A Decarboxylase Encoded at the *Cochliobolus heterostrophus* Translocation-Associated *Tox1B* Locus Is Required for Polyketide (T-toxin) Biosynthesis and High Virulence on T-cytoplasm Maize

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Genes at two unlinked loci (*Tox1A* and *Tox1B*) are required for production of the polyketide T-toxin by *Cochliobolus heterostrophus* race T, a pathogenic fungus that requires T-toxin for high virulence to maize with T-cytoplasm. Previous work indicated that *Tox1A* encodes a polyketide synthase (PKS1) required for T-toxin biosynthesis and for high virulence. To identify genes at *Tox1B*, a wild-type race T cDNA library was screened for genes missing in the genome of a *Tox1B* deletion mutant. The library was probed, first with a 415-kb *NotI* restriction fragment from the genome of the *Tox1B* mutant, then with the corresponding 560-kb fragment from the genome of wild type. Two genes, *DEC1* (similar to acetate decarboxylase-encoding genes) and *RED1* (similar to genes encoding members of the medium-chain dehydrogenase/reductase superfamily), were recovered. Targeted disruption of *DEC1* drastically reduced both T-toxin production and virulence of race T to T-cytoplasm maize, whereas specific inactivation of *RED1* had no apparent effect on T-toxin production (as determined by bioassay) or on virulence. *DEC1* and *RED1* map within 1.5 kb of each other on *Tox1B* chromosome 6;12 and are unique to the genome of race T, an observation consistent with the hypothesis that these genes were acquired by *C. heterostrophus* via a horizontal transfer event.

Additional keywords: *Mycosphaerella zeae-maydis*, race O.

T-toxin is a family of polyketides produced by *Cochliobolus heterostrophus* race T that is required for high virulence of the fungus to maize containing Texas male sterile (T) cytoplasm (Yang et al. 1996; Yoder et al. 1997). The only other known form of *C. heterostrophus* is race O, which does not produce T-toxin and is mildly virulent on T-maize. In crosses between naturally occurring isolates of races T and O, ability to produce T-toxin usually segregates as a single genetic locus designated

Tox1; however, in crosses between certain mutants of race T which lack the ability to produce T-toxin, *Tox1* segregates as two unlinked loci that map to two different chromosomes (Kodama et al. 1999). One locus, *Tox1A* on chromosome 12;6, contains a gene (*PKS1*) that encodes a polyketide synthase (PKS) necessary for both T-toxin biosynthesis and high virulence (Yang et al. 1996). The other locus, *Tox1B* on chromosome 6;12, is the subject of the investigation reported here.

Evidence for the existence of genes at *Tox1B* came by an unexpected route. In an attempt to clone PKS-encoding genes in addition to *PKS1* that might be involved in T-toxin biosynthesis, degenerate primers matching conserved motifs in acyltransferase (AT) domains of known PKS-encoding genes were used in polymerase chain reactions (PCR) with genomic *C. heterostrophus* race T DNA as template. Several clones were generated and sequenced to confirm their identities as AT domains. Four of these were mapped by probing electrophoretically separated chromosomes; each hybridized to a different chromosome, indicating four different putative PKS genes. Targeted disruption of each gene caused no discernible change in phenotype (i.e., T-toxin production and virulence of each mutant were normal), suggesting that either these genes are nonfunctional or their products are involved in biosynthesis of polyketides other than T-toxin (Rose 1996). However, among the mutants generated by transformation of the wild type with one of the AT PCR products was one in which the vector integrated at an ectopic site, yet it produced little or no T-toxin activity. Molecular analysis of this mutant revealed that it had a large deletion at the site of vector integration, and that the deletion mapped at *Tox1B* (Kodama et al. 1999). The vector cosegregated with the *Tox*– phenotype in progeny of crosses between the mutant and wild type; therefore, we inferred that the deletion covered one or more genes required for T-toxin biosynthesis. To identify these genes, an experiment was designed to recover sequences present in wild type but missing in the *Tox1B* deletion mutant. In this article, we describe two closely linked genes at *Tox1B*, *DEC1* (predicted to encode a decarboxylase), which is required for T-toxin production, and *RED1* (predicted to encode a reductase), which is not.

RESULTS

Identification of T-toxin-deficient mutant C4.PKS.13.

In separate work, we identified several *C. heterostrophus* PKS genes by PCR amplification of conserved AT domains (Rose 1996). In an attempt to specifically disrupt the genomic counterpart of one of these (L15, a 279-bp partial AT fragment cloned in pT15) (Table 1), one hygromycin B-resistant trans-

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formant (C4.PKS.13) was obtained that had reduced T-toxin production (Fig. 1A) and low virulence on T-cytoplasm maize (Fig. 1B). No other transformants generated with pT15 were *Tox*⁻. Crosses of C4.PKS.13 to race O yielded 100% *Tox*⁻ progeny, indicating the mutation mapped at *Tox1*. Crosses to race T produced progeny segregating 1:1 for parental types (*Tox*⁻; *hygR*:*Tox*⁺; *hygS*) indicating a single mutation tagged with the transformation plasmid (pT15; Table 1). Crosses between *Tox*⁻ mutant C4.PKS.13 and *Tox1A*⁻ mutants produced progeny segregating 3:1 (*Tox*⁻; *hygR*:*Tox*⁺; *hygS*) for failure versus ability to produce T-toxin, indicating independent segregation of two unlinked genes required for toxin production (Kodama et al. 1999). This result established that the mutation in C4.PKS.13 is genetically unlinked to *Tox1A*.

DNA gel blot analysis revealed a single copy of pT15 in the genome of C4.PKS.13 at an ectopic site (i.e., integration of the vector did not occur by homologous recombination between L15 sequences on the vector T15 and those on the chromosome. Both the restriction fragment (Fig. 2A) and the chromosome (Fig. 2B) bearing the native copy of L15 were undis-

turbed in C4.PKS.13. Chromosome 6;12, marked with *Tox1B*-linked restriction fragment length polymorphism marker B88 (Tzeng et al. 1992), also hybridized to L15 in the mutant strain (Fig. 2B). The pT15 and genomic DNA flanking it were recovered by plasmid rescue. Sequence analysis revealed that pT15 integrated in C4.PKS.13 by a recombination event that occurred within the f1 origin of replication on the plasmid and that integration of pT15 did not interrupt an open reading frame (ORF) with a start codon; in fact, there was no ORF longer than 13 amino acids within several hundred base pairs of the integration site. Thus, there is no DNA encoding a PKS at the pT15 insertion site even though transformation was with a vector carrying an AT fragment from a putative PKS gene and one transformant (C4.PKS.13) failed to produce T-toxin. Sequencing indicated that genomic DNA on both flanks of the pT15 integration site is approximately 70% A+T.

