APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Mar. 2002, p. 1305–1311 Vol. 68, No. 3
0099-2240/02/$04.00+0 DOI: 10.1128/AEM.68.3.1305–1311.2002

Cloning, Characterization, and Transcription of Three Laccase Genes from *Gaeumannomyces graminis* var. *tritici*, the Take-All Fungus

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Received 1 May 2001/Accepted 30 November 2001

***Gaeumannomyces graminis* var. *tritici*, a filamentous ascomycete, is an important root pathogen of cereals that causes take-all disease and results in severe crop losses worldwide. Previously we identified a polyphenol oxidase (laccase) secreted by the fungus when induced with copper. Here we report cloning and partial characterization of three laccase genes (*LAC1*, *LAC2*, and *LAC3*) from *G. graminis* var. *tritici*. Predicted polypeptides encoded by these genes had 38 to 42% amino acid sequence identity and had conserved copper-binding sites characteristic of laccases. The sequence of the *LAC2* predicted polypeptide matched the N-terminal sequence of the secreted laccase that we purified in earlier studies. We also characterized expression patterns of these genes by reverse transcription-PCR. *LAC1* was transcribed constitutively, and transcription of *LAC2* was Cu inducible. All three genes were transcribed in planta; however, transcription of *LAC3* was observed only in planta or in the presence of host (wheat) plant homogenate.**

Laccases (EC 1.10.3.2) are widely distributed oxidoreductases that catalyze the biological oxidation-reduction of polyphenols with a concomitant reduction of molecular oxygen to water. They are blue copper oxidases, which are characterized by having four bound copper atoms, of which at least one is contained in a type 1 site or "blue" copper site (29). Cu-binding domains are highly conserved among laccases. Recently, numerous genes coding for laccase-like proteins have been cloned and characterized (6, 7, 11, 18–20, 25–27, 32, 57).

In higher plants, laccases are involved in lignification of xylem tissues (10, 26); however, various functions have been reported for fungal laccases. Laccase of the animal pathogen *Cryptococcus neoformans* oxidizes dihydroxyphenylalanine into a melanin-like pigment (37, 51). *Aspergillus nidulans* laccase is required for pigment biosynthesis during conidial development and maturation (48). In the white-rot basidiomycetes *Trametes versicolor*, *Lentinus edodes*, and *Pleurotus ostreatus*, laccase is involved in lignin degradation (2, 9, 21). Laccase from *Pycnoporus cinnabarinus* functions in both lignin degradation and the biosynthesis of cinnabarinic acid, an antimicrobic with activity against a variety of bacterial species (14, 16, 44). Other functions, such as oxidation of humic acids (7, 40) and oxidation of Mn^{2+} to Mn^{3+} (5, 24) have also been proposed for fungal laccases. Nevertheless, the biological role of the majority of fungal laccases remains uncertain.

Many filamentous fungi produce several laccase isozymes encoded by multiple genes. For example, *Rhizoctonia solani* has four laccase genes (50), *T. versicolor* has five (53), the basidiomycete I-62 has three (27), *Agaricus bisporus* has two (42), *P. ostreatus* has at least three (20), and *Podospora anserina* has at least four (18). Quite often these genes are expressed under different environmental conditions (18, 28, 40, 42, 57) or during different stages of the fungal life cycle (18, 28, 48). Differential expression of laccase genes implies that laccases have alternative functions, but to our knowledge this has not been shown.

Many *G. graminis* var. *tritici* isolates secrete at least one laccase (13, 47). We recently purified and partially characterized a secreted laccase from *G. graminis* var. *tritici* (13). The active protein has an apparent molecular mass of 190 kDa, consists of two heavily glycosylated 60-kDa subunits, and is inducible by copper and xylidine. The purified protein catalyzed the polymerization of 1,8-dihydroxynaphthalene, a natural fungal melanin precursor, into high-molecular-weight melanin and catalyzed the oxidation of the dye poly B-411, a lignin-like polymer. We report here cloning and partial characterization of three laccase genes from *G. graminis* var. *tritici*.

MATERIALS AND METHODS

Strains, media, and culture conditions. *G. gramnis* var. *tritici* strain DM528 was isolated by D. Mathre from Montana wheat (*Triticum aestivum*) and purified by single-spore isolation. Cultures are available from the U.S. Department of Agriculture (Peoria, Ill.) agricultural fungus collection. The fungal strain was maintained on Luria-Bertani (LB) medium (38) plus 1.2% (wt/vol) agar at 24 to 26°C. Long-term stocks were maintained on potato dextrose broth (PDB; Difco, Detroit, Mich.) slants with 1.2% (wt/vol) agar stored at 4°C.

