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A cyclic peptide synthetase gene required for pathogenicity of the fungus *Cochliobolus carbonum* on maize

(HC-toxin/phytotoxin/*Helminthosporium*/plant disease)

DANIEL G. PANACCIONE, JOHN S. SCOTT-CRAIG, JEAN-ALAIN POCARD*, AND JONATHAN D. WALTON†

Michigan State University–Department of Energy Plant Research Laboratory, East Lansing, MI 48824-1312

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ABSTRACT **Specificity in many plant–pathogen interactions is determined by single genes in pathogen and host. The single locus for host-selective pathogenicity (*TOX2*) in the fungus *Cochliobolus carbonum* governs production of a cyclic tetrapeptide named HC-toxin. We have isolated a chromosomal region, 22 kilobases (kb) long, that contains a 15.7-kb open reading frame (*HTS1*) encoding a multifunctional cyclic peptide synthetase. The 22-kb chromosomal region is duplicated in toxin-producing isolates of the fungus but is completely absent from the genomes of toxin-nonproducing isolates. Mutants of the fungus with disruptions in both copies of *HTS1*, at either of two different sites within *HTS1*, were engineered by DNA-mediated transformation. Disruption of both copies at either site resulted in loss of ability to produce HC-toxin and loss of host-selective pathogenicity, but the mutants displayed different biochemical phenotypes depending on the site of disruption. The results demonstrate that *TOX2* encodes, at least in part, a large, multifunctional biosynthetic enzyme and that the evolution of host range in *C. carbonum* involved the insertion or deletion of a large piece of chromosomal DNA.**

The interactions between pathogenic microorganisms and plants have been intensively studied genetically, but the underlying chemical, biochemical, and cellular factors controlled by resistance and pathogenicity genes are poorly understood. The host-selective toxins are among the few known agents of specificity. These low molecular weight natural products are produced by certain plant pathogenic fungi and determine both host range and virulence of the organisms that produce them (1, 2). In three species of the genus *Cochliobolus* (imperfect state *Helminthosporium* or *Bipolaris*) that have been studied genetically, production of their characteristic host-selective toxins is controlled by single but different genetic loci. These loci are called *TOX1* in *Cochliobolus heterostrophus*, *TOX2* in *Cochliobolus carbonum*, and *TOX3* in *Cochliobolus victoriae* (3). The molecular nature of the *TOX* loci has remained unknown.

HC-toxin, the host-selective toxin produced by *C. carbonum* race 1 that is required for pathogenicity of this fungus on maize, is a cyclic tetrapeptide with the structure cyclo(D-Pro-L-Ala-D-Ala-L-Aeo), where Aeo is 2-amino-9,10-epoxy-8-oxodecanoic acid (4–6). We have identified and purified two enzymes involved in biosynthesis of HC-toxin (7, 8). One enzyme, HC-toxin synthetase 1 (HTS-1), has a molecular mass of ≈220 kDa, catalyzes ATP/PP$_i$ exchange in the presence of L-proline, and epimerizes L-proline to D-proline. The second enzyme, HTS-2, has an apparent molecular mass of 160 kDa, catalyzes L-alanine-dependent and D-alanine-dependent ATP/PP$_i$ exchange, and epimerizes L-alanine to D-alanine. Both of these enzymes are detected only in race 1 (Tox$^+$) isolates of *C. carbonum* and their activities segregate genetically with *TOX2* (7). We have undertaken a molecular genetic analysis of HC-toxin biosynthesis with the goals of understanding the nature of the economically important *TOX* loci of *Cochliobolus* and the evolution of new races in this and related pathogens.

MATERIALS AND METHODS

Nucleic Acid Manipulations. Isolation of fungal DNA and construction of the genomic DNA library in phage λEMBL3 were as described (9). Subcloning was done into pBluescript (Stratagene) or pUC18 (BRL). Probes were labeled with ^{32}P by random priming and were present in hybridizations at 2 × 10^5 cpm/ml. Hybridizations were done overnight at 65°C in 5× SSPE (1× SSPE = 150 mM NaCl/10 mM NaH_2PO_4/1 mM EDTA, pH 7.4)/7% SDS/0.5% nonfat dry milk/0.1 mg of denatured salmon sperm DNA per ml. Blots were washed in 2× SSPE/0.1% SDS; the final wash was at 65°C for 1 hr.

