

Tri1 in *Fusarium graminearum* Encodes a P450 Oxygenase†

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Gibberella zeae (asexual state *Fusarium graminearum*) is a major causal agent of wheat head blight and maize ear rot in North America and is responsible for contamination of grain with deoxynivalenol and related trichothecene mycotoxins. To identify additional trichothecene biosynthetic genes, cDNA libraries were prepared from fungal cultures under trichothecene-inducing conditions in culture and in planta. A gene designated *LHI* that was highly expressed under these conditions exhibited only moderate (59%) similarity to known trichothecene biosynthetic cytochrome P450s. To determine the function of *LHI*, gene disruptants were produced and assessed for trichothecene production. Gene disruptants no longer produced 15-acetyldeoxynivalenol, which is oxygenated at carbon 7 (C-7) and C-8, but rather accumulated calonectrin and 3-deacetylcalonectrin, which are not oxygenated at either C-7 or C-8. These results indicate that gene *LHI* encodes a cytochrome P450 responsible for oxygenation at one or both of these positions. Despite the relatively low level of DNA and amino acid sequence similarity between the two genes, *LHI* from *G. zeae* is the probable homologue of *Tri1*, which encodes a cytochrome P450 required for C-8 oxygenation in *F. sporotrichioides*.

Trichothecenes are potent inhibitors of protein synthesis and virulence factors in wheat head scab (4). Many of the genes required for trichothecene biosynthesis are clustered in a 26-kb region in both the *Fusarium sporotrichioides* and *F. graminearum* genomes (2, 11). Sequence differences have been identified in the gene clusters between T-2 toxin-producing strains of *F. sporotrichioides* and deoxynivalenol (DON)-producing strains of *F. graminearum* that can account for their different trichothecene phenotypes (3, 12, 12, 13). For example, the genes responsible for C-4 oxygenation and acetylation, *Tri13* and *Tri7*, respectively, are missing or inactive in the *F. graminearum* DON-producing strains but are present in T-2 toxin-producing strains of *F. sporotrichioides* and nivalenol-producing strains of *F. graminearum*. Not all trichothecene biosynthetic genes are in the cluster, and three additional trichothecene-related genes have been identified that are physically separated from the main cluster. *Tri101*, which encodes trichothecene C-3 acetyltransferase (10, 15), is located on linkage group 3, while the main cluster is located on linkage group 1 (9). With the release of the *F. graminearum* genomic sequence (www.genome.wi.mit.edu), *Tri101* has been located on contig 1.321 whereas the main cluster is on contig 1.159. A second small group of at least two trichothecene-related genes that encode the C-8 oxygenase (*Tri1*) and the C-8 acyltransferase (*Tri16*) in *F. sporotrichioides* also is known (18, 21). These researchers could not identify a homologue of *Tri1* in *F. graminearum* by Southern analysis.

A putative homologue of *Tri1* in *F. graminearum*, *LHI*, was found when we examined an expressed sequence tag (EST) database derived from a variety of *F. graminearum* expression libraries. In order to determine the function of this gene, disruption mutants were made and analyzed. This is the first characterization of a possible multifunctional oxygenase required for trichothecene biosynthesis.

MATERIALS AND METHODS

Strains and growth conditions. Originally collected from maize caryopsis in Ottawa, Ontario, Canada, by G. A. Neish in 1981, *F. graminearum* wild-type strain DAOM180378 was provided to us by C. Babcock of the Canadian Collection of Fungal Cultures (CCFC/DAOM), Agriculture & Agri-Food Canada, Ottawa. The *F. graminearum* wild-type strain, Z-3639 (NRRL 29214; USDA/ARS Culture Collection, Peoria, Ill), was isolated from scabby wheat by R. Bowden (Kansas State University) (1) and was maintained on V-8 juice agar (26) slants. *F. graminearum* LH1D3, LH1D41, LH1D44, LH1D47, LH1D59, LH1D65, and LH1D72 (available from the Fungal Genetics Stock Center [FGSC9497 to FGSC9503]) contain disrupted sequences of *LHI*, as described below. Transgenic strains GzTri3D-11 (FGSC9488) and GzTri11D-12 (FGSC9496) were constructed from Z-3639 with disrupted sequences of *Tri3* (17) and *Tri11* (16), respectively, as described for *F. sporotrichioides*. All fungal cultures were initially grown on V-8 juice agar plates under an alternating 12-h, 25°C light–12-h, 22°C dark cycle. Fungal cultures were maintained on V-8 slants. Spores or agar plugs of fungal cultures were stored long term at –80°C in 10% glycerol.

Culture conditions and cDNA library construction. The Fg03 cDNA library was prepared from a fungal culture (strain DAOM180378) grown under conditions designed to promote trichothecene production, based on a protocol of Miller and Blackwell (19). A 3.5-ml macerated suspension of potato dextrose agar (Difco Laboratories, Detroit, Mich.)-grown mycelium in distilled water was used to inoculate 50 ml of first-stage medium (3 g of NH₄Cl, 2 g of MgSO₄ · 7H₂O, 0.2 g of FeSO₄ · 7H₂O, 2 g of KH₂PO₄, 2 g of peptone, 2 g of yeast extract, 2 g of malt extract, and 20 g of glucose in 1 liter of distilled water) in a 250-ml flask and grown at 28°C in the dark (with shaking at ~250 rpm) for 3 days. The culture was ground in a Waring blender and concentrated by centrifugation (20 min, 1,000 × g, 4°C), and 50% of the supernatant was removed. A 3.5-ml aliquot of the concentrated culture was used to inoculate 50 ml of second-stage production medium (modified MYRO) [1 g of (NH₄)₂HPO₄, 3 g of KH₂PO₄, 0.2 g of

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MgSO₄ · 7H₂O, 5 g of NaCl, 40 g of sucrose, and 10 g of glycerol in 1 liter of distilled water] in 250-ml flasks and grown under the same conditions as first-stage medium for 8 days. The mean DON concentration of the cultures was determined by competitive direct enzyme-linked immunosorbent assay (25) to be 165 µg/ml (courtesy of M. Savard, Agriculture & Agri-Food Canada, Ottawa). The culture was harvested and partially dried by filtration through Whatman 1MM filter paper on a Büchner funnel and stored at -70°C until use.