For protein purification, fungal cultures were grown in LB liquid medium supplemented with 400 μM $CuSO_4$ with constant agitation (200 rpm) at 23 to 25°C. To determine the effects of different growth media on gene expression, the fungus was grown in LB broth with and without 400 μM $CuSO_4$ for 4 to 5 days, in PDB for 4 to 5 days, and in starvation basal medium for 10 days (43). For Cu induction experiments, the fungus was grown in Fahraeus minimal liquid medium (17) with sucrose substituted for glucose; $CuSO_4$ was added to a final concentration of 400 μM on the second day of incubation.

Wheat plants were infected with *G. graminis* var. *tritici* as described earlier with a few modifications (23). Briefly, wheat seeds were surface sterilized with 1% $AgNO_3$ for 10 min, washed 10 times with sterile water, and planted in sterile vermiculite with fungal inoculum in magenta boxes (Sigma-Aldrich, St. Louis, Mo.). The inoculum consisted of approximately one agar plug (1-cm diameter) per seed. Agar plugs were cut from young mycelia grown on solid LB medium and spread near seeds approximately 2 cm below the surface. Control (uninfected) wheat plants were treated with sterile LB agar plugs. All plants were grown with a 12-h photoperiod with 3,000 lx.

For plant homogenate induction experiments, wheat plants were grown aseptically in magenta boxes on sterile vermiculite for 7 to 10 days. From 10 to 20

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plants were harvested, quick-frozen and ground in liquid N_2, and resuspended in 20 ml of sterile water. The plant suspension was filter sterilized and added to fungal cultures to a final concentration of 30% (vol/vol).

Laccase purification and sequencing. The enzyme was purified from 5-day-old copper-induced LB cultures with a Bio-Rad (Hercules, Calif.) Prep Cell apparatus and eluted after 6 to 7 h as determined by 2,6-dimethoxyphenol assays, as described previously (13). Samples of laccase (30 μl) in 2× loading buffer (13) were heated at 95°C and electrophoresed on a sodium dodecyl sulfate (SDS)–7.5% (wt/vol) polyacrylamide gel electrophoresis (PAGE) gel using the Tris-tricine discontinuous gel system (39). The gel was electroblotted onto a polyvinylidene difluoride membrane (Millipore, Bedford, Mass.) using the Bio-Rad semidry electroblotter according to the manufacturer's instructions. The membrane was stained in 0.15% (wt/vol) Coomassie blue R250 in 50% (vol/vol) methanol for 5 min, destained in 50% methanol, washed in water, and air dried, and the protein was subjected to Edman degradation. Protein sequencing was performed on an Applied Biosystems 475 protein sequencer (Applied Biosystems, Foster City, Calif.) by the Laboratory for Bioanalysis and Biotechnology at Washington State University (Pullman, Wash.).

DNA and RNA purification. Fungal genomic DNA was prepared as previously described (30). RNA was purified from fungal mycelia grown in different culture media, from 10-day-old infected wheat plants, and from 10-day-old uninfected control plants. Infected and uninfected wheat plants and filtered mycelia were quick-frozen and ground in liquid N_2. Total RNA was purified with a Qiagen (Chatsworth, Calif.) RNeasy plant minikit according to the manufacturer's instructions. All RNA preparations were treated with RQ1 DNase (Promega, Madison, Wis.) for 2 h at 37°C, according to the manufacturer's instructions, to remove any contaminating DNA, and repurified with the same kit.

Genomic library construction and screening. A custom genomic library of *G. graminis* var. *tritici* was obtained from Stratagene (La Jolla, Calif.). The library was constructed in vector λ DASH II and propagated in *Escherichia coli* XL1-Blue MRA(P2). A subcloned laccase gene from *Neurospora crassa* (kindly provided by H. Inoue) was used as a probe for genomic library screening. Briefly, a 4.5-kb *Bgl*II DNA fragment was excised from the pBL1 plasmid (56), gel purified using the QIAquick gel extraction kit (Qiagen), and labeled with digoxigenin-11-dUTP (Boehringer Mannheim, Indianapolis, Ind.) by the random-primed method according to the manufacturer's instructions. Approximately 20,000 plaques were screened by plaque hybridization (38) with the probe at a concentration of 25 ng/ml under high-stringency conditions: 5× SSC (1× SSC is 150 mM NaCl, 15 mM sodium citrate, pH 7)–1% blocking reagent (Boehringer Mannheim)–0.1% *N*-lauroylsarcosine–0.2% SDS at 65°C overnight. Filters were washed twice in 2× SSC–0.1% SDS at room temperature and twice in 0.5× SSC–0.1% SDS at 65°C.