A deletion at the C4.PKS.13 insertion site.

Previous work (Kodama et al. 1999) revealed that C4.PKS.13 chromosome 6;12, carrying *Tox1B*, sustained a deletion of ap-

Table 1. Vectors and clones used in this study

Designation	Size (kb)	Characteristics ^a	Reference
pUCATPH	5.1	REMI transformation vector; the <i>hygB</i> cassette, encoding resistance to hygromycin B, can be excised as a 2 kb <i>XbaI/XmnI</i> or a 2.4-kb <i>SalI</i> fragment	Lu et al. 1994
pL15	4.2	TA cloning vector pCRII (Invitrogen) carrying a 279-bp PCR product amplified from <i>Cochliobolus heterostrophus</i> strain C4, encoding a PKS acyl transferase fragment	Rose 1996
pT15	6.6	Fungal transformation vector. pL15 carrying the 2.4-kb <i>SalI</i> <i>hygB</i> fragment from pUCATPH in the <i>XhoI</i> site	Rose 1996
pC4-3	3.8	pBluescript SK ⁻ (Stratagene) carrying an 814-bp <i>C. heterostrophus</i> strain C4 cDNA cloned as an <i>EcoRI/XhoI</i> fragment; cDNA encodes the carboxy terminus of <i>RED1</i>	This study
pC4-4	3.8	pBluescript SK ⁻ (Stratagene) carrying an 839-bp <i>C. heterostrophus</i> strain C4 cDNA cloned as an <i>EcoRI/XhoI</i> fragment; cDNA encodes <i>DEC1</i>	This study
pC4-3Shyg	5.8	Fungal transformation vector; pC4-3 carrying a blunt-ended 2-kb <i>XmnI/XbaI</i> <i>hygB</i> resistance cassette from pUCATPH inserted in a unique <i>StuI</i> site in the <i>RED1</i> -encoding cDNA	This study
pC4-4Shyg	5.8	Fungal transformation vector; pC4-4 carrying a blunt-ended 2-kb <i>XmnI/XbaI</i> <i>hygB</i> resistance cassette from pUCATPH inserted in a unique <i>SnaBI</i> site in the <i>DEC1</i> -encoding cDNA	This study
pHR1	11.4	Self-ligated <i>HindIII</i> fragment from transformant C4[C4-3Shyg]3U carrying vector pC4-3Shyg; contains genomic copies of <i>DEC1</i> and <i>RED1</i>	This study

^a PCR = polymerase chain reaction and PKS = polyketide synthase.

Fig. 1. A, T-toxin production by wild-type and mutant strains of *Cochliobolus heterostrophus*. An indicator plate overlaid with T-toxin-sensitive *Escherichia coli* cells was inoculated with mycelial plugs and incubated overnight at 30°C. Halos surrounding inocula indicate T-toxin activity. Inocula are (from top to bottom of the plate): race T *Tox1*⁺ control strain C4; mutants C4.PKS.13, C4.PKS.14, and C4.PKS.15 (each as a pair of inocula); race O *Tox1*⁻ control strain C5. Halos produced by C4.PKS.13 are like the *Tox1*⁻ control strain. The other two mutants produce wild-type levels of T-toxin. **B,** Virulence of wild-type and mutant strains on maize. Three representative leaves are shown for each fungal strain, from the left: race T (*Tox1*⁺) strain C4, race O (*Tox1*⁻) strain C5, *Tox*⁺ progeny of C4.PKS.13 × wild-type *Tox1*⁺ tester strain C9, mutant C4.PKS.13, *Tox*⁻ progeny of C4.PKS.13 × wild-type *Tox1*⁺ tester strain C9. Symptoms caused by C4.PKS.13 and its *Tox*⁻ progeny are similar to those of race O, which is *Tox1*⁻.

proximately 100 kb. To refine the estimate of the deletion size, embedded chromosomes of strains C5 (race O), C4 (race T), and C4.PKS.13 were digested with the rare-cutting enzyme *NotI* and separated in agarose. Most fragments were less than 300 kb in size and not resolved (Fig. 3A). However, clear polymorphisms in the 400- to 750-kb size range were observed between near-isogenic wild-type strains C4 and C5 and between C4 and *Tox1B* mutant C4.PKS.13. Two large fragments (approximately 700 and 500 kb), evident in C4, were missing in C5 (Fig. 3A). In C4.PKS.13, the approximately 500-kb fragment was replaced by an approximately 400-kb fragment; the 700-kb fragment remained unchanged (Fig. 3A). Additional gels, run under conditions that better resolve fragments in this size range, indicated the sizes of the C4 *NotI* fragments are approximately 700 and 560 kb and the C4.PKS.13 fragment is approximately 415 kb (data not shown).

To determine if the unique *NotI* fragments were associated with the vector insertion site in C4.PKS.13, and also with the *Tox1* locus, a gel carrying *NotI*-digested chromosomes was blotted and probed with vector pT15 minus the L15 insert. Bands of approximately 415 and 70 kb hybridized only in C4.PKS.13 (Fig. 3B). The transformation plasmid (pT15; Table 1), inserted in the genome of C4.PKS.13, has one *NotI* site in the vector portion of the plasmid; therefore, probing with the vector is expected to yield two fragments which should add up to approximately 560 kb. However, the sum of sizes of the two hybridizing fragments is 485 kb (415 + 70 kb), less than the expected 560 kb, indicating that an approximately 75-kb deletion occurred on chromosome 6;12 of C4.PKS.13.

Cloning of cDNAs missing in C4.PKS.13.

