from other known non-ribosomal peptide synthetases.

Attempts to disrupt the peptide synthetase gene of D. chlamydosporia

To test whether the cloned non-ribosomal peptide synthetase gene from *D. chlamydosporia* was chlamydocin synthetase, an attempt was made to develop a

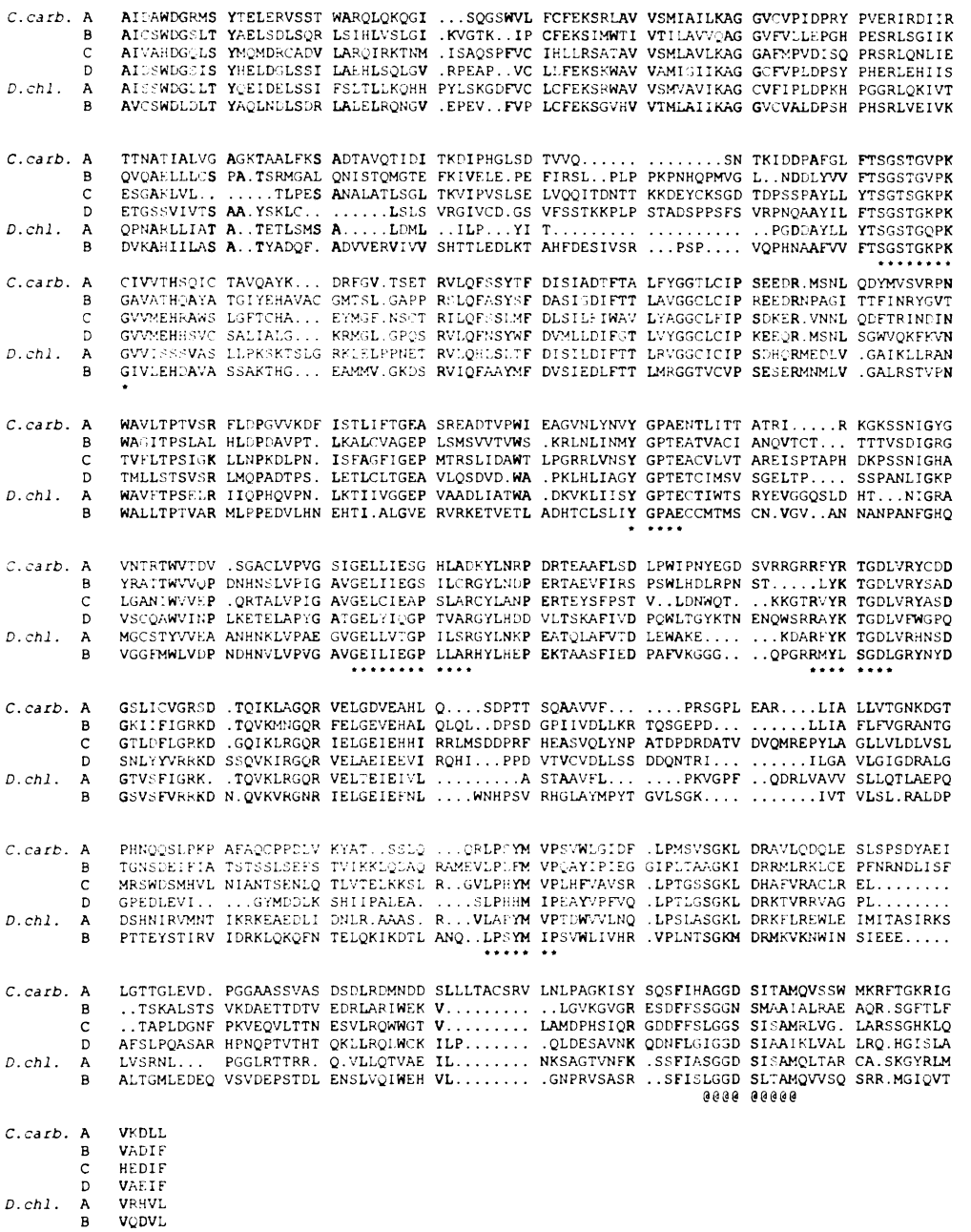

Figure 7. Alignment of amino acid sequences of the four conserved domains of *C. carbonum* HTS and two conserved domains from *D. chlamydosporia*. Conserved amino acids are highlighted; putative adenylate formation, ATP binding and thioester binding motifs are shown by asterisks and @, respectively. *C. carb., C. carbonum; D. chl., D. chlamydosporia.*

transformation system for *D. chlamydosporia*. Three potential markers were tested: hygromycin B resistance, neomycin resistance and acetamide utilization. *D. chlamydosporia* was found to be resistant to Hygromycin. Several transformation vectors incorporating neomycin resistance and acetamide utilization markers were used but all attempts at transformation failed (data not shown).

Materials and Methods

Fungal culture growth and maintenance

Fungal strains used in this study are described in Table 1. Conidia of all fungal strains were stored at -80° C in 25% (v/v) glycerol and grown on V8 juice agar plates. For DNA extraction from *C. carbonum*, mycelial agar plugs (0.5 cm^2) were inoculated into 1-L Erlenmeyer flasks containing 125 ml modified Fries' medium (Scheffer and Ullstrup, 1965). Cultures were incubated at room temperature (21-23° C) without shaking for four days. For DNA extraction from other species, spores and hyphae were scraped off the Petri dish and the resulting suspension in 0.1% Tween-20 was used to inoculate a flask. Cultures were then agitated at room temperature for two days (*Cyl. macrosporum)* or four days (*P. guttulata*, *D. chlamydosporia* and all *Helicoma* species).

DNA manipulations

Genomic DNA was isolated from fungal mycelia as described (Pitkin et al., 1996). Southern blot analysis was performed as described (Sambrook et al., 1989).

For PCR, 1 to 3 μg DNA was used as template. The reaction mixture contained

0.2 mM of each dNTP, 1 μM of each primer, and 0.5 to 1 units of *Taq* polymerase (Promega, Madison, WI, USA) with buffer supplied by the manufacturer, in a total volume of 100 μl. The reactions were carried out in a DNA Thermal Cycler model 480 (Perkin-Elmer Corp., Norwalk, CT, USA) programmed as follows: 94° C for 4 min; 25 cycles of 94° C for 1 min, 37° C for 2 min, 72° C for 3 min; 72° C for 6 min. Aliquots from the completed reactions were fractionated on 2% agarose gels. PCR products of the predicted sizes were isolated and used as templates to reamplify under the reaction conditions outlined above.

D. chlamydosporia genomic DNA library in phage λEMBL3 was constructed using the Undigested EMBL3 Cloning Kit (Stratagene, La Jolla, CA) and the Gigapack II Gold Packaging Extract (Stratagene) according to the manufacturer's instructions. The library was screened as described (Sambrook et al., 1989).

The DNA sequence was determined by automated fluorescent sequencing performed by the MSU-DOE-PRL Plant Biochemistry Facility using an ABI Catalyst 800 (Applied Biosystems Division, Foster City, CA) for Taq cycle sequencing and an ABI 373A Sequencer (Applied Biosystems Division) for analysis of the products.

Toxin extraction and epoxide detection

HC-toxin and other epoxide-containing toxins were purified from 24-day culture filtrates by solvent extraction as described by Walton et al. (1982). Fractions were lyophilized, resuspended in methanol and loaded onto silica gel TLC. TLC plates were developed in CH_2Cl_2:acetone (1:1, [v/v]) and epoxide groups were detected by spraying plates with 4-(*p*-nitrobenzyl)-pyridine (*p*-NBP) (Hammock et al., 1974).

Chapter 2

REDUCED VIRULENCE CAUSED BY MEIOTIC INSTABILITY OF THE *TOX2* CHROMOSOME OF *COCHLIOBOLUS CARBONUM*

Abstract

In some HC-toxin-producing ($Tox2^+$) isolates of *Cochliobolus carbonum*, the known HC-toxin biosynthetic genes are located on a chromosome of 3.5 Mb, whereas in other isolates the genes are on a chromosome of 2.2 Mb. Crosses between $Tox2^+$ and $Tox2^-$ isolates and between isolates in which the *TOX2* genes were on chromosomes of different size yielded progeny that had lost one or more copies of one or more *TOX2* genes. Of 200 progeny analyzed, eight (4%) had lost at least one *TOX2* gene. All eight still had at least one functional copy of *HTS1*, *TOXA*, *TOXC*, and *TOXE*. The deletion strains were characterized with respect to virulence, HC-toxin production, HTS enzyme activity, and size of the *TOX2* chromosome. Most deletion strains could be explained by simple chromosome breaks resulting in the loss of major contiguous portions (0.8 Mb to 1.4 Mb) of the 3.5-Mb *TOX2* chromosome, but at least one strain had apparently undergone an internal deletion. Most strains were still completely virulent ($Tox2^+$), but two displayed a novel phenotype of reduced virulence (RV), defined as an ability to cause small lesions that expanded at a reduced rate and an inability to colonize plants systemically. Although the RV strains produced no detectable HC-toxin in culture, the RV phenotype was dependent on the presence of a functional copy of *HTS1*. We propose that the RV strains still produce a low level of HC-toxin, at least in planta, and that the

RV strains are missing one or more unknown genes that have a role in, but are not absolutely required for, HC-toxin production.

Introduction

Isolates of *C. carbonum* that produce the cyclic tetrapeptide known as HC-toxin are exceptionally virulent on maize of genotype *hm1/hm1*. These strains are called $Tox2^{+}$ or race 1. Race 2 ($Tox2^{-}$) strains of *C. carbonum* are non-virulent and do not produce HC-toxin. HC-toxin production is controlled by a complex locus, *TOX2*, that contains multiple copies of multiple genes (Walton, 1996; Walton et al., 1998). The genes of *TOX2* include *HTS1*, encoding a large multifunctional non-ribosomal peptide synthetase; *TOXA*, encoding an HC-toxin efflux carrier; *TOXC*, encoding a fatty acid synthase β subunit; and *TOXE*, encoding a regulatory protein (Panaccione et al., 1992; Scott-Craig et al., 1992; Pitkin et al., 1996; Ahn and Walton, 1997; Ahn and Walton, 1998). The genes of *TOX2* are absent from toxin non-producing ($Tox2^{-}$) isolates.

The synthesis of HC-toxin is catalyzed in part by HTS, a non-ribosomal peptide synthetase encoded by the *HTS1* gene (Walton and Holden, 1988; Panaccione et al., 1992). HTS catalyzes the activation of the four amino acids found in HC-toxin and probably polymerizes and cyclizes the HC-toxin tetrapeptide. HTS also epimerizes L-Ala to D-Ala and L-Pro to D-Pro (Walton and Holden, 1988). *HTS1* is absent from $Tox2^{-}$ isolates and is, thus, $Tox2^{+}$ -unique. Disruption of both, but not only one or the other, copies of *HTS1* results in strains that do not produce HC-toxin and have lost their virulence (Panaccione et al., 1992).

Three other $Tox2^{+}$ -unique genes, *TOXA, TOXC* and *TOXE,* have been found to be

involved in HC-toxin biosynthesis and efflux (Pitkin et al., 1996; Ahn and Walton, 1997, Ahn and Walton, 1998). The alkyl side chain of Aeo (2-amino-9,10-epoxy-8-oxodecanoic acid) may be produced in part by the gene product of the *TOXC* gene, whose predicted amino acid sequence is similar to the β subunit of fungal fatty acid synthases (Ahn and Walton, 1997). Disruption of the *TOXC* gene results in strains that do not produce HC-toxin and are non-virulent.

The predicted amino acid sequence of the protein encoded by *TOXA* gene is very hydrophobic and shows amino acid and secondary structure similarities to integral membrane pumps of small molecules and antibiotics. *TOXA* probably encodes an HC-toxin efflux pump (Pitkin et al., 1996). Each of the two copies of *TOXA* is tightly linked to a corresponding copy of *HTS1*, forming a *TOXA/HTS1* cluster. The transcriptional start sites of *TOXA* and *HTS1* (in both copy 1 and copy 2 of *TOXA/HTS1*) are 386 bp apart, and these two genes are transcribed in opposite directions (Pitkin et al., 1996). *TOXA* may be essential for the protection of the fungus against HC-toxin by transporting it out of the cell, because it has proven impossible to recover mutants with both copies of *TOXA* disrupted (Pitkin et al., 1996).

TOXE, encoding a regulatory protein, is present in two copies in most naturally occurring $Tox2^+$ isolates. This gene is also essential for HC-toxin biosynthesis, its disruption turns off transcription of *TOXA*, *TOXC* and *TOXD*, another $Tox2^+$ -unique gene with unknown function (Ahn and Walton, 1998).

In some isolates, including the strain SB111, all of the *TOX2* genes except one copy of *TOXE* are located on a chromosome of 3.5 Mb (which is the largest known chromosome in any isolate of *C. carbonum*). In SB111, the other copy of *TOXE* is on a

0.7-Mb chromosome. In other Tox2$^+$ isolates, such as CC141, all of the known *TOX2* genes, including both copies of *TOXE*, are on a chromosome of 2.2 Mb (Ahn and Walton, 1996; Canada and Dunkle, 1997). The 3.5-Mb *TOX2* chromosome of SB111 also contains single-copy DNA that is common to Tox2$^+$ and Tox2$^-$ isolates. In Tox2$^-$ isolates and in Tox2$^+$ isolates such as CC141, this single-copy DNA is on a chromosome of 2.0 Mb. Together, the data are consistent with the 3.5-Mb and 0.7-Mb chromosomes of SB111 being related by a reciprocal translocation to the 2.2-Mb and 2.0-Mb chromosomes of other Tox2$^+$ isolates (Ahn and Walton, 1996).

TOX2 genes have been physically mapped on the 3.5 Mb *Tox2* chromosome of strain SB111 (Ahn and Walton, 1996) (Figure 8). A chromosome of this size is not found in Tox2$^-$ strains of *C. carbonum*, and at least part of it is completely missing from Tox2$^-$ strains (Ahn and Walton, 1996).

Among the progeny from a cross between SB111 and SB114 (a related Tox2$^-$ isolate), one progeny, called 243-7, was found to have a truncated 3.5-Mb chromosome and to lack at least one copy of each of the known *TOX2* genes. This chapter describes the molecular genetic, biochemical and pathogenic phenotype of 243-7, as well as the isolation and characterization of other isolates with deletions of the 3.5-Mb *TOX2* chromosome.

The truncated *TOX2* chromosome of 243-7 has been partially described (Figure 8) (Ahn and Walton, 1996). By PFGE, the chromosome of 243-7 is 2.1 Mb instead of the parental 3.5 Mb, and the parental 1-Mb *Pac*I fragment on which both copies of *HTS1* are located is reduced to 0.28 Mb (Ahn and Walton, 1996). Isolate 243-7 is missing copy 1 of *TOXA/HTS1* (these two genes are tightly clustered; Pitkin et al., 1996); copy 3 of

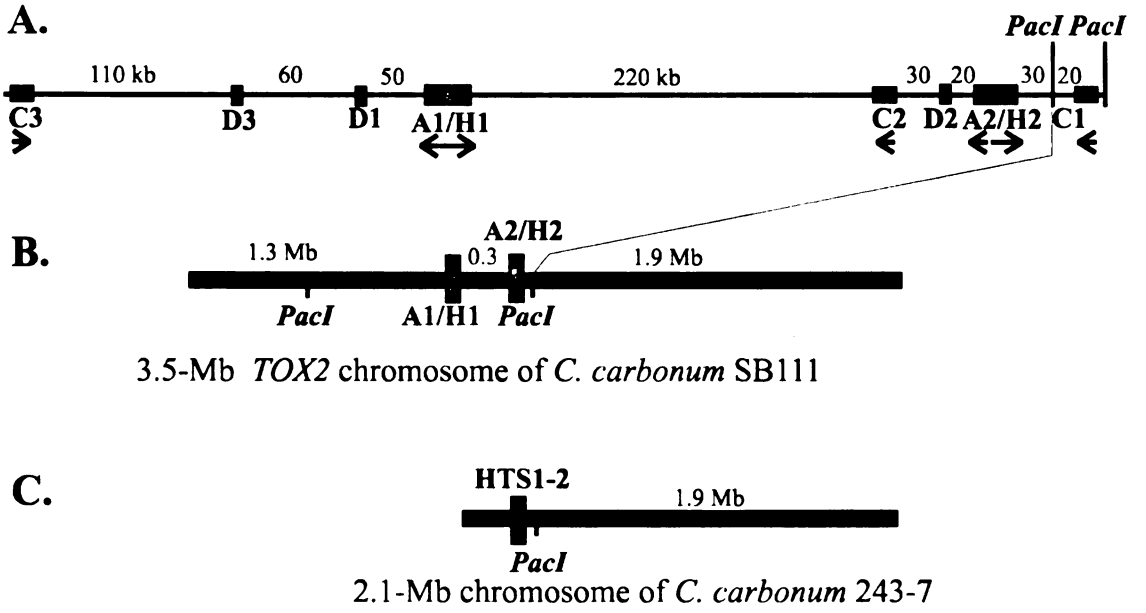

Figure 8. Physical maps of the *TOX2* chromosome in SB111 and 243-7 (after Ahn and Walton, 1996). **(A)** Detailed map of the region of the *TOX2* chromosome containing the *HTS1* genes. Distances are given in kb. A1/H1 indicates *TOXA*-1 (*TOXA* copy 1) and *HTS1-1* (*HTS1* copy 1). C1, C2 and C3 indicate copies 1, 2 and 3 of *TOXC*, respectively. D1, D2 and D3 indicate copies 1, 2 and 3 of *TOXD*, respectively. Arrows indicate the direction of transcription of the genes. **(B)** Map of the entire 3.5-Mb *HTS1*-containing (*TOX2*) chromosome of the Tox2$^+$ (race 1) strain, SB111. **(C)** Deduced map of 243-7, a strain bearing truncated *TOX2* chromosome. Lines connect the corresponding sites in **(A)** and **(B)**. Distances are given in Mb. *Pac*I indicates known restriction sites of the rare-cutting restriction endonuclease *Pac*I.

TOXC, and copies 1 and 3 of *TOXD* (Figure 8). The evidence is consistent with 243-7 having undergone a simple chromosome break, resulting in the loss of 1.4 Mb of contiguous DNA extending from just to the right of copy 1 of *TOXA/HTS1* to the left-hand end of the chromosome (Figure 8) (Ahn and Walton, 1996). Strain 243-7 still has at least one functional copy of each of the known *TOX2* genes, including the regulatory gene *TOXE* (Ahn and Walton, 1998).

Results and discussion

Disease phenotype of 243-7

1. Inoculation of 2-week-old seedlings.

Pathogenicity of strain 243-7 was compared to 367-2, a typical $Tox2^{+}$ isolate, and 243-10, a typical $Tox2^{-}$ isolate, on maize of genotype *hm1/hm1* (Multani et al., 1998) when the plants were 25 cm tall and had four true leaves. No obvious differences in lesion morphology or size were observed two days post-inoculation, but at four days the development of lesions on the leaves infected with 243-7 was clearly intermediate to that caused by 367-2 and 243-10 (Figure 9A). The lesions caused by 243-7 were fewer than those caused by 367-2 and more variable in size and shape. Isolate 243-10 caused only small flecks, typical of a resistance response in this disease. At 7 days, the intermediate disease phenotype of 243-7 was still clear. Plants infected with the $Tox2^{+}$ isolate 367-2 were completely dead as the fungus colonized the entire plant, including the stalks, whereas plants infected with the $Tox2^{-}$ isolate 243-10 were completely healthy (Figure 9B). The plants infected with 243-7, on the other hand, showed some lesions of highly

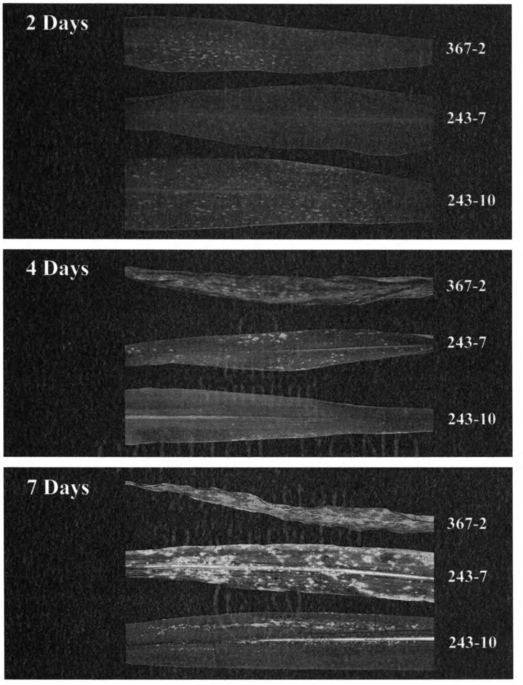

Figure 9. Strain 243-7 exhibits a new pathogenicity phenotype on maize. Race 1-susceptible maize leaves were inoculated with strains 367-2 (Tox2$^+$, race 1), 243-7 (RV) and 243-10 (Tox2$^-$, race 2) strains of *C. carbonum*. **(A)** Time course of infection. Individual leaves are shown at two, four and seven days post inoculation. Note that the lesions on the 367-2 (Tox2$^+$, race 1) infected leaf have not spread significantly after four days post inoculation due to the desiccation of the leaf.