Plaques that hybridized with the *N. crassa* laccase probe were selected and purified, and fungal DNA fragments were subcloned into the *Not*I site of plasmid pGEM-5Zf(+) (Promega) according to standard protocols (38). Both strands of putative laccase genes and their 5′ and 3′ untranslated regions were sequenced by the primer-walking approach (38).

RT-PCR. Single-tube Access reverse transcription (RT)-PCR (Promega) was used to monitor the differential transcription of laccase genes. Two micrograms of total RNA (determined spectrophotometrically at A_{260}) was used for each RT-PCR. Reactions were carried out in 25-μl volumes with 1× AMV/*Tfl* reaction buffer (0.2 mM deoxynucleoside triphosphates, 2.5 mM each primer, 1 mM $MgSO_4$, 2.5 U of avian myeloblastosis virus [AMV] reverse transcriptase, and 2.5 U of *Tfl* DNA polymerase). RT-PCR parameters were 45 min at 48°C and 3 min at 94°C, followed by 40 cycles of 1 min at 94°C, 1 min at 50 to 60°C (depending on the specific primer pair), and 1 min at 72°C, followed by 1 cycle of 7 min at 72°C.

Primers specific for each laccase gene were designed based on the sequences reported in this article. *LAC1*-specific primers were 5′-GAGCTGGAACGCGA TGGCTA-3′ and 5′-GCATCATCCGCTACGACAAG-3′, with an annealing temperature of 55°C. *LAC2*-specific primers were 5′-CGCATCATCTTTTGTG CTCC-3′ and 5′-AGCGCCAACTACGACGAGGA-3′, with an annealing temperature of 52°C. *LAC3*-specific primers were 5′-GCTATGCGGCACCAGCC TTA-3′ and 5′-GTGGCCGTGCAGGTGGAT-3′, with an annealing temperature of 50°C.

To verify that the same amounts of total mRNA were used for each RT-PCR, transcription of the housekeeping DNA polymerase γ (*POLG*) gene from *G. graminis* var. *tritici* was monitored with primers 5′-ATGAGCAGGTGGAGGT AGTCA-3′ and 5′-CGCAGCTGCTGAGACAGTTCAA-3′, which were designed after we sequenced a *POLG* gene fragment of *G. graminis* var. *graminis*. Sequences of the primers used for determining intron positions and for sequencing are available upon request. Amplification products were separated on 2% agarose gels in TBE (90 mM Tris, 90 mM boric acid, 2 mM EDTA), stained with ethidium bromide, and visualized under UV light. For each RT-PCR, a negative control PCR without AMV reverse transcriptase was performed to rule out DNA contamination. PCR products were subcloned into the pCR4-TOPO vector (Invitrogen, Carlsbad, Calif.) and sequenced using M13 forward and reverse primers to verify their identity.

Competitive PCR. Transcript levels of *G. graminis* laccase genes were determined by competitive RT-PCR (4, 42, 57). A plasmid containing subcloned *LAC1* from *G. graminis* var. *tritici*, a plasmid containing a subcloned *G. graminis* var. *graminis LAC2* gene that contains one intron not present in *G. graminis* var. *tritici*, and a plasmid containing a subcloned portion of the *G. graminis* var. *graminis POLG* gene with a 100-bp *Kpn*I-*Xho*I insertion were used as competitor templates. Competitor templates with introns or an insertion were larger than cDNA templates and separated from cDNA PCR products on agarose gels. Serial 10-fold dilutions of competitor plasmids were made and added to RT-PCRs. Two micrograms of total RNA purified from fungal cultures growing in the presence or absence of Cu was used for each RT-PCR. Three independent spectrophotometric measurements were performed to determine the concentration for each RNA preparation. *LAC2*-specific primers were 5′-TGGCAGTGC ATGATCCAG-3′ and 5′-ATCGGC/GCAGCGCTACGACGT-3′, with an annealing temperature of 50°C. RT-PCR conditions, cycling parameters, and *LAC1*- and *POLG*-specific primers were the same as for other RT-PCR experiments.