A cDNA library of race T strain C4 was plated and transferred to filters, after which the filters were probed with the C4 560-kb *NotI* fragment and then (following stripping) with the C4.PKS.13 415-kb *NotI* fragment (represented by *Tox1B*::*hygR* ascospore 1213-R-65). No clear differential hybridization was observed. Plaques in the regions of the majority of hybridizing signals (433 plaques) were collected and pooled. Plaque lift filters (containing approximately 10,000 plaques each) of this sub-library were probed again, first with the 415-kb and then with the 560-kb *NotI* fragments. Three clones hybridized, on duplicate filters, with the 560-kb probe only. Restriction enzyme digestion of their excised recombinant pBluescript vectors, designated pC4-1, pC4-3, and pC4-4, revealed that pC4-1 and pC4-4 were identical and different from pC4-3. Radiolabeled cDNA inserts of pC4-3 and pC4-4 hybridized to a single 5.6-kb *HindIII* fragment of C4 genomic DNA, indicating that the genes encoding these cDNAs are closely linked (data not shown). When chromosomes of C5, C4, and 1213-R-65 were probed with these inserts, both hybridized to *Tox1B* chromosome 6;12 in C4; neither hybridized to chromosomes of the mutant 1213-R-65 or to C5 (race O) chromosomes (Fig. 4).

Targeted gene disruption using *Tox1B*-unique cDNAs.

To disrupt the genomic counterparts of cDNAs C4-3 and C4-4, strain C4 was transformed with pC4-4Shyg (carrying C4-4) or pC4-3Shyg (carrying C4-3) (Table 1), each linearized (with *SacI/XhoI*) to promote gene replacement via double

Fig. 2. Native location of fragment L15 (a partial polyketide synthase acyltransferase domain) is not on *Tox1B* chromosome 6;12. **A**, Genomic DNA from strains C5 (lanes 3, 6, and 9), C4 (lanes 4, 7, and 10), and C4.PKS.13 (lanes 5, 8, and 11), was digested with *EcoRI* (lanes 3-5), *PstI* (lanes 6-8), and *XbaI* (lanes 9-11), separated on an agarose gel, blotted, and the gel probed with polymerase chain reaction-amplified L15. In each digest, the same signal is seen in DNA of all three strains, indicating an intact copy of native L15; there is an additional signal (open arrows) in each of the digests of C4.PKS.13 DNA, indicating that L15 did not integrate at its native location in this transformant but rather at a different location. Lanes 1, 13; lambda/*HindIII* markers; lanes 2, 12; empty. **B**, Chromosomes of *Cochliobolus heterostrophus* strains C5, C4, and C4.PKS.13 were separated by CHEF gel electrophoresis, blotted, and probed with chromosome 6;12-specific, *Tox1B*-linked, fragment B88 (Tzeng et al. 1992); the filter was stripped and probed with L15. B88 hybridized to chromosome 6;12 in strains C4 and C4.PKS.13 (left, black arrow) and to chromosome 12 in strain C5, as expected (Tzeng et al. 1992). L15 did not hybridize to chromosome 6;12 in strains C4, or to chromosome 12 in strain C5, but rather to a group containing chromosomes 1, 2, and 3, unresolved in this gel, in all three strains (right, black arrow). One of these three chromosomes carries the native copy of L15 in each strain. In C4.PKS.13, L15 also hybridized to chromosome 6;12 (right, open arrow), confirming its ectopic location at *Tox1B* in this mutant. The top band in each lane is the well.

crossover events. Of 40 pC4-4Shyg transformants, 33 were Tox⁻ (a subset is shown in Fig. 5A). DNAs from C4 and C5 plus nine transformants (six Tox⁻ and three Tox⁺) were probed with radioactively labeled C4-4; the six Tox⁻ strains had disruptions of the C4-4 counterpart, whereas the three Tox⁺ strains had ectopic vector integrations (Fig. 5B). Two Tox⁺ and three Tox⁻ transformants were assayed for virulence on T-cytoplasm maize. Symptoms produced by the Tox⁺ transformants were indistinguishable from those produced by wild-type strain C4, whereas those caused by the Tox⁻ transformants were like symptoms of race O (Fig. 6; plant assays of only one Tox⁺ and one Tox⁻ strain are shown), indicating that the gene represented by C4-4 is required for T-toxin production and high virulence on T-cytoplasm maize.

In contrast, 39 pC4-3Shyg transformants all produced wild-type levels of T-toxin (a subset is shown in Fig. 5C); 10 of 10 transformants examined by PCR analysis (Fig. 5D) and 9 of these examined by DNA gel blot analysis were confirmed to have deletions of the C4-3 counterpart. Two Tox⁺ transformants with disrupted C4-3 counterparts were assayed on plants and found to cause wild-type race T symptoms on T-cytoplasm maize (Fig. 6; only one is shown). Thus, the gene represented by C4-3 does not appear to be required for T-toxin biosynthesis or for high virulence.

Cloning genomic sequences by plasmid rescue.

Genomic copies of the genes represented by C4-3 and C4-4 were obtained by plasmid rescue following transformation of

strain C4 with a circular version of the transformation vector, pC4-3Shyg. A transformant (C4{C4-3Shyg}3U) was identified which had a single copy of the vector integrated by a single crossover homologous recombination event. The cDNA inserts of both pC4-3 and pC4-4 are on a single 5.6-kb genomic *Hind*III fragment, and pC4-3Shyg lacks *Hind*III sites; therefore, this enzyme was used to digest C4{C4-3Shyg}3U genomic DNA, which was then self-ligated and used to transform *Escherichia coli*. *Hind*III digestion of plasmids isolated from amp^R transformants revealed an 11.4-kb plasmid (pHR1) with a single *Hind*III site, wild-type copies of ORFs represented by C4-4 and C4-3, and a second disrupted copy of the ORF represented by C4-3 (Fig. 7A).

Sequence analysis of *Tox1B*.

Sequence comparisons of pHR1 and the cDNA inserts in pC4-3 and pC4-4 revealed that the pC4-4 insert corresponds to a 759-bp intronless ORF on pHR1, while the pC4-3 insert corresponds to the 3' end (677 bp) of an intronless 1,056-bp ORF (Fig. 7B). The two ORFs are divergently transcribed and separated by 1,522 bp of noncoding sequence. The G+C-content of the two ORFs is 46 and 44%, while the intervening DNA is 40% G+C. At the 3' end of C4-4 is a 1,500-bp A+T-rich region (28% G+C) (Fig. 7B). BLASTP and PSI-BLAST searches with the translated C4-4 counterpart showed high similarity to acetoacetate decarboxylase from the nitrogen-fixing symbiotic bacterium *Mesorhizobium loti*, the anaerobic bacteria *Clostridium acetobutylicum* and *C. beijerinckii*, the purple bacterium *Ralstonia solanacearum*, the actinomycete *Streptomyces nogalater*, and the hyperthermophilic, sulfate-reducing archaeon *Archaeoglobus fulgidus*. The *Cochliobolus heterostrophus* gene was designated *DEC1* (Fig. 8). BLASTP and PSI-BLAST searches revealed that the translated C4-3 counterpart is most similar to various NADPH-dependent oxidoreductases (data not shown). Similarities also were observed with the enoyl reductase domains of fatty acid and polyketide synthases as well as with alcohol dehydrogenases. All four of these enzymes (or enzymatic domains) belong to the medium-chain dehydrogenase/reductase gene (MDR) super-