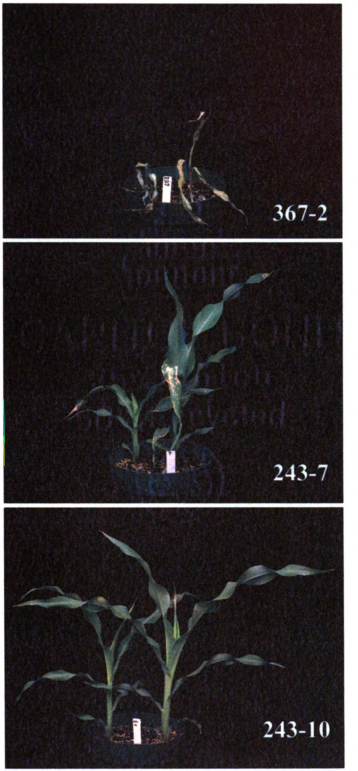

Figure 9 (B). Plants at seven days post inoculation.

variable size and irregular outline but the plants remained alive and continued to grow Figure 9B). Isolate 243-7 was never observed to invade the stalks of infected plants or to kill plants. We call this intermediate disease phenotype "RV" for "reduced virulence".

Inoculation of plants that were 10 cm (two true leaves) or 16 cm tall produced the same infection phenotype as older plants.

2. Inoculation of very young seedlings.

When seedlings with only the flag leaf and one true leaf were inoculated with the same three isolates at conidial concentrations of either 10^4/ml or 5 x 10^4/ml, all of the plants survived and grew until 6 days post-inoculation (Figure 10). The plants inoculated with 367-2 or 243-7 developed lesions only on the tips of the first true leaves, which had been present at the time of inoculation. Plants inoculated with 243-7 had consistently fewer lesions than those sprayed with 367-2. At 6 or 7 days post-inoculation, plants infected with 367-2 but not 243-7 began to lose turgor and became grayish in color, and they collapsed and died 2-3 days later without developing any lesions on the leaves that had not been directly inoculated. Plants inoculated with 243-10 or 243-7 continued to grow normally for at least 3 weeks.

3. Inoculation of seeds.

Maize seeds (nine per treatment planted three per 15-cm diameter pot) were soaked in a suspension of conidia from strains 267-2, 243-10, and 243-7 (10^4/ml in 50 ml 0.1% Tween-20) for 1.5 hr. Plants in all treatments emerged at the same time. Plants infected with 367-2 were brown in color from the first day of appearance, grew to a

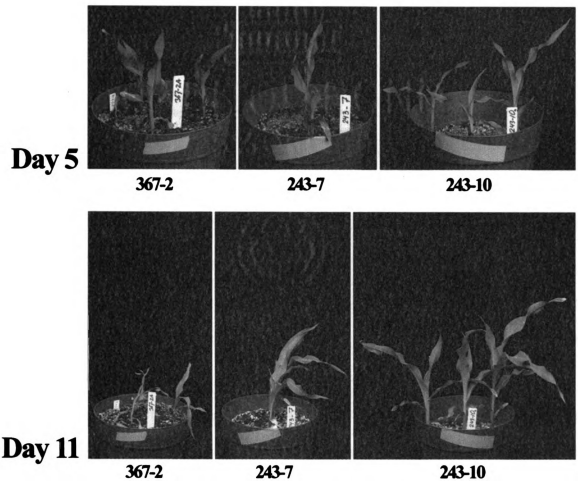

Figure 10. Disease phenotype after inoculation of very young seedlings. Seedlings 1 in. in height with only the flag leaf and one true leaf were inoculated with strains 367-2 ($Tox2^+$, race 1), 243-7 (RV) and 243-10 ($Tox2^-$, race 2) of *C. carbonum* at conidial concentration of 10^4/ml. Plants are shown at 5 and 11 days post inoculation. Note a few lesions on the lower leaves of 243-7-infected plant.

height of only 1-2 cm, and died 3-4 days after appearance (Figure 11). Plants from seeds inoculated with 243-7 or 243-10 had the same appearance as non-inoculated plants and never developed any visible symptoms.

4. Soil inoculation.

After planting the seeds (three per 15-cm diameter pot), the soil was watered with a suspension of conidia at concentrations of 10^4/ml, 5 x 10^4/ml, or 10^5/ml in a volume of 10 ml. The pots were covered with plastic bags overnight. Plants inoculated with conidia of isolate 367-2 at 5 x 10^4/ml or 10^5/ml looked healthy for the first 3 days after their appearance above ground, but then became greyish in color, wilted, and died by day 10 without the development of any visible leaf lesions (Figure 12). Plants inoculated with 367-2 at 10^4 conidia/ml remained healthy. Plants infected by soil inoculation with isolates 243-10 or 243-7 showed no symptoms at any concentration at any time point.

In conclusion, the pathogenicity tests indicated that 243-7 has an intermediate virulence between wild type $Tox2^+$ and $Tox2^-$ isolates. When inoculated directly onto leaves, isolate 243-7 can cause disease, but the lesions are fewer, more irregular in outline, and more variable than those caused by $Tox2^+$ isolates (Figure 9 A, B). Isolate 243-7 never caused wilting or death of seedlings, and could not cause any disease when inoculated onto seeds either directly or through the soil. The most probable explanation for this is that isolate 243-7 cannot infect stems and therefore cannot infect or spread systemically. It can cause disease only in leaf tissue that has come into direct contact with the primary inoculum.

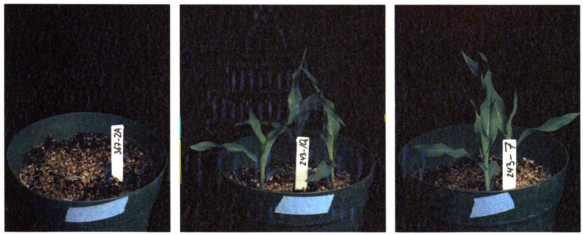

Figure 11. Disease phenotype after inoculation of seeds. Plants are shown at 5 days after emerging (12 days after infected seeds were planted). Note that 367-2-infected plant can be barely seen because it grew only 1 in. in height before desiccation.

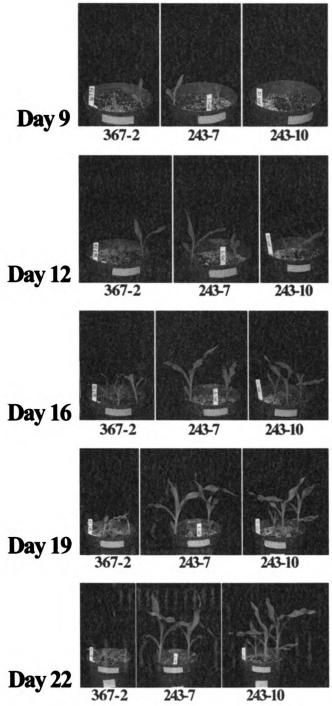

Figure 12. Disease phenotype after soil inoculation. After planting the seeds, the soil was watered with a suspension of conidiospores of strains 367-2 ($Tox2^+$, race 1), 243-7 (RV) and 243-10 ($Tox2^-$, race 2) at a concentration of 5 x 10^4/ml, in a volume of 10 ml. Time course of infection is shown (the day of inoculation is the same day the seeds were planted). Plants emerged at seven days after the seeds were planted and the soil inoculated.

Saprophytic growth, maize leaf penetration and toxin phenotype of strain 243-7

Strain 243-7 sporulated normally in culture. It also grew slightly faster on Petri plates and in race tubes than wild type (wild type strain 367-2 had a linear growth rate of ~ 0.79 cm/day, strain 243-7, ~ 0.84 cm/day) (Figure 13). Therefore, the RV disease phenotype of 243-7 is not due to reduced growth.

Appressorium formation and penetration of the maize leaf by strain 243-7 was compared to that of the wild type. After inoculation of the susceptible maize plants or individual excised leaves with strains 367-2 ($Tox2^+$), 243-7 (RV) or 243-10 ($Tox2^-$), leaves were collected at 18, 24 and 48 hr post-inoculation and stained in 0.1% cotton blue in lactophenol. The number of germinated spores that formed appressoria and penetrated the intact leaf surface, those that penetrated through stomata and those that failed to penetrate the leaf were counted under the microscope. At 24 and 48 hr, all three strains penetrated the leaf at the same rate (results not shown). Therefore, strain 243-7 is not deficient in appressorium formation and leaf penetration.

By analyzing culture filtrates by HPLC followed by TLC for the presence of HC-toxin, J. Pitkin showed that if 243-7 produces any HC-toxin in culture, it does so at a level less than 1% of the wild type, which is undetectable by the TLC method (data not shown).

Disruption of* HTS1 *in isolate 243-7 eliminates the RV phenotype

Two explanations for the RV phenotype seemed possible. One, supported by the in vitro HC-toxin analysis, was that strain 243-7 had completely lost the ability to produce HC-toxin and its residual virulence was due to a different factor that is

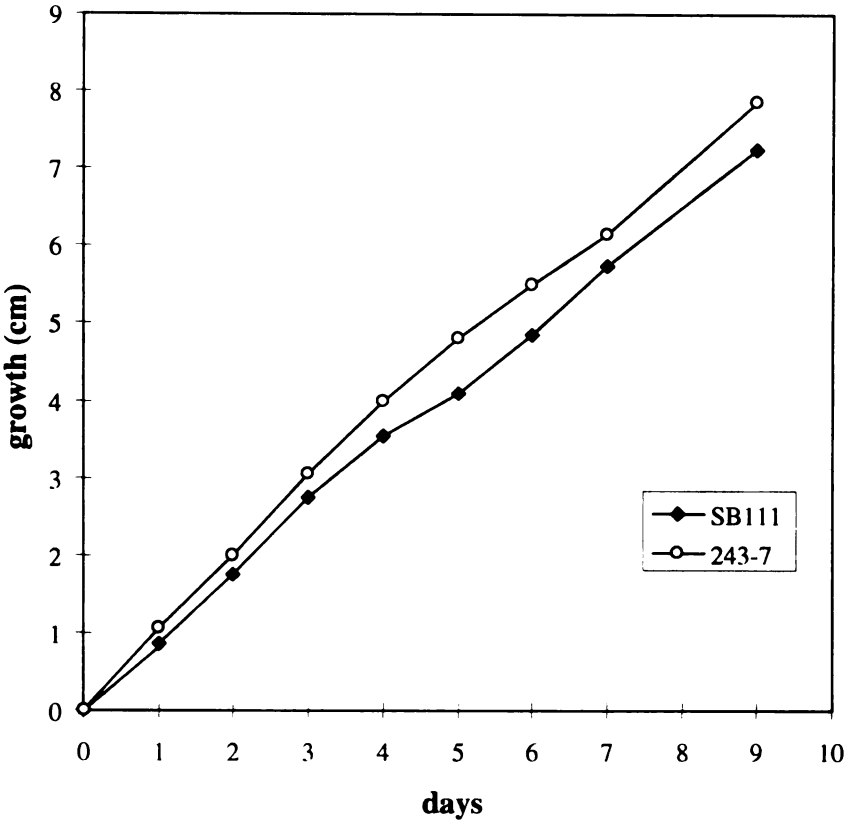

Figure 13. Growth of strain 243-7 compared to SB111. SB111 (Tox2$^+$, race1, virulent) and 243-7 (RV) strains were grown in horizontal race tubes containing potato dextrose agar. Data points are averages of duplicate samples.

genetically linked to the *TOX2* locus. An alternative hypothesis was that 243-7 produces a very low level of HC-toxin, at least in planta, that is sufficient to allow some colonization of the plant. To test the involvement of HC-toxin in the RV phenotype, the remaining copy of *HTS1* (copy 2, see Figure 8, p. 34) in strain 243-7 was disrupted by transformation-mediated homologous integration, creating an *HTS1*-null strain.

Plasmid pCC119, which contains a 0.6-kb fragment from the 5' end of *HTS1* and the *hph* gene for hygromycin resistance (Panaccione et al., 1992), was linearized at a unique *Xho*1 restriction site and transformed into *C. carbonum* strain 243-7. Five hygromycin-resistant transformants were analyzed. The restriction map of the wild-type *HTS1* locus (3' end) and the predicted map resulting from integration of a single or multiple copies of pCC119 are shown in Figure 14A, B and C, respectively. Southern blot analysis (Figure 14D) indicates that the wild-type 17.1-kb band disappears in the disruption mutants, and the new pattern of hybridization (12.5-, 10.6- and 6.0-kb bands) corresponds to the integration of pCC119 at *HTS1* in single (560-2) or multiple (560-1, 3, 5, and 6) copies (Figure 14D).

Pathogenicity of the transformants was compared to that of 367-2 (wild type $Tox2^{+}$), 243-10 (wild type $Tox2^{-}$) and 243-7 (RV). All five transformants were completely avirulent, that is, their disease phenotype was indistinguishable from that of the wild type $Tox2^{-}$, as shown in Figure 15 for transformant 560-2. Thus, the residual virulence of 243-7 is dependent on *HTS1*. The simplest explanation for this result is that 243-7 still produces a low level of HC-toxin, at least in planta, and that this low level of HC-toxin production accounts for its residual virulence.

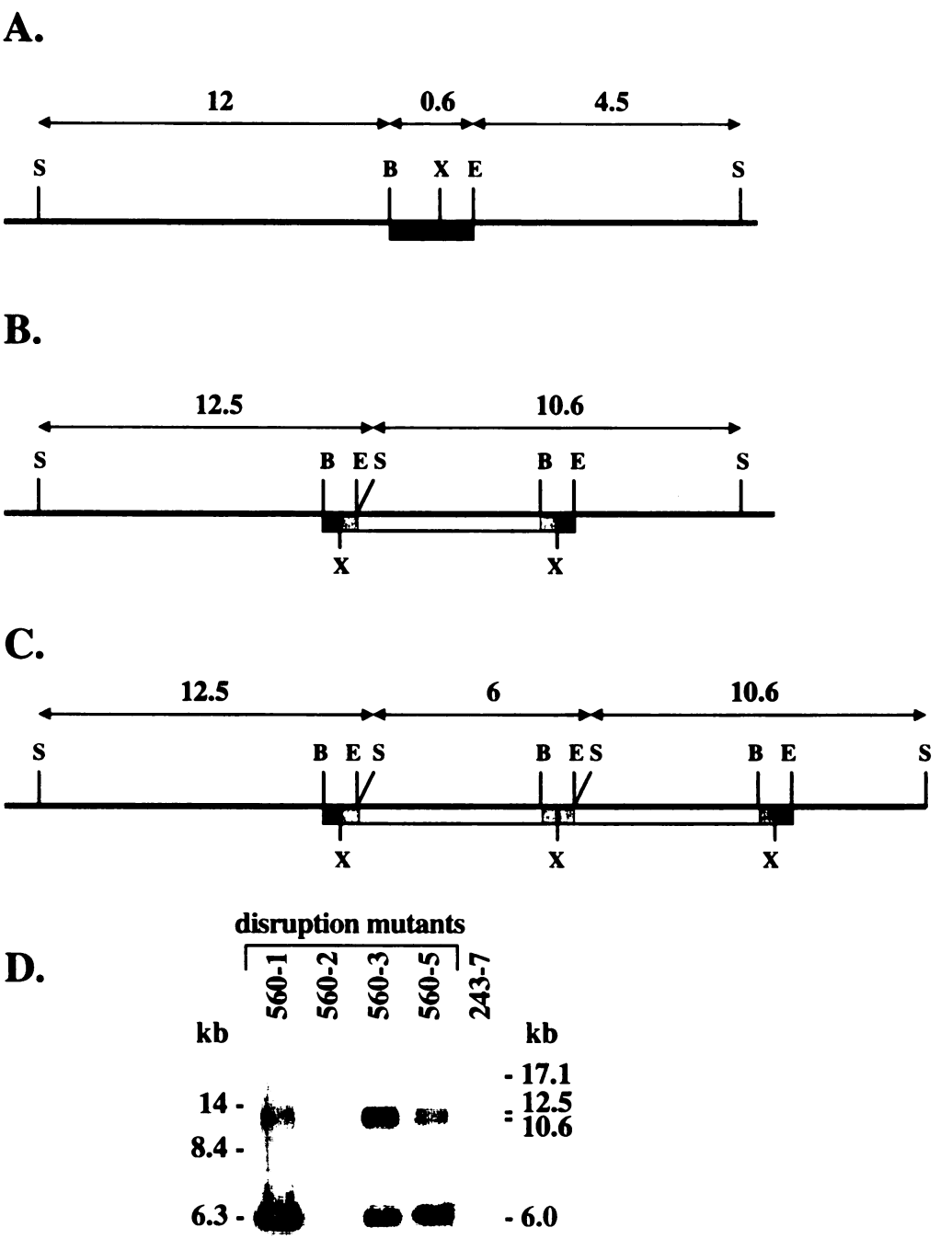

Figure 14. Analysis of *HTS1* disruption mutants. **(A)** Restriction map of wild type *HTS1* copy 1 locus. **(B)** and **(C)** Predicted restriction maps of *HTS1* with insertion of a single **(B)** and double **(C)** copies of pCC119. Predicted fragment sizes are indicated in kilobases. **(D)** Southern blot of genomic DNA from 243-7 and *HTS1* disruption mutants 560-1, 560-2, 560-3 and 560-5. DNA was cut with *Sal*I and the filter was probed with the insert of pCC119. The black boxes indicate the genomic copy of the *Bam*HI/*Eco*RI fragment used to construct pCC119, and the unshaded boxes indicate pCC119 bearing the same fragment indicated by the shaded boxes. B, *Bam*HI: E, *Eco*RI; S, *Sal*I; X, *Xba*I.

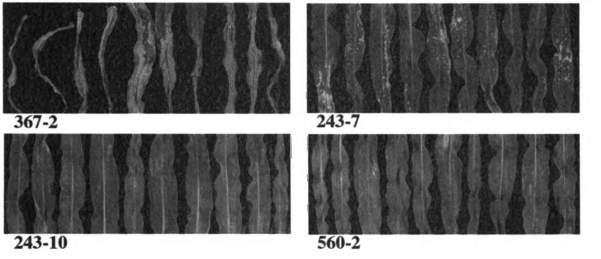

Figure 15. Disruption of *HTS1* in 243-7 makes this strain avirulent (Tox2$^-$). Race 1-susceptible maize plants were inoculated with strains 367-2, 243-7, 243-10 and 560-2 (*HTS1* disruption mutant). Five plants per strain were inoculated, and from these five plants, 10 leaves were collected at 7 days post inoculation.

Isolation and initial characterization of additional strains with chromosomal aberrations

The molecular evidence suggests that the 1.4-Mb of DNA that is deleted from 243-7 contains genetic material that is necessary for wild type rates of synthesis of HC-toxin and hence for full virulence. In order to obtain an indication of the rarity of the chromosome break event that had occurred in 243-7, i.e., of the instability of the *TOX2* chromosome, we examined progeny from additional crosses for deletions within the *TOX2* chromosome. Another rationale for searching for new isolates with deletions in the *TOX2* locus is that the analysis of such strains could help determine the presence and location of additional genes necessary for HC-toxin production or virulence. For these crosses (Figure 16), parental strains were chosen that differed from each other with respect to the *TOX2* chromosome, either in presence or size, on the presupposition that this would promote abnormal behavior of the *TOX2* chromosome during meiosis. The parents in cross 373 were $Tox2^{+}$ isolates with *TOX2* chromosomes of different size (3.5 and 2.5 Mb), and the parents in cross 512 were a $Tox2^{+}$ isolate with a 3.5 Mb *TOX2* chromosome and a $Tox2^{-}$ isolate lacking any chromosome of 3.5-Mb and all of the known *TOX2* genes (Ahn and Walton, 1996; Canada and Dunkle, 1997).

Thirty-four progeny were screened in cross 373 and 110 in cross 512. Progeny were initially screened by DNA blot analysis using *TOXC* as a probe (a subset is shown in Figure 17A), because copies 1 and 3 of *TOXC* form the boundaries of the mapped ~540 kb region of the *TOX2* chromosome (Figures 8, 21). Strains lacking one or more copies of *TOXC* were analyzed further by Southern blotting for *TOXA* and *TOXD* RFLPs (Figure 17B,C).