DNA sequencing and analysis. Sequencing reactions were performed using the ABI PRISM Big Dye terminator cycle sequencing ready reaction kit (Applied Biosystems) according to the manufacturer's instructions. Sequences were analyzed on an Applied Biosystems 310 genetic analyzer using a 47-mm capillary column. Sequences were determined in both directions, edited, and assembled using the Sequencher 3.1.1 program (Gene Codes Corporation, Ann Arbor, Mich.). Sequence homology searches were performed using the BlastX search engine (1).

Nucleotide sequence accession numbers. Nucleotide sequences of the *G. graminis* var. *tritici LAC1*, *LAC2*, and *LAC3* genes were deposited in the EMBL nucleotide sequence database under accession numbers AJ417685, AJ417686, and AJ417687, respectively.

RESULTS

Cloning and analysis of laccase genes. We identified and subcloned three putative laccase genes that were designated *LAC1*, *LAC2*, and *LAC3* (55). The overall exon-intron structures of all three laccase genes in *G. graminis* var. *tritici* were distinct. *LAC1* contained a 1,977-bp open reading frame (ORF) that was interrupted by two introns of 87 and 80 bp. *LAC2* contained a 1,731-bp ORF with no introns, and *LAC3* contained a 2,212-bp ORF that was interrupted by six introns ranging from 63 to 87 bp. Positions of putative introns were determined by analysis of nucleotide sequence and later verified by partial sequencing of corresponding cDNAs.

Characterization of deduced laccases. We predicted that *LAC1*, *LAC2*, and *LAC3* encoded polypeptides of 609, 577, and 581 amino acids, respectively. The deduced amino acid sequences showed 35.8, 35.4, and 48.5% identity with *N. crassa* laccase (EMBL P06811), respectively, and 42.6% (Lac1-Lac2), 38.1% (Lac1-Lac3), and 39.9% (Lac2-Lac3) identity with each other. All three predicted gene products contained four conserved putative Cu-binding sites that are characteristic of laccases (Fig. 1). The deduced primary polypeptides also began with 18- to 20-amino-acid signal sequences typical of extracellular enzymes as predicted by the Neilsen et al. algorithm (31), and they contained six to eight potential glycosylation sites as predicted by the Hansen et al. algorithm (22) (Fig. 1).

N-terminal peptide sequence analysis. In liquid LB culture, when induced with Cu, *G. graminis* var. *tritici* produced only