Fig. 3. *Not*I restriction fragment length polymorphisms distinguish race T strain C4 from race O strain C5 and mutant C4.PKS.13 from its progenitor strain C4. **A**, Chromosomes of wild-type *ToxI*⁻ strain C5 (lane 3), *ToxI*⁺ strain C4 (lane 4), and *ToxIB*⁻ mutant C4.PKS.13 (lane 5) were digested with *Not*I, separated in an agarose gel, and the gel stained with ethidium bromide. Lane 1; Lambda DNA concatamer size markers; lane 2; 100-kb ladder size markers (corresponding sizes on left in kilobases). The approximately 700 and 500 (560)-kb fragments (asterisks) seen in the C4 lane are missing in C5 (lane 3). The C4.PKS.13 lane has the 700-kb fragment, but the 500 (560)-kb fragment is missing and replaced with a 415-kb fragment (open arrow). **B**, Deletion size in mutant C4.PKS.13 estimated by *Not*I digestion. Chromosomes of wild-type *ToxI*⁻ strain C5 (lane 2), *ToxI*⁺ strain C4 (lane 3), and *ToxIB*⁻ mutant C4.PKS.13 (lane 4) were digested with *Not*I, separated in an agarose gel, blotted, and the filter probed with vector pT15 minus the L15 insert. No hybridizing bands were seen in C5 and C4 lanes. In the C4.PKS.13 lane, bands of approximately 415 and 70 kb hybridized. The sum of these bands (485 kb) compared with the size of the native band (560 kb) indicates the deletion in C4.PKS.13 is approximately 75 kb. Lanes 1 and 5 are lambda DNA concatamer size markers. Arrow indicates the well.

Fig. 4. cDNAs recovered by differential screening are unique to wild-type race T (*ToxI*⁺). Chromosomes of race O strain C5, race T strain C4, and *ToxIB*⁻ mutant 1213-R-65 (a *ToxIB*⁻; *hygB*^R progeny of C4.PKS.13 X C9) were separated in agarose, after which the gel was blotted and the filter probed with inserts from either pC4-3 or pC4-4. Both probes hybridized to chromosome 6:12 in C4 (black arrow) only, indicating that these genes are missing in both C5 and *ToxIB*⁻ mutant 1213-R-65. Both probes hybridized to the same chromosome indicating they are linked. Upper signal is the sample well.

family (Persson et al. 1994). The gene corresponding to C4-3 was called *RED1*.

BLASTX searches identified a region 3' of *RED1* (44% G+C) with high similarity to the carboxyl terminal third of Tc1/mariner transposases (Fig. 7B). However, three stop codons and one frameshift occur within this region of transposase similarity. Additionally, there are no apparent terminal inverted repeats, characteristic of Tc1/mariner transposons, flanking the transposase (data not shown).

BLASTN searches revealed three regions in the sequenced region of the *Tox1B* chromosome (Fig. 7B) (base pairs 642 to 490, 865 to 880, and 2,718 to 2,733) with high similarity (80.3, 100, and 100%, respectively) to nucleotide sequences flanking the coding sequence of *PKS1* (accession #U68040; base pair positions 9,614 to 9,766, 286 to 301, and 739 to 754, respectively), the PKS encoding gene at locus *Tox1A* required for T-toxin biosynthesis (Yang et al. 1996). Between *DEC1* and *RED1* is a 43-bp imperfect inverted repeat (base pairs

3,440 to 3,483 and 3,543 to 3,500) encompassing a 23-bp perfect inverted repeat (base pairs 3,440 to 3,462 and 3,543 to 3,521), separated by 16 bp (Fig. 7B).

Distribution of *DEC1*, *RED1*, and *PKS1*.

The *EcoRI/XhoI* cDNA inserts of pC4-4 (encoding *DEC1*) and pC4-3 (encoding *RED1*) and a 5-kb *HindIII/XbaI* fragment from the *PKS1* clone R.C4.350LF5P1 (Yang et al. 1996) were used to probe DNA gel blots of genomic DNA from a collection of *C. heterostrophus* race O and race T field isolates. All three probes hybridized only with race T DNA, indicating race O lacks homologs of these genes (Fig. 9). Low stringency hybridization of these probes with DNA isolated from several close relatives of *C. heterostrophus* and unrelated *Mycosphaerella zeae-maydis* revealed that (i) the *DEC1* probe did not hybridize with any DNA examined, (ii) the *RED1* probe hybridized very weakly to two bands in *C. spicifer* DNA, and (iii) the *PKS1* probe hybridized very weakly with