Fungal Transformation Procedures and Constructs. *C. carbonum* strains were maintained and cultured as described (7). Transformation of *C. carbonum* to hygromycin resistance was as described (9). Transformants capable of using acetamide as a sole nitrogen source were selected on a medium (pH 5.2) consisting of (per liter) 342 g of sucrose, 2 g of KH_2PO_4, 2.5 g of $MgSO_4{\cdot}7H_2O$, 1.25 g of $CaCl_2{\cdot}2H_2O$, 2.1 g of CsCl, 0.6 g of acetamide, and 7 g of agarose. All transformed strains were purified by isolation of single conidia.

Gene disruption constructs for the 5′ region of *HTS1* were prepared as follows: (*i*) fragment 119 (Fig. 1) was subcloned as a *Bam*HI/*Sal* I (*Sal* I site from vector) fragment into *Bam*HI/*Sal* I-digested pUCH1 (10) to create pCC119. This plasmid was linearized at an *Xho* I site internal to fragment 119 before transformation. (*ii*) Fragment 119 was subcloned into *Bam*HI/*Sal* I-digested pBluescript, and then this plasmid was digested with *Sal* I and *Kpn* I and ligated with a *Sal* I/*Kpn* I fragment containing the *amdS* gene of *Aspergillus nidulans* (11), which confers the ability to use acetamide as a sole nitrogen source. The resulting plasmid (pCC129) was linearized with *Xho* I before transformation.

The constructs for disrupting the 3′ region of *HTS1* consisted of (*i*) fragment 121 (Fig. 1) subcloned as an *Eco*RV/*Sal* I (*Sal* I site from vector) fragment into *Sma* I/*Sal* I-digested pUCH1 to create pCC121, and (*ii*) fragment 121 subcloned into *Sma* I/*Sal* I-digested pBluescript, followed by ligation with the *Sal* I/*Kpn* I fragment containing the *amdS* gene. The resulting plasmid (pCC128), as well as pCC121, was linearized at a unique *Xho* I site internal to fragment 121 before transformation.

Analysis of Transformants. The pathogenicity of *C. carbonum* isolates was tested on the susceptible maize inbred K61

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*Present address: Université de Nice-Sophia Antipolis, Laboratoire de Biologie Végétale, Unite de Recherche Associée, Centre National de la Recherche Scientifique, 1114, Parc Valrose, 06034 Nice Cedex, France.

†To whom reprint requests should be addressed.