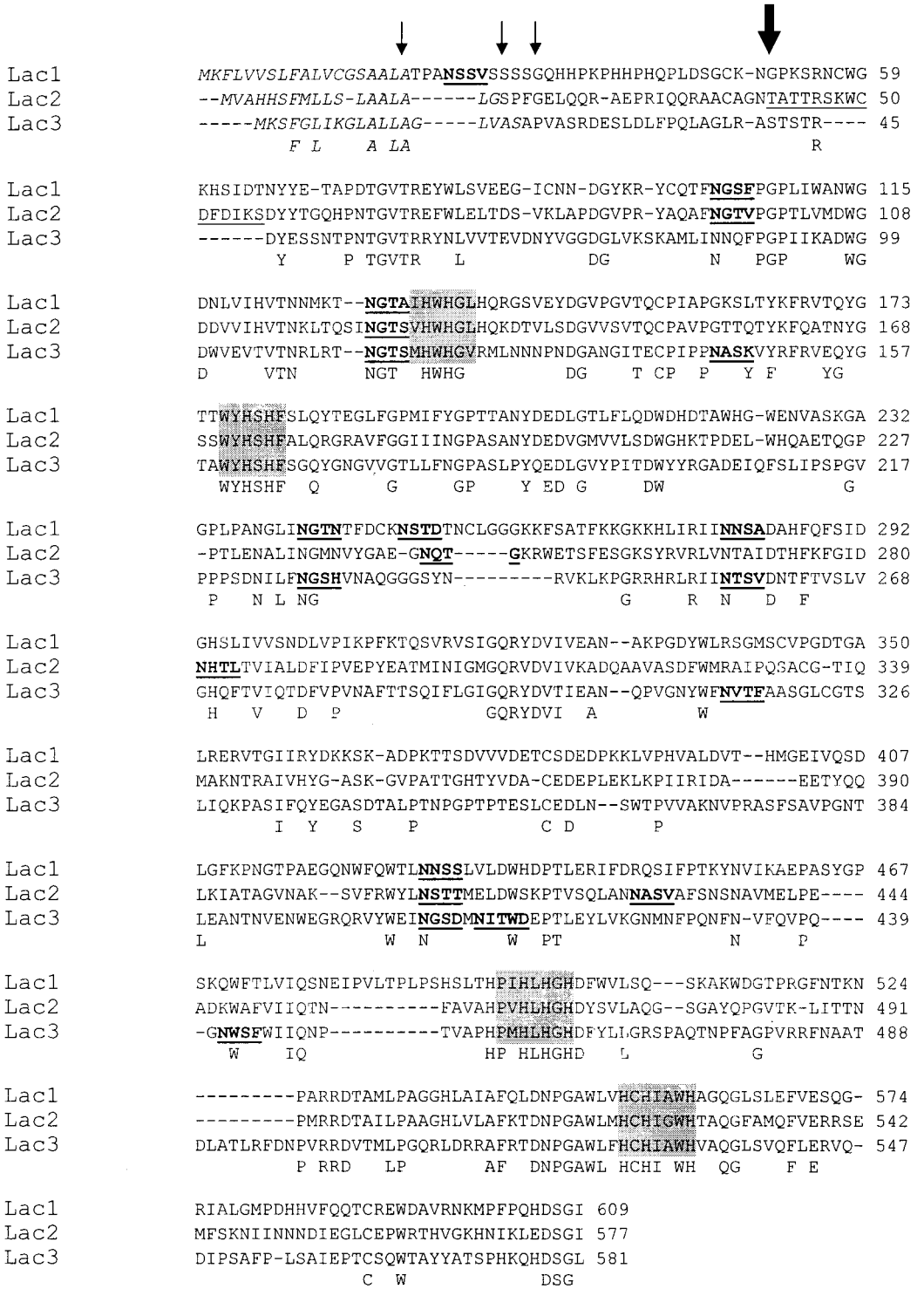

FIG. 1. Alignment of the deduced Lac1, Lac2, and Lac3 amino acid sequences, performed with the ClustalW method (46). Four putative copper-binding domains are shaded. Possible N-glycosylation sites are in bold and underlined, putative signal sequences are shown in italic, suggested cleavage sites are marked with thin arrows, the cleavage site in mature Lac2, determined by protein sequencing, is shown with a thick arrow, and the sequenced polypeptide is underlined.

one major secreted laccase protein. We purified this protein according to the previously described protocol (13) and subjected it to Edman degradation and sequencing. The first 15 amino acid residues at the amino terminus were TATTRS KWCDFDIKS. This sequence was identical to residues 41 to 56 of the precursor protein encoded by *LAC2* (Fig. 1) and implied that the protein undergoes posttranslational proteolytic cleavage at position 40.

Characterization of the upstream regulatory regions of *LAC1*, *LAC2*, and *LAC3*. Sequence analysis of 5′ nontranscribed regions of *LAC1*, *LAC2*, and *LAC3* revealed typical promoter sequences (49). Transcription of many laccase genes is regulated by copper ions (8, 18, 34). We screened the 5′ nontranscribed regions of three laccase genes for similarity with consensus eukaryotic sequences known to mediate transcriptional activation by copper ions (45). Metal-responsive elements (MRE) are found in the upstream regions of the *Saccharomyces cerevisiae* Cu-metallothionein and CuZn-superoxide dismutase genes (45). Putative MRE sequences are also present in several promoter regions of previously characterized laccase genes (18, 28). We located four putative MRE consensus sequences situated around the TATA box of the *LAC2* gene promoter and two consecutive putative MRE sequences located 751 and 690 bp upstream of the TATA box of the *LAC1* gene promoter.

ACE1 is a eukaryotic copper-responsive transcription factor that activates Cu-dependent transcription of the *S. cerevisiae* metallothionein genes (45). We identified one putative ACE1 consensus sequence located 108 bp upstream of the TATA box in the *LAC1* gene promoter and two consecutive ACE1 consensus sequences flanking the CT-rich box in the promoter of *LAC2* downstream of the TATA box. We did not identify any MRE or ACE1 elements in the *LAC3* gene promoter.

Regulation of laccase transcription. We used an RT-PCR approach to characterize transcription of the three laccase genes of *G. graminis* var. *tritici*. Total RNA was purified from young actively growing mycelia, at the peak of secreted laccase activity. The transcription pattern of the *LAC1* gene is shown in Fig. 2A. Fungal cultures were grown in starvation medium (lane 2), Fahraeus minimal medium with and without Cu (lanes 3 and 4, respectively), PDB (lane 5), LB with and without Cu (lanes 6 and 7, respectively), LB supplemented with sterile plant homogenate (lane 8), and the host wheat plant (lane 9). With primers specific for *LAC1*, similar levels of the *LAC1* transcript were observed in all conditions tested.