The Fg05 cDNA library was prepared with RNA isolated from mycelium (strain DAOM180378) cultured on cornmeal. Cornmeal powder (25 g) was hydrated with 12 ml of water in a 250-ml flask and inoculated with mycelium washed from two confluent V-8 agar plates. The culture was grown in the dark at 25°C without agitation and harvested after 11 days. The mean DON concentration was determined by competitive direct enzyme-linked immunosorbent assay to be 46 µg/ml.

Total fungal RNA was isolated by using the standard TRIzol method (Invitrogen, Carlsbad, Calif.), and poly(A) RNA was isolated by using the PolyAtract system (Promega, Madison, Wis.). In order to construct a cDNA library with a more complete representation of mRNAs, the Fg03 library was prepared by using a modified subtractive suppression hybridization (SSH) (5) protocol that reduces the bias toward the more abundant mRNA species. This normalization protocol involved allowing the mRNA to hybridize to itself, and as the more abundant mRNA species would hybridize more easily to each other, they would form a dimer that would be eliminated from the PCR amplification step that followed. Consequently, the mRNA population is normalized so that the more abundant mRNA species are decreased to a level similar to that of the less abundant species. The protocol was a modified version of the PCR-Select cDNA subtraction kit (Clontech Laboratories, Inc., Palo Alto, Calif.) whereby cDNA was not digested with RsaI prior to hybridizations and no subtraction with an alternate mRNA population was attempted. The Fg05 cDNA library was not normalized and was constructed by using the GeneRacer kit (Invitrogen). For both Fg03 and Fg05 cDNA libraries, cDNA was cloned undirected into the pGEM-T vector (Promega) and transformed into SURE 2 cells (Stratagene, La Jolla, Calif.).

Sequencing and gene analysis. Single-pass 5'- or 3'-end sequencing of random cDNA clones was conducted with a LI-COR 4200 sequencing system (LI-COR, Lincoln, Neb.) with an average read length of 619 nucleotides. Redundant ESTs were grouped into contigs with the Seqman II assembly program (DNASTAR Inc., Madison, Wis.) with a minimum match of 90% over a length of 30 bp. Bioinformatic analyses were performed with the Lasergene software package (DNASTAR, Inc.). BLAST searches were done with the TBLASTN program (<http://www.ncbi.nlm.nih.gov/BLAST>) by using the default parameters.

Infected wheat heads. Wheat heads (cv. Roblin) at mid-anthesis stage were sprayed with a conidial suspension of *F. graminearum* (10⁵ spores/ml). Following inoculation, plants were kept for 2 days at high humidity in a dew chamber (15°C walls, 20°C water, 30°C air) without light. The infected plants were then kept for 2 days under misting conditions with a 10-s mist every 20 min at 25°C. The resulting fungal mycelial tissue was sampled and stored at -70°C.

Solid rich medium. Mycelia were grown on V-8 agar plates for 7 days at 28°C with 14 h of light to obtain a culture grown on rich medium. Mycelia were harvested from plates with 0.6 M MgSO₄, ground under liquid N₂, and stored at -70°C until further use.

Stripped wheat leaves. Leaves (100 g) from 39-day-old wheat plants were soaked in oxalate buffer at 95°C for 48 h. The leaves were placed on Miracloth (Calbiochem, La Jolla, Calif.), rinsed with water, and ground in liquid N₂. The ground tissue was resuspended twice in methanol, separated through filtration, and suspended in chloroform with stirring overnight at room temperature. The ground leaf tissue was rinsed two times with ethanol and three times with water, and excess water was absorbed by Whatman filter paper. An aliquot of the ground leaf tissue (5 g) was autoclaved and added to 500 ml of 1× synthetic low-nutrient agar (20), with 1 g of sodium acetate per liter added, to form the medium. This medium was inoculated with two agar plugs of *F. graminearum* DAOM180378 and shaken at 200 rpm at 28°C for 6 days. The culture was harvested and partially dried by vacuum filtration through sterile Miracloth (Calbiochem) and stored at -70°C until use.

Northern analyses. Total RNA was isolated by the standard TRIzol method (Invitrogen) from the (trichothecene-producing and cornmeal) cultures described above.

Total RNA (10 µg) was transferred to Magnacharge membrane (Osmonics Inc., Westborough, Mass.) in accordance with the manufacturer's instructions. A 650-bp EcoRI fragment from cDNA clone Fg03_09a11 was random prime labeled with the Prime-a-Gene Labeling System (Promega) and [³²P]dCTP (Amersham Biosciences, Piscataway, N.J.) for use as a hybridization probe. Essentially, hybridizations were done in 50% formamide buffer overnight at 42°C. Final

TABLE 1. DNA primers used in this study

Primer	Sequence 5'→3'	Gene
247	GGTCAACATGATGTCAGG	<i>hyg</i>
719	GACGTTGTAAAACGACGGCCAGTG	Plasmid
1113	ATGTTTCAATATTCCTATGGC	<i>FgTri11</i>
1114	TCATCTTGGGTCAAGGTAC	<i>FgTri11</i>
1199	CGGATCATCTATCCTTGAG	<i>FgTri11</i>
1207	GGAGGGCCTAGTTTAGG	<i>FgTri11</i>
1211	CGTGCCCTGGGGACCAACAC	<i>hyg</i>
1212	CACAGCCGTGGTTGGTGGGG	<i>hyg</i>
1275	CAACCATCAAGATGAGCGC	<i>FgTri3</i>
1276	CCTCGAGAAACCTATGTACC	<i>FgTri3</i>
1277	GTTCTAATAACACAGAGC	<i>FgTri3</i>
1278	GTCCTTGGAGTACCACCG	<i>FgTri3</i>
1328	CACAGGAAACAGCTATGACC	Plasmid
1457	GTCATGGCTCTCATCACCAG	<i>FgLHI</i>
1459	GCCTCCCCTCTGGCCGATG	<i>FgLHI</i>
1462	GTAGCAGTGCCTCTGTGAG	<i>FgLHI</i>
1464	CATGGCGCGCCAGGCGGCGTGTCTC ^a	<i>FgLHI</i>
1466	GTCAACAAGTGCGAGATC	<i>FgLHI</i>

