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Research Note

Virulence Gene Expression During Conidial Germination in *Cochliobolus carbonum*

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The fungal pathogen *Cochliobolus carbonum* race 1 produces a host-selective toxin (HC-toxin) that is responsible for increased virulence on susceptible genotypes of maize. The toxin is synthesized by a peptide synthetase, which is a product of the *HTS1* gene. Because the toxin is not stored in dormant conidia, early expression of *HTS1* is crucial for extensive colonization of susceptible leaf tissue. To detect the *HTS1* transcript and determine the onset of *HTS1* gene expression, we analyzed RNA preparations from ungerminated and germinated conidia by reverse transcription–polymerase chain reaction using oligonucleotide primers within the 15.7-kb open reading frame of *HTS1*. With primer pairs near both the 3′- and the 5′-termini, amplified products of the *HTS1* transcript were detected in RNA prepared from dormant conidia. With all primer pairs used, the quantities of transcript increased substantially during germ tube emergence and elongation, indicating that expression of *HTS1* is up regulated during spore germination. Digestion with restriction endonucleases confirmed the identity of the amplified products. Amplification of the constitutively expressed β-tubulin transcript, which is processed to remove introns, as well as the absence of amplification products with primers spanning the *HTS1* coding sequence established that cDNA was amplified and not contaminating genomic DNA.

Additional keywords: Bipolaris zeicola, host–pathogen interaction, *Helminthosporium carbonum.*

The ability of fungi to cause diseases on specific plant species or host genotypes (pathogenicity) and the capacity of a given pathotype or race of the pathogen to cause a greater amount of damage to certain host genotypes (virulence) are determined by a number of factors in both of the interacting organisms. In some fungal pathogens, these characteristics are influenced directly by low molecular weight metabolites that are phytotoxic to a limited number of potential host plants (Yoder 1980). The maize pathogen *Cochliobolus carbonum* Nelson (anamorph: *Helminthosporium carbonum* Ullstrup = *Bipolaris zeicola* (Stout) Shoemaker) race 1 produces a host-selective toxin (HC-toxin) that is responsible for increased virulence on susceptible genotypes (Scheffer and Ullstrup1973). Production of this virulence factor is required for extensive colonization of susceptible leaf tissue (Comstock and Scheffer 1973). Although conidia from the non-toxin-producing race 2 germinate and penetrate the leaf at rates that are initially indistinguishable from those of race 1, the intercellular hyphae stop growing soon thereafter (Comstock and Scheffer 1973) and ultimately incite smaller lesions on most maize lines and hybrids. Addition of HC-toxin to conidial inoculum from the less-virulent race results in the formation of larger lesions similar to those caused by race 1 isolates on leaves of susceptible genotypes (Cantone and Dunkle 1990; Comstock and Scheffer 1973), indicating that the non-producers are pathogenic but less virulent than the toxin-producing race 1.

Synthesis of HC-toxin, a cyclic tetrapeptide comprised of cyclo-(D-proline-L-alanine-D-alanine-L-2-amino-8-oxo-9,10-epoxydecanoic acid) (Pope et al. 1983; Walton et al. 1982) is directed by a large (approximately 574 kDa), multifunctional peptide synthetase, which catalyzes the activation of amino acids by ATP/PP_i exchange and the subsequent thioesterification and polymerization of activated amino acids (Walton 1987; Walton and Holden et al. 1988). The synthetase enzyme is encoded by the *HTS1* gene located within the *Tox2* locus (Scott-Craig et al. 1992), a 22-kb fragment that is duplicated in the genome of race 1 isolates but absent from isolates of non-toxin-producing races (Panaccione et al. 1992). One copy of the 15.7-kb *HTS1* open reading frame (ORF) has been sequenced and shown to be devoid of introns and to contain four amino acid-activating domains, designated A, B, C, and D, which share some homology with similar domains in other peptide synthetases of bacteria and fungi (Scott-Craig et al. 1992).