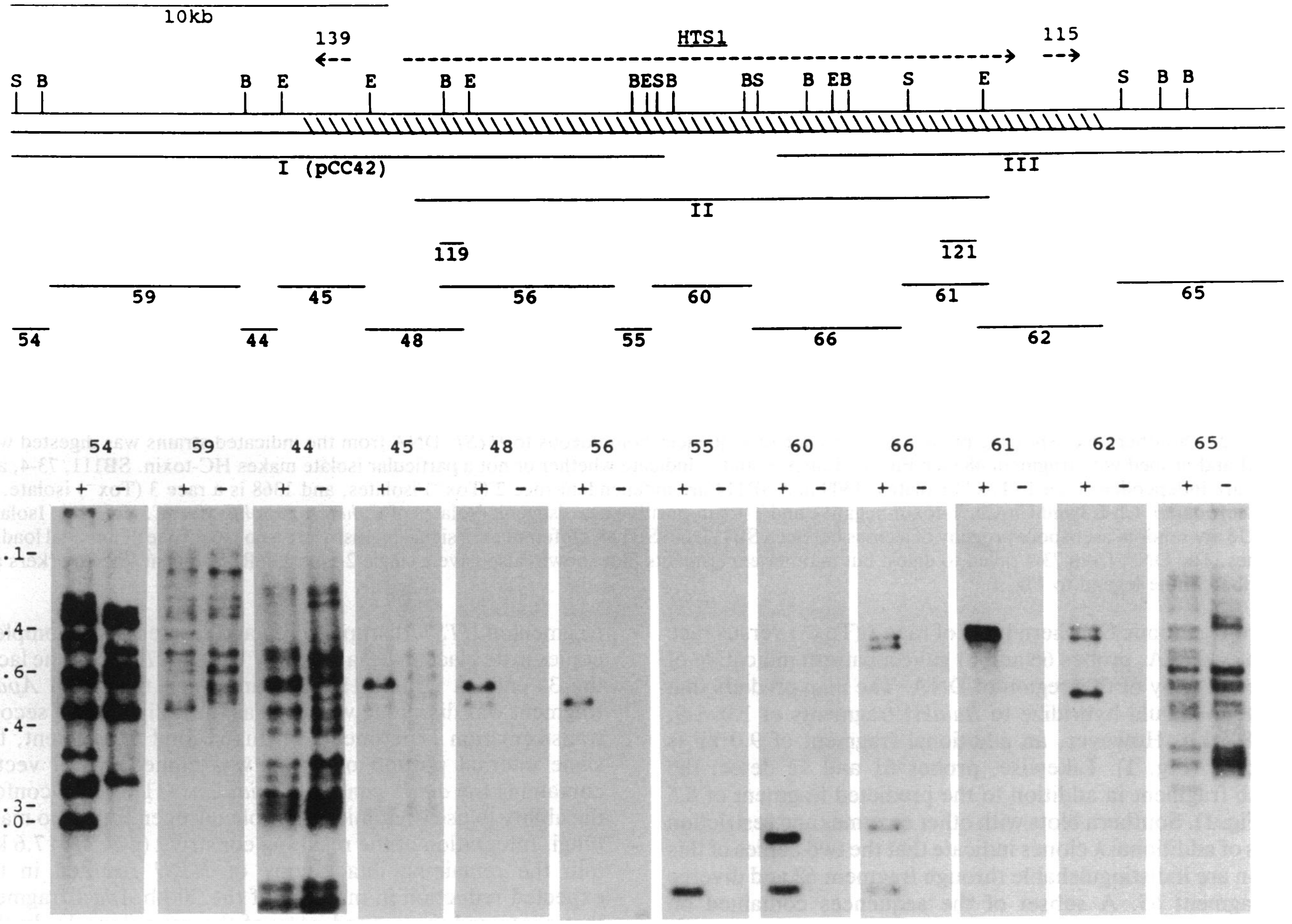

FIG. 1. Restriction map of copy 1 of *HTS1* and flanking DNA from *C. carbonum* race 1. (*Upper*) Restriction map derived from overlapping λ clones I (pCC42), II, and III. B, *Bam*HI; E, *Eco*RI; S, *Sal* I. Hatching indicates the 22-kilobase (kb) region unique to race 1. Known genes or transcribed regions are indicated by dashed arrows. (*Lower*) Series of Southern blots of total genomic DNA from *C. carbonum* SB111 (lanes +, Tox$^+$) and *C. carbonum* SB114 (lanes −, Tox$^-$) digested with *Bam*HI, transferred to Zeta-Probe membrane (Bio-Rad), and probed with different DNA fragments. Relative mobility of *Hin*dIII-digested bacteriophage λ DNA (sizes in kb) is indicated on the left. The 22-kb, race 1-unique region is present as two copies in Tox$^+$ isolates (see Figs. 3 and 4); the map represents copy 1.

(genotype, *hm/hm*). Leaves of 2-week-old seedlings were sprayed to saturation with suspensions of 1 × 10^4 conidia per ml. Inoculated plants were incubated in a clear plastic bag for 18 hr and then on a greenhouse bench. HTS-1 and HTS-2 activities in extracts, partially purified by ammonium sulfate precipitation, were assayed as described (7) except that amino acids, when present, were at 20 mM. HC-toxin was solvent extracted from culture filtrates and analyzed by TLC as described (12).

RESULTS

Absence of the Gene for HTS-1 and Flanking DNA in HC-Toxin Nonproducers. A cDNA encoding part of HTS-1, previously identified in an expression library with anti-HTS-1 antibody (13), was used as a probe to isolate a clone for the gene (*HTS1*) that encodes HTS-1. A genomic library of the *C. carbonum* race 1 isolate SB111, constructed in λEMBL3, was screened with the cDNA, and a 16-kb *Sal* I/*Sal* I insert from one positive recombinant bacteriophage was subcloned to create pCC42 (Fig. 1).

Southern hybridization analyses of DNA from SB111 compared to DNA from race 2 (Tox$^-$) isolate SB114 showed that approximately half of the insert of pCC42 was found only in SB111 (probes 45, 48, 56, and 55; Fig. 1). Probes 44, 59, and 54 detected multiple bands, indicative of moderately repeated DNA common to both isolates. By using subcloned fragments from the right-hand end of pCC42 as a starting point to "walk" along the SB111 chromosome, two additional overlapping genomic λ clones (II and III; Fig. 1) were obtained. DNA hybridizing to probes 60, 66, 61, and 62 was present in SB111 but absent from SB114 (Fig. 1). Together with the insert from pCC42, they define a contiguous region of 22 kb of DNA that is unique to the race 1 isolate SB111. The orientation and presumptive position of *HTS1*, based on a large open reading frame identified by sequence analysis (J.S.S.-C., D.G.P., and J.D.W., unpublished data), is indicated by the labeled arrow in Fig. 1. As at the left border, repeated DNA common to race 1 and race 2 is present at the right border of the 22-kb, race 1-unique region (Fig. 1).