^a The underlined sequence is an *AscI* site.

washes were done with 0.1× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)-0.1% sodium dodecyl sulfate at 65°C. The blot was exposed to Kodak (Rochester, N.Y.) BioMax MS film with a Kodak BioMax TranScreen-HE intensifying screen.

Gene disruption and transformations. To make the disruption vector for *F. graminearum* *LHI*, the gene (GenBank AY339129) was amplified from strain Z-3639 with *Pfu* polymerase (Stratagene) by following the manufacturer's recommendations (95°C for 2 min, 95°C for 30 s, 52°C for 30 s, and 72°C for 2 min for 30 cycles, followed by 72°C for 10 min) with primers 1459 and 1464 (Table 1), the latter containing an *AscI* site (underlined), with a PTC-100 thermocycler (MJ Research, Watertown, Mass.). The resulting 1,281-bp fragment was purified from an agarose gel (UltraClean; MoBio, Solana Beach, Calif.) and cloned into pCR-Blunt II-TOPO (Invitrogen). This plasmid was cut with *AscI*, and an *AscI* fragment containing the promoter 1-*hygB* selectable marker (30) was inserted. This insertion formed a disruption vector (pLHID-1) consisting of a truncated portion of the coding region lacking 268 bp from the 5' end and 202 bp from the 3' end of *LHI*, followed by the hygromycin selectable marker.

The disruption vectors for *FgTri11* and *FgTri3* were made by inserting the promoter1-*hygB* selectable marker into the middle region of each gene, thereby disrupting the formation of an intact, functional protein. *FgTri11* was amplified from strain Z-3639 with *Pfu* polymerase under the conditions described earlier, with primers 1113 and 1114 (Table 1). The 1.73-kb fragment was inserted into pT7-Blue3 with the Perfectly Blunt Cloning Kit (Novagen, EMD Biosciences). The newly created vector was cut with the restriction enzyme EcoRV at a unique site in the *FgTri11* sequence, 724 bp from the start site and 1,014 bp from the stop site; treated with CIAP; and ligated with the *SacI*/*XmnI* fragment containing the promoter1-*hygB* marker from the vector pUCH4 (14). Transformation of fungal protoplasts was performed as previously described (22).

Disruption analysis. Fungal transformants were analyzed with PCR and Southern techniques (24). To confirm the disruption of *LHI*, primers outside of the cloned sequence (1457 and 1466) were paired with internal ones (247, 719, and 1328) in order to detect true insertions versus ectopic insertions (Fig. 1). The same technique was used for confirmation of the disruption of *FgTri11* and *FgTri3* with primers specific to their sequences (Table 1).

In the Southern analysis, genomic DNAs of the *F. graminearum* wild-type and transformants strains were digested with *XbaI* and *HindIII* (New England Biolabs, Beverly, Mass.), blotted to NYtran SuperCharge membrane (Schleicher & Schuell, Keene, N.H.), and hybridized to a ³²P-labeled probe (Prime-A-Gene; Promega) consisting of 443 bp of the coding region (overlapping exons 1 through 4) of *LHI*. Primers 1459 and 1462 (Table 1) were used to amplify the probe from the Z-3639 template. For analysis of *FgTri11* disruptants, the genomic DNA was restricted with *PvuII*/*NcoI*, while *BglII* was used for *FgTri3* disruptants.

Trichothecene toxin assays. Mutant strains were maintained on slants of V-8 juice agar with hygromycin B (300 µg/ml). Liquid cultures were grown on GYEP medium (5% glucose, 0.1% yeast extract, 0.1% peptone; 20 ml in 50-ml Erlenmeyer flasks). Liquid cultures of *F. graminearum* were inoculated with a 20-mm mycelial plug cut from 1-week-old cultures grown on V-8 juice agar. All liquid