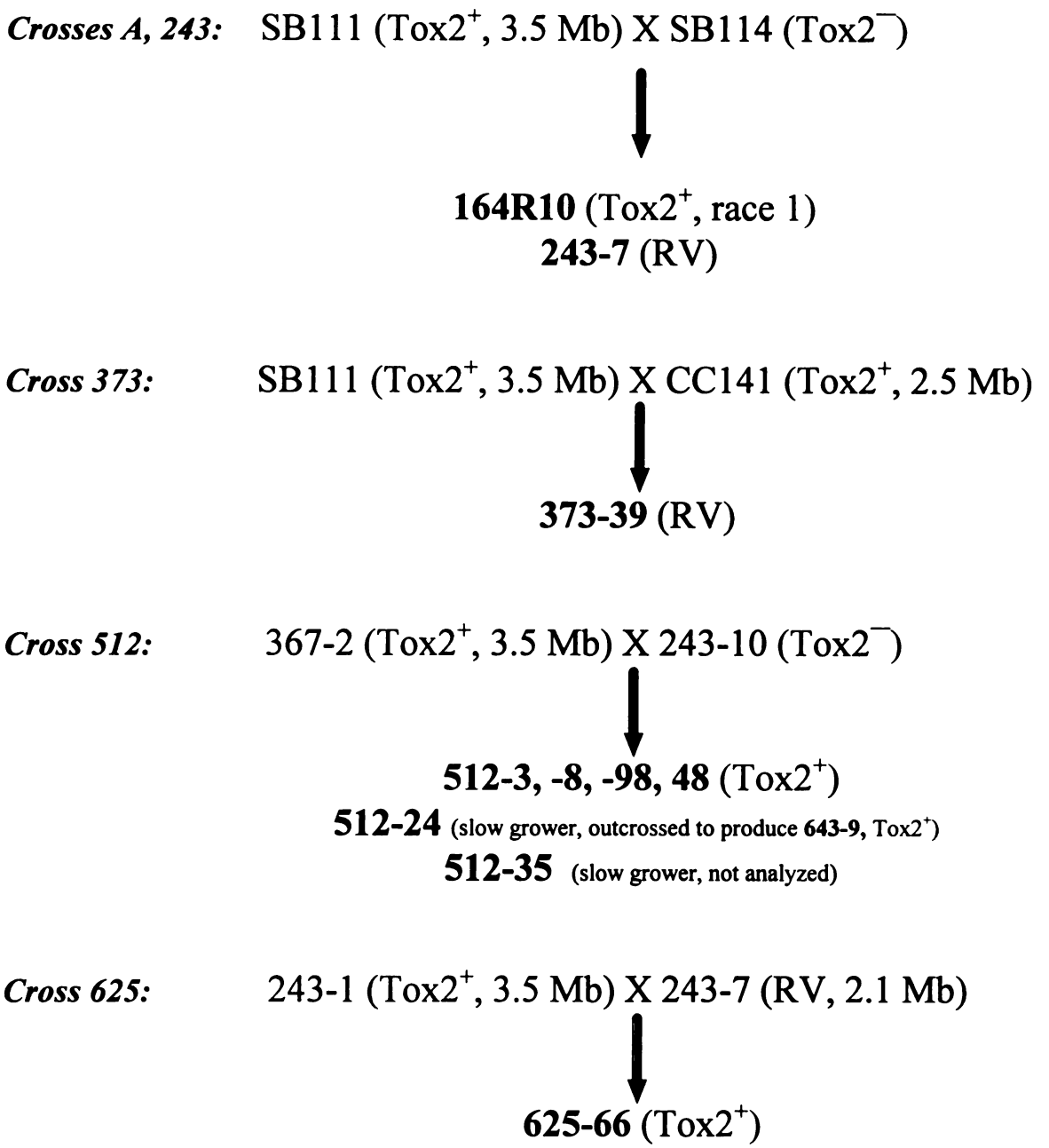

Figure 16. Crosses to generate deletion strains. Crosses were made between Tox2$^+$ and Tox2$^-$ *C. carbonum* strains and between Tox2$^+$ strains with different *TOX2* chromosome sizes. The size of the *TOX2* chromosome in the Tox2$^+$ parents is given in parentheses. The relevant deletion-bearing progeny from the crosses are indicated below the arrows with the race phenotype indicated in parentheses. Cross A was performed by S. Briggs (Walton, 1987). Strains 243-1, 243-7 and 243-10 are progeny of a cross between SB111 (Tox2$^+$; ATCC 90305) and SB114 (Tox2$^-$; Walton 1987). Strain 367-2 was derived from a progeny (243-1) of the same cross by backcrossing it three times to SB111 (J. Pitkin, unpublished).

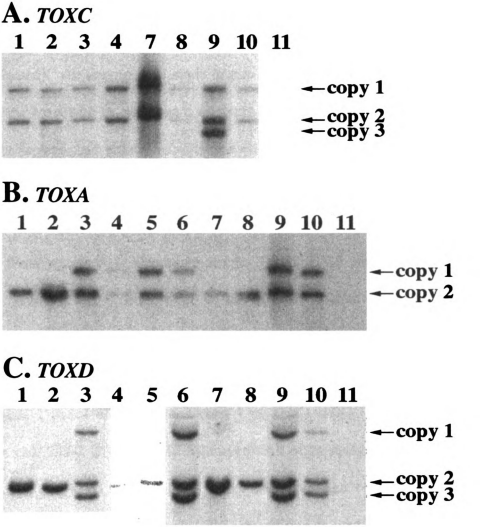

Figure 17. Southern blot analysis of the strains used in this study. Lanes: 1, 243-7; 2, 373-39; 3, 512-3; 4, 512-48; 5, 625-66; 6, 512-8; 7, 643-9; 8, 164R10; 9, 367-2A; 10, 512-98; 11, 243-10a. **(A)** *TOXC* RFLP. Genomic DNA was cut with *Xho*I and the filter was probed with *TOXC* internal fragment. *TOXC* copy 1 restriction fragment is 18 kb, copy 2, 14 kb; copy 3, 12 kb. **(B)** *TOXA* RFLP. Genomic DNA was cut with *Bgl*I and probed with *TOXA* internal fragment. *TOXA* copy 1 restriction fragment is 15 kb, copy 2 restriction fragment is 12 kb. **(C)** *TOXD* RFLP. Genomic DNA was cut with *Bam*HI and probed with *TOXD* internal fragment. *TOXD* copy 1 restriction fragment is 15 kb; copy 2, 9 kb; copy 3, 8 kb.

progeny (512-24, 512-35, 512-48) were found to lack copy 3 of *TOXC*. However, eight additional progeny from cross 512 had an extremely faint *TOXC*-3 band on Southern blots (data not shown), suggesting that they were not homogenous (either heterokaryons or contaminated). An attempt was made to purify these deletion strains away from the contaminating karyotype by two rounds of single conidiospore isolation, but all resulting single spore isolates had either the wild type RFLP pattern (harboring all three *TOXC* copies) or presented the same RFLP pattern as the original isolate (very faint *TOXC*-3 band). However, some of these isolates formed sectors frequently when grown on a Petri plate, and these sectors were also examined for *TOXC* RFLP pattern after single spore isolation. Through this process, three of the original isolates gave rise to nuclearly homogenous strains bearing deletions (512-3, 512-8, 512-98).

In summary, two isolates (373-39 and 512-24) were found that lack copy 3 of *TOXC*, copies 1 and 3 of *TOXD*, and copy 1 of *TOXA/HTS1* (Table 2). Thus, these strains resemble 243-7 in their *TOX2* haplotype. All other strains that lacked copy 3 of *TOXC* (512-3, 512-8, 512-35, 512-48, and 512-98) still had copy 1 of *TOXA/HTS1*. Strain 512-48 lacks copies 1 and 3 of *TOXD* in addition to copy 3 of *TOXC*. *TOXE* is present in one copy in all the strains tested (164R10, 512-3, 512-48, 243-7, 373-39, 643-9), while wild-type strains SB111 and CC141 have two copies of this gene (Figure 18).

Growth tests measuring either radial growth on Petri plates or linear growth in race tubes of various isolates in parallel indicated that the growth rates of 367-2, 243-7, 243-10, 373-39, 512-48, and 512-8 were statistically indistinguishable (data not shown).

Isolates 512-24 and 512-35, on the other hand, grew slowly in culture. To test whether this slow growth was related to the loss of DNA from the *TOX2* chromosome,

Table 2. Summary of data on *C. carbonum* strains used in this study.

Strain	Genes Missing[b]	Phenotype[c]	HTS activity[d]	HTS protein[e]	HC-toxin[f]	*Pac*I fragment size (kb)[g]	*TOX2* chromosome size (Mb)[h]
SB111 367-2	all present	V	+	+	+	1000	3.5
CC141	all present	V	+	+	+	ND	2.2
243-10	none present	AV	–	–	–	–	–
243-7	H1, A1, C3, D1, D3	RV	+	+	–	280	2.1
560-2[a]	H1, H2::hyg, A1, C3, D1, D3	AV	–	–	–	ND[i]	ND
373-39	H1, A1, C3, D1, D3	RV	+	+	–	190	ND
643-9	H1, A1, C3, D1, D3	V	+	+	ND	290	ND
164R10	H1, A1, C3, D1, D3	V	+	+	+	900	3
512-48	C3, D1, D3	V	+	+	+	400	2.7
625-66	C3, D1, D3	V	+	+	ND	490	ND
512-3	C3	V	+	+	+	450	2.3
512-8	C3	V	+	+	ND	ND	ND
512-98	C3	V	+	+	ND	ND	ND
512-35	C3	ND, slow grower	ND	ND	ND	ND	ND
512-98D	A1, A2, H1, H2, C3, C1, D1, D2, D3	AV	ND	ND	ND	ND	ND

[a]243-7 with remaining copy of *HTS1* mutated by targeted gene disruption
[b]H = *HTS1*; A= *TOXA*; C = *TOXC*; D=*TOXD*. Numbers indicate the particular copy (see Figures 8, 21). Data on *TOXE* (see Figure 18) are not included in this Table.
[c]V, virulent; RV, reduced virulence; AV, avirulent (see Figure 9 A,B).
[d]HC-toxin synthetase activity (HTS) measured by D-Ala-depedent ATP/PP_i exchange (see Figure 25 and Table 3)
[e]HTS protein detected by immunoblotting (see Figure 24)
[f]HC-toxin production in culture, analyzed by TLC (J. Pitkin, unpublished data).
[g]size of *Pac*I fragment hybridizing to *HTS1* as measured by pulsed-field gel electrophoresis (see Figure 20)
[h]size of chromosome containing all copies of *HTS1*, *TOXA*, *TOXC*, and *TOXD* (J. Ahn, unpublished)
[i]ND - not determined

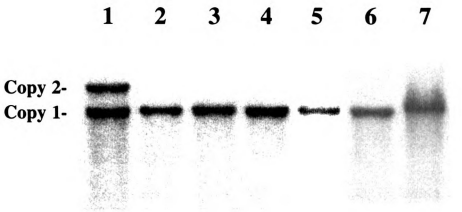

Figure 18. Southern blot analysis of *TOXE* RFLP in selected deletion strains. Genomic DNA was cut with *Bam*HI and probed with *TOXE* internal fragment. Lanes: 1, CC141; 2, 164R10; 3, 243-7; 4, 373-39; 5, 512-3; 6, 512-48; 7, 643-9.

512-24 was outcrossed to strain 243-6 (Tox2$^+$, normal growth). Ten progeny were isolated and their growth rates were assessed. Five of them grew at the same rate as the wild type. These five were analyzed by Southern blotting and two (643-8 and 643-9) had the same genetic composition as the parental strain 512-24. Therefore, in these two isolates the chromosomal aberration segregated away from the slow-growth phenotype.

Slow-growing strain 512-35 was not outcrossed and not analyzed further, because it belonged to the same deletion class as strains 512-3, 512-8 and 512-98 based on RFLP analysis.

An additional strain, 512-98D, was isolated from a sector (derivative of strain 512-98). It lacked all the *Tox2* genes except *TOXC*-1 (Figure 19).

The isolates with deletions of the *TOX2* chromosome, including 643-9, were analyzed further. Isolate 164R10, which, like 243-7, is a progeny of SB111 and SB114 (Figure 16) was also included. Isolate 164R10 has been used in other experiments from this laboratory (Ahn and Walton, 1997); it lacks copy 3 of *TOXC,* copies 1 and 3 of *TOXD*, and copy 1 of *TOXA/HTS1*, but has a wild type Tox2$^+$ virulence phenotype and produces HC-toxin in culture.

To better estimate the sizes of deletions on *TOX2* chromosome, some of the strains were analyzed by pulsed-field gel electrophoresis after digesting chromosomal DNA with the rare-cutting restriction endonuclease *Pac*I (Figure 20). Available markers allow the determination of the size of only one relevant *Pac*I fragment, the one containing both copies of *HTS1* (Figures 8, p. 34 and 21, p. 58). Additional experiments performed by J. Ahn (unpublished data) determined the *TOX2* chromosome sizes for some of the strains (summarized in Table 2).

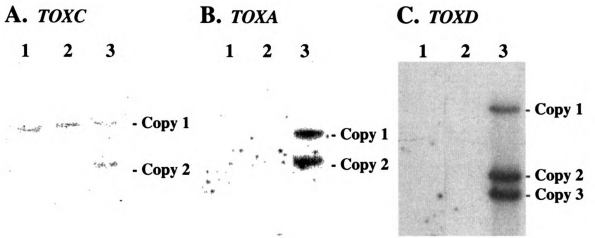

Figure 19. Southern blot analysis of strain 512-98D. **(A)** *TOXC* RFLP. Genomic DNA was cut with *Xho*I and the filter was probed with a *TOXC* internal fragment. Lanes: 1 and 2, two different single-spore isolates of strain 512-98D; 3, strain 243-7 (harboring *TOXC* copies 1 and 2). **(B)** *TOXA* RFLP. Genomic DNA was cut with *Bgl*I and probed with *TOXA* internal fragment. Lanes: 1 and 2, two different single spore isolates of strain 512-98D; 3, wild type Tox2$^+$ strain 367-2. **(C)** *TOXD* RFLP. Genomic DNA was cut with *Bam*HI and probed with *TOXD* internal fragment. Lanes: 1 and 2, two different single spore isolates of strain 512-98D; 3, wild type Tox2$^+$ strain 367-2.

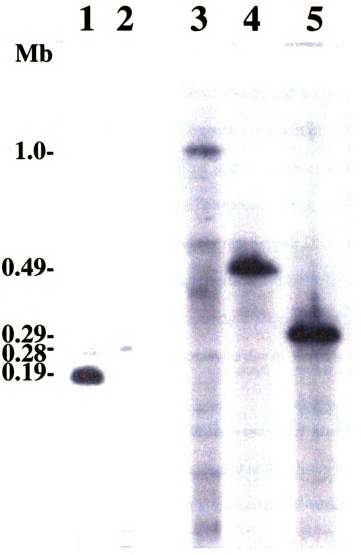

Figure 20. Physical mapping of *TOX2* chromosomes in representatives of wild type and deletion strains. DNA was cut with *Pac*I, separated by pulsed field gel electrophoresis, blotted, and probed with a fragment of the *HTS1* gene. The locations of relevant *Pac*I sites are shown in Figure 1. Lane 1, 373-39; lane 2, 243-7; lane 3, 367-2; lane 4, 635-66; lane 5, 643-9.

Maps of *TOX2* chromosomal deletions were constructed based on all available data. They are shown in Figure 21. In some of the strains, chromosomal deletions may be internal and in others, terminal; and some strains may have translocations in addition to deletions. Strain 164R10 is an example of a putative translocation or large internal deletion because it has a 3.0-Mb *TOX2* chromosome and a *Pac*I digestion fragment of 900 kb while missing all the known *TOX2* genes to the left of *TOXC*-2. Another factor contributing to the imprecision of the maps is that in the cases of simple truncations, telomeres must be added to the end of the chromosome after the break occurs. Lack of available markers does not allow us to tell how much repair DNA might have been added and how much of the DNA between the markers is native DNA (Figure 21). The fact that in strains 512-48 and 625-66, the *Pac*I restriction fragments are larger than predicted based on the genes missing in these strains, may be due to one or both of these factors.

Co-segregation of the RV phenotype with the chromosomal aberration

To test whether the RV phenotype is linked to the chromosomal deletion in strain 243-7, it was crossed to the wild type $Tox2^+$ isolate 243-1, which is a sibling of 243-7 from cross 243. Fifty-three progeny of this cross were isolated and analyzed. Twenty-seven were found to have three copies of *TOXC* and be fully virulent, thus resembling the wild type parent, 243-1. Another 25 progeny were missing copy 3 of *TOXC* and had an RV disease phenotype, thus resembling the RV parent, 243-7. One progeny (625-66) was missing copy 3 of *TOXC* but had a fully virulent ($Tox2^+$) disease phenotype. It was subsequently found to have two copies of *TOXA/HTS1* and one copy (copy 2) of *TOXD* (Figure 17). Thus, it had a genetic composition different from both parents and had

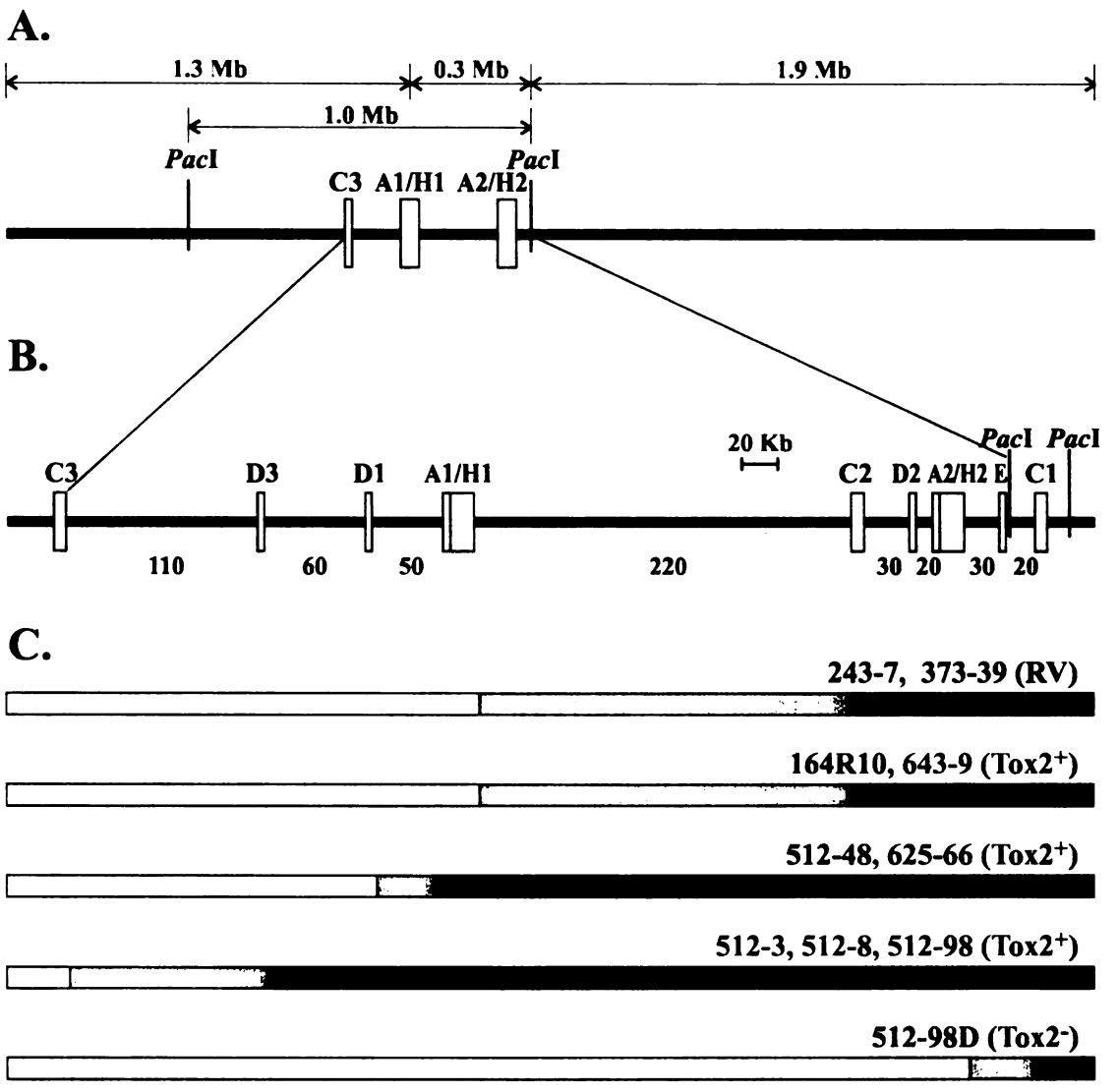

Figure 21. Physical maps of the *TOX2* chromosome. **(A)** Map of the entire 3.5 megabase *TOX2* chromosome of the wild type Tox2$^+$ strain, SB111 (after Ahn and Walton, 1996). Distances are given in megabases. *Pac*I indicates known *Pac*I restriction sites. **(B)** Detailed map of the region of the *TOX2* chromosome containing *TOX2* genes (after Ahn and Walton, 1996, with the addition of *TOXE*). Distances are given in kilobases. A1/H1 indicates *TOXA*-1 (*TOXA* copy 1) and *HTS1-1* (*HTS1* copy 1). C1, C2 and C3 are copies 1, 2 and 3 of *TOXC*, respectively. D1, D2 and D3 are copies 1, 2 and 3 of *TOXD*. E indicates *TOXE* copy 1. Lines connect the corresponding sites in **(A)** and **(B)**. **(C)** Deduced maps of deletions in strains bearing *TOX2* chromosome aberrations in relation to the defined *TOX2* region (~540 Kb from C3 to C1) of SB111. Data were derived from Figures 17 - 20. The maps are not meant to imply anything about the presence or absence of DNA outside the region shown.