Several other isolates of *C. carbonum*, including the progeny of a cross between SB111 and SB114, as well as isolates of *C. heterostrophus* and *C. victoriae*, were examined for the presence of DNA that would hybridize with the race 1-unique DNA. Among all the isolates tested, including the progeny of the cross, DNA homologous to probe 48 (or other tested probes including 60, 66, 61, and 62) was found only in Tox$^+$ isolates of *C. carbonum* (Fig. 2) and, thus, is genetically linked to *TOX2*.

Clustering and Duplication of Genes Within the Race 1-Unique DNA. To look for additional genes transcribed from the race 1-unique DNA, probes 45, 48, 56, 55, 60, 66, 61, and 62 were used to screen the λgt11 cDNA library of SB111. Two additional transcribed regions (dashed arrows in Fig. 1) were identified. Arrows 115 and 139 represent the size, position, and orientation of the two additional cDNAs.

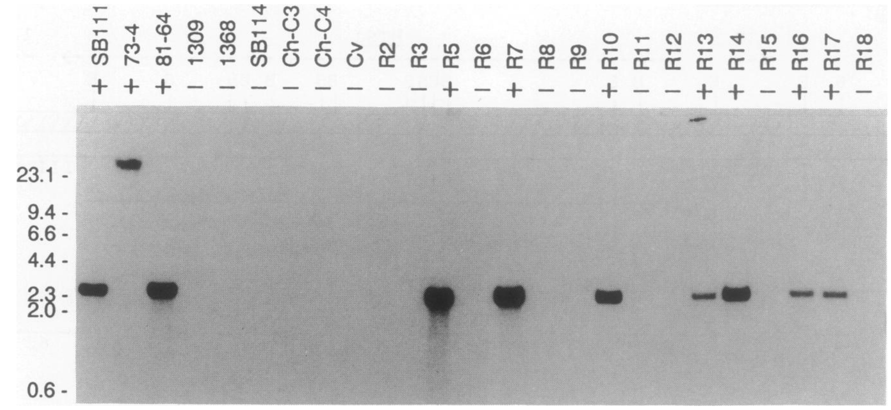

FIG. 2. Southern blot showing presence or absence of sequences homologous to *HTS1*. DNA from the indicated strains was digested with *Eco*RI and probed with fragment 48 (see Fig. 1). Lanes + and − indicate whether or not a particular isolate makes HC-toxin. SB111, 73-4, and 81-64 are independent race 1 (Tox$^+$) isolates, 1309 and SB114 are independent race 2 (Tox$^-$) isolates, and 1368 is a race 3 (Tox$^-$) isolate, all of *C. carbonum*. Ch-C3 and Ch-C4, T-toxin-negative and T-toxin-positive near-isogenic isolates of *C. heterostrophus*; Cv, *C. victoriae*. Isolates R2–R18 are random ascospore progeny of a cross between SB111 and SB114. Differences in signal intensity are a consequence of unequal loading of lanes. The DNA from 73-4 failed to digest but in other experiments (not shown) also gave a single 2.5-kb *Eco*RI fragment. Size markers are described in the legend to Fig. 1.

In the genomic Southern blots of race 1 (Tox$^+$) versus race 2 (Tox$^-$) DNA, probes 66 and 61 gave a pattern indicative of a second copy of this region of DNA. The map predicts that probe 66 should hybridize to *Bam*HI fragments of 1.0, 1.9, and 8.5 kb. However, an additional fragment of 9.0 kb is detected (Fig. 1). Likewise, probes 61 and 62 detect the 9.0-kb fragment in addition to the predicted fragment of 8.5 kb (Fig. 1). Southern blots with other enzymes and restriction maps of additional λ clones indicate that the two copies of this region are indistinguishable through fragment 62 and diverge in fragment 65. A subset of the sequences contained on fragment 62 are present in at least one additional copy, as evidenced by the hybridization of probe 62 to a fragment of 5.5 kb in Fig. 1.