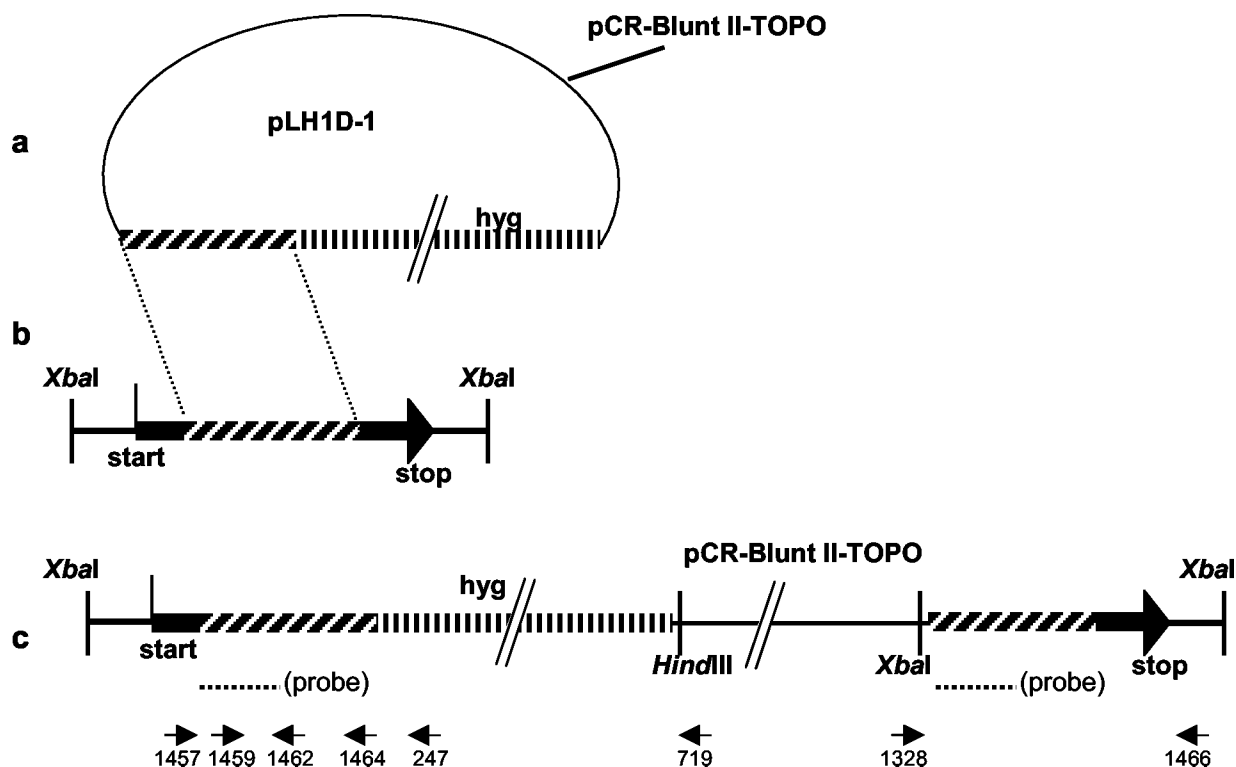


FIG. 1. Disruption of *LHI* in *F. graminearum*. (a) Diagram of disruptant plasmid pLH1D-1. The hatched segment on the left represents a 1,281-bp fragment of *LHI*. The vertically lined segment on the right represents a promoter 1-*hygB* fragment. (b) Diagram of the Z-3639 genomic DNA containing the *LHI* region. "Start" and "stop" are the ends of *LHI*. (c) Diagram of the *LHI* region of a transformant resulting from a single crossover event. The binding position of the probe used for Southern analysis is indicated; arrows indicate the positions of primers. Primer sequences are given in Table 1.

cultures were grown at 28°C in the dark and shaken at 200 rpm. Liquid cultures of *F. graminearum* were harvested after 7 days and extracted with ethyl acetate by vortexing in a conical tube, and the concentrated extract was analyzed by gas chromatography (GC) as described previously (17). To isolate sufficient quantities of the compounds produced by the *F. graminearum* mutant strains for spectral analyses, putative mutant strains were grown on cornmeal cultures. Cornmeal cultures were prepared by inoculating cornmeal (25 g moistened with 11 ml of water) in 300-ml Erlenmeyer flasks with spores washed from V-8 juice agar plates. The inoculated cornmeal was incubated in the dark for 7 days at 28°C. Cultures were extracted overnight with 150 ml of ethyl acetate. Compound identifications were confirmed by GC-mass spectrometry (MS).

Feeding studies. Liquid cultures of transgenic strains LH1D41, LH1D59, GzTri3D-11, and GzTri11D-12 were prepared as described above for Fg03 cDNA library construction. Second-stage production cultures (20 ml in 50-ml Erlenmeyer flasks) were amended with an acetone solution containing one of four 15-ADON precursors—isotrichodermin, 8-hydroxy-3-decalonectrin, 7,8-dihydroxycalonectrin, or calonectrin (250 μM final concentration). Isotrichodermin was isolated and purified from FsTri11D cultures (16). 7,8-Dihydroxycalonectrin and calonectrin were isolated and purified from GzTri8D mutant (14) strains, and 8-hydroxy-3-decalonectrin was isolated and purified from FsTri13D mutant strains (2). Aliquots (5 ml) were removed from amended cultures after 2 to 6 days, extracted with ethyl acetate (2 ml), and analyzed by GC-MS for conversion to 15-ADON.

Chemical analyses. GC measurements were made by flame ionization detection with a Hewlett-Packard 5890 gas chromatograph fitted with a 30-m fused silica capillary column (DB1; 0.25 μm; J&W Scientific Co., Palo Alto, Calif.). For routine screening of the trichothecene toxin phenotype, the column was held at 120°C at injection, heated to 210°C at 15°C/min and held for 1 min, and then heated to 260°C at 5°C/min and held for 8 min. Low-resolution mass spectra were obtained by GC-MS with the same temperature program and a Hewlett-Packard 5891 mass-selective detector fitted with a DB-5-MS column (15 m by 0.25 mm [film thickness]).

Nucleotide sequence accession numbers. The seven EST sequences representing *LHI* have been deposited in the National Center for Biotechnology Information GenBank database under the following accession numbers: Fg03_05c04, CF075160; Fg03_05d01, CF075163; Fg03_08d01, CF075159; Fg03_09a03, CF075158; Fg03_09a11, CF075161; Fg03_09g12, CF075162; Fg05_04m07, CF120339.

RESULTS

We constructed an *F. graminearum* EST database derived from two cDNA libraries, Fg03 and Fg05, prepared from RNA produced by trichothecene-producing liquid cultures and solid substrate cultures, respectively. The analysis revealed numerous genes that were only found in the Fg03 and Fg05 libraries and not represented by previously released *F. graminearum* ESTs (28). Two sequence contigs were identified whose best BLAST match was a P450 monooxygenase from *F. sporotrichioides* (TRI1 protein, AAK77933). These two contigs contained six ESTs from the Fg03 library and one EST from the Fg05 library. Further sequencing of the seven clones representing the above ESTs confirmed that they were overlapping and were derived from a single gene, tentatively named *LHI*. A cDNA consensus sequence of these seven ESTs could potentially code for a 512-residue protein exhibiting 59% amino acid identity with FsTRI1.