Fig. 5. T-toxin production is reduced by disruption of the genomic counterpart of cDNA C4-4 but not C4-3. **A** and **C**, T-toxin activity of wild-type and gene-disrupted mutants. Wild-type race T strain C4 (*Tox1*⁺, with halo) and race O strain C5 (*Tox1*⁻, without halo) controls are at the top of each plate. **A**, After transformation of C4 with pC4-4Shyg, 40 transformants were assayed for T-toxin. Most of them (33) were Tox⁻ (**B**), and shown by DNA gel blot to represent homologous integration events; five of the transformants are shown. Three are Tox⁻ [middle row, left to right: C4(C4-4Shyg)1, C4(C4-4Shyg)3, C4(C4-4Shyg)4] and two are Tox⁺ [bottom row, left to right: C4(C4-4Shyg)11 and C4(C4-4Shyg)36]. Tox⁺ transformants represent ectopic integration events. Note that one of the *dec1*-deleted mutants (middle row, left), actually has a small halo, suggesting a leaky phenotype. **C**, After transformation of C4 with pC4-3Shyg, 39 transformants were assayed for T-toxin, and all were Tox⁺; 10 are shown (row 2, from left: red 12-1, red 15-2, red 17-1, red 18-1; row 3, from left: red 25-1, red 30-1, red 37-1, red 42-1; row 4, from left: red 46-1, red 50-1). **B** and **D**, Identification of homologous and ectopic recombination events. **B**, *HindIII*-digested genomic DNA (blotted and probed with pC4-4) of wild-type *Tox1*⁺ strain C4 (lane 3) and mutants recovered after transforming C4 with pC4-4Shyg. Lanes 4–6 contain DNAs from ectopic transformants [C4(C4-4Shyg)11, C4(C4-4Shyg)36, C4(C4-4Shyg)40; DNA in lane 5 is undigested]. Lanes 7–12 contain DNAs from homologous transformants [C4(C4-4Shyg)1–6]; the signal in each lane reflects the size of the native band plus the size of the disruption vector. The native band (5.6 kb), seen in C4 (lane 3) is absent. Lanes 2 and 13 are empty. **D**, Polymerase chain reaction (PCR) products generated using primers specific to the ends of cDNA C4-3 (*RED1*-specific primers). Lane 1 contains the 1-kb DNA ladder; lane 2 contains a reaction mixture with no DNA template; Lanes 3 and 5 have the products from genomic DNAs of strains C5 (race O) and C4 (race T), respectively; lane 4 has a product generated with pC4-3Shyg as template; and lanes 6–8 contain PCR products generated using genomic DNAs from three transformants (red 25-1, red 17-1, and red 12-1) as templates. Wild-type strain C4 produced a product of 0.8 kb; for each transformant, the product size increased to 2.8 kb, which is the size amplified from pC4-3Shyg. Race O did not yield a product.

two bands in DNA from *S. turcica* and one in *M. zeae-maydis* (Fig. 10).

DISCUSSION

Transformant C4.PKS.13 is unique in our collection of induced *Tox1*⁻ mutants (Kodama et al. 1999; Yang et al. 1996). It is the only one in which the *Tox*⁻ mutation maps at *Tox1B*. Molecular analyses revealed that two mutational events occurred at the same site in C4.PKS.13: vector insertion and deletion of approximately 75 kb of chromosomal DNA. The genes *DEC1* and *RED1* were found in the chromosomal region covered by the deletion. Despite the usefulness of C4.PKS.13 in revealing the organization of the *Tox1* locus (Kodama et al. 1999), it is not clear how the mutation in this transformant occurred. The transformation vector used to generate C4.PKS.13 carried a 279-bp sequence (L15) corresponding to part of an AT domain of a *C. heterostrophus* PKS gene, yet there are reasons to conclude that integration did not occur by homologous recombination. First, sequencing of the integration site in C4.PKS.13 showed that vector integration did not involve the PKS AT domain on the vector as would be expected for homologous recombination, but rather the f1 origin of replication, which has no homologous sequence in the *C. heterostrophus* genome. Second, only one copy of L15 can be detected in the *C. heterostrophus* genome at the level of a DNA gel blot, and that copy, which is not linked to *Tox1*, is undisturbed in C4.PKS.13 (Fig. 2B). Third, the *C. heterostrophus* genome has been sequenced (Torrey Mesa Research Institute, San Diego, CA, U.S.A.) and L15 mapped; sequence analysis

Fig. 7. Genetic organization in the *Tox1B* region of chromosome 6;12. **A**, Sequences corresponding to C4-4 and C4-3 were recovered together on a single *HindIII* fragment, as pHRI, from a transformant of strain C4 in which the circular form of the vector pC4-3Shyg was integrated by homologous recombination at the native C4-3 sequence. This resulted in a recombinant chromosome with two copies of C4-3 (one wild type and one disrupted by *hygB*) separated from each other by vector sequences, and a wild-type copy of C4-4. **B**, Sequencing of the insert in pHRI (5,613 bp) and database searches revealed that C4-4 corresponds to a decarboxylase-encoding gene designated *DEC1* (759 bp, open arrow) and C4-3 corresponds to a reductase-encoding gene designated *RED1* (1,056 bp, open arrow); the genes are transcribed in opposite directions and are separated by 1.5 kb, which contains inverted repeats as described in the text (arrows above the line). Beyond the 3' end of *RED1* is a 760-bp sequence with similarity to a transposase. The small open boxes represent sequences similar or identical to regions of the *PKS1* 3' flank. Percentage of G+C is indicated below each region.

Fig. 6. Virulence of *Cochliobolus heterostrophus* to T-cytoplasm maize is reduced by disruption of *DEC1* but not *RED1*. From left to right, pairs of leaves from plants inoculated with strains: C5 race O, C4 race T, *dec1*-deletion mutant C4(C4-4Shyg)3, *DEC1*⁺ strain C4(C4-4Shyg)11 (ectopic integration), and *red1*-deletion mutant C4(C4-4Shyg)12-1 on plants. Note the reduced symptoms caused by the *dec1*-deletion mutant are like those of C5, indicating that *DEC1* is required for high virulence while the symptoms caused by the *red1*-deletion mutant are like those of C4, indicating that *RED1* is not involved in virulence of *C. heterostrophus*.

indicates that L15 is indeed part of a PKS gene, unlinked to *Tox1*. Thus, pT15 did not insert at *Tox1B* and concomitantly cause a large deletion as a result of an interaction with its homolog in the genome. Although the origin of the *Tox1B* mutation in C4.PKS.13 remains a mystery, analysis of the DNA deleted in this mutant is essential for understanding the molecular nature of the *Tox1* locus.

The requirement of a functional *DEC1* gene for T-toxin activity implies that a decarboxylase is involved in production of active T-toxin molecules. How? Among the possibilities are the notions that the decarboxylase is needed for synthesis of a T-toxin precursor (e.g., a substrate for PKS1) analogous to the product of the chain-initiation decarboxylase of polyketide-producing *Streptomyces* (Bisang et al. 1999), or that a T-toxin intermediate requires decarboxylation for activity or for secretion from the cell. Although neither alternative has been proven, we favor the second of these because of the precedent established for the *Clostridium acetobutylicum* decarboxylase (Highbarger et al. 1996; Westheimer 1969). The substrate for this enzyme is acetoacetate (a diketide) and its products are acetone and carbon dioxide (Fig. 11). If the action of DEC1 is similar, we can imagine a model in which the product of PKS activity is a hypothetical T-toxin precursor with an even-numbered carbon chain (e.g., C₄₂), the carboxyl end of which would match the structure of acetoace-

tate. DEC1 would convert this molecule to T-toxin and carbon dioxide. The model could explain why native T-toxin, which is a family of 10 to 15 linear polyketide molecules, consists entirely of odd-numbered carbon chains (C₃₅ to C₄₅), none of which terminates with a carboxyl function (Yoder et al. 1997). An experimental test of this hypothesis would be examination of metabolite profiles of wild-type race T, race O, and the *dec1*- mutant. The mutant, although incapable of T-toxin production, as determined by the microbial assay (Fig. 5A), may still produce T-toxin-like molecules but with even numbers of carbon atoms.