Restriction fragment length polymorphisms were detected only in fragments that span the junction between single-copy and repeated DNA at the right border of the 22 kb of unique DNA and never within the unique sequences (Fig. 1), indicating that the entire region of unique DNA was duplicated. To find polymorphisms at the left border of the unique DNA, DNA samples from SB111 were digested with 22 different restriction endonucleases and hybridized with probe 48. Only one of the enzymes tested, *Apa* I, gave a pattern that allowed detection of a duplication of this region. Although probe 48 contains no *Apa* I sites, two *Apa* I fragments, one 20 kb long and the other 30 kb long, are detectable in genomic Southern blots (Fig. 3, lane 2). The duplication of this 22-kb region, as indicated by polymorphisms in the DNA that flanks it, has been observed in all six independent race 1 isolates examined. The presence of this precise duplication is confirmed by data from gene disruption experiments.

Disruption of *HTS1*. To confirm the identity of *HTS1* and test its role in HC-toxin biosynthesis, we selectively mutated this gene by homologous integrative transformation. Because of the duplication of this region, the gene disruptions were performed in two successive rounds of transformation with two different selection systems. One copy of *HTS1* was disrupted with a construct (pCC119; 5.8 kb) consisting of an internal portion of *HTS1* (fragment 119; Fig. 1) cloned into a transformation vector containing a gene that confers resistance to hygromycin B (10). Because pCC119 contains no *Apa* I sites, homologous recombination of this plasmid into one copy of *HTS1* or the other was indicated by a reduction in the mobility of either the 20- or the 30-kb *Apa* I fragments that hybridize with probe 48 (Fig. 3, lanes 3 and 4). Homologous recombination of pCC119, which contains an internal fragment of *HTS1*, disrupts *HTS1* and creates two incomplete copies in its place. One lacks the 5′ end of *HTS1* and one lacks the 3′ end. A strain, 119X3.1, in which the 30-kb *Apa* I fragment was disrupted was used as the recipient in a second transformation experiment. In this second experiment, the same internal portion of *HTS1* was cloned into a vector containing the *amdS* gene of *A. nidulans* (11), which confers the ability to use acetamide as a sole nitrogen source to many fungi. Integration of the resulting construct (pCC129; 7.6 kb) into the remaining intact copy of *HTS1* resulted in the expected reduction in mobility of the 20-kb *Apa* I fragment that contained the second copy of the gene (lane 1). In this way, strains containing a disruption in one or the other copy of *HTS1*, two disruptions in the same copy of *HTS1*, and disruptions in both copies of *HTS1* were obtained.

Strains mutated near the 3′ end of *HTS1* were engineered by homologous recombination of transformation vectors containing a fragment from this region of the gene (fragment 121 in Fig. 1). The two copies of the 3′ end of *HTS1* are distinguishable as 5.5- and 8.8-kb *Sal* I fragments in SB111 (Fig. 4, lane 2). First, a plasmid (pCC121; 6.2 kb) conferring hygromycin resistance was used to disrupt individual copies of the 3′ end of *HTS1*, indicated by the disappearance of the 8.8-kb *Sal* I fragment (lane 3) or the 5.5-kb *Sal* I fragment (lane 4) in genomic Southern blots of transformed strains. Homologous recombination of pCC121 into the 8.8-kb *Sal* I

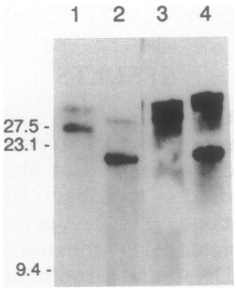

FIG. 3. Southern blot showing single and double disruptions of the 5′ region of *HTS1*. Genomic DNA from strains 129X9.4 (lane 1), SB111 (lane 2), 119X17.1 (lane 3), and 119X3.1 (lane 4) was digested with *Apa* I and probed with fragment 48 (see Fig. 1). Strains 119X17.1 and 119X3.1 have alternate copies of *HTS1* disrupted; 129X9.4 has disruptions in both copies of *HTS1*. Differences in the intensity of the 30-kb *Apa* I band are due to variation in the quality of the high molecular weight DNA. Size markers are described in the legend to Fig. 1.