Expression analysis of *LHI*. A 650-bp EcoRI fragment from the Fg03_09a11 EST clone was used as a probe to investigate

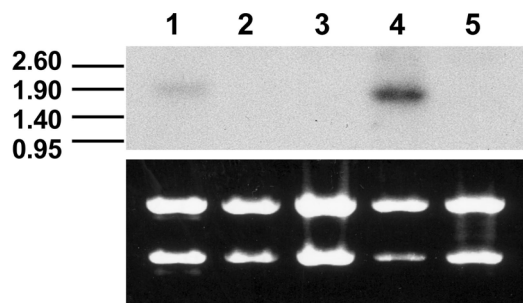


FIG. 2. Northern blot analysis of *LHI* with the ^{32}P -labeled gene-specific Fg03_09a11 probe hybridized with total RNA isolated from *F. graminearum* grown under the following conditions: lane 1, infected wheat heads; lane 2, stripped wheat leaves in liquid culture; lane 3, solid rich medium; lane 4, trichothecene-producing liquid culture; lane 5, solid cornmeal substrate. The blot was exposed to film for 5 h at -70°C . The corresponding ethidium bromide-stained gel (showing strong 25S and 18S rRNA bands) prior to blotting is shown under the hybridization panel. Sizes of markers are indicated in kilobases on the left.

the expression of *LHI* under various growth conditions. Expression of a single transcript of approximately 1.9 kb was detected in the trichothecene-producing liquid culture and, at a lower level, in *Fusarium*-infected wheat heads (Fig. 2).

Disruption of *LHI* in *F. graminearum*. Seven of 72 hygromycin-resistant colonies resulting from transformation of Z-3639 with pLH1D-1 had a trichothecene phenotype that differed from that of Z-3639 and accumulated two compounds in larger relative amounts than did wild-type strain Z-3639: calonectrin and 3-decalonectrin. (Fig. 3). In addition, these seven transformant strains produced no detectable 15-ADON or DON. The remaining 65 hygromycin-resistant strains produced 15-ADON and a small amount of 3-decalonectrin and were indistinguishable from wild-type Z-3639 (Fig. 3).

The seven transformants with the altered trichothecene phenotype were confirmed to be *LHI* disruptants by PCR analysis (Fig. 4a). Primers 1457 and 1464 (Fig. 1) made a band of approximately 1.5 kb in both wild-type strain Z-3639 and transformant LH1D-59 (Fig. 4a, lanes 2). When primer 1457 (located outside of the region cloned into the disruption vector) was paired with primer 247 (located in the *hyg* sequence), no band was seen in Z-3639 while a band just slightly larger than 1.5 kb was seen in LD1-59 (Fig. 4a, lanes 3). When primer 1466, located downstream of the 3' end of the gene (Fig. 1), was paired with primer 1328 (located in the vector sequence), no band was seen in Z-3639 while a band of approximately 1.7 kb was seen in the transformant (Fig. 4a, lanes 4). A control reaction for the presence of the *hygB* gene (primers 1211 and 1212; Fig. 4a, lanes 5) showed a band in LH1D-59 but not in Z-3639. The sizes of the PCR products are consistent with disruption of *LHI* via a single homologous recombinational event between the plasmid and chromosomal *LHI* sequences. Both hygromycin and plasmid sequences were detected within the coding region of *LHI*, showing that the gene had been disrupted.

The PCR results were confirmed by Southern analysis (Fig. 4b). The wild-type *F. graminearum* strain gave a band of about 3 kb (lane 1). Transformant FgLH1D-59 (lane 2) gave bands of about 4.4 and 2.3 kb. As shown in Fig. 1c, there is a HindIII site

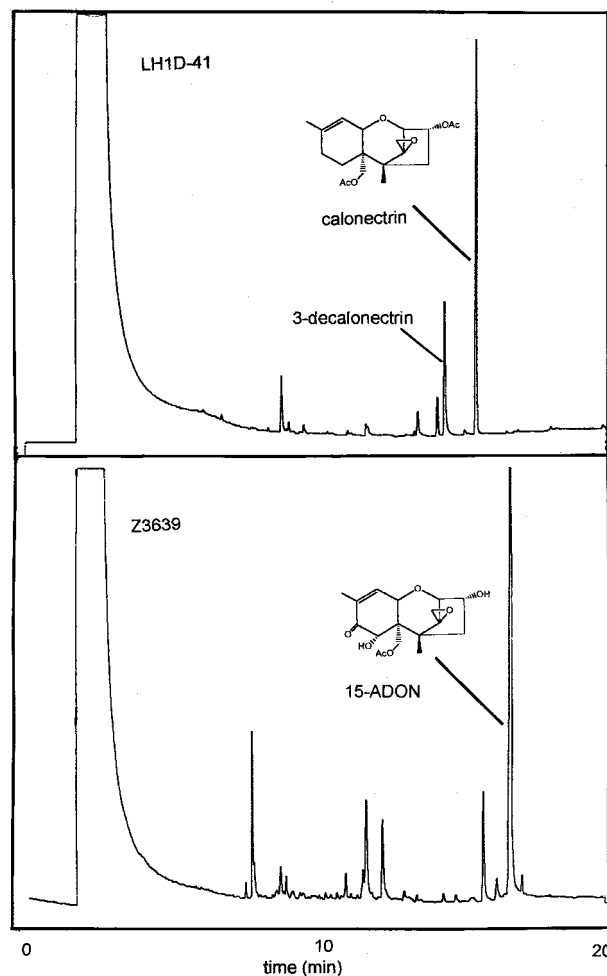


FIG. 3. GC chromatograms of extracts from 7-day-old liquid cultures of *F. graminearum* mutant strain LH1D-41 and wild-type strain Z-3639. OAc or AcO, acetate.

located in the plasmid just after the 3' end of the *hygB* gene so that an XbaI/HindIII fragment identified by the probe would be a total of 2.5 kb (*hygB*) plus 1.9 kb (*LHI*). The smaller 2.3-kb band seen is the 5' end of the insertion or gene (Fig. 1c). The 3-kb band seen in the wild type is not present in the transformant. Ectopic insertions of the plasmid did occur in some transformants, as can be seen in Fig. 4b (lane 3). In this transformant, there are the expected 4.4- and 2.3-kb bands, as well as a third band of 3.8 kb. This extra band results from an ectopic insertion of the plasmid as digestion of the plasmid alone gives a 3.8-kb band (Fig. 4b, lane 4). Transformants selected for further study carried the single insertion that caused disruption of *LHI*.