In certain assays, *dec1*- mutants had greatly reduced, but not zero, T-toxin activity; these mutants produced halos much smaller than those of their race T progenitor, yet they were still discernable (Fig. 5A). This observation suggests that the *dec1*- mutation is leaky. Despite this fact, mutants cause symptoms on T-maize that are indistinguishable from those caused by race O, which does not produce T-toxin. The cause of *dec1*- leakiness can only be surmised; perhaps there is another enzyme that can perform the decarboxylation reaction on T-toxin, albeit inefficiently, or the precursor may itself have T-toxin activity, or it may fail to be secreted.

Deletion of *RED1* had no discernible effect on the phenotype of *C. heterostrophus* race T; growth, reproduction, T-toxin production, and virulence all were normal in *red1*-

Fig. 8. Alignment of the DEC1 amino acid sequence with those of *Mesorhizobium loti* (NP_103754.1, NP_104702.1, NP_106500.1), *Clostridium acetobutylicum* (NP_149328.1; JN0487), *Ralstonia solanacearum* (NP_522619.1), *C. beijerinckii* (AAD54946), *Streptomyces nogalater* (AAG42848), and *Archaeoglobus fulgidus* (NP_070005.1). Conserved amino acids are shaded. The conserved catalytic lysine residue is indicated by an asterisk.

deletion mutants. This observation suggests either that *RED1* has no role in T-toxin production or that there is another protein in the *C. heterostrophus* race T capable of fully compensating for loss of *RED1*. The latter possibility deserves serious consideration, because *RED1* belongs to a large family of MDR enzymes. It is notable that an unrelated maize pathogen, *M. zeae-maydis*, produces a T-cytoplasm-specific polyketide (PM-toxin), like *C. heterostrophus* T-toxin (Yoder 1973). The gene (*MzPKS1*) involved in PM-toxin biosynthesis has been cloned and is approximately 60% identical at the amino acid level to *C. heterostrophus* *PKS1* (the faint band in Figure 10 corresponds to *MzPKS1* of *M. zeae-maydis*) (Yun 1998). In contrast to *C. heterostrophus* *PKS1*, *MzPKS1* is tightly linked to two reductase-encoding genes (*MzRED1* and *MzRED2*), both of which are required for production of PM-toxin; neither shows similarity to *C. heterostrophus* *RED1* (Yun 1998).

We reported previously (Yang et al. 1996) that codon usage in *PKS1*, as measured by the frequency of a 'C' in the third position of synonymous codons, is unlike that of other known genes in *Cochliobolus* spp. and unlike that of genes from most other fungi (available at that time), suggesting *PKS1* might be of alien origin. *DEC1* and *RED1* have been analyzed similarly; their codon usage closely resembles that of *PKS1*. Since the *C. heterostrophus* genome has been sequenced, we performed a genome-wide analysis of codon preference in ORFs > 500 bp. If one considers the 15 amino acids for which there is a possibility of a 'C' in the third position of their synonymous codons, 14 out of 15 'prefer' a 'C'. If one considers the subset of eight amino acids for which there is a possibility of any one of the four nucleotides in the third position of their synonymous codons, 7 out of 8 prefer a 'C'. In sharp contrast, equivalent numbers for *PKS1*, *DEC1*, and *RED1* are 4 out of 15 for all three genes, 2 out of 8 for *PKS1* and *DEC1*, and 0 out of 8 for *RED1* (B. G. Turgeon, unpublished data).

Several additional observations, detailed previously (Yang et al. 1996), argue that *Tox1* may be the functional equivalent of a pathogenicity island (Hacker and Kaper 2000) that arrived relatively recently in the *C. heterostrophus* genome via a horizontal transfer event. Similar lateral transfers have been suggested for genes controlling production of HC-toxin, a peptide

virulence factor of *C. carbonum* race 1 (Ahn et al. 2002; Walton 2000), and the *PEP* genes of *Nectria haematococca* that are required for its pathogenicity to pea (Han et al. 2001; Rosewich and Kistler 2000). In the case of *C. heterostrophus*, we assume race O is ancestral to race T. This is based primarily on the observation that race O was discovered in the field long before race T (Ullstrup 1970) and that mating type genes were unevenly distributed in the field population of race T when it first appeared in the early 1970s, but reached a 1:1 equilibrium a few years later (Alcorn 1975; Leonard 1973). The hypothesis for a horizontal transfer event is derived from the facts that (i) *PKS1*, *DEC1*, and *RED1* are unique to the genome of race T and all map at the *Tox1* locus; and (ii) comparison of chromosome sizes in race O and race T indicates that the race T genome is approximately 1.2 Mb larger than the race O genome, and that the extra DNA in race T maps at *Tox1* (Chang and Bronson 1996; Tzeng et al. 1992). This large segment of DNA may have destabilized the race O chromosome into which it inserted, thus promoting a reciprocal translocation that placed part of the extra DNA on chromosome 12:6 and the remainder on chromosome 6:12 in race T. We have designated these two components of the *Tox1* locus *Tox1A* on chromosome 12:6 (which contains *PKS1*) and *Tox1B* on chromosome 6:12 (which contains *DEC1* and *RED1*) (Kodama et al. 1999). The model predicts that these three genes once belonged to a contiguous cluster of genes. It appears that there are other, as yet undiscovered, genes in the 1.2-Mb insert required for T-toxin biosynthesis, because *PKS1* will not complement the T-toxin deficiency of a mutant deleted for approximately 700 kb at *Tox1A*, and *DEC1* will not complement mutant C4.PKS.13, which is deleted for 75 kb at *Tox1B* (Zhu 1999).

MATERIALS AND METHODS

Strains, media, transformation, and crosses.

