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

(Host-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

Communicated by Anton Lang, April 20, 1992 (received for review February 5, 1992)

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 (HTSI) 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 HTSI, at either of two different sites within HTSI, 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, the HC-toxin synthetase 1 (HTS-1), has a molecular mass of ~220 kDa, catalyzes ATP/PPi 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/PPi exchange, and epimerizes L-alanine to D-alanine. Both of these enzymes are detected only in race 1 (Tox1) 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 AEMBL3 were as described (9). Subcloning was done into pBluescript (Stratagene) or pUC18 (BRL). Probes were labeled with 32P by random priming and were present in hybridizations at 2 × 106 cpm/ml. Hybridizations were done overnight at 65°C in 5× SSPE (1× SSPE = 150 mM NaCl/10 mM NaH2PO4/1 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 5× 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 KH2PO4, 2.5 g of MgSO4.7H2O, 1.25 g of CaCl2.2H2O, 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 3′ region of HTSI were prepared as follows: (i) fragment 119 (Fig. 1) was subcloned as a BamHI/SalI (SalI site from vector) fragment into BamHI/SalI-digested pUC119 (10) to create pCC119. This plasmid was linearized at an XhoI site internal to fragment 119 before transformation. (ii) Fragment 119 was subcloned into BamHI/SalI-digested pBluescript, and then this plasmid was digested with SalI and KpnI and ligated with a SalI/KpnI 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 XhoI before transformation.

The constructs for disrupting the 3′ region of HTSI consisted of (i) fragment 121 (Fig. 1) subcloned as an EcoRV/SalI (SalI site from vector) fragment into SmaI/SalI-digested pUCH1 to create pCC121, and (ii) fragment 121 subcloned into SmaI/SalI-digested pBluescript, followed by ligation with the SalI/KpnI fragment containing the amdS gene. The resulting plasmid (pCC128), as well as pCC121, was linearized at a unique XhoI site internal to fragment 121 before transformation.

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

*Present address: Université de Nice-Sophia Antipolis, Laboratoire de Biologie Végétale, Unité de Recherche Associée, Centre National de la Recherche Scientifique, 1114, Parc Valrose, 06034 Nice Cedex, France.
†To whom reprint requests should be addressed.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. §1734 solely to indicate this fact.
A clonal DNA different (genotype, hm/hm). Inoculated activities in HC-Toxin were region acids, previously identified gene 18 hr pared create antibody (13), DNA common carbonum race screened ments (probes 45, SB111). FIG. 1. regions hybridization Southern Bio-Rad, and probed with different DNA fragments. Relative mobility of HindIII-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 (HTSI) 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 SalI/SalI 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 HTSI, 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 Agt11 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.
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 region of this region of DNA. The map predicts that probe 66 should hybridize to BamHI 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, ApaI, gave a pattern that allowed detection of a duplication of this region. Although probe 48 contains no ApaI sites, two ApaI 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 HTSI. To confirm the identity of HTSI and test its role in HC-toxin biosynthesis, we selectively mutared 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 HTSI was disrupted with a construct (pCC119; 5.8 kb) consisting of an internal portion of HTSI (fragment 119; Fig. 1) cloned into a transformation vector containing a gene that confers resistance to hygromycin B (10). Because pCC119 contains no ApaI sites, homologous recombination of this plasmid into one copy of HTSI or the other was indicated by a reduction in the mobility of either the 20- or the 30-kb ApaI fragments that hybridize with probe 48 (Fig. 3, lanes 3 and 4). Homologous recombination of pCC119, which contains an internal fragment of HTSI, disrupts HTSI and creates two incomplete copies in its place. One lacks the 5' end of HTSI and one lacks the 3' end. A strain, 119X3.1, in which the 30-kb ApaI fragment was disrupted was used as the recipient in a second transformation experiment. In this second experiment, the same internal portion of HTSI 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 HTSI resulted in the expected reduction in mobility of the 20-kb ApaI 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 HTSI, two disruptions in the same copy of HTSI, and disruptions in both copies of HTSI were obtained.

Strains mutated near the 3' end of HTSI 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 HTSI are distinguishable as 5.5- and 8.8-kb SalI 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 HTSI, indicated by the disappearance of the 8.8-kb SalI fragment (lane 3) or the 5.5-kb SalI fragment (lane 4) in genomic Southern blots of transformed strains. Homologous recombination of pCC121 into the 8.8-kgb SalI

---

**Fig. 2.** Southern blot showing presence or absence of sequences homologous to HTSI. DNA from the indicated strains was digested with EcoRI 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 EcoRI fragment. Size markers are described in the legend to Fig. 1.

**Fig. 3.** Southern blot showing single and double disruptions of the 5' region of HTSI. 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 HTSI disrupted; 129X9.4 has disruptions in both copies of HTSI. Differences in the intensity of the 30-kb ApaI band are due to variation in the quality of the high molecular weight DNA. Size markers are described in the legend to Fig. 1.
fragments 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/PPi 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 ATP/PPi exchange (Fig. 6). Strain 129X9.4, with both copies of HTS1-5' disrupted, has only background levels of both HS-1 and HS-2 (Fig. 6). Strain 128X8.2, which has disruptions in both copies of HTS1-3', has ~60% of the HTS-1 and HS-2 activities of SB111 (Fig. 6).

**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 HS-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
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.

We thank Steve Briggs (Pioneer Hi-Bred), Olen Yoder (Cornell University), Kurt Leonard (University of Minnesota), and Robert Scheffer (Michigan State University) for fungal isolates. We also thank Michael Hynes (University of Melbourne, Australia) and Olen Yoder for fungal transformation vectors. This work was supported by the Department of Energy, Division of Biological Energy Research, and the National Science Foundation.