HC-toxin, like other host-selective toxins that contain an epoxide moiety (e.g., AK-toxin and AF-toxin produced by strains of *Alternaria alternata* pathogenic to pears and strawberries, respectively), is not present in mature, dormant conidia of *C. carbonum* (Dunkle et al. 1991). However, HC-toxin is released into infection droplets on maize leaves and then accumulates within enlarging lesions (Dunkle et al. 1991). Thus, the toxin synthesis that accompanies spore germination

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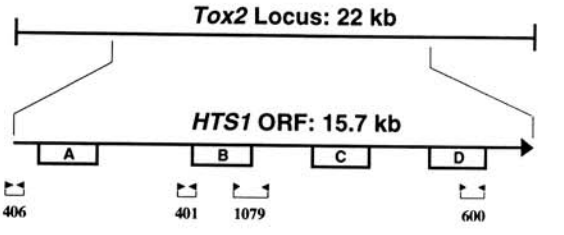
requires either de novo transcription of the *HTS1* gene or translation of stable transcripts stored in the spore.

Synthesis of protein and RNA begins within the first 15 min of spore germination in *C. carbonum* (Cantone and Dunkle 1991), but the products of these biosynthetic activities have not been identified. Furthermore, little is known about specific genes that are expressed by a pathogen during the infection process and disease cycle. Diseases in which genes are known to directly influence the outcome of host-pathogen interactions, such as the *HTS1* gene in *C. carbonum* and the avirulence gene *avr9* in *Cladosporium fulvum* (Van Kan et al. 1991), provide excellent systems to analyze the expression of specific genes. As a prelude to studying gene expression in the host-pathogen interaction, we analyzed the conidia for *HTS1* expression during germination, an early stage of pathogenesis. In our preliminary experiments with standard Northern analyses, we were unable to detect the transcript of *HTS1* in RNA preparations from germinated conidia (unpublished data). Scott-Craig et al. (1992), likewise could not detect the transcript in RNA preparations from 4-day-old cultures. Consequently, we applied reverse transcription-polymerase chain reaction (RT-PCR) to assay for the presence and expression of *HTS1*, because the method is capable of detecting rare transcripts in small amounts of starting material (Kawasaki et al. 1988; Rappolee et al. 1988).

To assay for the presence of the HC-toxin synthetase transcript, we designed primer pairs that are located inside the *Tox2* locus. Figure 1 shows the positions of the primers relative to the *HTS1* ORF and to the four putative amino acid-binding domains and indicates the sizes of the amplified products generated by each primer pair. Three primer pairs were distributed along the length of the *HTS1* gene and used to amplify sequences within the coding region. Because the *HTS1* gene lacks introns, we designed a fourth pair of primers that spans the 5′ translation start site with the 5′ sense primer located in the noncoding region of the *Tox2* locus. This pair of primers thus served as a control to detect the presence of DNA, if present in our RNA preparations.

Total RNA was extracted (Chirgwin et al. 1979) from conidia at a final concentration of 2.5×10^5 conidia per milliliter incubated for various periods of time in the dark at 24°C in 50 ml of 0.2× modified Fries' medium (Pringle and Scheffer 1963). Under these conditions, the conidia germinated synchronously, reaching >95% germination by 3 h, and the germ tubes formed an intertwining thallus by 12 h. For analysis of ungerminated conidia, precautionary measures were included to prevent activation of transcription during the harvesting and washing of spores (Bonnen and Brambl 1983) and to assure that the transcript detected was, in fact, present in dormant spores. First, the spores were harvested with ice-cold



Primer Sequences	A/S[a]	Primer Sites[b]	Product Length (bp)
5′-ACGGCACAGTTGCGTGAGATG	S	- 218 to 187	406
5′-AGCATGGTCTGGAGGGGGTAA	A		
5′-GCCAAAACGCCCTACCAATGT	S	5,282 to 5,682	401
5′-TCCATCCCAACTGCAAATAGC	A		
5′-TTGGTCCGGTACAGCGC	S	6,679 to 7,757	1,079
5′-ATTCCTGTCGAGGCCAG	A		
5′-CCGGGGAACTAACACCTTCTT	S	14,203 to 14,802	600
5′-TGATTCCAGAAGCGTATGTGC	A		

Fig. 1. Diagram of the *HTS1* gene in the *Tox2* locus of *Cochliobolus carbonum* race 1 showing the locations of the primers used, the sizes of fragments amplified from RNAs by reverse transcription-PCR, and the regions of the conserved amino acid-activating domains (labeled A through D). The primers at the 5′ end of *HTS1* span the coding sequence and the upstream nontranscribed region, yielding a 406-bp product with DNA, but not RNA as substrate. [a]A = Antisense sequence of DNA; S = sense sequence of DNA; [b]5′ end relative to the *HTS1* transcription start site.