■ = region of the chromosome remaining
□ = region of the chromosome missing
▭ = region of the chromosome where the break point maps

apparently arisen as a de novo aberration during the cross. It belongs to the same class as 512-48 (Figure 21). Two versions of the *TOX2* chromosome - wild type 3.5-Mb (presumed when all three copies of *TOXC* were present) and truncated 2.1-Mb (presumed when copy 3 of *TOXC* was missing) segregated as expected for homologous chromosomes, at a ratio not statistically different from 1:1 ($\chi^2 = 0.077$; df=1; $0.70 < P < 0.90$).

We also tested if any of the progeny from cross 373 (Figure 16) had an RV phenotype despite not having *TOX2* deletions detectable by RFLP. All 34 progeny from this cross were screened for pathogenicity. All except 373-39 were fully virulent ($Tox2^+$). Strain 373-39 had an RV phenotype. These results show that the RV phenotype cosegregates with deletion of the chromosomal region containing copy 1 of *TOXA/HTS1*, copy 3 of *TOXC*, and copies 1 and 3 of *TOXD*, and is therefore presumed to be due to the deletion.

Discussion of* TOX2 *chromosome segregation and instability

The *TOX2* chromosome of *C. carbonum* is a large supernumerary chromosome, and at least part of it is conditionally dispensable. Supernumerary chromosomes in filamentous fungi are extra chromosomes found only in some isolates of a given species and are composed primarily of DNA not found in all representatives of this species (Covert, 1998). Sometimes they carry genes encoding detectable phenotypes. In *Nectria haematococca,* the *PDA1-1* chromosome carries a number of genes contributing to high virulence on garden pea: the *PDA1-1* gene (Kistler and Van Etten, 1984a, 1984b; Miao et al., 1991b; Wasmann and Van Etten, 1996), and the *PEP* genes (Van Etten et al., 1997;

Covert, 1998). The *MAK1PDA6-1* chromosome of *N. haematococca* carries *MAK1*, a gene associated with high virulence on chickpea (Miao and Van Etten, 1992; Covert et al., 1996; Enkerli et al., 1998), and *PDA6-1*, one of the pisatin detoxification genes (Miao et al., 1991a). The *TOX2* chromosome of *C. carbonum* carries genes conferring high virulence on susceptible maize, an adaptive trait important in some, but not all, growth conditions. By definition, supernumerary chromosomes do not carry any essential genes, and the *TOX2* chromosome is not known to carry any. It does, however, contain some DNA common to $Tox2^{+}$ and $Tox2^{-}$ isolates (e.g., marker G242 [Ahn and Walton, 1996]; marker Q [Canada and Dunkle, 1997], *EXG2* [J. Ahn, unpublished data]). The 3.5-Mb *TOX2* chromosome may be a product of a translocation between a 2.2-Mb *TOX2* chromosome found in some wild-type isolates and a 2.0-Mb essential chromosome (Ahn and Walton, 1996). The proposed translocation pattern is shown in Figure 22. It implies that ca. 1.6 Mb of the *TOX2* chromosome containing the *TOX2* locus and everything to the left of it (i.e., conditionally dispensable "*TOX2* module") may be joined to either a 1.9- or a 0.6-Mb chromosomal segment and thus form the 3.5- or 2.2-Mb *TOX2* chromosome. The chromosomal 1.9-Mb segment contains markers G242, *EXG2* (Ahn and Walton, 1996 and unpublished data) and Q (Canada and Dunkle, 1997); and the 0.6-Mb segment contains marker CC62 (Ahn and Walton, 1996). A reciprocal event joins a putative 0.1-Mb segment to either the 0.6- or 1.9-Mb segment and results in chromosomes of 0.7 or 2.0 Mb. The chromosomal segments of 1.9, 0.6 and 0.1 Mb may or may not be dispensable and may carry essential genes. In the latter case, one or more of them must be present in the genome either as part of the 3.5- or 2.2-Mb *TOX2* chromosomes or as part of the 2.0- or 0.7-Mb chromosomes. This could be tested by

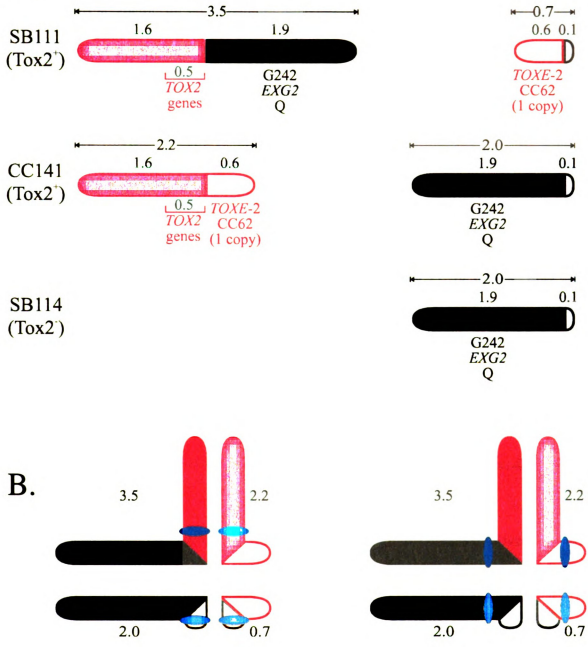

Figure 22. Proposed translocation involving two naturally occurring variants of *TOX2* chromosome. **(A)** Karyotypes of naturally occurring isolates. Known markers are indicated below the corresponding chromosomal segments. Maps are derived from all available data (Ahn and Walton, 1996; Canada and Dunkle, 1997; this work). **(B)** Cruciform structure that can form during meiosis I in the cross between SB111 and CC141 (cross 373). Two outcomes are possible depending on the location of the centromeres, indicated by blue ovals. Chromosome sizes and distances are given in Mb.

screening *TOX2*$^-$ cross progeny for the presence or absence of these chromosomes by CHEF or by screening for corresponding markers on Southern blots. Markers available on the 0.6-Mb segment are one of the copies of an internal fragment of CC62 (or A2B3) and *TOXE*-2. Based on the data from Southern blots (Figure 18 and DNA blots probed with A2B3, results not shown), these markers are missing in some strains (164R10, 243-7, 373-39, 512-3, 512-48, 643-9) and in independent Tox2$^-$ isolates (e.g., SB114). Thus, the available data suggests that the 0.6-Mb segment is most likely dispensable.

All these data put together suggest an intriguing hypothesis. The original *TOX2* chromosome may have been the 2.2-Mb chromosome (1.6-Mb *TOX2* module plus 0.6-Mb segment). It contained all of the genes essential for HC-toxin biosynthesis and no genes essential for growth, and was dispensable. It may have moved from another species and may have contained genes that were essential for its organism of origin but that had counterparts in *C. carbonum* and thus became dispensable. It is not clear, though, what could have been the species of origin and why it might have produced HC-toxin.

The *TOX2* chromosome contains much repetitive DNA (Ahn and Walton, 1996). Some of the repetitive sequences are Tox2$^+$-unique (e.g., CC115 transposase-like sequence, see Appendix B), while others are also found elsewhere in the genome (e.g., Fcc1 transposon, Panaccione et al., 1996). Similarly, in two other well-studied species, *N. haematococca* and *Colletotrichum gloeosporioides,* moderately and highly repeated DNA sequences are found on the supernumerary chromosomes, and some of these sequences are chromosome-specific, while others are shared with other chromosomes (Enkerli et al., 1997; Masel et al., 1993; 1996). This fact suggests that the *TOX2*

chromosome and the supernumerary chromosomes from *N. haematococca* and *C. gloeosporioides* have a different evolutionary history relative to the rest of the genome and lends further evidence to the horizontal transfer hypothesis.

The mechanism of the proposed horizontal chromosomal transfer remains to be elucidated, but there is evidence that in *C. gloeosporioides,* the supernumerary chromosome is transferred between asexual isolates at a frequency of 10^{-7} (Masel et al., 1996; Covert, 1998). It was suggested that such transfer may be analogous to the conjugative transfer of plasmids in bacteria (Covert, 1998).

Unlike other supernumerary chromosomes from sexually reproducing fungal species, for which the information is available (Miao et al., 1991a; Orbach et al., 1996; Xu and Leslie, 1996; Tzeng et al., 1992), the *TOX2* chromosome of *C. carbonum* does not exhibit non-Mendelian segregation ratios. In crosses between $Tox2^{+}$ and $Tox2^{-}$ isolates, a segregation ratio of 1:1 was first demonstrated by Nelson and Ullstrup (1961). To confirm this in light of the data on the non-Mendelian segregation of the supernumerary chromosomes in other fungal species, the segregation ratio in cross 512 with respect to the presence or absence of the *TOX2* chromosome was determined and compared with the Mendelian ratio of 1:1 which was expected based on earlier data (Nelson and Ullstrup, 1961). For this purpose, we can assume that the *TOX2* chromosome is missing in those progeny in which none of the copies of *TOXC* are present (although it is possible that in some of them, the whole *TOX2* locus was deleted with the rest of the chromosome remaining). In 59 progeny, the *TOX2* chromosome was present, and in 51 it was missing, which is not statistically different from the expected Mendelian ratio of 1:1 ($\chi^2 = 0.582$; df=1; $0.3 < P < 0.5$). In a cross between a wild type

Tox2$^+$ strain and an RV strain (243-7) bearing a truncated but presumably otherwise unaltered *TOX2* chromosome (cross 625), the wild-type and truncated chromosomes (as detected by Southern blot *TOXC* RFLP patterns) also segregated at a Mendelian ratio of 1:1, as expected for homologous chromosomes. In a cross between two wild type parental strains with different naturally-occurring variants (3.5- and 2.2-Mb) of the *TOX2* chromosome (cross 373), none of the progeny lost the chromosome, as determined by hybridization to *TOXC* probe. If the 3.5-Mb and 2.2-Mb *TOX2* chromosomes segregate independently of each other and also independently of the 2.0- and 0.7-Mb chromosomes with which they have sequences in common (Ahn and Walton, 1996; Canada and Dunkle, 1997), 25% of the progeny would be expected to lose the *TOX2* locus altogether, and another 25% to harbor both the 3.5-Mb and 2.2-Mb *TOX2* chromosomes. Although we did not determine by CHEF chromosome separation whether these two variants of *TOX2* chromosome segregated at a 1:1 ratio, the absence of Tox2$^-$ progeny suggests that, despite the fact that these two naturally occurring *TOX2* chromosome variants are homologous only in part, they pair up during meiosis as opposed to assorting independently. A similar conclusion was reached by Canada and Dunkle (1997). Furthermore, seven progeny of cross 373 were analyzed by PFGE (J. Ahn, unpublished data), and 11 progeny of a similar cross were analyzed by Canada and Dunkle (1997), and none were found to harbor two *TOX2* chromosomes.

However, we have to consider the possibility that one or both of the non-parental progeny classes (harboring none or both *TOX2* chromosomes) are inviable. We assume that duplications of the chromosomal segments within the *C. carbonum* genome are not lethal, similar to what is known for some other fungi (e.g., Perkins, 1974; Tzeng et al.,

1992). However, both of these classes may be missing some of the chromosomal segments (the progeny containing both *TOX2* chromosomes may be missing the 0.1-Mb "tip" segment located on the other chromosomes involved in translocation). In the case of the two *TOX2* chromosomes segregating independently of each other, there are three possible segregation patterns for the other chromosomes involved in the translocation. These possibilities are:

1. All four chromosomes segregate independently of each other. In this case, ¼ of all progeny will have both *TOX2* chromosomes and ¼ will have none. In each of these classes, ¼ will have all three of the other chromosomal segments present on the non-*TOX2* chromosomes. Thus, 1/16 of all progeny will be missing the *TOX2* locus but be viable and detectable by Southern blots. Of the class with two *TOX2* chromosomes, ¾ will have all chromosomal segments (some in more than two copies) on different chromosomes, and so presumably will be viable. Because neither of these are found among the progeny, we may conclude that this scenario is not possible.

2. The 3.5-Mb chromosome pairs with the 2.0-Mb chromosome, and the 2.2-Mb chromosome pairs with the 0.7-Mb chromosome (using the 0.6-Mb segment for a homologous region). Because the progeny classes that lack both of the *TOX2* chromosomes and that harbor both of the *TOX2* chromosomes will be missing the "tip" segment in addition to the *TOX2* module, and this can be lethal, this scenario cannot be excluded.

3. Only one or the other of the above pairs form, and the remaining two chromosomes segregate independently. In this case, ¼ of all the progeny will have both *TOX2* chromosomes, and ¼ will have none. In each of these classes, ½ will have all

three of the other chromosomal segments present and so presumably be viable. Thus, 1/8 of all the progeny will be missing the *TOX2* locus but be viable and detectable by Southern blots. Because this is not the case, we may conclude that this scenario is not possible.

The possible (# 2) scenario could be tested by using each of the available probes (*TOX2* genes, G242, *EXG2* and CC62). Assuming the progeny classes missing both *TOX2* chromosomes or harboring both *TOX2* chromosomes are inviable, ½ of the remaining progeny will contain all segments (having the 2.0- and 2.2-Mb chromosomes present); another ½ will lack the 1.9-Mb segment (and thus will lack G242 and *EXG2* markers) while containing the 3.5-Mb *TOX2* chromosome (this class may also be inviable). It would be possible to distinguish between this outcome and the scenario where the two *TOX2* chromosomes act as homologs (with the other pair acting as homologs or independently).

Finally, if these four chromosomes form a cruciform pattern typical for balanced translocations in meiosis, the results would be indistinguishable from those with two independent homologous pairs. The actual outcome will depend on the centromere position. If centromeres are on the *TOX2* module and on the "tip", the resulting progeny will be the same as in the case when two *TOX2* chromosomes (3.5- and 2.2-Mb) form a pair and two other chromosomes (2.0- and 0.7-Mb) form a pair. Alternatively, if the centromeres are on the 1.9- and 0.6-Mb segments, the resulting progeny will be the same as in the case when the 3.5-Mb chromosome pairs with 2.0-Mb chromosome (using the 1.9-Mb segment as a homologous region), and 2.2-Mb chromosome pairs with 0.7-Mb chromosome (using 0.6-Mb segment for a homologous region).

Still, it is unlikely that both the progeny class lacking the *TOX2* chromosome altogether and the one containing both *TOX2* chromosomes are inviable (because scenario #2 requires that the "tip" segment is indispensable), and so the 2.2- and 3.5-Mb *TOX2* chromosomes probably behave as homologs. This, in turn, would mean that the centromere is located on the 1.6-Mb *TOX2* module, and the one in the second pair of homologs is on the 0.1-Mb "tip" (first option in Figure 22B, p. 60, as opposed to the second option). On the other hand, from the data collected so far it appears that breaks in the *TOX2* chromosome are random, but usually to the left of *TOXC*-2. Since at least some of the strains (e.g., 243-7) appear to be missing the entire left segment of the *TOX2* chromosome (1.4 Mb in case of 243-7, with a breakpoint between *HTS1*-1 and *HTS1*-2 at ~20 kb from *HTS1*-1), the centromere must lie to the right of *HTS1*-1, and probably to the right of *HTS1*-2 (since the *TOX2* chromosome size in strain 512-98D was not determined, we cannot be sure it has a simple truncation). Assuming that the ca. 1.3-Mb segment of the *TOX2* chromosome to the left of and including *HTS1*-1 is dispensable, the centromere is between *HTS1*-1 and *TOXC*-1. Further, if the segment between A1/H1 and D2 is dispensable, the centromere is very close to *TOXC*-1. This region is also the location of DNA common to $Tox2^{+}$ and Tox^{-} isolates (Ahn and Walton, 1996).

We found that in some progeny truncated versions of this chromosome or other rearrangement patterns arose. In this work, a total of ~200 progeny from three crosses were analyzed by Southern blotting, and of these eight (4.0%) had deletions of one or more genes within the ~540 kb region of *TOX2*. Adding previously analyzed progeny from crosses A and 243 (Figure 16), out of a total of ~220 progeny analyzed, 10 (4.5%) had deletions within the *TOX2* locus. Pulsed-field gel electophoresis analysis of *TOX2*

chromosomes in these strains shows that major chromosomal rearrangements (truncations, translocations or major deletions) had taken place. This form of meiotic instability of supernumerary chromosomes has been also detected in *Nectria haematococca* and *Gibberella fujikuroi* (Miao et al., 1991a; Xu and Leslie, 1996). It was noted (Covert, 1998) that it is possible that such rearrangements are detected only in supernumerary chromosomes and not in essential chromosomes simply because the aberrations in the essential chromosomes are lethal. Interestingly, the data for yeast show that about 7% of haploid meioses (or 3.5% of all haploid progeny) produced chromosomes that differed by more than 10 kb from their wild-type counterparts (Loidl and Nairz, 1997).

One possible mechanism that can generate these chromosomal aberrations is via nonallelic recombination facilitated by repetitive sequences. This mechanism is well studied in yeast (e.g., Jinks-Robertson and Petes, 1986; Loidl and Nairz, 1997). Such recombination may occur between homologous as well as nonhomologous chromosomes and may produce major deletions and translocations.

In our experiments, some of the progeny in cross 512 showed a pattern of hybridization with the *TOXC* probe (two strong bands, one extremely faint band) suggesting nuclear non-homogeneity. Since in some cases this pattern persisted through several rounds of single-spore re-isolation, it may be due to the existence of heterokaryons among the progeny. The observation that such strains sometimes formed sectors when grown on plates and some of these sectors gave rise to pure isolates with deletions or complete loss of the *TOX2* chromosome further suggests that these heterokaryons can spontaneously resolve. The existence of heterokaryons among single

conidiospore isolates of *C. carbonum* recently also was suggested by observations on *C. carbonum* strains with disruptions of cell wall degrading enzyme genes (J. Scott-Craig, unpublished data). One possible way for the heterokaryons observed in this work to arise is that the *TOX2* chromosome rearrangement occurs in the early mitotic divisions of a germinating ascospore and the resulting heterogenous nuclei do not separate into different daughter cells. The fact that sometimes *C. carbonum* strains form sectors with de novo *TOX2* chromosome rearrangements (isolate 512-98D) proves that such rearrangements can be produced during mitosis (mitotic instability).

Pathogenicity phenotype and* TOX2 *gene expression in strains with chromosomal aberrations

Pathogenicity assays were done on strains with detectable deletions within the defined region between the two flanking copies of *TOXC* (summarized in Table 2). Strain 373-39 had an RV disease phenotype, and strains 512-3, 512-48, 643-9, 625-66 and 164R10 had a $Tox2^+$ (fully virulent) phenotype.

Isolates 512-3, 512-48 and 164R10 were tested for HC-toxin production by J. Pitkin (unpublished data summarized in Table 2). All produced HC-toxin in culture at levels comparable to the wild type $Tox2^+$ strains.

One possible reason for the putative low levels of HC-toxin in RV strains was that one or more of the HC-toxin biosynthetic genes was underexpressed. To test this hypothesis, we tested all the deletion strains for the presence of *TOXC, TOXA* (J. Pitkin, unpublished data) and *TOXE* (Figure 23) mRNA by Northern blotting and found that these genes are expressed at a level similar to wild type in all the strains used in this

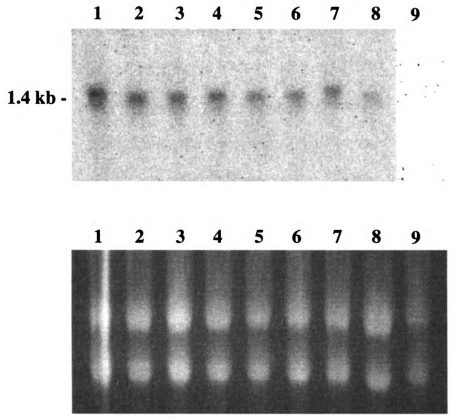

Figure 23. Northern blot analysis of *TOXE* expression in the *TOX2* deletion strains. **(A)** Total RNA was probed with *TOXE* internal fragment. **(B)** The same RNA gel stained with ethidium bromide before transferring RNA to nitrocellulose filter. Lanes: 1, SB111; 2, CC141; 3, 164R10; 4, 243-7; 5, 373-39; 6, 512-3; 7, 512-48; 8, 560-2 (243-7 with remaining copy of *HTS1* mutated by targeted gene disruption); 9, 243-10 (Tox2$^-$).

study.