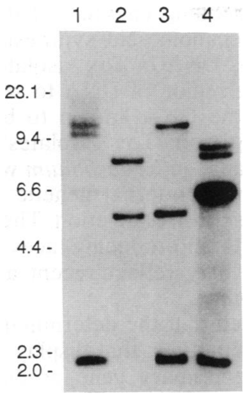

FIG. 4. Southern blot showing single and double disruptions of the 3′ region of *HTS1*. Genomic DNA from strains 128X8.2 (lane 1), SB111 (lane 2), 121X3.1 (lane 3), and 121X4.1 (lane 4) was digested with *Sal* I and probed with fragment 121 (see Fig. 1). Strains 121X3.1 and 121X4.1 have disruptions in alternate copies of *HTS1*; 128X8.2 has both copies of *HTS1* disrupted. Size markers are described in the legend to Fig. 1.

fragment resulted in the appearance of 2.1- and 12.9-kb *Sal* I fragments (lane 3) due to a single *Sal* I site in the vector. Similarly, the disruption of the 5.5-kb *Sal* I fragment created 2.1- and 9.6-kb *Sal* I fragments in its place (lane 4). The presence of the strongly hybridizing band of 6.2 kb (lane 4) indicates that multiple copies of pCC121 have integrated in tandem in this strain.

Strain 121X3.1 (Fig. 4, lane 3), in which the 8.8-kb *Sal* I fragment had been disrupted, was used as the recipient in a second transformation experiment. In this second experiment, a plasmid (pCC128; 8.0 kb) containing fragment 121 (Fig. 1), and the *amdS* gene as a selectable marker, disrupted the remaining copy of *HTS1* in 121X3.1. This second disruption is indicated by the disappearance of the 5.5-kb *Sal* I fragment and the appearance of an 11.4-kb *Sal* I fragment, in addition to a second 2.1-kb *Sal* I fragment, in a genomic Southern blot of this strain (Fig. 4, lane 1).

Phenotypes of Mutants. The pathogenicity of the mutant strains created by gene disruption was tested on *C. carbonum* race 1-susceptible maize. Strain 119X3.1, with one copy of the 5′ end of *HTS1* (*HTS1-5′*) disrupted, caused lesions identical to those produced by the race 1 isolate SB111 (Fig. 5), as did strains with disruptions in the alternate copy of *HTS1-5′*. Inoculation with strain 129X9.4, which has both copies of *HTS1-5′* disrupted, resulted in only small chlorotic flecks indistinguishable from those produced by the race 2 isolate SB114 (Fig. 5), indicative of a nonpathogenic interaction. Similar to the mutants containing single disruptions of *HTS1-5′*, strains with either copy of the 3′ end of *HTS1* (*HTS1-3′*) disrupted retained race 1 pathogenicity. Strain 128X8.2, with disruptions in both copies of *HTS1-3′*, was nonpathogenic (Fig. 5).

The inability of the two nonpathogenic mutants to produce HC-toxin was confirmed by analyzing chloroform extracts of culture filtrates for toxin by silica TLC followed by detection with an epoxide indicator. The mutant strains with both copies of either *HTS1-5′* or *HTS1-3′* disrupted produced no detectable HC-toxin. However, the strains in which only one copy of *HTS1-5′* or *HTS1-3′* had been disrupted retained the ability to produce toxin.

Strain 129X3.1, which has one intact and one disrupted copy of *HTS1-5′*, had approximately one-half the HTS-1 activity, measured as ATP/PP$_i$ exchange in the presence of L-proline, of the parental race 1 strain SB111 (Fig. 6). Surprisingly, this strain also had a proportional reduction in HTS-2 activity measured as L-alanine-dependent or D-alanine-dependent ATP/PP$_i$ exchange (Fig. 6). Strain 129X9.4, with both copies of *HTS1-5′* disrupted, has only background levels of both HTS-1 and HTS-2 (Fig. 6). Strain 128X8.2, which has disruptions in both copies of *HTS1-3′*, has ≈60% of the HTS-1 and HTS-2 activities of SB111 (Fig. 6).