Cloning hosts were *E. coli* strains DH5 α and XL1-blue (Stratagene, La Jolla, CA, U.S.A.). *C. heterostrophus* laboratory strains used in this study were C4 (*Tox1*⁺; *MAT*-2), C5 (*Tox1*⁻; *MAT*-1), C9 (*Tox1*⁺; *MAT*-1), C4.PKS.13 (*Tox1B*⁻; *MAT*-2; *hygB*), and 1213-R-65 (*Tox1B*⁻; *MAT*-1; *hygB*). C4, C5, and C9 are members of a near-isogenic set of strains produced by a series of backcrosses (Leach et al. 1982). C4.PKS.13 (this study) is a transformant of strain C4; 1213-R-65 is a *Tox1B*⁻ progeny of a cross between C4.PKS.13 and C9. The *Tox1B* mutation in C4.PKS.13 and 1213-R-65 is tagged with *hygB*. Strains C4.PKS.14, and C4.PKS.15 are *Tox*⁺ transformants of strain C4, transformed with pT15 (Table 1).

Field isolates used for hybridization queries included (i) *C. heterostrophus* race T isolates Hm80 and Hm92 from North Carolina, Hm338 and Hm#9 from New York, 19679-2 from Australia, and Pr1X412 from Mexico; (ii) *C. heterostrophus* race O isolates Hm648 from Florida, Hm404 from Costa Rica, Hm540 from North Carolina, and Haw288 from Hawaii; (iii) *Cochliobolus* spp. isolates: *C. sativus* A20, *C. carbonum* race 1 A, *C. victoriae* HvW, *C. spicifer* D5-7, and *Bipolaris sacchari* 764-1 (an asexual relative of *Cochliobolus*); and (iv) isolates of other genera: *Setosphaeria turcica* NK2, *S. rostrata* 32197, and *Mycosphaerella zeae-maydis* 24 (isolate details available upon request).

E. coli strains were grown in either Luria broth plus ampicillin at 100 μ g/ml (LA) or LA plus kanamycin at 50 μ g/ml (LAK) (Sambrook et al. 1989). NZY medium used in plating the cDNA library was prepared according to specifications given in the ZAP-cDNA Synthesis Kit (Stratagene). Media, growth conditions, and storage of *C. heterostrophus* have been described (Turgeon et al. 1985), as have procedures for mating

Fig. 9. *DEC1* and *RED1* are found in the genomes of *Cochliobolus heterostrophus* race T isolates but not race O lab and field isolates. Genomic DNAs were digested with *EcoRI*, separated in agarose, blotted, probed successively with *PKS1* (top panel), *DEC1* (middle panel), and *RED1* (bottom panel); filters were stripped between probes. DNAs from race T (*Tox1*⁺) isolates are in lanes 3 (C4), 4 (Hm80), 5 (Hm92), 6 (Hm338), 8 (Hm#9), 9 (19679-2), and 10 (Pr1X412); DNAs from race O (*Tox1*⁻) isolates are in lanes 7 (Hm648), 11 (C5), 12 (Hm404), 13 (Hm540), and 14 (Haw288). Lanes 1 and 16 = lambda *Hind*III markers; lane 2 is empty.

(Leach et al. 1982) and transformation (Turgeon et al. 1993). Complete medium (CM; Leach et al. 1982), CM with xylose (CMX; Tzeng et al. 1992), CM without salts (CMNS), minimal medium (MM; Leach et al. 1982), and Fries medium (Pringle and Braun 1957) were described previously.

Fig. 10. Among fungal pathogens, *DEC1* and *RED1* are unique to the genome of *Cochliobolus heterostrophus* race T. Genomic DNAs were digested with *EcoRI*, separated in agarose, blotted, probed successively with *PKS1* (top panel), *DEC1* (middle panel), and *RED1* (bottom panel); filters were stripped between probings. DNAs were from *C. heterostrophus* races C4 (lanes 3 and 4) and C5 (lane 5), *C. sativus* isolate A20 (lane 6), *C. carbonum* race 1 isolate A (lane 7), *C. victoriae* isolate HvW (lane 8), *C. spicifer* isolate D5-7 (lane 9), *Bipolaris sacchari* isolate 764-1 (lane 10), *Setosphaeria turcica* isolate NK2 (lane 11), *S. rostrata* isolate 32197 (lane 12), and *Mycosphaerella zeae-maydis* isolate 24 (lane 13). A strong signal can be seen only in DNA from *C. heterostrophus* race T. Lanes 1 and 15 = lambda *HindIII* markers; lanes 2 and 14 are empty. Note that there is a faint signal in the top panel in lane 11 (*S. turcica*), lane 12, (*S. rostrata*, visible in longer exposures) and lane 13 (*M. zeae-maydis*). The latter has been shown to correspond to the *M. zeae maydis* *PKS1* gene (Yun 1998). Bands in lanes 11 and 12 have not been investigated. We also observed a weak signal in the bottom panel, lane 9, upon longer exposures.

Nucleic acid manipulations.

Purification of genomic DNA from *C. heterostrophus* has been described (Turgeon et al. 1993). Isolation of plasmid DNA, agarose gel electrophoresis, restriction enzyme digestions, DNA gel blot analyses, and screening of plasmid clones were done according to standard protocols (Sambrook et al. 1989). DNA fragments, recovered from gels, were purified using GeneClean (Qbiogene, Carlsbad, CA, U.S.A.). Vectors and clones used in this study are described in Table 1.

DNA sequences were determined at the Cornell DNA Sequencing Facility using *TaqCycle* automated sequencing with DyeDeoxy terminators (Applied Biosystems, Inc., Foster City, CA, U.S.A.). Primers for sequencing included T3 and T7, as well as custom-made primers corresponding to previously determined sequences synthesized by the Cornell University DNA Services facility (primer sequences available on request). DNA sequences were assembled using either SEQED (Applied Biosystems, Inc.) or SEQMAN (DNASTAR, Inc., Madison, WI, U.S.A.). Similarity searches of the nonredundant database maintained by the National Center for Biotechnology Information (NCBI) were performed using BLASTX, BLASTP, or PSIBLAST (Altschul et al. 1990). Amino acid sequence alignments were made using MEGALIGN (DNASTAR, Inc.).

cDNA library preparation.