Because the HTS mRNA is too large to be reliably detected by Northern blotting (Scott-Craig et al., 1992), we tested the deletion strains for the presence of HTS and HTS enzyme activity. HTS was partially purified by ammonium sulfate fractionation from mycelial pads and analyzed by Western blotting using anti-HTS-1 polyclonal antibodies (Scott-Craig et al., 1992) (Figure 24). HTS protein was detected in all of the strains. To compare HTS activity in RV and wild type $Tox2^+$ strains, cultures of 367-2, 243-7, 373-39 and 243-10 were grown in parallel, HTS was partially purified from mycelial pads by ammonium sulfate fractionation and anion exchange chromatography, and the HTS activity was compared in the fractions containing maximum activity (Figure 25). $Tox2^+$ and RV isolates had comparable HTS activity, the differences between isolates being considered within the range of experimental variation (Walton, 1987). Therefore, the defect in HC-toxin production in RV strains is probably not related to a defect in HTS. HTS activity was also measured in most other deletion strains ($Tox2^+$) and was also found to be comparable to that of the wild type (Table 3; summarized in Table 2, p. 52).

In conclusion, no changes in expression of any known *TOX2* genes were detected in RV strains or any other strains with chromosomal aberrations.

Possible mechanisms that may cause the RV phenotype

The reduced virulence phenotype in some isolates of *C. carbonum* was found to be associated with large deletions in the *TOX2* chromosome. Because the disease phenotype of the RV strains became race 2 (non-virulent) when the remaining copy of *HTS1* was disrupted in strain 243-7, we conclude that the RV strains produce at least

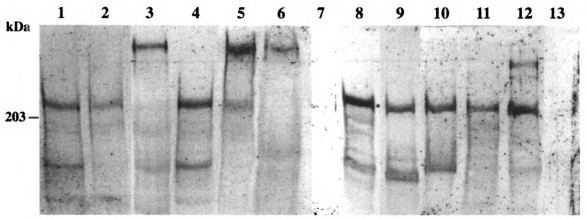

Figure 24. Western blot of HTS preparations from different strains used in this study. Protein extracts were purified through ammonium sulfate precipitation, resolved on a 6% acrylamide SDS-PAGE gel, and transferred to nitrocellulose as described by Scott-Craig et al., 1992. The filter was probed with anti-HTS-1 antiserum (raised against HTS-1, the 220-kDa portion of HTS that contains L-proline-activating activity and is one of the HTS breakdown products, Scott-Craig et al., 1992). The difference in size of the protein to which the antibody binds is the same as is normally observed when different preparations from the same wild type strain are compared (Scott-Craig et al., 1992). It is explained by the breakdown of the native HTS (570 kDa) into "HTS-1" (~200-220 kDa), "HTS-2" (~160 kDa), and some other breakdown products. HTS is extremely prone to this partial breakdown and two major breakdown products were originally thought to be two different enzymes (Walton and Holden, 1988). Lanes: 1, SB111; 2, 643-9; 3, 164R10; 4, 512-8; 5, 373-39; 6, 243-7; 7, 512-3; 8, 512-48; 9, 367-2; 10, 512-98; 11, 625-66; 12, CC141; 13, 243-10.

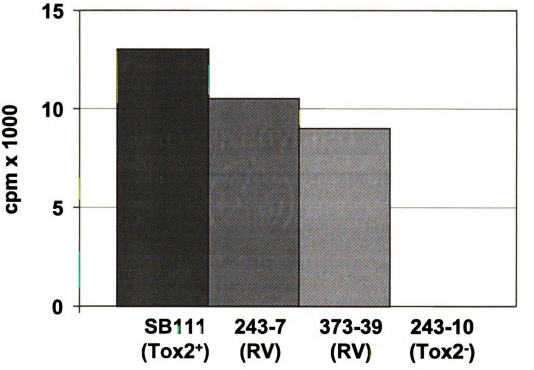

Figure 25. HC-toxin synthetase (HTS) activity in the RV isolates of *C. carbonum*. The protein extracts from isolates SB111 (Tox2$^+$), 373-39 (RV), 243-7 (RV), and 243-10 (Tox2$^-$) were desalted, and nine mg protein from each preparation was used for anion exchange HPLC (Walton and Holden, 1988). Ten µl of each 1-ml fraction was assayed for alanine-dependent ATP/PP_i exchange. The reaction mixes contained 300,000 cpm [^{32}P]pyrophosphate per reaction (125 µl). The results for the peak fractions (either fraction 20 or 21) are shown.

Table 3. HC-toxin synthetase (HTS) activity in isolates of *C. carbonum*.

D-alanine and L-proline-dependent ATP/PP_i exchange was measured in HTS preparations partially purified through ammonium sulfate precipitation and desalted. Specific activity was calculated as pmol PP_i/min/μg protein. For each reaction, 10 μg protein in 25 μl of extraction buffer (Walton and Holden, 1988) was added to 100 μl reaction buffer (Walton and Holden, 1988) containing 642,313 cpm of [^{32}P]PP_i, and reaction mixes were incubated for 20 min at 30° C.

	L-Pro -dependent specific activity	D-Ala -dependent specific activity
Strain		
243-7	13.6	6.8
643-9	19.5	14.6
164R10	7.3	2.9
367-2A	18.5	10.2
512-8	16.1	6.8
625-66	12.2	4.9

some small amount of HC-toxin *in planta* which accounts for the residual virulence. However, we could not detect HC-toxin in culture filtrates of RV strains. It is possible that RV strains produce less than 1% of the wild type-level of HC-toxin. Alternatively, these strains may be producing HC-toxin only in planta. We propose that reduced amounts of HC-toxin are responsible for the reduced virulence phenotype.

It is known (Panaccione et al., 1992) that both copies of *HTS1* are active in wild-type race 1 strains, and when one or the other is disrupted, the remaining one is sufficient to sustain a race 1 (virulent) phenotype. Therefore, the RV phenotype is not due simply to the loss of one of the copies of *HTS1*.

Several explanations are possible for the reduction in HC-toxin production in RV strains. The presence and levels of activity of HC-toxin synthetase (HTS) in RV strains were similar to those in wild-type virulent strains. Therefore, the low level of HC-toxin production is not due to a low level of HTS. One possibility is that HTS in the RV strains is defective in some step other than amino acid activation, such as thioesterification, epimerization, or peptide bond formation. Such a defect would not be detectable by ATP/PP_i exchange assay or Western blotting. However, the fact that both copies of *HTS1* are fully functional in the $Tox2^+$ parents of RV strains and each of the copies alone produce a fully virulent phenotype makes this explanation unlikely. It would require two independent mutational events during or after meiosis in two different crosses to produce two strains (243-7 and 373-39) with identical phenotypes.

Another possibility is the loss of a gene involved in some step of HC-toxin biosynthesis other than those catalyzed by *HTS1* and *TOXC*. For the fungus to retain a residual level of HC-toxin production there should be another gene whose product can

partially substitute for the missing enzyme. The absence of this gene may be partially complemented by another enzyme, presumably encoded by a housekeeping gene. This partial complementation would result in the low levels of HC-toxin production. For example, the putative α subunit of *TOXC* may have a housekeeping homologue involved in primary fatty acid synthesis that may be responsible for the residual levels of HC-toxin if the $Tox2^+$ -specific gene (*TOXC*) is deleted. We also know that *C. carbonum* has peptide synthetase genes other than *HTS1* (Nikolskaya et al., 1995). These peptide synthetases may have their own corresponding set of biosynthetic genes (e.g., alanine racemase), and they could also play a role in partially substituting for the gene missing in RV strains. Basal levels of HC-toxin may also be produced if one of the two copies of a biosynthetic gene was partly defective, and the fully functional copy was lost. It is also possible that the gene predicted to be missing in RV strains may actually be present but inactivated by the proximity to the new telomere.

It is also possible that RV isolates have a defect in the regulation of expression of genes other than *HTS1* or *TOXC* involved in HC-toxin biosynthesis. Changes in regulation may be due to the loss of a positive activator gene that controls their expression. This may result in basal expression of such HC-toxin biosynthetic gene(s), decreased HC-toxin production and a reduction in virulence. So far one $Tox2^+$ -unique gene (*TOXE*) encoding a promoter-binding protein has been found (Ahn and Walton, 1998; K. Pedley, unpublished data). *TOXE*, however, is not responsible for the RV phenotype because it is necessary for the transcription of *TOXA, TOXC* and *TOXD,* is present in RV strains, and is expressed in these strains at wild-type levels (Figures 18 and 23).

Alternatively, HC-toxin of much reduced activity (e.g., lacking an epoxide group) may be produced by RV strains. In this case, it may be not detectable by the epoxide-specific assay used after TLC separation. Both the epoxide group and the 8-carbonyl group of the Aeo side chain are known to be important for biological activity of HC-toxin (Ciuffetti et al., 1983, Kim et al., 1987). Current evidence indicates that the site of action of HC-toxin is histone deacetylase, an enzyme that reversibly deacetylates core histones while they are assembled in chromatin. HC-toxin was shown to inhibit histone deacetylase (Brosch et al., 1995). Recently, apicidin, a novel cyclic tetrapeptide, was also shown to inhibit histone deacetylase and to have strong cytostatic activity similar to HC-toxin (Darkin-Rattray et al., 1996) (Figure 1, p. 5). Apicidin lacks the epoxide group but has the 8-carbonyl group on the 2-amino-8-oxo-decanoic acid moiety, similar to HC-toxin. This makes it plausible that an HC-toxin derivative without the epoxide group, while lacking the biological activity in the standard root growth assay, may still possess residual activity during the plant-pathogen interaction. Such altered HC-toxin can be produced if the RV strains lack gene or genes required for the epoxide group biosynthesis.

In conclusion, to account for the low level of HC-toxin produced by RV strains, the gene or genes that are presumed missing could have a structural (biosynthetic) or regulatory function. The deleted structural gene or genes could be partially substituted for by other genes that encode enzymes with overlapping function.

Cosmids containing the chromosomal region adjacent to* HTS1/TOXA-*1* *do not complement the RV phenotype

Since it appears to be most likely that the RV phenotype is due to a missing gene or genes, we attempted to narrow down the region where such gene(s) may be located by comparing putative maps of all available strains with chromosome aberrations. From the available data on strains 512-48 and 625-66 (Figure 21, p. 58; Table 2, p. 52), it appears that insofar as at least one of these deletion isolates is missing all of its DNA to the left of *TOXD*-1, this segment of the *TOX2* chromosome is not required for HC-toxin biosynthesis. Since all the data is consistent with strain 243-7 having a simple terminal deletion of the entire segment to the left and including *HTS1*-1 (Ahn and Walton, 1996), it is likely that there are no essential genes on this ca. 1.3 Mb of DNA. Thus, the left segment of the *TOX2* chromosome appears to be conditionally dispensable.

The maps of the *TOX2* chromosomes in the strains with chromosomal aberrations are consistent with the hypothesis that a putative gene missing in RV strains maps to the region immediately adjacent to *HTS1/TOXA*-1 (Figure 21). Based on the *TOX2* chromosome and *Pac*I restriction fragment sizes, the breakpoint in strain 243-7 maps to ca. 20 kb to the right of *HTS1*-1 (Ahn and Walton, 1996). It is important to remember that the exact breakpoint in this and other strains is unknown, because available markers do not give sufficient resolution. The *Pac*I restriction fragment size in the second RV strain 373-39 (ca. 190 kb) is smaller than in 243-7 (ca. 280 kb), so this strain should be missing the same region adjacent to *HTS1/TOXA*-1 plus 90 kb to the right. Strain 643-9 has a *Pac*I digestion fragment of ca. 290 kb and is fully virulent (Figures 20, p. 56; and 21, p. 58). The putative gene is predicted to be present in strain 643-9 and missing in

243-7 and 373-39, in which case it must be located within ca. 10 kb to the right of *HTS1*-1. Allowing for the possibly imprecise distances determined by PFGE analysis, this region can be expanded to ca. 20 kb. However, the *TOX2* chromosomes in some of these strains may bear translocations and/or large internal deletions rather than having undergone a single, simple chromosome break. (Strain 164R10 is an obvious example and therefore was excluded from the above assessment of the location of the putative missing gene).

To test the hypothesis that the missing gene is located in this region, complementation experiments were conducted. They were designed to determine if the RV phenotype could be restored to wild-type (virulent) by introducing the chromosomal region in question back into 243-7. Chromosomal walking is often impossible in *C. carbonum* because of the abundance of repetitive DNA. A *C. carbonum* cosmid library constructed by J. Scott-Craig (unpublished data) was screened with two $Tox2^+$-unique DNA fragments, CC61 and a *TOXA* internal fragment. These two fragments are located in the rightmost and leftmost parts of the *HTS1/TOXA* cluster, respectively. By selecting cosmid clones that hybridized to one of these probes and not the other, we obtained cosmids that extended to the right or to the left of *TOXA/HTS1*, and that did not contain the complete copy of *TOXA/HTS1*. Among these cosmids, we selected by RFLP analysis those that originated from copy 1 of *HTS1/TOXA*, and not from copy 2. The resulting four cosmids (5E8, 7F6, 14F6, 15A7) covering the region immediately to the right of *HTS1/TOXA*-1 were transformed into 243-7. Hygromycin-resistant transformants were generated (5, 3, 20 and 6 transformants for 5E8, 7F6, 14F6 and 15A7, respectively). They were isolated and tested for pathogenicity. All exhibited the RV phenotype and

none showed wild-type race 1 phenotype. Thus, these cosmids failed to complement the RV phenotype.

These results indicate that either the putative missing gene is not located in this 20 kb region, or the cosmids used are chimeric. The map of the putative chromosomal breaks is imprecise because the region between *HTS1*-1 and *TOXC*-2 in RV strains does not necessarily correspond to this region in wild type. It may have originated by a translocation, or a telomere may have added the critical "extra" DNA to the detectable *Pac*I fragment. In both of these cases, the actual chromosomal region missing in strain 243-7 may be much larger than 20 kb. More markers between *TOXC*-2 and *HTS1*-1 could help to determine if this is the case.

Materials and methods

Fungal culture growth and maintenance

Conidia of *Cochliobolus carbonum* SB111 ($Tox2^+$; ATCC 90305), SB114 ($Tox2^-$; Walton, 1987), 367-2 (Görlach et al., 1998), CC141 (Ahn and Walton, 1996), 243-7 (Ahn and Walton, 1996), and others (this study: Figure 16; Table 2) were stored at -80° C in 25% (v/v) glycerol and grown on V8 juice agar plates. For DNA or protein extraction, mycelial agar plugs (0.5 cm^2) were inoculated into 1-L Erlenmeyer flasks containing 125 ml modified Fries' medium (Scheffer and Ullstrup, 1965). Cultures were incubated at room temperature (21-23° C) without shaking for four days.

Fungal crosses were performed as described for *Cochliobolus heterostrophus* (Yoder, 1988; Tzeng et al., 1992). Individual ascospores were picked and progeny were

put through two rounds of single-spore re-isolation to insure nuclear homogeneity.

For growth rate comparison, cultures were grown in horizontal race tubes containing potato dextrose agar. Averages of duplicate samples were used to calculate growth rate. Radial growth was measured on V8 juice agar plates.

Pathogenicity tests and leaf penetration tests

All pathogenicity tests were done in a greenhouse. Plants were grown in 30-cm pots (three plants per pot) in soil composed of Bactomix (Michigan Peat Co., Houston, TX) and sand, 4:1 (v/v). Leaves of maize inbred Pr (genotype *hm1/hm1*), typically at the 4-leaf stage (~25 cm tall) were sprayed with an atomizer with a suspension of conidia (typically $5x10^4$/ml) suspended in 0.1% Tween-20 and covered with plastic bags overnight. Development of symptoms was observed each day for 7 to 21 days. Every pathogenicity test was done at least three times, and in each of these experiments, one to three pots were used to inoculate with each strain tested.

Seed and soil inoculation was done as described on pp. 38 and 40. Each of these experiments was done twice, and 3 pots were used for each strain and each treatment.

For leaf penetration tests, maize plants at the 4-leaf stage or individual excised leaves were inoculated with a suspension of conidia ($5x10^4$/ml) in 0.1% Tween-20. For the whole plant inoculation, the general pathogenicity test protocol was followed and leaves were examined at appropriate time points. For individual leaf inoculation, leaf segments were wetted with the spore suspension, placed in closed Petri dishes on water-saturated filter paper, and incubated at room temperature. To stain the fungal hyphae, a solution of 0.1% cotton blue in lactophenol (20% phenol, 20% lactic acid, 40% glycerol,

20% water [v/v]) was diluted 1:3 (v/v) with 95% ethanol. Leaf segments were placed in 25 ml of the resulting mix, boiled for 1 min, cooled and boiled again for 30 sec, and incubated for 48 hr at room temperature. After washing in water several times, leaves were mounted on glass slides with 50% glycerol in H_2O and observed under a microscope at the magnification of X100.

Fungal transformations

Preparation and transformation of protoplasts was as described (Scott-Craig et al., 1990). Transformants able to grow on V8 juice agar containing 100 units/ml hygromycin (Calbiochem, La Jolla, CA) were purified by two rounds of single-spore isolation to obtain nuclear homogeneity.

Nucleic acid manipulations

Fungal genomic DNA and total RNA was isolated as described by Pitkin et al. (1996). Southern and Northern blot analysis was done as described by Sambrook et al. (1989).

Pulsed-field gel electophoresis

Chromosomal DNA was prepared as described by Orbach et al. (1988). Agarose plugs containing DNA were immersed in 1 ml of TE (10 mM Tris, 1 mM EDTA, pH 8.0) and chilled on ice for 30 min. TE was replaced with 1 ml of NDS buffer (0.45 M EDTA, 10 mM Tris pH 7.5, 1% lauroyl sarcosine) containing 2 mg/ml Proteinase K and plugs were incubated for 48 h at 50° C, then rinsed with 50 mM EDTA. Plugs containing 1 to 2

μg DNA were rinsed with 1 ml of TE, then TE was replaced with the appropriate restriction endonuclease buffer and agarose plugs were incubated on ice for 1 hr. The buffer was replaced with the fresh buffer containing 30 units of *Pac*I restriction enzyme; the plugs were incubated on ice overnight and then for 2 hr at 37°. After digestion, enzyme and buffer were removed, the plugs were washed with TE and loaded onto the CHEF gel.

Contour-clamped homogenous field (CHEF) electrophoresis (CHEF-DR II, Bio-Rad) was performed as described by Ahn and Walton (1996). Chromosomal DNA was fractionated by CHEF on a 1% chromosomal-grade agarose (Bio-Rad) gel at 170 V with a 2- to 25-sec switching time for 22 hr and then a 60- to 120-sec switching time for 24 hr. After staining with ethidium bromide for photographing, the gels were destained and transfer and hybridization were performed as described by Ahn and Walton (1996).

Protein manipulations and HTS activity assays

HTS enzyme extraction, partial purification by ammonium sulfate precipitation and anion exchange HPLC, and assay by ATP/PP_i exchange were done as described (Lee and Lipmann, 1975; Walton, 1987; Walton and Holden, 1988). Protein was measured by the method of Bradford (1976). For Western blot analysis, loading buffer (62.5mM Tris-HCl, pH 6.8, 10% glycerol, 2% SDS, 5% [v/v] β-mercaptoethanol, 0.0025% Bromphenol Blue) was added to the desalted samples and they were heated to 95° C for 5 min. Resolving gels were prepared with an acrylamide concentration of 6% (acrylamide/bisacrylamide ratio 30:0.8). Electrophoresis was performed in a mini-PROTEAN minigel apparatus (Bio-Rad Laboratatories, Richmond, California) using high

molecular weight prestained standards (Bio-Rad Laboratories). After electrophoresis, proteins were transferred to nitrocellulose using an LKB Multiphor II transfer unit. The nitrocellulose was blocked with 0.3% Tween-20, incubated first with polyclonal anti-HTS-1 antibody (Scott-Craig et al., 1992) at 1:500 dilution and then with goat anti-mouse IgG coupled with alkaline phosphatase (Sigma) at 1:1000 dilution.

Chapter 3

EXG1, A NOVEL β-1-3-EXOGLUCANASE FROM *COCHLIOBOLUS CARBONUM*

Abstract

Results described in this chapter were published as Nikolskaya et al., 1998.