LH1D-41 and LH1D-59 were selected for feeding studies (Table 2). These cultures accumulate calonectrin and could convert 7,8-dihydroxycalonectrin to 15-ADON but could not convert 8-hydroxy-3-decalonectrin to 15-ADON (Table 2). The GzTri3D and GzTri11D mutant strains, which accumulate 15-decalonectrin and isotrichodermin, respectively (see Fig. 6), could convert calonectrin, 8-hydroxy-3-decalonectrin, and 7,8-dihydroxycalonectrin to 15-ADON (Table 2).

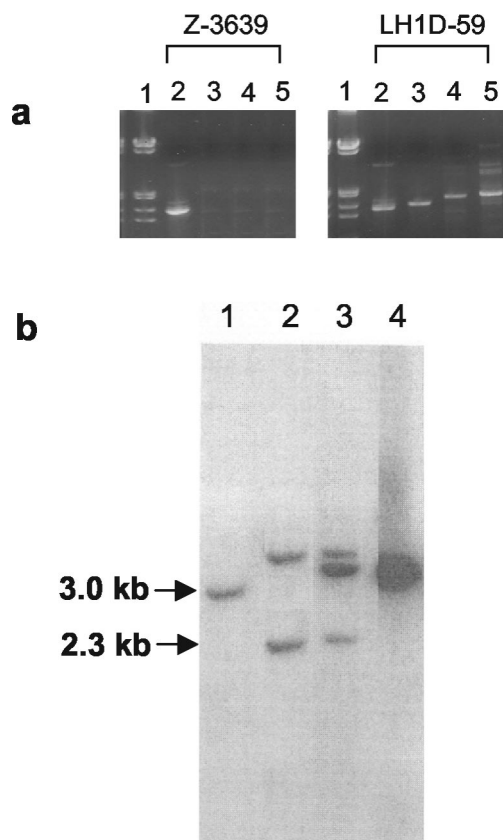


FIG. 4. Analysis of Z-3639 and LH1D transformants. (a) Ethidium bromide-stained gel of PCR products. Lanes: 1, molecular weight standards of lambda DNA cut with EcoRI and HindIII; 2, primers 1457 and 1464; 3, primers 1457 and 247; 4, primers 1466 and 1328; 5, primers 1211 and 1212. Locations of primers are indicated in Fig. 1. (b) Southern blot analysis of Z-3639, transformants LH1D-59 and LH1D-3, and vector pLH1D-1. Genomic and plasmid DNAs were digested with XbaI/HindIII and probed with an *LHI* fragment prepared by using primers 1459 and 1462. Lanes: 1, Z-3639; 2, LH1D-59; 3, LH1D-3; 4, pLH1D-1.

Sequence characteristics of *LHI*. The sequence including and surrounding *LHI* was examined. *LHI* is found on 79.5-kb contig 1.4 (scaffold 1) of the *F. graminearum* genome sequence (*Fusarium graminearum* Sequencing Project, Whitehead Institute/MIT Center for Genome Research [http://www.genome.wi.mit.edu]). *LHI* contains four introns located at bp 340 (54 bp long), bp 500 (62 bp long), bp 630 (48 bp long), and bp 1254 (52 bp long). One of the *LHI* clones, Fg05_04m07, was generated by the cDNA library construction method that selects for the isolation of capped messages. If the 5' end of clone

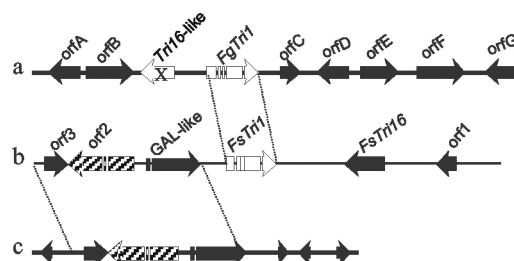


FIG. 5. Genomic maps of the *FsTri1-Tri16* gene cluster and homologous regions in *F. graminearum*. Open reading frames and direction of transcription are indicated by arrows. Dotted lines indicate the sequence conserved between *F. sporotrichioides* and *F. graminearum*, although the sequences conserved between *FsTri16* and the *FgTri16*-like region are not indicated to simplify the figure. (a) *F. graminearum* genomic contig 1.4 sequence, nucleotides 26000 to 44000. Translated and predicted proteins: orfA, GAL4-like transcription factor; orfB, sugar transporter; orfC, unknown; orfD, WW domain-containing oxidoreductase; orfE, flavodoxin reductase; orfF, amino acid transporter. (b) *F. sporotrichioides* *Tri1-Tri16* gene cluster (17,529 bp; GenBank accession no. AY217783). (c) *F. graminearum* genomic contig 1.196, nucleotides 273000 to 284000.

Fg05_04m07 represents the transcription start site, there is a 48-bp 5' leader sequence in front of the predicted translation start site and a TATA box sequence (general eukaryotic transcription binding site) at position -41. There are three TRI6 binding sites (5'YNAGGCC at -157, -213, and -307) (8), all in the inverse orientation, and one PacC binding site (5'GC-CARG at -439) within 500 bp of the transcription start site of *LHI*. TRI6 is a zinc finger transcriptional activator of the trichothecene biosynthetic pathway (8), and PacC is a general fungal zinc finger transcription factor that regulates gene expression by ambient pH (27).