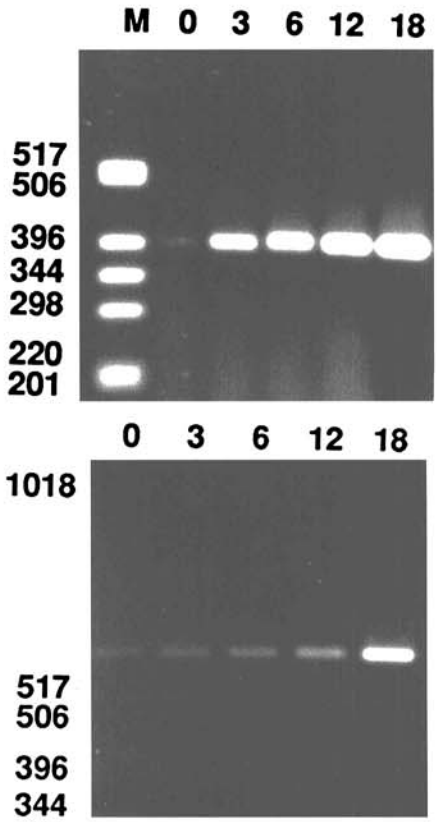


Fig. 2. Detection of *HTS1* transcript by RT-PCR amplification of RNA prepared from *Cochliobolus carbonum* conidia incubated for increasing periods of time in dilute modified Fries' medium from 0 (ungerminated conidia) to 18 h. The primers used resulted in amplification of a 401-bp fragment (top) or a 600-bp fragment (bottom). Molecular markers (M) are shown in the upper figure, and their sizes and migration are indicated in bp in the margins. RNA preparations were digested with RNase-free DNase I (Dilworth and McCarrey 1992) and then incubated with reverse transcriptase and random primers (shown in Fig. 1) to synthesize cDNA with the GeneAmp RNA PCR kit (Perkin Elmer, Norwalk, Connecticut). The cDNAs were amplified in a Perkin Elmer model 480 thermal cycler operating at the fastest heating and cooling rates and programmed for one cycle of 1 min 15 s at 94°C, 35 cycles of 45 s at 94°C, 45 s at 54°C (for primers to amplify the 401-bp fragment) or 60°C (for primers to amplify the 600 bp fragment), 72°C for 1 min 15 s, and followed by a final 7-min extension at 60°C.

0.05% Tween 20 containing 10 mM Na azide and kept on ice during the brief period prior to disruption of the spores. Second, the spores (1.25×10^7) were harvested and disrupted (Van Etten and Freer 1978) in the absence of carbon and nitrogen sources and in the presence of Na azide at concentrations that inhibited germination. In separate experiments, we determined that inclusion of 1 to 50 mM Na azide in the spore harvesting and disruption media did not interfere with RT-PCR (data not shown).

RNA purified through CsCl was incubated with random hexamers and reverse transcriptase to produce cDNAs, which were then amplified with *Taq* polymerase and gene-specific primers. The primer pair nearest the 5′ end of the *HST1* coding region produced a 401-bp fragment, and the pair near the 3′ end produced a 600-bp fragment (Fig. 2). The amplified products were readily visible by staining with ethidium bromide (EtBr) after electrophoresis on agarose gels. The presence of these amplification products indicated that the *HTS1* transcripts are preserved in ungerminated conidia and are present in germinated conidia at all times analyzed. The intensity of the EtBr-stained product increased with time of germination and germ tube growth. In a separate experiment, we confirmed that the amount of amplified product, as indicated by the intensity of the EtBr-stained band, was proportional to the amount of initial RNA over the range of 0.065 to 1.5 µg per reaction (data not shown). Because we analyzed equal amounts of RNA (1.0 µg) from conidia at each time of germination, these results indicate that the amount of *HTS1* transcript is greater in germinated conidia than in ungerminated conidia and suggest that the *HTS1* gene is expressed at increasing rates during germination and germ tube elongation.