Genomic and cDNA copies of *EXG1*, a gene encoding an exo-β-1,3-glucanase from the filamentous fungus *Cochliobolus carbonum*, were isolated. The gene contains two introns of 50 and 53 bp, and the mRNA has a 3' untranslated region of 159 bases. The deduced protein product, EXG1p, has a predicted 17-amino acid signal peptide but apparently undergoes a second processing event resulting in the removal of a total of 42 amino acids. At the time (1995) of submission of the *EXG1* sequence to GenBank (accession # L48994), the deduced amino acid sequence of EXG1p was not closely related to any other known protein, thus being a novel glucanase. Later, three glucanase gene sequences from mycoparasitic fungi became available that belong to the same family: BGN13.1, an endo-β-1,3-glucanase from *Trichoderma harzianum*, and exo-β-1,3-glucanases from *T. harzianum* and one from *Ampelomyces quisqualis*. BGN13.1, in contrast to EXG1p and the other two exoglucanases from this family, is acidic rather than basic, and its distribution of cysteine residues is biased towards the carboxy half of the protein. EXG1p and the other two exo-β-1,3-glucanases from this family contain two imperfect copies of a 23-amino acid motif that is found in several other proteins that interact with polysaccharides, including plant and bacterial polygalacturonases, phage

neck appendage protein, phage endoneuramidase, and bacterial mannuronan epimerase.

Introduction

Extracellular cell-wall degrading enzymes are widespread among plant pathogenic and saprophytic microorganisms. During plant pathogenesis, cell wall degrading enzymes may contribute to penetration, ramification, and the acquisition of nutrients from plant wall polymers (Walton, 1994).

β-1,3-Glucanases occur widely in bacteria, fungi and higher plants. In fungi, they have been proposed to have one or more functions. The nutritional utilization of β-1,3-glucans is one obvious possible function (e.g., Stahmann et al., 1992; Lorito et al., 1994), but they have also been proposed to be involved in autodigestive loosening of the wall to promote wall expansion and hence growth (Nobela et al., 1988). Although plants normally contain only small amounts of β-1,3-glucan, during plant pathogenesis β-1,3-glucanases could have a role in penetration through papillae, which are pathogen-induced wall appositions containing the β-1,3-glucan known as callose (Schaeffer et al., 1994).

The filamentous fungus *Cochliobolus carbonum* produces many extracellular cell wall degrading enzymes, including at least one enzyme with β-1,3-glucanase activity (Walton, 1994). The major β-1,3-glucanase activity of *C. carbonum,* EXG1p, an exo-acting enzyme, has been purified (Van Hoof et al., 1991). A partial genomic clone of the gene encoding EXG1p, *EXG1*, was isolated by H. Schaeffer and used to create, by targeted gene disruption, a strain of *C. carbonum* mutated in *EXG1*. Disappearance of the major chromatographic peak of exo-β-1,3-glucanase activity in the mutant strain indicated that the correct protein had been purified and that the correct gene had been

disrupted (Schaeffer et al., 1994). The *exg1* mutant grew much less well than wild type on β-1,3-glucan as sole carbon source but had unaltered pathogenicity on maize (Schaeffer et al., 1994). The *exg1* mutant strain had significant residual β-1,3-glucanase activity due to MLG1p, an endo-acting enzyme that can degrade both β-1,3-β-1,4-(mixed-linkage) and β-1,3-glucans (Van Hoof et al., 1991; Görlach et al., 1998), *EXG2* (J. Ahn, unpublished data), and perhaps to other β-1,3-glucanases as well.

Results and discussion

Analysis of genomic and cDNA clones of EXG1

A partial genomic copy of *EXG1* was previously isolated using PCR primers based on the experimentally determined sequences of N-terminal and internal tryptic peptides of EXG1p (Schaeffer et al., 1994). To obtain a clone containing the entire coding region, a 650-bp *Bgl*II fragment internal to this open reading frame was used as a probe to screen a genomic DNA library made in phage lambda. Two overlapping fragments that hybridized to the probe were subcloned from a single lambda clone and a total of 4048 bp was sequenced on both strands (Figure 26).

The same *Bgl*II fragment was used to screen a cDNA library made from mRNA from *C. carbonum* grown on maize cell walls (Pitkin et al., 1996). Two overlapping cDNA clones were isolated and the sequences of both strands determined. Genomic and cDNA sequences were entirely colinear with the exception of two introns of 50 and 53 bp (Figure 27). The introns contain conserved 5' (consensus GTAMGH, where M = A or C, and H = C, T or A) and 3' (consensus YAG, where Y = C or T) splice junctions as well as

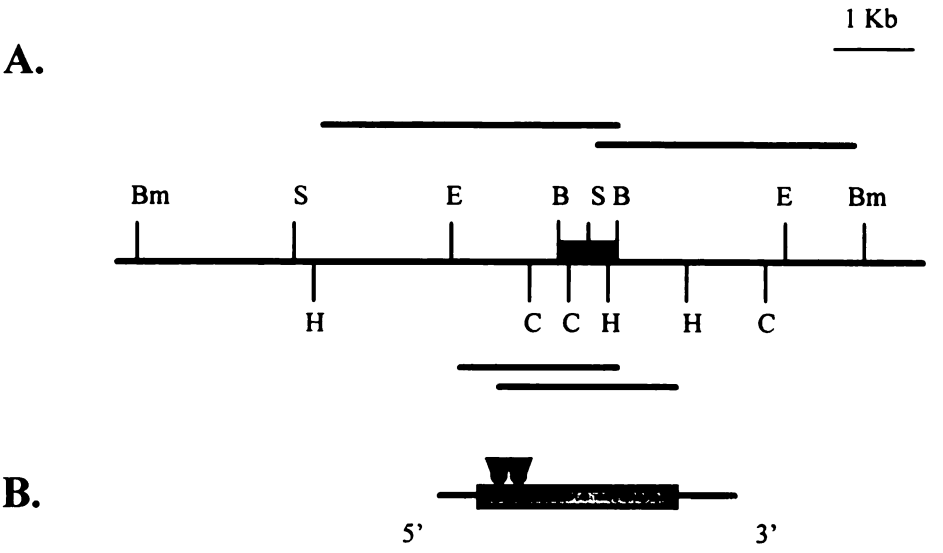

Figure 26. Restriction map of *EXG1* genomic and cDNA clones. **(A)** Genomic restriction map of the *EXG1* locus. Location of the two overlapping genomic fragments and of the two overlapping cDNA clones are shown above and below the restriction map, respectively. *Bgl*II fragment is shown as a black box. **(B)** Sequenced region with open reading frame shown as a shaded box. Location of the two introns is shown by triangles. B, *Bgl*II; Bm, *Bam*HI; C, *Cla*I; E, *Eco*RV; H, *Hind*III; S, *Sal*I.

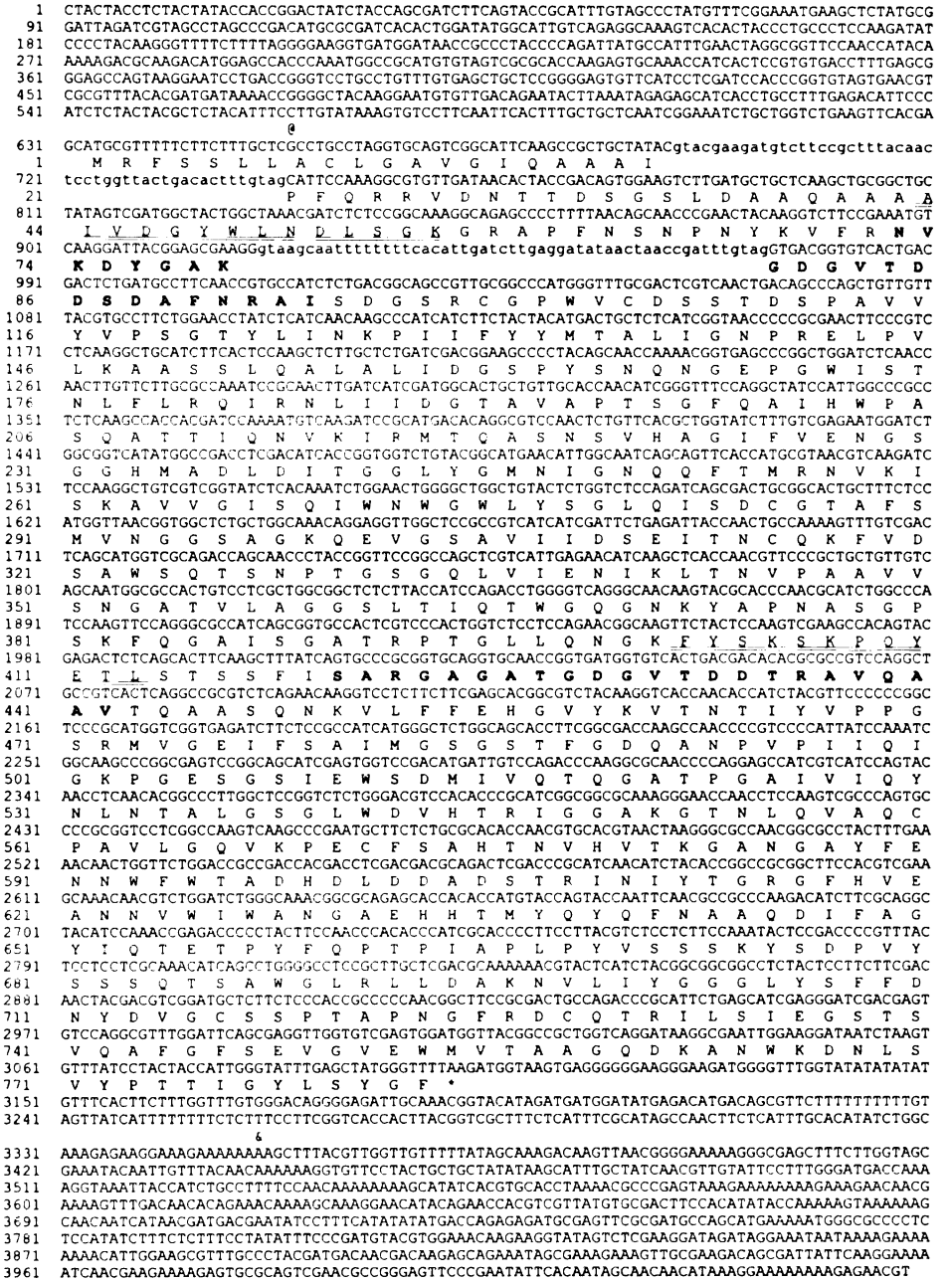

Figure 27. Nucleotide and deduced amino acid sequences of *EXG1*. Intron sequences are shown in lower case, and conserved splice sites are underlined. The conserved motifs are highlighted. The start of the longest cDNA and the polyadenylation site are indicated underneath the corresponding nucleotide by @ and &, respectively. The double underlined regions are the peptide sequences obtained from amino acid sequencing of the purified protein (Schaeffer et al., 1993), the first one corresponds to the amino terminus of the mature protein. The sequence was submitted to GenBank in 1995 (accession number L48994).

internal consensus sequences RCTRAC, where R = A or G (Orbach et al., 1986; Ballance, 1986) (Figure 27). Based on the sequences of two independent cDNAs, the polyadenylation site is 159 bp downstream of the stop codon, TAA. The first ATG codon between the start of the longest cDNA and the experimentally determined N-terminus of the mature protein (at nucleotide 633) is assumed to be the translational start site. This conclusion is supported by the lack of any other in-frame Met residues, numerous stop codons in all frames upstream of the cDNA start, and the presence of a strongly predicted signal peptide from amino acids 1 through 17 (see below).

EXG1 encodes three peptides derived directly from the purified protein, indicated by double underlining in Figure 27 (Schaeffer et al., 1994). The predicted size of the mature EXG1p protein is 79 kDa, in reasonable agreement with the size determined by SDS-PAGE, 73 kDa (Van Hoof et al., 1991). A signal peptide cleavage site is predicted between amino acids 17 and 18 by the SignalP program (Nielsen et al., 1997). Because the mature protein starts at amino acid 42 (Van Hoof et al., 1991), EXG1p probably undergoes a second processing event, but there is not a pair of basic residues at this position indicative of processing by a KEX2- like protease. There are three potential N-glycosylation sites (NXS/T) at amino acids 233, 333, and 773, but glycosylation of the mature protein was not detected (Van Hoof et al., 1991).

EXG1p is a novel β-1,3-glucanase

At the time when these results were submitted to GenBank, analysis using BLAST indicated that EXG1p had some overall amino acid sequence similarity to BGN13.1, an endo-acting β-1,3-glucanase produced by the mycoparasitic fungus *Trichoderma*

harzianum (de la Cruz et al., 1995). The two sequences are only 29% identical overall, and the largest contiguous stretch of identical amino acids is seven (AASQNKV, starting at position 450) (Figure 28). Unlike BGN13.1, the distribution of the cysteine residues of EXG1p is not biased toward the carboxy terminal half of the protein. Furthermore, EXG1p is an acidic protein, with an experimentally determined as well as predicted pI of 6.3 (Van Hoof et al., 1991), whereas BGN13.1 is basic, with an experimentally determined pI of 7.7 to 8.0 and a predicted pI of 7.5 (de la Cruz et al., 1995).

Recently, two new β-1,3-exoglucanases that belong to the same novel family were sequenced: one from *Trichoderma harzianum* (Cohen-Kupiec et al., 1998) and another from *Ampelomyces quisqualis* (Rotem et al., 1998) (Figure 28). They are 42% and 38% identical to EXG1p and have pI's of 4.7 and 4.9, respectively. Thus, these proteins are more similar to EXG1p than is BGN13.1. It was proposed that cysteine residues that occur predominantly in the C-terminal portion of BGN13.1 may play a role in cell wall binding (de la Cruz et al., 1995). Some of these residues are conserved throughout the members of the EXG1p family (Figure 28), but others are not. Furthermore, in the three exoglucanases, unlike in BGN13.1, the distribution of cysteine residues is not biased towards the carboxy half of the protein. Whether the carboxy-terminal domain of any or all of the glucanases from the EXG1p family is a cell wall-binding domain has to be determined experimentally.

The deduced amino acid sequences of β-1,3-glucanases from barley and yeast are conserved around the nucleophilic active site, V(S/G)E(S/T)GWP(S/T) (Chen et al., 1993). EXG1p contains a sequence, GEPGWIS, starting at amino acid 167, that is somewhat similar (GEXGWXS) to this motif. The Glu, second Gly, and final Ser

Figure 28. Comparison of the predicted amino acid sequence of *C. carbonum* EXG1p with other β-1,3-glucanases from the same novel family. The sequences of EXG1p, *Trichoderma harzianum* β-1,3-exoglucanase (Cohen-Kupiec, 1998), *Ampelomyces quisqualis* β-1,3-exoglucanase (Rotem et al., 1998), and *T. harzianum* β-1,3-endoglucanase (BGN13.1, De la Cruz et al., 1995) were compared using LASERGENE software (DNASTAR, Inc., Madison, WI, USA). Identical amino acids are highlighted, conserved cysteine residues are indicated by asterisks, and the N-termini of mature proteins are shown in bold.

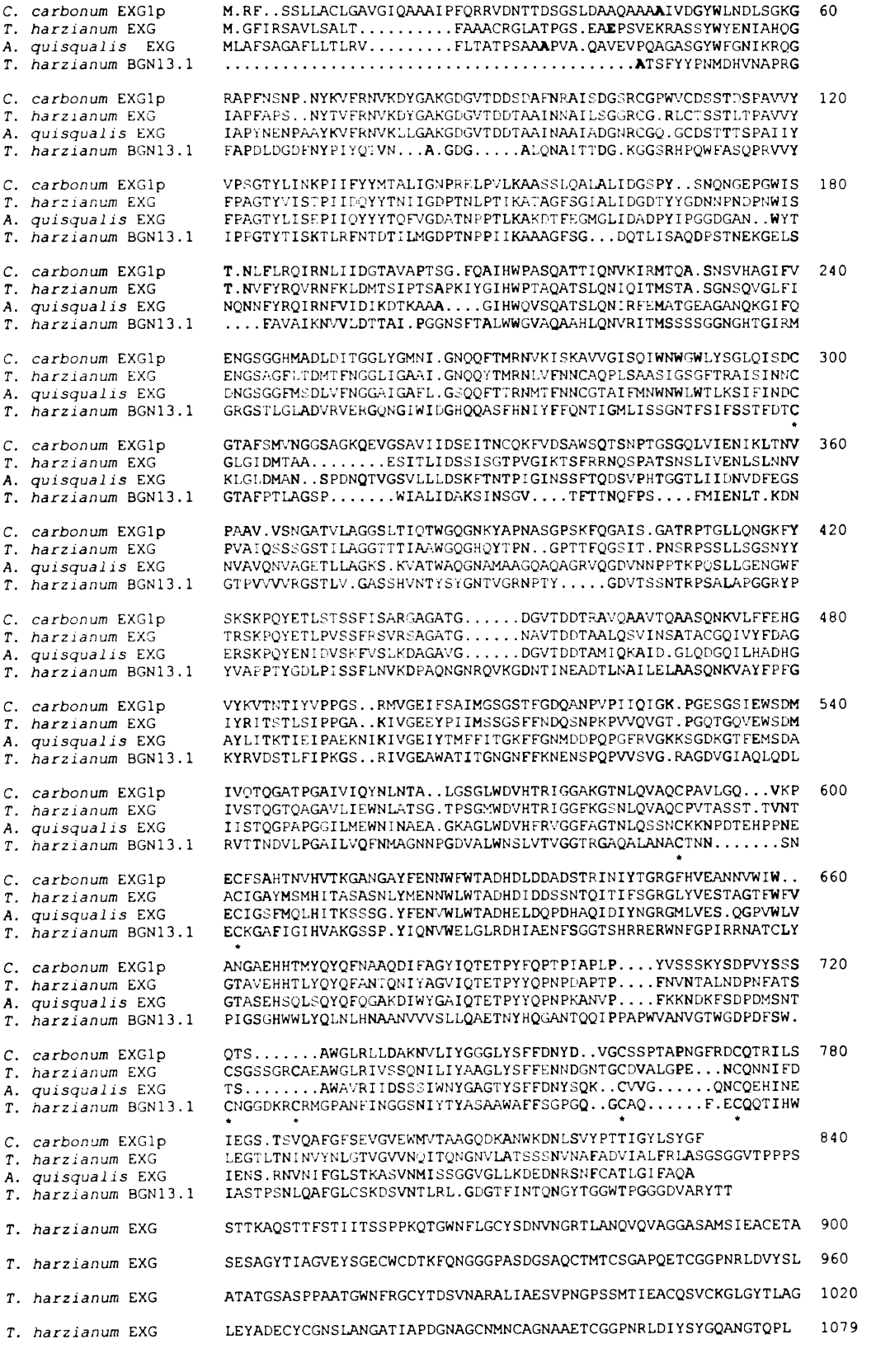

Figure 28. Comparison of the predicted amino acid sequence of *C. carbonum* EXG1p with other β-1,3-glucanases from the same novel family.

residues of this motif are also conserved in a comparable position (NEKGELS) in BGN13.1 (de la Cruz et al., 1995). However, in the barley and yeast β-1,3-glucanases this motif is located toward the C-terminus, whereas in EXG1p and BGN13.1 it is closer to the N-terminus. β-1,3-Glucanases from other plants, fungi and bacteria do not have this motif at the active site (Keitel et al., 1993; Mackenzie et al., 1997).