The flanking sequences contain numerous open reading frames (Fig. 5). However, there are no public EST sequences representing any of the predicted genes within 20 kb of *LHI*. Open reading frames immediately upstream of the region illustrated in Fig. 5 exhibit similarity to sugar utilization genes. An examination of the published *F. graminearum* genome sequence suggests that a *TRI16*-like sequence is present next to *LHI*, but as a pseudogene (Fig. 5).

DISCUSSION

Fusarium sporotrichioides *Tri1* (*FsTri1*) encodes a P450 oxygenase that converts triacetoxyscirpenol to 3-acetylneosolaniol in T-2 toxin biosynthesis (Fig. 6). The isolation of other 8-hydroxytrichothecenes (e.g., 8-hydroxyisotrichodermin, 8-hy-

TABLE 2. Summary of feeding experiments with mutant strains blocked in trichothecene biosynthesis

Strain	Gene blocked	Acetone	Isotrichodermin	Calonectrin	8-Hydroxy-3-decalonectrin	7,8-Dihydroxycalonectrin
GzTri3D-11	<i>Tri3</i>	15-Decal	15-Decal	15-ADON	15-ADON	15-ADON
GzTri11D-12	<i>Tri11</i>	3-OAc ^a	3-OAc	15-ADON	15-ADON	15-ADON
LH1D-41	<i>LHI</i>	Calonectrin	Calonectrin	Calonectrin	Calonectrin	15-ADON
LH1D-59	<i>LHI</i>	Calonectrin	Calonectrin	Calonectrin	Calonectrin	15-ADON

^a OAc, acetate.

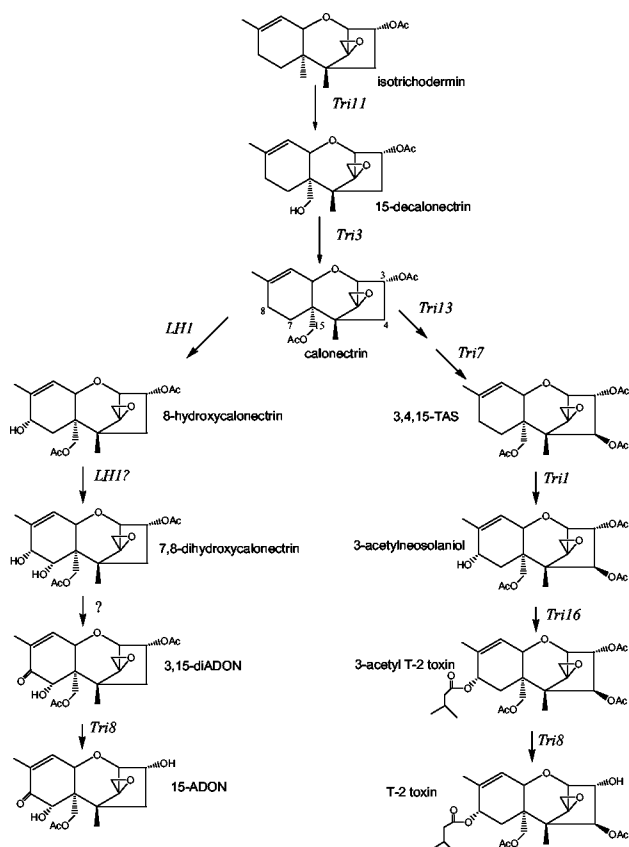


FIG. 6. Proposed pathway from calonectrin to 15-ADON used by *F. graminearum* (left side) or to T-2 toxin used by *F. sporotrichioides* (right side). OAc or AcO, acetate. 3,15-diADON, 3,15-diacetyl-DON; 3,4,15-TAS, 3,4,15-diacetoxyscirpenol.

droxydecalonectrin, 2,3,8,11-isotrichotetraol) from *Fusarium* suggested that TRI1 may have a wide substrate specificity (18). *FsTri1* is located in a small cluster adjacent to an acyltransferase gene responsible for adding the isovalerate group to the C-8 position. Although there is great similarity between the major trichothecene gene clusters of *F. sporotrichioides* and *F. graminearum*, DON-producing strains of *F. graminearum* lack functional *Tri7* and *Tri13* genes that are involved in hydroxylation and esterification at C-4 (2, 3, 12). The structures of T-2 toxin and 15-acetyldeoxynivalneol (Fig. 6) also significantly differ at the C-7 and C-8 positions. 15-ADON and DON have a keto group at C-8 and a hydroxyl at C-7, while T-2 toxin has no substituent at C-7 and an isovaleryl ester at C-8. The location of the genes involved with the C-8 position in *F. sporotrichioides* in a small, unique cluster suggests that other *Fusarium* species may have significant differences or changes in these enzymatic capabilities.

LHI was represented by six cDNA clones derived from a DON-producing *F. graminearum* liquid culture and one clone isolated from *F. graminearum* grown on cornmeal. Northern analysis revealed detectable expression in trichothecene-producing liquid culture and infected wheat heads but not in cultures grown on rich medium. Expression of *LHI* was not detected by Northern analysis in mycelia grown on cornmeal

even though the specific culture yielding this RNA contained 210 μg of DON per ml (M. Savard, personal communication). However, these Northern results mirror the relative abundance of *Tri* genes in the Fg03 and Fg05 cDNA libraries. Other trichothecene biosynthetic genes are highly represented in the trichothecene-producing Fg03 library (e.g., there are 13 FgEST clones representing the *Tri4* gene), but the Fg05_04m07 clone is the only putative trichothecene biosynthetic gene observed in the sequenced clones of the Fg05 library. It is possible that the fungal culture grown on cornmeal has progressed past the stage of *LHI* gene expression.