Transcription patterns of *LAC2* are shown in Fig. 2B. The *LAC2* gene was transcribed in Fahraeus minimal medium supplemented with Cu (lane 3), in LB medium with or without Cu (lanes 8 and 10, respectively), and in planta (lane 12). It was transcribed at a very low level in Fahraeus minimal medium without copper (lane 5), and was not transcribed in starvation medium (lane 2) or in PDB (lane 7).

Transcription of *LAC3* was observed only in planta (Fig. 2C, lane 2) or in culture medium with homogenized, sterile plant tissue (Fig. 2C, lane 3). The *LAC3* transcript was detected in infected root, leaf, and stem tissues (data not shown).

Transcription patterns of the housekeeping *POLG* gene are shown in Fig. 2D. Similar high levels of housekeeping gene transcripts were observed in all conditions tested.

Regulation of *LAC1* and *LAC2* transcription with copper. The results of the discriminatory *LAC1*/*LAC2* competitive RT-PCR from Fahraeus minimal medium cultures with and without 400 μM $CuSO_4$ are shown in Fig. 3. *LAC1* transcription was independent of copper; cultures grown with and without $CuSO_4$ had the same level of *LAC1* transcript (Fig. 3A). In contrast, transcription of *LAC2* was increased at least 10-fold in the presence of 400 μM $CuSO_4$ (Fig. 3B) and corresponded to an 8-fold increase in secreted laccase activity (data not

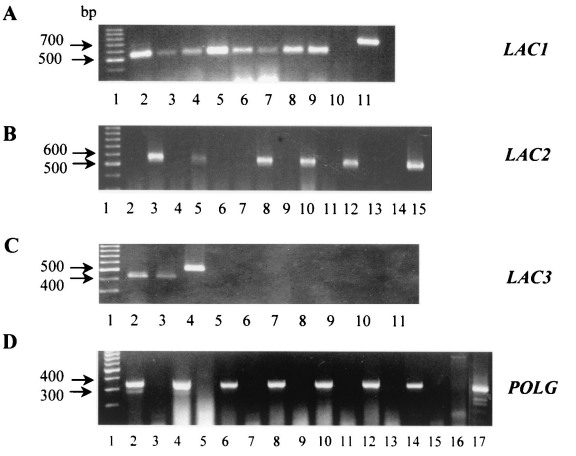

FIG. 2. (A) Transcription patterns of the *LAC1* gene. RT-PCR was performed with *LAC1*-specific primers, and total RNA was isolated from *G. graminis* grown in starvation medium (lane 2), minimal medium supplemented with 400 μm $CuSO_4$ (lane 3), minimal medium without copper (lane 4), PDB (lane 5), LB medium supplemented with 400 μM $CuSO_4$ (lane 6), LB medium without copper (lane 7), and LB medium supplemented with sterile plant homogenate (lane 8). Lane 9 shows RT-PCR with *LAC1*-specific primers and RNA purified from wheat plants infected with *G. graminis*; lane 10 shows RT-PCR with *LAC1*-specific primers and RNA purified from uninfected control wheat plants. Lane 11 shows amplified genomic *LAC1*, which contains two introns. Lane 1 shows a 100-bp ladder (Promega) as molecular size markers. (B) Transcription patterns of the *LAC2* gene. RT-PCR was performed with *LAC2*-specific primers and total RNA isolated from *G. graminis* grown in starvation medium (lane 2); minimal medium supplemented with 400 μM $CuSO_4$ (lane 3); minimal medium without copper (lane 5); PDB (lane 7); LB medium supplemented with 400 μm $CuSO_4$ (lane 8); and LB medium without copper (lane 10). Lane 12 shows RT-PCR with primers specific for *LAC2* and RNA purified from the wheat plants infected with *G. graminis*; lane 14 shows RT-PCR with primers specific for *LAC2* and RNA purified from the uninfected control wheat plants; and lane 15 shows amplified genomic *LAC2*, which has no introns. Control reactions without reverse transcriptase are shown in lanes 4, 6, 9, 11, and 13. Lane 1 shows 100-bp ladder molecular size markers. (C) Transcription patterns of the *LAC3* gene. RT-PCR was performed with *LAC3*-specific primers, and total RNA was isolated from wheat plants infected with *G. graminis* var. *tritici* (lane 2); *G. graminis* var. *tritici* grown in minimal medium supplemented with 30% (vol/vol) sterile plant homogenate (lane 3); minimal medium with 400 μM $CuSO_4$ (lane 6); minimal medium without copper (lane 7); starvation medium (lane 8); PDB (lane 9); LB with 400 μM $CuSO_4$ (lane 10); and LB without copper (lane 11). Lane 4 shows amplified genomic *LAC3*, which contains an intron. Lane 5 shows RT-PCR with primers specific for *LAC3* and RNA purified from the uninfected control wheat plants. Lane 1 shows 100-bp ladder molecular size markers (Promega). (D) Transcription patterns of the *POLG* gene. RT-PCR was performed with *POLG*-specific primers and total RNA isolated from *G. graminis* grown in starvation medium (lane 2); PDB (lane 4); minimal medium supplemented with 400 μM $CuSO_4$ (lane 6); minimal medium without copper (lane 8); LB medium supplemented with 400 μM $CuSO_4$ (lane 10); and LB medium without copper (lane 12). Lane 14 shows RT-PCR with primers specific for *POLG* and RNA purified from the wheat plants infected with *G. graminis*; lane 16 shows RT-PCR with primers specific for *POLG* and RNA purified from the uninfected control wheat plants; lane 17 shows amplified genomic *POLG*, which has no introns. Control reactions without reverse transcriptase are shown in lanes 3, 5, 7, 9, 11, 13, and 15. Lane 1 shows 100-bp ladder molecular size markers.