EXG1p has two copies of a motif shared with other proteins that interact with polysaccharides

EXG1p contains two imperfect copies of a 23-amino acid sequence (NVKDYGAKGDGVTDDSDAFNRAI and SARGAGATGDGVTDDTRAVQAAV), starting at amino acid 72 (equivalent to amino acid 29 in the mature protein) and 425, respectively (Figures 26, 28). The two copies are 48% identical. A related amino acid sequence is found in recently sequenced β-1,3-exoglucanases from *T. harzianum* and from *A. quisqualis*, and in several other proteins, including viral endoneuroaminidase (sialidase), viral neck appendage protein, bacterial mannuronan epimerase, and several plant and bacterial polygalacturonases (Figure 29). In all enzymes that have a single copy of this motif, it is found near the N-terminus. New members of the EXG1p family from *T. harzianum* and from *A. quisqualis* contain two copies of this motif located at positions similar to those of EXG1p (Figures 26, 27). Of the five known mannuronan epimerases of *Azotobacter vinelandii*, two contain a 385-amino acid duplicated module, and each module contains a copy of the EXG1p motif in its N-terminal part (Ertesvåg et al., 1995; Figure 29). Interestingly, β-1,3-glucanases of the EXG1p family are much bigger than other β-1,3-glucanases, and two copies of the conserved motifs may belong to two

EXG1p, *C. carbonum*, Motif 1	NVKDYGAKGDGVTDDSDAFNRAI
EXG1p, *C. carbonum*, Motif 2	SARGAGATGDGVTDDTRAVQAAV
EXG, *Trichoderma*, Motif 1 (Cohen-Kupiec, 1998)	NVKDYGAKGDGVTDDTAAINNAI
EXG, *Trichoderma*, Motif 2 (Cohen-Kupiec, 1998)	SVRSAGATGNAVTDDTAALQSVI
EXG, *Ampelomyces*, Motif 1 (Rotem et al., 1998)	NVKLLGAKGDGVTDDTAAINNAI
EXG, *Ampelomyces*, Motif 2 (Rotem et al., 1998)	SLKDAGAVGDGVTDDTAMIQKAI
NE, Phage K1 (Gerardy-Schan et al., 1995)	SLKDFGAKGDGKTNDQDAVNRAI
NE, Phage K1 (Long et al., 1995)	SLKDFGAKGDGKTNDQDAVNAAM
NE, Phage K1F (Petter and Vimr, 1993)	DARGWGAKGDGVTDDTAALTSAL
NAP, Phage PZA (Paces et al., 1985)	SVKTYGAKGDGVTDDIKAFEKAI
ME, *Azotobacter*, Motif 1 (Ertesvag et al., 1995)	NVKDFGALGDGVSDDTAAIQAAI
ME, *Azotobacter*, Motif 2 (Ertesvag et al., 1995)	NAKDFGALGDGASDDRPAIQAAI
PG, *Solanum* (Kalaitzis et al., 1995)	NVQNYGAKSDGKTDSSKAFLNAW
PG, *Prunus* (Lester et al., 1994)	NVASLGAKADGKTDSTKAFLSAW
PG, *Actinidia* (Atkinson and Gardner, 1993)	NVDDFGAKGDGR-DDTKAFEKAW
PG, *Arabidopsis* (Quigley, 1993)	DVKASGAKGDGKTDDSAAFAAAW
PG, *Gossypium* (John and Petersen, 1994)	VVAKFGAKADGKTDLSKPFLDAW
PG, *Brassica* (Petersen et al., 1996)	SVSNFGAKGDGKTDDTQAFKKAW
PG, *Erwinia* (He and Collmer, 1990)	NITQYGAKGDGTTLNTSAIQKAI
Consensus	NVKxFGAKGDGKTDDTxAFxxAI
	S R Y V W

Figure 29. Alignment of the amino acid sequences of the two 23-amino acid motifs of EXG1p with the corresponding related sequences of other proteins. Conserved amino acids are indicated by shading. Abbreviations: EXG, β-1,3-exoglucanase; NE, endo-N-acetylneuraminidase; NAP, neck appendage protein; ME, mannuronan C-5-epimerase E1; PG, polygalacturonase. The dash in the sequence of *Actinidia* PG represents an introduced gap of one amino acid.

domains within these enzymes, each motif being at the N terminus of the corresponding putative domain. However, we could not find any other internal similarities between the putative first and second domains of EXG1p.

None of the other 16 known *C. carbonum* cell wall degrading enzymes, including PGN1p (encoding endopolygalacturonase) (Scott-Craig et al., 1990), PGX1p (exopolygalacturonase) (Scott-Craig et al., 1998a), and MLG1p (which has activity against both β-1,3 and β-1,3-β-1,4 glucans) (Görlach et al., 1998), contains any detectable sequences related to this motif. This motif is also not apparent in BGN13.1 of *T. harzianum*, although amino acid boxes GDG and NVKD occur at positions corresponding to the EXG1p motif first and second copies, respectively (Figure 28).

Most of the proteins with the EXG1p motif, including both repeats of EXG1p, also have the sequence YVPXG seven to 22 amino acids downstream from the 23-amino acid motif.

Although the proteins sharing the conserved 23-amino acid motif of EXG1p have different enzymatic activities, they have in common the fact that they all interact with polysaccharides. β-1,3-Glucanase, N-acetylneuraminidase, polygalacturonase, and mannuronan epimerase catalyze the structural modification of polysaccharides, whereas the viral neck appendage protein is involved in binding to cell surface carbohydrates (Villanueva and Salas, 1981). Therefore, these conserved motifs may be important for binding to polysaccharides. The sequence similarity between two of these types of enzymes, polygalacturonases and mannuronan epimerase, has been noted in relation to the fact that polygalacturonases can bind alginate, the substrate of mannuronan epimerase (Ertesvåg et al., 1995; Gupta et al., 1993).

β-1,3-Glucanase A1 from *Bacillus circulans* has two imperfect repeats of 100 amino acids at its N-terminus that are involved in binding to the substrate (Watanabe et al., 1992). The sequences of these repeats appear to be unrelated to those of the EXG1p repeats.

Effect of carbon source on expression of EXG1

An internal 650-bp *Bgl*II fragment was used to probe a blot of RNA isolated from *C. carbonum* grown on media with different carbon sources. The 2.7-kb mRNA corresponding to *EXG1* was detected when *C. carbonum* was grown on laminarin, a laminarin-rich extract of extract of the seaweed *Laminaria saccharina*, oat bran, and maize cell walls, but not when the fungus was grown on 2% sucrose as a carbon source (Figure 30). Like other cell wall degrading enzyme genes of *C. carbonum* and other fungi, *EXG1* mRNA is more abundant when grown on its substrate and is scarce when grown on sucrose (e.g., Scott-Craig et al., 1990; Sposato et al, 1995).

Materials and Methods

Fungal culture growth and maintenance

Conidia of the strains of *C. carbonum* race 1 isolate SB111 (ATCC #90305) were stored at -80° C in 25% glycerol and grown on V8 juice agar plates. For DNA extraction, mycelial agar plugs (ca. 0.5 cm^2) were inoculated into 1-L Erlenmeyer flasks containing 125 ml modified Fries' medium (Scheffer and Ullstrup, 1965). Cultures were incubated at room temperature (21-23° C) without shaking for four days. For RNA extraction, the

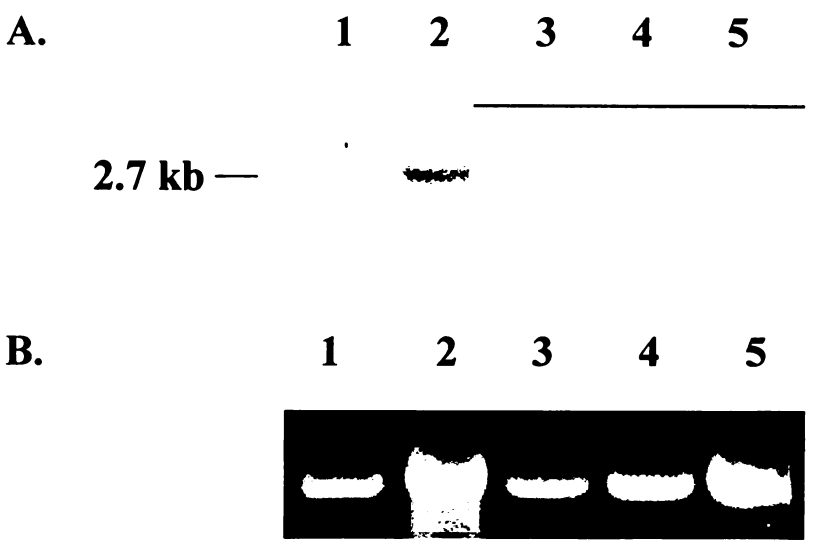

Figure 30. Northern blot analysis of *EXG1* expression in *C. carbonum* grown on different carbon sources. Total RNA was extracted from *C. carbonum* grown for 8 days on 2% (w/v) sucrose (lane 1), 1% oat bran (lane 2) or for 12 days on 1% maize cell walls (lane 3), 1% ground *Laminaria saccharina* (Sigma, St. Louis, MO) (lane 4), or 1% laminarin (lane 5) as a sole carbon source. The blot was probed with *Bgl*II fragment internal to *EXG1* (upper panel). The lower panel shows the ethidium bromide staining of the ribosomal RNA band from the same gel. The age of culture for each substrate was the time of maximum expression of *EXG1* for the given substrate based on a time course experiment (data not shown).

same culture conditions were used except that the media contained 2% (w/v) sucrose, 1% maize cell walls, 1% oat bran, 1% laminarin or 1% ground *Laminaria saccharina* (Sigma, St. Louis, MO) as sole carbon source, and the cultures were incubated for 8 or 12 days. Maize cell walls were prepared as previously described (Sposato et al., 1995).

Nucleic acid manipulations

Fungal genomic DNA was isolated as described by Pitkin et al. (1996). Southern blot analysis was done as described (Sambrook et al., 1989). Total *C. carbonum* RNA was isolated as described (Pitkin et al., 1996), and RNA blotting was done following standard techniques (Sambrook et al., 1989).

A *C. carbonum* genomic library (Scott-Craig et al., 1990) was screened using a *Bgl*II fragment internal to the partial *EXG1* clone described by Schaeffer et al. (1994). A *C. carbonum* cDNA library constructed from the fungus grown on maize cell walls as a carbon source (Pitkin et al., 1996) was screened using the same *Bgl*II fragment. Screening of the cDNA and genomic libraries was done by standard methods (Sambrook et al., 1989).

DNA sequencing

Sequencing was done using nested exonuclease III deletions made using the Erase-a-base kit (Promega, Madison, WI, USA). The sequence of both strands was determined by automated fluorescent sequencing at the MSU-DOE-PRL Plant Biochemistry Facility using an Applied Biosystems (Foster City, California) Catalyst 800 for Taq cycle sequencing and an Applied Biosystems 373A Sequencer for analysis of the

products. DNASIS software (Hitachi, San Bruno, CA) and the Wisconsin Computer Genetics Software (Wisconsin Package, 1996) were used to analyze the data. Database searching was done using BLAST (Altschul et al., 1993; Altschul et al., 1997).

APPENDICES

APPENDIX A

AMINO ACID SPECIFICITY OF THE FUNCTIONAL DOMAIN A OF THE *COCHLIOBOLUS CARBONUM* HC-TOXIN SYNTHETASE

Introduction

Non-ribosomal peptides (and some linear peptides) are synthesized by a class of enzymes known as non-ribosomal peptide synthetases. These enzymes catalyze thioesterification of their amino acid substrates via ATP/PP_i exchange and the formation of amino acid adenylate intermediates (reviewed by Kleinkauf and von Döhren, 1990; Kleinkauf and von Döhren, 1996; Cane et al., 1998). Multifunctional non-ribosomal peptide synthetases, those that catalyze activation of more than one amino acid, are organized into units, one for each amino acid substrate. Within these units, conserved amino acid activating domains are approximately 600 amino acids in length and contain highly conserved motifs known or believed to be involved in aminoacyl adenylation, ATP binding, thioester binding, and epimerization (Stachelhaus and Marahiel, 1995; Kleinkauf and von Döhren, 1996; Cane et al., 1998) (see also Chapter 1). HC-toxin synthetase (HTS) from *C. carbonum* contains four such domains, which is consistent with the number of amino acids in HC-toxin. The amino acid specificity of these putative functional domains is not known, although they are presumed to contain motifs involved in substrate selection.

Results and discussion

Domain A of HTS activates L-Proline

In order to understand the structure/function relationship of the domains of HTS and, possibly, the putative chlamydocin synthetase, expression of individual domains and subsequent assaying of their amino acid-dependent ATP/PP_i exchange activity was attempted. In the experiments utilizing the *E.coli*/pET expression system and *Baculovirus* system (K. Akimitsu, unpublished data), the desired protein domains were expressed and their identity was confirmed by Western blotting. However, in this study it was found that unlike the native HTS, the expressed proteins were in the insoluble fraction and no ATP/PP_i exchange activity could be detected (data not shown). The expressed proteins could be solubilized only by harsh methods (6M urea or 1% SDS) which caused them to lose enzymatic activity irreversibly (data not shown). Chaperonins GroES and GroEL were expressed in *E. coli* simultaneously with the desired HTS domains as an attempt to achieve native protein folding/activity, but no change in solubility/activity was found (results not shown).

The expression system for individual HTS (and other possible peptide synthetase) domains described here utilized as a host organism a $Tox2^-$ strain of *C. carbonum* (strain 243-10) that lacks the whole *HTS1*-containing *TOX2* chromosome. Expression was driven by a strong constitutive promoter from the *Cochliobolus heterostrophus GPD1* gene encoding glyceraldehyde-3-phosphate dehydrogenase (Van Wert and Yoder, 1992). The native *HTS1* promoter could not be used because it may be regulated by *TOX2* - specific regulators which may be missing in $Tox2^-$ isolates. The entire 5' 5-kb region of

Figure 31. Design of pGPD18, the vector for HTS domain A expression driven by the *GPD1* promoter. The resulting construct, pGPD18, contains the *PGN1* internal fragment that can be linearized using a unique internal *Not*I site for homologous integration into the Tox2$^-$ genome. Because *Sal*I is the only site in pGPDA suitable for cloning, pPGE#3 plasmid had to be made so that the *PGN1* fragment could be available as a *Sal*I/*Sal*I fragment. Distances are given in kilobases. Restriction enzyme sites used in constructing the plasmids are shown in bold. P, *GPD1* promoter from *Cochliobolus heterostrophus*; PG, *C. carbonum PGN1* internal fragment (Scott-Craig et al., 1990); HYG^R, the cassette conferring hygromycin B resistance (*C. heterostrophus* promoter 1 driving the expression of the *hph* gene encoding hygromycin phosphotransferase; Schafer et al. 1989); A, *Apa*I; B, *Bam*HI; C, *Cla*I; E, *Eco*RI; H, *Hind*III; Hp, *Hpa*I; K, *Kpn*I; N, *Not*I; P, PstI; S, *Sal*I; Sc, *Sac*I; Sm, *Sma*I; Sn, *Sna*BI; Sp, SpeI; X, *Xho*I; Xb, *Xba*I.

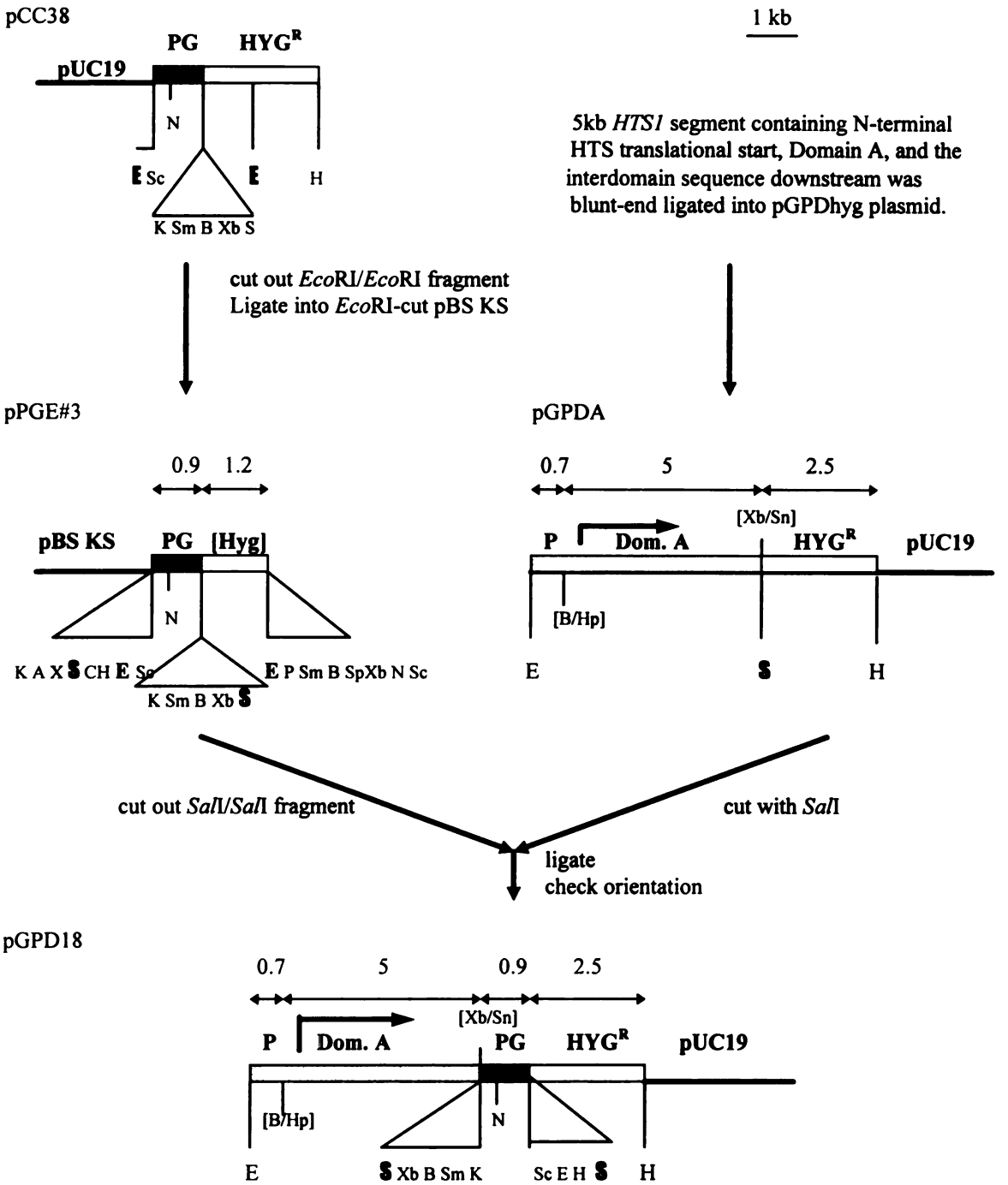

Figure 31. Design of pGPD18, the vector for HTS domain A expression driven by the *GPD1* promoter.

HTS1 containing the native translational start site was fused to the *GPD1* promoter (Figure 31, construct pGPDA, T. Black, unpublished results). For selection of transformants, the cassette conferring hygromycin B resistance was used (Schafer et al., 1989). This cassette is a *SalI/HindIII* fragment containing the *C. heterostrophus* promoter 1 driving the expression of the *hph* gene encoding hygromycin phosphotransferase. To increase transformation efficiency, the HTS domain expression vector had to be introduced into the $Tox2^{-}$ recipient strain via homologous integration. For this purpose, a fragment of an unrelated non-essential *C. carbonum* gene (*PGN1*, encoding endopolygalacturonase, Scott-Craig et al., 1990) was engineered into the pGPDA construct (Figure 31) utilizing *Sal*I, the only restriction site in pGPDA linkers that is unique throughout the construct and thus available for cloning.

The resulting construct, pGPD18, was linearized by cutting with *Not*I, a unique restriction enzyme site internal to the *PGN1* fragment and introduced into wild type *C. carbonum* 243-10 ($Tox2^{-}$) strain by transformation of protoplasts as described by Scott-Craig et al. (1990). Five hygromycin B resistant transformants (551-1 through -5) were obtained and characterized by DNA blot analysis (Figure 32A). Figure 32B depicts the wild type *PGN1* locus and Figure 32C and D shows the predicted maps for single or multiple integration events of pGPD18 into *PGN1*, respectively. The DNA gel blot analysis of transformants T551-3 and T551-5 was consistent with a single insertion event, and transformants T551-1, T551-2, and T551-4 - with multiple insertion events. Additional DNA blot analysis (not shown) confirmed that the integrated pGPDA18 construct was intact in all transformants, containing the entire *GPD1/HTS1* (Domain A) fusion.

Figure 32. Southern blot analysis of pGPD18 integration into the *PGN1* locus of the Tox2$^-$ strain 243-10. **(A)** DNA blot of wild type Tox2$^-$ strain 243-10 (lane 6) and five transformants, 551-1 through 5 (lanes 1 - 5). Genomic DNA was digested with *Hind*III and the filter was probed with the 0.9-kb *Kpn*I/*Sac*I *PGN1* internal fragment. **(B)** Restriction map of the wild type *PGN1* locus (Scott-Craig et al., 1990). **(C)** and **(D)** Predicted restriction maps of the *PGN1* locus with a single and multiple (double) insertion of the pGPD18 expression vector, respectively. Distances are given in kilobases. *GPD1* promoter/*HTS1* domain A fusion construct is indicated by shaded boxes, *PGN1* fragment introduced as part of pGPD18 vector is indicated by solid black boxes, and genomic copy of the same fragment is indicated by dark gray boxes. P, *GPD1* promoter (Van Wert and Yoder, 1992); PG, *C. carbonum PGN1* gene internal fragment (Scott-Craig et al., 1990); HYG^R, the cassette conferring hygromycin B resistance (containing *C. heterostrophus* promoter 1 driving the expression of the *hph* gene encoding hygromycin phosphotransferase; Schafer et al. 1989); E, *Eco*RI; H, *Hind*III; K, *Kpn*I; N, *Not*I; S, *Sal*I; Sc, *Sac*I.