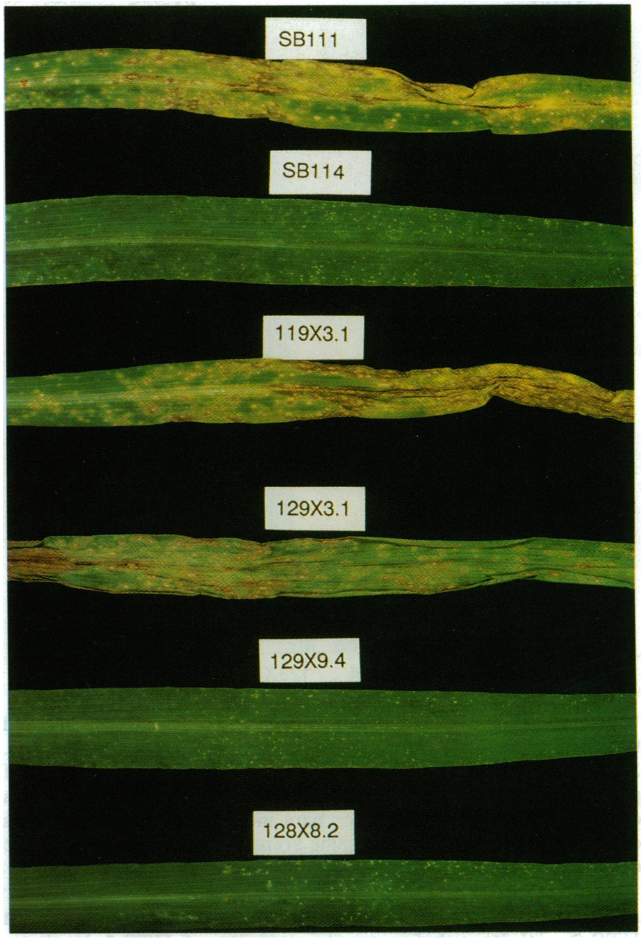

FIG. 5. Lesions incited by various strains of *C. carbonum* on HC-toxin-sensitive maize (genotype, *hm/hm*). Photograph was taken 5 days postinoculation. SB111 is a wild-type race 1 isolate; SB114 is a wild-type race 2 isolate; 119X3.1 has a single copy of *HTS1-5′* disrupted; 129X3.1 has both *HTS1-5′* disruption constructs integrated into a single copy of *HTS1-5′*; 129X9.4 has disruptions in both copies of *HTS1-5′*; 128X8.2 has both copies of *HTS1-3′* disrupted.

DISCUSSION

We have cloned a gene, *HTS1*, that is required for biosynthesis of HC-toxin and pathogenicity of *C. carbonum* on maize. *HTS1* is duplicated, has no homology with DNA from Tox$^-$ isolates of *C. carbonum*, and segregates genetically with the *TOX2* locus. We conclude that *HTS1* must be part of *TOX2* and that *TOX2* is a complex locus containing, as a minimum, two copies of a gene (*HTS1*) encoding a multifunctional biosynthetic enzyme. The lack of pathogenicity in the toxin-nonproducing strains created by gene disruption further substantiates the role of HC-toxin as an essential pathogenicity determinant in this disease interaction.

Analysis of HTS-1 and HTS-2 activities in mutants created by gene disruption demonstrates some interesting features of HC-toxin biosynthesis. First, an apparent effect of gene dosage can be observed in strain 129X3.1. This strain, which retains only one of the two copies of *HTS1*, has half the HTS-1 activity of the wild-type strain (Fig. 6) but still makes HC-toxin and is fully pathogenic (Fig. 5). Second, whenever

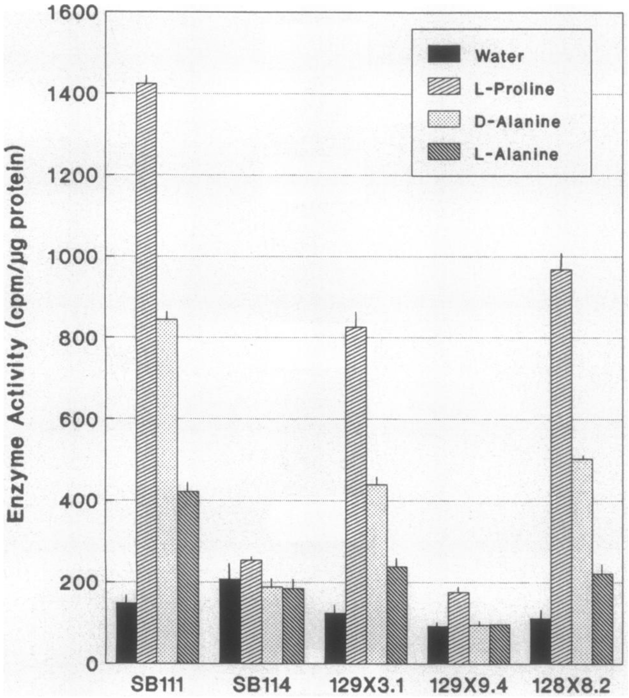

FIG. 6. HTS-1 (L-proline activating) and HTS-2 (D-alanine and L-alanine activating) activities in various strains of *C. carbonum*. Activity given is the amount of ^{32}P incorporated into ATP per µg of protein in the presence of water or the indicated amino acids at a concentration of 20 mM. Error bars indicate the range of values observed in two assays of the same preparation. Strains assayed are described in the legend to Fig. 5.