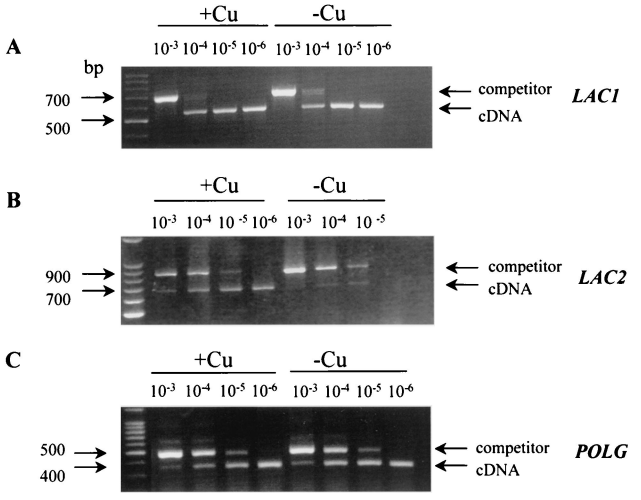

FIG. 3. Transcription of *LAC1* (A), *LAC2* (B), and *POLG* (C) in the presence and absence of 400 μM $CuSO_4$. The amplified competitive templates and cDNAs are indicated with arrows. The amounts of the competitive templates are indicated above the gels (in micrograms per microliter). A 100-bp ladder (Promega) was used as molecular size markers.

shown). Transcription of *POLG* was also independent of copper and indicated that the same amounts of mRNA were used for each RT-PCR. (Fig. 3C).

Figure 3 demonstrates that in the presence or absence of copper, *LAC1* was transcribed at a level (10^{-3} to 10^{-4} μg per μg of total RNA) similar to or higher than that of *POLG*; in the absence of copper, *LAC2* was transcribed at a level (10^{-5} μg per μg of total RNA) lower than that of *POLG*, and in the presence of the inducer, *LAC2* was transcribed at a level similar to that of *POLG* (10^{-4} μg per μg of total RNA). Transcription of laccase genes was not induced by zinc or manganese (data not shown). All competitive PCR experiments were repeated three times with similar results.

DISCUSSION

We identified and partially characterized three laccase genes from a plant-pathogenic fungus, *G. graminis var. tritici.* Each gene had a distinct structure, with different numbers of introns, ranging from zero (*LAC2*) to six (*LAC3*). Intron positions were also unique for each gene and did not overlap between the genes. The deduced protein sequences showed 38 to 42% amino acid sequence identity.

Laccases are characterized by having four conserved copper-binding sites. They contain one type 1 (T1) Cu atom, bound as a mononuclear center that gives the proteins their blue color, as well as type 2 (T2) and type 3 (T3) Cu atoms, which together form a trinuclear center. The T1 site functions as a primary electron acceptor, extracting electrons from the substrate. Electrons are then transferred to the T2/T3 center, where reduction of molecular oxygen to water takes place (12, 29). According to a recent analysis of the *Coprinus cinereus* laccase crystal structure (12) and site-directed mutagenesis studies of *Myceliophthora thermophila* and *R. solani* laccases (52), a pentapeptide segment located downstream of the second conserved histidine in the T1 site (H560 in Lac1, H537 in Lac2, and H533 in Lac3; Fig. 1) has a major effect on the redox potential and specificity of the enzyme. The amino acid composition in this region was unique for all three of the deduced laccases in *G. graminis* var. *tritici* (Fig. 1), which implies that the enzymes have different substrate specificities and probably different functions in the fungus.