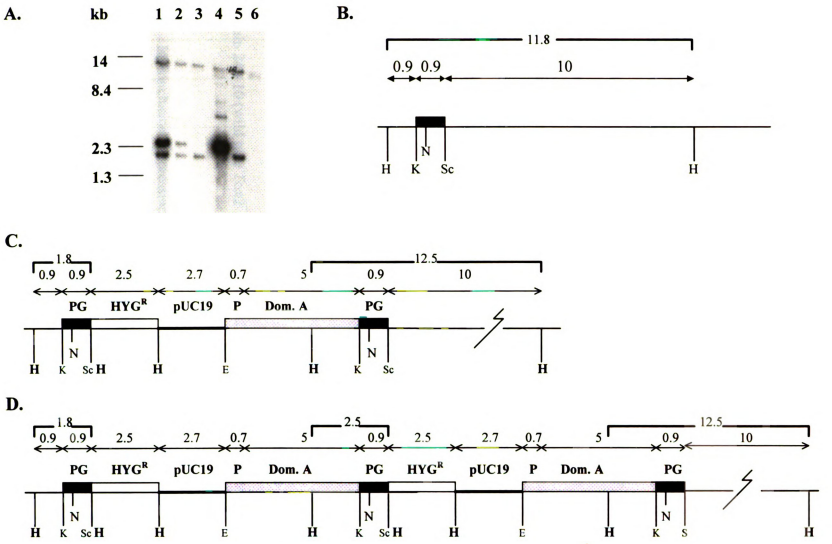

Figure 31. Southern blot analysis of pGPD18 integration into the *PGN1* locus of the Tox2$^-$ strain 243-10.

Transformants 551-1, -2, -3, and -5 were further analyzed by Northern blot (Figure 33) and the 6.6 kb mRNA hybridizing to the *HTS1* domain A internal fragment was detected in all of them. To test these strains for the presence of HTS domain A protein and any ATP/PP_i exchange activity, cultures of T551-1, T551-3, SB111 (wild type $Tox2^+$), and 243-10 (wild type $Tox2^-$) were grown in parallel, and protein was extracted from mycelial pads and partially purified by ammonium sulfate fractionation and anion exchange chromatography (Walton and Holden, 1988). ATP/PP_i exchange in wild type strains and transformants T551-1 and T551-3 was compared in the fractions containing maximum activity. The results are given in Table 4. Expressed protein was also analyzed by Western blotting using anti-HTS-1 polyclonal antibodies (Scott-Craig et al., 1992) (Figure 34). These results show that HPLC fraction 17 of protein preparations from $Tox2^+$ wild type strain SB111, as well as from transformants T551-1 and T551-3, contained protein recognized by anti-HTS-1 antibodies. The same fraction contained L-Pro-dependent and D-Ala-dependent ATP/PP_i exchange activity in SB111, whereas in both transformants it contains only L-Pro-dependent ATP/PP_i exchange activity. No ATP/PP_i exchange activity or protein recognized by anti-HTS-1 antibodies was detected in strain 243-10 (wild type $Tox2^-$) which was used as a recipient strain for transformation T551.

In conclusion, expression of domain A of HTS driven by the *GPD1* promoter from *C. heterostrophus* in a $Tox2^-$ strain was confirmed by Northern and Western blotting, and this domain was shown to have L-Pro-dependent ATP/PP_i exchange activity. This result is consistent with the fact that, according to the partial peptide sequence obtained from proteolytic fragments of HTS (Panaccione et al., 1992),

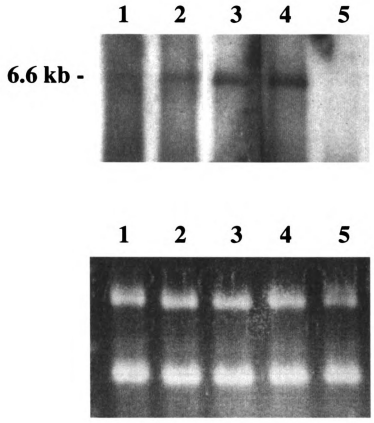

Figure 33. Northern blot analysis of transformants bearing pGPD18 expression vector. Total RNA was extracted from four-day-old mats and separated on a 1% agarose gel containing formaldehyde and transferred to nitrocellulose filter. Upper panel: The filter was probed with a DNA fragment internal to domain A of *HTS1*. Lower panel: same gel stained with ethidium bromide before the transfer, showing the ribosomal RNA bands. Lanes: 1 - 4, transformants 551-1, -2, -3, and -5, respectively; 5, 243-10 ($Tox2^-$ recipient strain).

Table 4. ATP/PP_i exchange activity in *C. carbonum* bearing pGPD18 expression vector. L-Proline and D-alanine-dependent ATP/PP_i exchange was measured in the peak fraction (fractions 15 and 17) of anion exchange HPLC (Walton and Holden 1988). One ml of each protein preparation was chromatographed, and 25 µl of each 1-ml fraction assayed. The reaction mixes contained ca. 105,000 cpm [^{32}P]pyrophosphate per reaction (total volume 125 µl).

		Kcpm incorporated	
Isolate	Fraction	L-Pro-dependent ATP/PP_i exchange	D-Ala-dependent ATP/PP_i exchange
SB111[a]	15	28	3.4
	17	80	61
243-10[b]	15	5	3
	17	3.5	0.8
T551-1	15	14.6	2.8
	17	22	0.35
T551-3	15	8.8	2.1
	17	9.6	0.6

[a] wild type $Tox2^+$ strain
[b] wild type $Tox2^-$ strain

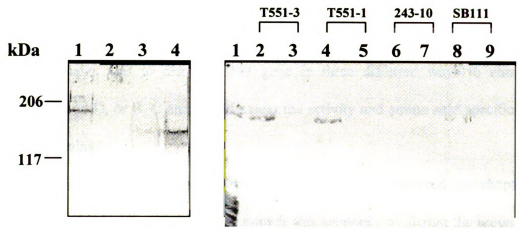

Figure 34. Western blot analysis of protein extracts from transformants bearing pGPD18 expression vector. (A) Protein extracts were purified through ammonium sulfate precipitation, desalted, resolved on a 6% acrylamide SDS-PAGE gel, and transferred to nitrocellulose as described by Scott-Craig et al. (1992). The filter was probed with anti-HTS-1 antiserum (Scott-Craig et al., 1992). Lanes: 1, SB111 (wild type $Tox2^+$); 2, 243-10 (wild type $Tox2^-$); 3, T551-3; 4, T551-1. **(B)** Anion exchange HPLC (Walton and Holden, 1988) fractions containing ATP/PP_i exchange activity (fractions 15 and 17) were resolved on a 6% acrylamide SDS-PAGE gel, transferred to nitrocellulose, and treated with anti-HTS-1 antiserum. Lanes: 1, SB111 preparation not fractionated by HPLC; 2 and 3, T551-3 fractions 17 and 15; 4 and 5, T551-1 fractions 17 and 15; 6 and 7, 243-10 fractions 17 and 15; 8 and 9, SB111 fractions 17 and 15.

L-Pro-dependent activity resides in domains A or B, and L-Ala and D-Ala-dependent activity - in domains C and/or D.

Sequential disruption of HTS1 functional domains

Another approach undertaken to determine the amino acid specificity of HTS functional domains was to disrupt *HTS1* gene in three different ways to eliminate domains D, C and D, or B, C and D and assess the activity and amino acid specificity of remaining domains.

Before $Tox2^+$ strains with only one copy of *HTS1* were discovered (see chapter 2), to create a strain with only one functional copy it was necessary to disrupt the second one so that no protein is produced by it. To make the second copy disruption and subsequent selection easier, for the disruption of the first copy acetamide utilization as sole nitrogen source was used as a selectable marker, and for the second copy disruption hygromycin B resistance was used as a selectable marker. Strain 553-11 was created by disrupting copy 1 of *HTS1* with the *C. carbonum* transformation vector pCC129 (Panaccione et al., 1992), and transformants were selected for acetamide utilization. The insertion of pCC129 disrupts *HTS1* at the 5' end (at the beginning of domain A).

Strain 553-11 was used as a recipient for three different step-wise disruptions of the second copy of *HTS1* (Table 5). Three transformation vectors were designed to contain the *HTS1* sequences for the homologous integration at the beginning of the domains B, C, or D (vectors pHTS1, pHTS2, and pHTS3, respectively). Surprisingly, very few of the recovered transformants had homologous integration of pCC129 into copy 2 of *HTS1* (Table 5). These transformants were analyzed for the presence of the

Table 5. Analysis of the transformants for the step-wise disruption of *HTS1*.

Transformation number and vector	Domains remaining	Number of Hyg^R transformants analyzed	Transformants with homologous integrations into *HTS1* copy 2	Antibody binding to protein on Western	ATP/PP_i exchange activity
T593 pHTS1	A	20	3	none or very weak anti-HTS-1[a]	L-Pro [c]
T594 pHTS2	A and B	29	3	anti-HTS-1[a,b]	L-Pro [c]
T595 pHTS3	A, B, and C	26	none	n/a	n/a

[a] Weak antibody hybridization to the ca. 150 kDa band.

[b] Weak antibody hybridization to the ca. 200 kDa band.

[c] Weak exchange activity detected by ATP/PP_i assay (ca. 1/5 to ¼ of the wild type control).

truncated HTS protein and for HTS activity (summarized in Table 5). When strain 164R10 was found to harbor only one copy of *HTS1* (see Chapter 2), the same transformation vectors were used for the step-wise disruption in this strain, but none of the transformants had homologous integration of the vector.

Overall, only very low levels of the truncated HTS protein products were produced by these transformants, if at all. The transformants showed some L-Pro-dependent ATP/PP_i exchange activity, which is expected because this activity was shown to reside in domain A by the expression experiments (see above), and this domain remains intact in all of the transformants. Based on the sequence comparisons, Stachelhaus and Marahiel (1995) and Kleinkauf and von Döhren (1996) have suggested that domain A activates L-Pro and epimerizes it (converting to D-Pro), domain B activates L-Ala, domain C activates D-Ala, and domain D activates Aeo. The results described here confirm that domain A activates L-Pro. The L-Ala-dependent activity of the second domain may be harder to detect, since there always is more background L-Ala-dependent activity. The function of domains C and D remains unconfirmed.

APPENDIX B

CC115, A TRANSPOSASE-LIKE SEQUENCE FROM *COCHLIOBOLUS CARBONUM*

In the *C. carbonum TOX2* locus, immediately downstream from *HTS1,* there is a segment of *TOX2* -unique DNA that was subcloned as CC62 (Panaccione et al., 1992). CC115 is a 1.2-kb cDNA clone obtained from a *C. carbonum* cDNA library by screening it with CC62. CC115 was sequenced on both strands (Figure 35) and was not found to contain a contiguous open reading frame. This means that either the corresponding gene is nonfunctional and is not translated, or the cDNA is chimeric. However, the deduced amino acid sequence of the part of one short open reading frame shows high similarity to the transposase sequences from the transposons found in various filamentous fungi (Figure 35). Another putative transposon from *C. carbonum,* named Fcc1 (Panaccione et al., 1996), also shows sequence similarity to CC115. However, although Fcc1 appears especially prevalent on the *TOX2* chromosome, it is also present on most of the chromosomes in the *C. carbonum* genome. The CC115 DNA sequence, on the other hand, is $Tox2^{+}$-unique. Chromosome-specific repeated sequences from supernumerary chromosomes are known in *Nectria haematococca* (Enkerli et al., 1997; Covert, 1998) and *Colletotrichum gloeosporioides* (Masel et al., 1993; 1996). In both of these fungi, there are also repeated sequences that are common to both essential and supernumerary chromosomes. In addition, in *C. gloeosporioides*, repeated sequences that are present on the essential chromosomes but not on the supernumerary chromosome have also been

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1     CGTTGCTGTCGGCCATTAGATCAAACTAGGTGGAAGGAATTGCTGTTGGTGAGCATGACG
                                  *  V  E  G  I  A  V  G  E  H  D
61    TGTTTATAACATCAACCCCGTCAGGTTGGAGCAATAATGCCGTTAGGCTAGCATGGCTTG
      V  F  I  T  S  T  P  S  G  W  S  N  N  A  V  R  L  A  W  L
121   AGCAAGTTTTTGATCGCTGCACGAGGAACAAACCAGGGAGATGGCGATTACTTATCCTTG
      E  Q  V  F  D  R  C  T  R  N  K  P  G  R  W  R  L  L  I  L
181   ATGGCCATGGATCTCACGTCACGCCCGAGTTTATTGAGTATTGCAATCGCCATAGAATAC
      D  G  H  G  S  H  V  T  P  E  F  I  E  Y  C  N  R  H  R  I
241   TCCTTATGGTGTTTCCTCCTCATTCAACTCACACACTGCAACCGCTTGATGTGGTGATGT
      L  L  M  V  F  P  P  H  S  T  H  T  L  Q  P  L  D  V  V  M
301   TTAAACCACTCTCCACCAGCTACTCAAATGAGCTCACTAATCACCTCTACAACGCCCAAG
      F  K  P  L  S  T  S  Y  S  N  E  L  T  N  H  L  Y  N  A  Q
361   GCCTCGTCTCAGTCAAGAAGGGAGACTTTTTTCCGCTGTTCTGGCGAGCCTGGAGCTCAT
      G  L  V  S  V  K  K  G  D  F  F  P  L  F  W  R  A  W  S  S
421   CCTCTACTAAAAAATAATATCTTGAAGGCCTTTAGCGCCACTGGTATATGGCCAGCAGAT
      S  S  T  K  K  *  Y  L  E  G  L  *
481   CCCGACGTTATACTCAAAAGTTTAGTTCAACACCTGATAAGAGCCACCGCAAGCGATCTC
541   GGCTCTCTCCAAGTGATTGGAATCACCTGAGGCAGCTAGTATGAGAAGCTGCTGAAGATG
601   GAGCTGAGAGTGGAGTTAAAAAGCTTAGTGCCCTACTCCATCATCTCCAGGTTCAGAATG
661   AGCTATTGCGTCATGAGATGGAGGGATTGAGAGCAGCTCTTTCACAAAAACAGAAGCATA
721   AAGGCAAGGGCAAAGCTCTAAATCTTCAATAACGCAAGGAGTATCATGGCGGAGCGGTCT
781   TCTGGTCACCTCGTAAGTTCCGCGAAGCTCGAGCTCGAGAAGCAGTTCGTGAGCGCGAGG
841   AAGTAGAGGAGAAACTCCAGAAAGCACAGGCTAAGAAGGACTGCGAGGAGACTCAGTTGT
901   GGCGTCAAGTTGAGCGCGAGGAGAAGCGCACTGAACGATTGAGACTTAATGAGATGCGTA
961   AGCTTGAGCGAGCTGAGAAAGCAGCTGAACGCGCGCGTAAAAAAGAAGCTCGCAACACTG
1021  AAAAATCTCAACACCAAGCTCAAAAGCGTAAGCGTACAGCCTCACGAGTGCCCACCTCTA
1081  AGAACAAGCGTCAAAAACTATCAGTGCTGGATGGAGCTCGCGATGGAGCTGCATCTACTT
1141  CATCATCTATCCCGCCAAAGATCACGACGCGAGGCCGCAGCGTTAACGTCCCGCAGAAAT
1201  TTAGATAGCACAAACTAACCACAAGTATTTGGTTACTGTAAATCCAAGTTTTATATATAT
1261  TAAAAAAAAAAAAAAAA
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Figure 35. Nucleotide and partial deduced amino acid sequences of CC115. The part of the open reading frame that is similar to fungal transposase sequences is highlighted.

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C. carbonum CC115  EGIAVGEHDV.FITSTPSGWSNNAVRLAWLEQVFDRCTRN.KPGRWRLLILDGHGSHVTP
    Magnaporthe 1  PTDLSPFDNWQF.HATENGWTNNQTAIEWLKKVFIPYTQPLTP.EKRLLVLDGHGSHITD
    Magnaporthe 2  EGG.LP.DTWRL.KPTVNGWTDNETGLDWVQ.HFDNHTKSRTKGVYRMLVLDGHGSHRSP
      A. nidulans  TG..LP.PDWRF.EISTNGWTTNEISLRWLQKQFIPSTEHRTRGRYQLLVLDGHGSHLTP
         Botrytis  PIKLDNYEGWEF.TATDNGWTTDSTGLEWLKEVFIPQSAPTRPKEARLLVLDGHGSHETT
          A. niger  EGQSIP.PTWRF.EVSDNGWTTDKIGLRWLQKHFIPLIRGKSVGKYSLLVLDGHGSHLTP
  C. carbonum Fot  ..HSLP.PDWTI.GVSENDWKTDELGVEWV.KHFNQHTVTRTAGVYRLLILDGHSSHATP
      Talaromyces  ..D.LP.DDWRI.NISDNGWTTDQIGLEWLKTHFIPYINGRTVGKYRMLILDGHGSHLTP
          Nectria  EEFKEI.ADWYY.ITSPNGWTDDHIGVEWLERVYLPQTMPADDSDARLIILDGHGSHATD

C. carbonum CC115  EFIEYCNRHRILLMVFPPHSTHTLQPLDVVMFKPLSTSYSNELTNHLYNAQGLVSVKKGD
    Magnaporthe 1  EFMLLCLQNNIQLLYLPPHSSHVLQPLDLSVFGPLKEAYRRQL.GFVSQFCCSTVIGKRN
    Magnaporthe 2  EFEGYCKDYNIIPLYLPAHSSHLTQPLDVGVFNVLKRAYGQKI.NDFIRAH.ITNISKVD
      A. nidulans  EFDQICTDHNIIPLCMPAHSSHLLQPLDIGCFAVLKRSYASLV.DQKMRLG.ISHIDKLD
         Botrytis  QFMLECFKNNIHLLFLPPHTSHVLQPPDLSIFSPLKKEYRYHL.NTLDSLADSTPIDKRN
          A. niger  EFDQSCAENEVIPICMPAHSSHLLQPLDVGCFSVLKRTYGGMV.QKQMQYG.RNHIDKLD
  C. carbonum Fot  EFDQFCTENKIITLCMPSHTSHLLQPLDVSCYSTLKRAYGREI.EELARHG.VYHVDKID
      Talaromyces  EFDHICTENNIIPVCMPPHSSHLLQPLDVGCFAVLKRHYGQLV.EQRMRLG.FNHIDKMD
          Nectria  EWMATCFLNNVYCCYLPAHCSHGLQPLDNGVFNASKAAYRREL.ENFASLTDSTPMDKVN

C. carbonum CC115  FFPLFWRAWSSSSTKK*YLEGL*R
    Magnaporthe 1  FLLCYRKARLKAFIAKTIQSGW.R
    Magnaporthe 2  FFLAFAAAYKKSMTKENMAGGF.R
      A. nidulans  FLAAYPQARISTFKLDTIRNSF.R
         Botrytis  FLACYQKARLKALTLRNITSGW.K
          A. niger  FLEVYPKAHQCALSKSNIISGF.R
  C. carbonum Fot  FLTVYTRIRPTAFTQQNIQAGF.Q
      Talaromyces  FLTAFPQARTVAYRAQTIRNSF.A
          Nectria  FIRAYAKARRVGMTEKNILSGW.R
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Figure 36. Comparison of the partial predicted amino acid sequence of *C. carbonum* CC115 with transposase sequences from filamentous fungi. Only the portion of CC115 that shows similarity to the transposase sequences, and the corresponding amino acid sequences of fungal transposases are shown. The sequences belong to the middle parts of the corresponding transposases, and the N-terminal and C-terminal parts are not shown. Highly conserved amino acids are highlighted. *Magnaporthe* 1, *Magnaporthe grisea* (Kachroo et al., 1994); *Magnaporthe* 2, *Magnaporthe grisea* (Farman et al., 1996); *A. nidulans, Aspergillus nidulans (Emericella nidulans)* (Kupfer et al., 1997); *Botrytis, Botrytis cinerea (Botryotinia fuckeliana)* (Levis et al., 1997); *A. niger, Aspergillus niger* var. *awamori* (Amutan et al., 1996; Nyyssonen et al., 1996); *C. carbonum* Fot, *C. carbonum* transposase (Fot1-like) sequence (Panaccione et al., 1996); *Talaromyces, Talaromyces stipitatus* (Cummings et al., 1998); *Nectria, Nectria haematococca* (Enkerli et al., 1997).

found, and at least one of them (*Cg*T1) appears to be a transposable element (Masel et al., 1993; 1996; He et al., 1996). Thus, in this fungus there are three sets of repeated transposon-like sequences: those that are unique to the supernumerary chromosomes, those that are unique to the essential chromosomes, and those that are common throughout the genome. The existence in *C. carbonum, N. haematococca,* and *C. gloeosporioides* of the chromosome-specific repeated sequences and/or transposable elements that are unique to the supernumerary chromosomes provides an additional argument in favor of the horizontal movement of the supernumerary chromosomes (horizontal movement of the *TOX2* module in the case of *C. carbonum*).

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