To obtain RNA, four 150-mm petri dishes, each containing 75 ml of Fries medium, were inoculated with conidia of C4 grown on CM plates (1×10^6 conidia per dish), then incubated in the dark at 30°C without shaking. After 36 h, as pigmentation first appeared, mycelium was harvested, blotted with paper towels, frozen at -80°C, and lyophilized. To extract RNA (Logemann et al. 1987), lyophilized mycelium was ground in liquid nitrogen and suspended (1 g per 30 ml) in RNA extraction buffer (8 M guanidinium hydrochloride, 20 mM MES, 20 mM EDTA, 50 mM mercaptoethanol at pH 7.0). Yield was approximately 5 mg of RNA per gram of dry mycelium. Poly(A)⁺ RNA was purified using the PolyAtract mRNA Isolation System (Promega Corp., Madison, WI, U.S.A.) as specified by the manufacturer. Yield was approximately 5 µg of poly(A)⁺ RNA from 1.1 mg of total RNA. To construct the cDNA library, the ZAP-cDNA Synthesis Kit and Gigapack II Gold Packaging Extract (Stratagene) were used as described by the manufacturer, starting with approximately 7 µg of

Fig. 11. One possible model for *DEC1* enzymatic activity. The model derives from the observation that the substrate for *Clostridium acetobutylicum* decarboxylase is acetoacetate and the products are acetone and CO₂ (Westheimer 1969). The carboxyl end of a hypothetical T-toxin precursor (C₄₂) would be structurally identical to acetoacetate. *DEC1* would recognize the carboxyl end of the polyketide as a substrate and decarboxylate it, yielding CO₂ and T-toxin (C₄₁).

poly(A)⁺ RNA in 35.5 µl of H₂O. The primary cDNA library contained 5.3×10^6 PFU in 530 µl; 0.7% were nonrecombinant as determined by blue/white screening. An aliquot of the primary library containing 1×10^6 PFU was amplified on agar medium in 41 petri dishes (100 mm) according to the Stratagene protocol. The titer was 1.8×10^8 PFU/ml. Platings of the amplified cDNA library were blotted to Colony/Plaque Screen filters (DuPont, Wilmington, DE, U.S.A.) and probed according to DuPont's protocol.

Preparation of chromosomes and isolation of *NotI* fragments.

Embedded protoplasts were prepared by described methods (Kodama et al. 1999). If chromosome degradation was detected when samples were checked by CHEF electrophoresis, embedded protoplasts were treated a second time with 10 mM Tris, pH 9.0, 0.5 M EDTA, 1% lauryl sarkosyl, and 2 mg of proteinase K/ml. For CHEF analyses of chromosomal digests, protoplast-containing agarose blocks (stored in 0.5 M EDTA) were cut into sections measuring 1 by 5 by 2 to 3 mm and rinsed 12 times in 50 mM EDTA, 2 to 3 times in Tris-EDTA, and 2 times in *NotI* or *SfiI* digestion buffer. Rinsed blocks were placed in fresh buffer with enzyme (typically 20 U per section) and incubated overnight at 37°C, then washed once with 0.5× 45 mM Trizma base, 45 mM boric acid, 1.25 mM EDTA pH 8 (TBE) prior to loading on a CHEF gel. CHEF gels were prepared using 90 ml of 0.8% pulse field certified agarose (BioRad) in 0.5× TBE poured in a 15-by-15-cm gel casting mold with a well-forming comb. Agarose-embedded chromosomal preparations were placed in wells and sealed with molten 0.8% agarose in 0.5× TBE. Electrophoresis was performed in a HEX-CHEF 6000 gel box (C.B.S. Scientific Co., Delmar, CA, U.S.A.) in 0.5× TBE at 12°C. CHEF gels were run at 150V for 24 h with a switching time of 50 sec. After electrophoresis, gels were stained with ethidium bromide, and photographed.

To obtain probes, *NotI* digests of agarose-embedded chromosomes were run on preparative CHEF gels as described above. *NotI* fragments (20 to 30 ng) were eluted from each of seven gels, each loaded with 22 to 24 DNA-containing agarose blocks. Approximately 50 ng were used to make each probe. Fragments were labeled with α -³²P-dCTP (6,000 Ci/mmol) using the Prime-It II Random Primer Labeling Kit (Stratagene), and added to hybridization solution (10% dextran sulfate, 1.5 M NaCl, 1% sodium dodecyl sulfate [SDS]) to a final concentration of 4×10^6 cpm/ml (determined by scintillation counting). Probes used for the enriched cDNA library were labeled with α -³²P-dCTP (3,000 Ci/mmol) and hybridization solutions contained 2×10^6 cpm/ml. Plaque lift filters were probed in plastic bags containing 8 ml of hybridization solution per filter at 65°C for approximately 24 h. Filters were then washed twice in 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) plus 0.1% SDS at 65°C for 15 min, once in 1× SSC plus 0.1% SDS at 65°C, and repeatedly in several washes of 0.5× SSC plus 0.1% SDS at 65°C until filter activity was reduced to 400 to 500 cpm as determined with a handheld survey meter. Filters were exposed to Kodak X-omat AR film for 2 to 3 days before development. Clones of interest were isolated from NZY agar plates by standard methods (Sambrook et al. 1989). Recombinant pBluescript KS(−) plasmids were recovered from phagemids by the in vivo excision protocol (Stratagene).

Plasmid rescue.

HindIII-digested genomic DNA (50 µg) from transformant C4{C4-3Shyg}3U was dissolved in 500 µl of 1× T4 DNA ligase buffer with 1,000 units of T4 DNA ligase (Promega Corp.) and incubated overnight at 16°C. The ligation reaction

was ethanol precipitated, dissolved in 10 µl of water, and 2 µl were used to transform *E. coli* using the BioRad Gene Pulser with the following settings: 200 ohms resistance; 25 µFD capacitance; and 2.5 kV. Transformants were recovered by plating on LAK agar. Plasmid DNA (pHR1, Table 1, Fig. 7) was prepared following standard protocols (Sambrook et al. 1989).

T-toxin and virulence assays.

Assays for T-toxin were performed as previously reported (Ciuffetti et al. 1992; Yang et al. 1996). For virulence assays, conidia were harvested in 0.5% Tween 20 solution from fungal strains grown on CMX agar plates at 20°C under 40-watt Sylvania 350 bulbs. Using aerosol canisters, 10 ml of each spore suspension (1×10^5 conidia/ml), were sprayed on approximately 2-week-old maize plants reared in a growth chamber. Maize inbreds used in this study were W64A-N and W64A-T. Plants were incubated in a mist chamber at 20°C overnight, held in a growth chamber for 5 days to allow symptom development, and then photographed.

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