HTS1 was disrupted, there was a proportional reduction in HTS-2 activity relative to the activity of HTS-1 in those strains (Fig. 6). Two possible explanations for the reduction in HTS-2 activity in these mutants are (*i*) that HTS-2 is encoded by *HTS1* and either becomes separated from HTS-1 by posttranslational processing or as an artifact of purification, or (*ii*) that these enzymes are encoded by separate genes but HTS-2 is unstable in the absence of HTS-1. Third, strain 128X8.2, a toxin nonproducer created by disrupting the 3′ region of both copies of *HTS1*, has levels of HTS-1 and HTS-2 activity equivalent to those in the strain 129X3.1 (Fig. 6), which has a single disruption in *HTS1-5′*. Because these levels of HTS-1 and HTS-2 activity were sufficient for HC-toxin production in 129X3.1, it is likely that strain 128X8.2 is unable to produce HC-toxin due to the loss of a function other than those catalyzed by HTS-1 or HTS-2.

From limited chromosome walking, we estimate that the two 15.7-kb copies of *HTS1* are at least 25 kb apart, putting the minimum size of *TOX2* at 56 kb. There may be additional genes that are also required for HC-toxin biosynthesis; for example, genes encoding enzymes that catalyze 2-amino-9,10-epoxy-8-oxodecanoic acid synthesis. Presumably, the lack of homologous DNA in Tox$^-$ isolates accounts for the ability of this large region of DNA to segregate as a single gene. That all of the DNA known to be part of *TOX2* is completely missing from Tox$^-$ isolates indicates that the evolution of host range in *C. carbonum* was not the result of point mutations or an internal genetic rearrangement but rather of a major insertion or deletion. The apparently sudden emergence of new toxin-producing races in species of *Cochliobolus* (1, 2, 14) may reflect recent acquisition of toxin biosynthetic capability.

It has been argued that the determinants of specificity in plant–pathogen interactions that display a gene-for-gene relationship must be primary gene products because these determinants are monogenically inherited, whereas the synthetic pathways for most secondary metabolites require multiple steps and hence multiple genes. That *TOX2*, which behaves as a single Mendelian gene, contains two copies of a gene encoding a multifunctional enzyme raises the possibility that pathogenicity or avirulence determinants in other plant pathogens are also secondary metabolites.

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1. Kohmoto, K. & Otani, H. (1991) *Experientia* **47,** 755–764.
2. Walton, J. D. (1990) in *Molecular Industrial Mycology*, eds. Leong, S. A. & Berka, R. M. (Dekker, New York), pp. 225–249.
3. Bronson, C. R. (1991) *Experientia* **47,** 771–776.
4. Walton, J. D., Earle, E. D. & Gibson, B. W. (1982) *Biochem. Biophys. Res. Commun.* **107,** 785–794.
5. Pope, M. R., Cuiffetti, L. M., Knoche, H. W., McCrery, D., Daly, J. M. & Dunkle, L. D. (1983) *Biochemistry* **22,** 3502–3506.
6. Kawai, M., Rich, D. H. & Walton, J. D. (1983) *Biochem. Biophys. Res. Commun.* **111,** 398–403.
7. Walton, J. D. (1987) *Proc. Natl. Acad. Sci. USA* **84,** 8444–8447.
8. Walton, J. D. & Holden, F. R. (1988) *Mol. Plant Microbe Interact.* **1,** 128–134.
9. Scott-Craig, J. S., Panaccione, D. G., Cervone, F. & Walton, J. D. (1990) *Plant Cell* **2,** 1191–1200.
10. Schäfer, W., Straney, D., Ciuffetti, L., Van Etten, H. D. & Yoder, O. C. (1989) *Science* **246,** 247–249.
11. Hynes, M. J., Corrick, C. M. & King, J. A. (1983) *Mol. Cell. Biol.* **3,** 1430–1439.
12. Rasmussen, J. B. & Scheffer, R. P. (1988) *Plant Physiol.* **86,** 187–191.
13. Walton, J., Scott-Craig, J. S. & Pocard, J.-A. (1990) *Phytopathology* **80,** 1009 (abstr.).
14. Leonard, K. J. (1973) *Phytopathology* **63,** 112–115.