The 17.5-kb genomic sequence containing the *Tri1* and *Tri16* gene cluster from *F. sporotrichioides* (AY217783) also contains three other open reading frames, as well as a potential coding region for a GAL4-like protein, in the following order: orf3, orf2, GAL4-like, *Tri1*, *Tri16*, orf1. Compared to *F. graminearum*, there is only the *Tri16*-like sequence near *LHI*. The orf3, orf2 (accession no. AA064249), and GAL4-like sequences in *F. graminearum* are conserved in the same order as in *F. sporotrichioides*; however, this group is located on contig 1.196 (scaffold 3) and is therefore separated from *LHI* and the *Tri16*-like sequence. The predicted orf1 sequence from *F. sporotrichioides* has not been conserved in *F. graminearum*.

When aligned with *FsTRI16*, the *F. graminearum* *Tri16*-like genomic sequence exhibits greater than 20 insertions and deletions ranging from 1 to more than 400 bp. As a result, the *F. graminearum* *Tri16*-like sequence could not code for a gene product with any significant homology with *FsTRI16* or other acetyltransferases. When the *LHI* protein sequence is aligned with the *FsTRI1* protein sequence, the predicted first residue of the *LHI* protein aligns with residue 23 of the *FsTRI1* protein and there is a single three-amino-acid gap in the *LHI* protein after residue 266. Although the sequence identity between *LHI* and *FsTri1* is only 59%, the fact that all four introns line up exactly suggests that the two sequences do have homology.

A comparison of the heme-binding motifs of several different P450s involved in the trichothecene biosynthetic pathway from *F. sporotrichioides* and *F. graminearum* shows the conservation of residues in this 20-amino-acid region. TRI4, a P450 that oxygenates C-2 early in the pathway, is the same in both species, while TRI11, which oxygenates C-15, differs at three amino acids and *FsTRI1* and *LHI* differ at five. Although it is not known how many amino acid changes it is possible to make within this 20-amino-acid region and still have a functional protein, a comparison of the sequences of *F. sporotrichioides* and *F. graminearum* TRI4, TRI11, and TRI1 indicates that 15 of the 20 amino acids may be changed and heme binding will still occur. The consensus sequence among the six proteins is FxxGxxxCxGxxxAxxxxxx. However, there are more significant differences when the total protein or nucleotide sequences are compared with those of the other P450s identified in trichothecene biosynthesis, as sequence conservation between the two species is usually greater than 75% (2). *FsTRI11* (492 amino acids) and FgTRI11 (488 amino acids) exhibit a predicted protein sequence identity of 90%. Similarly, TRI4 proteins from both species have a protein identity of 87%. In contrast, the protein identity of *FsTRI1* and *LHI*, at 59%, is much lower than expected, but this may explain why other researchers (18) were unable to detect a *Tri1* homologue in *F. graminearum*.

F. graminearum Z-3639 normally produces 15-ADON (Fig. 3) in liquid culture, but disruption of *LH1* results in the accumulation of calonectrin (Fig. 3) and 3-decalonectrin. 3-Decal onectrin is the predicted product of the *Tri8* esterase, which removes the C-3 acetyl group from a variety of trichothecenes (14), but also may result from acid hydrolysis of the C-3 acetyl group in the medium. The final sequence of the oxygenation and oxidation steps in 15-ADON-producing strains of *F. graminearum* is not known. Both 7,8-dihydroxycalonectrin (Fig. 6) and 15-deacetyl-7,8-dihydroxycalonectrin were precursors of 3-ADON in *F. culmorum* (7) cultures that also accumulated calonectrin and 7-hydroxycalonectrin. On the basis of these data, the authors proposed the following pathway: calonectrin → 7-hydroxycalonectrin → 7,8-dihydroxycalonectrin → 3,15-diacetyl-DON → 3-ADON. However, we have not found 7-hydroxycalonectrin in our mutant cultures derived from Z-3639. *F. graminearum* *Tri8* mutants accumulate several 3-acetylated trichothecenes: 3,15-diacetyl-DON, calonectrin, 8-hydroxycalonectrin, and 7,8-dihydroxycalonectrin (14). This pattern suggests that in *F. graminearum* Z-3639, oxygenation of calonectrin occurs first at C-8 and is followed by C-7 hydroxylation and oxidation of the C-8 hydroxyl group (Fig. 6).

The sequence differences between *FsTRI1* and *LH1* could result in *LH1* having functions in addition to C-8 hydroxylation. The feeding experiments with 8-hydroxy-3-decalonectrin were consistent with this hypothesis since mutants blocked at *Tri3* and *Tri11* could convert 8-hydroxy-3-decalonectrin and 7,8-dihydroxycalonectrin to 15-ADON, but *LH1* mutants could convert only 7,8-dihydroxycalonectrin (Fig. 6; Table 2). These results indicate that conversion to the carbonyl at C-8 is not blocked in the *LH1* mutant strains and that an additional P450 not encoded by *LH1* catalyzes this final oxidation step. If *LH1* were limited to C-8 hydroxylation, the *LH1* mutants would be expected to convert 8-hydroxy-3-decalonectrin to 15-ADON. For example, multifunctional oxygenases are used in successive oxygenations at the same carbon, as well as at different carbons, in *G. fujikuroi* gibberellin biosynthesis (6, 23, 29). Heterologous expression of *LH1* in *Fusarium verticillioides* or yeast and feeding experiments with additional substrates will allow us to definitively determine if *LH1* encodes a multifunctional oxygenase for both C-8 and C-7 hydroxylations or if an additional P450 gene is required for the second hydroxylation in 15-ADON biosynthesis. However, on the basis of the sequence similarity between *LH1* and *FsTri1* and the similarity in function of C-8 hydroxylation, we propose that *LH1* is the homologue of *FsTri1*.

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Names are necessary to report factually on available data; however, the U.S. Department of Agriculture neither guarantees nor warrants the standard of the products, and the use of the name by the U.S. Department of Agriculture implies no approval of the product to the exclusion of others that may also be suitable.

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