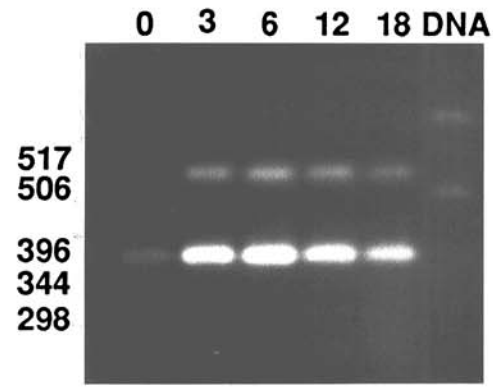


Fig. 3. Detection of the β-tubulin transcript by RT-PCR amplification of RNA prepared from *Cochliobolus carbonum* conidia incubated for increasing periods of time in dilute modified Fries' medium from 0 (ungerminated conidia) to 18 h. PCR amplification products of DNA preparations are shown in the last lane (DNA) for comparison with products of transcripts in the RNA preparations. The sizes and migration of molecular markers are indicated in bp in the margin. The β-tubulin primers were used in the RT-PCR reaction as described in Figure 2 with an annealing temperature of 60°C. Primers were designed from consensus sequences near the 5′-terminus of the respective genes in *N. crassa* (Orbach et al. 1986) and *Colletotrichum graminicola* (Panaccione and Hanau 1990). The sequence of the sense strand (DNA) primer was 5′-CTCCAAACCGGCCAATG-3′, and the sequence of the antisense strand (DNA) primer was 5′-GTGATCTGGAAANCCCTG-3′ with one degenerate nucleotide. These sequences in *N. crassa* and *C. graminicola* flank four introns and, thus, can be used to detect the presence of contaminating DNA in RNA preparations.

Digestion of the 401-bp amplified product with *Msp*I and with *Pvu*1 resulted in the expected sizes of digestion products (321 + 80 and 272 + 129, respectively) and confirmed that this fragment was derived from the *HTS1* transcript (data not shown). Furthermore, amplification products were not detected when RNA preparations from germinated conidia of a non-toxin-producing (race 2) isolate were analyzed (data not shown), indicating that the products detected were, in fact, unique to the toxin-producing race 1 and not products of nonspecific priming and amplification.

Because *Taq* polymerase from *Thermus aquaticus* can exhibit reverse transcriptase activity (Jones and Foulkes 1989), a control that simply excludes reverse transcriptase from the reaction mixture is an inadequate test for the presence of contaminating DNA. In our RNA isolation procedure, DNA that may have pelleted with RNA through CsCl was removed by digestion with RNase-free DNase. To confirm the efficacy of this step, we attempted to amplify RNA preparations with a pair of DNA-specific primers, those that span the translation start site (Fig. 1). The predicted 406-bp product was detected when purified *C. carbonum* DNA was added to the amplification reaction, but no product was amplified with the RNA preparations (data not shown). This experiment indicated that our RNA preparations were devoid of contaminating genomic DNA and that the products we detected by RT-PCR were derived from RNA.

RT-PCR with primers to amplify the tubulin gene(s) also confirmed the absence of DNA in the RNA preparations (Fig. 3). Two products were amplified when RNA was used in the reactions, and they were smaller than the corresponding products of DNA amplification presumably due to processing of the transcripts and splicing out of introns between the primers. The larger products, detected with DNA as the template, were not detected in the RNA preparations. The tubulin transcripts, like the *HTS1* transcript, were present in RNA preparations from ungerminated conidia.

During germination and germ tube elongation, the amount of the tubulin transcript increased rapidly to a relatively consistent level, indicating that those transcripts comprise a constant proportion of the total mRNA population once the spores have germinated.

Results presented here indicate that *HTS1* is expressed during sporulation and that the transcripts are stored in ungerminated conidia. Thus, the spores are prepared for a pathogenic existence in the event that they encounter susceptible maize leaf tissue. It is probable that, during sporulation, transcripts of genes that are expressed in the vegetative growth phase immediately preceding sporulation are incorporated into the developing spore and that the process does not include only those transcripts that are necessary for survival or germination of the spore. Transcripts of the β-tubulin were also detected in RNA from ungerminated conidia. However, because of the importance of tubulin in growth processes, the preservation of this transcript in dormant spores is perhaps expected and more easily reconcilable than preservation of the *HTS1* transcript. Unlike the *avr9* avirulence gene in *C. fulvum*, which is induced by nitrogen limitation and is not expressed under conditions that are optimal for vegetative growth (Van den Ackerveken et al. 1994), expression of the *HTS1* gene is apparently not suppressed by optimal nutritional conditions. Although we have not conducted exhaus-

tive studies of potential regulatory factors and conditions, we have found that the *HTS1* gene is expressed by conidia germinated in nutrient media over a range of pH from 3.5 to 8.0 (unpublished results). The possibility that the synthesis of HC-toxin is regulated beyond the transcriptional level cannot be ignored.

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