Recently, we purified and partially characterized a secreted, copper-induced laccase from *G. graminis* var. *tritici* (13). Here we show that the N terminus of this protein was identical to the predicted protein encoded by the *LAC2* gene. *LAC2* transcription was significantly induced by Cu and corresponded to a similar increase in the total laccase activity of induced cultures. In PDB or starvation medium, in which *LAC2* was not transcribed, a low level of secreted laccase activity was detected. This activity was independent of the copper concentration in the medium (data not shown) and likely corresponded to *LAC1* expression, since it was the only laccase gene transcribed in these conditions. However, we have not purified Lac1 or Lac3 laccases because of their low concentrations. Recently it was shown that extracellular proteases may affect the concentrations of secreted laccases (33), which could explain why we were unable to obtain larger amounts of Lac1 and Lac3.

Our study indicated that the laccase genes in *G. graminis* var. *tritici* were induced in different conditions. *LAC1* was transcribed constitutively in all conditions tested. Constitutive transcription has been reported for several fungal laccase genes (15, 54, 57). In contrast, transcription of *LAC2* was copper inducible. The promoter region of *LAC2* contained four putative MRE consensus sites, two of which were inverted and 51 bp apart. In addition, this promoter sequence contained two putative ACE1 binding sites situated between the TATA box and ATG codon.

Copper induction has been reported for the *P. anserina* (18), *T. versicolor* (25), and *P. ostreatus* (34) laccase genes. MRE consensus sequences are found in the promoter regions of two fungal laccase genes, those of *P. anserina* (18) and *Phanerochaete chrysosporium* (4). One consensus sequence that has some similarities with the binding site of the ACE1 transcription factor from *S. cerevisiae* was reported for the *lcc1* promoter from the basidiomycete PM1 (28). Nevertheless, the role of MRE and ACE1 sites in laccase promoters requires additional investigation, including site-directed mutagenesis to remove these sites.

Transcription of *LAC3* was observed only when the fungus was grown in association with the host plant. We were unable to induce *LAC3* transcription in a culture except by the addition of sterile, homogenized plant filtrate to the medium. To our knowledge, this is the first report of a fungal laccase gene whose transcription depends on the presence of a host. However, laccase from *Botrytis cinerea* is inducible in culture by the addition of pectin, suggesting that its expression is host dependent (35). This laccase also oxidizes plant stilbene phytoalexins in vitro and may be a pathogenicity factor (35). The animal pathogen *C. neoformans* expresses laccase that is involved in the biosynthesis of a melanin-like pigment in mouse tissues; laccase-negative *lac1* (Mel^-) knockout mutants of *C. neoformans* are defective in pigment production and nonpathogenic (37, 51).

Although the biological functions of several fungal laccases have been determined (14, 16, 35, 48), the functions of the

majority of laccases are still uncertain. However, their abundance suggests that they play important and diverse roles. *G. graminis* var. *tritici* produces melanized macrohyphae on its host's root surfaces, and a laccase(s) could be involved in melanin polymerization in these hyphae (41). A laccase(s) could also degrade lignin depositions produced by the plant in response to invasion, and/or oxidize-reduce phytoalexins and other toxic plant compounds. Other functions, such as sequestration or oxidation of manganese, required by the plant for lignin synthesis (36) are also possible.

Previously, we demonstrated that the major secreted laccase in culture, here shown to be encoded by *LAC2*, is capable of oxidizing lignin and polymerizing melanin precursors in vitro. The ability of laccase to oxidize or reduce polyphenolic compounds such as lignin or melanin precursors depends on the redox potential of the surrounding environment. Both functions can be carried out by the same enzyme in different situations; alternatively, oxidation and reduction may be split between different laccases expressed in different environments or compartments of the infected host. Mutants in which each of these genes are knocked out would help identify the functions of the individual laccases of *G. graminis* var. *tritici,* but thus far we have been unsuccessful in generating disruption mutants with cloned laccase genes. Homologous recombination with extraneous DNA is rare in *G. graminis* (3), and thus, alternative methods for reducing the expression of laccase genes may be required to determine their functions.

ACKNOWLEDGMENTS

We thank Bill Edens and Tim Magnuson for help with protein purification, Kathy Sheehan for critical reading of the manuscript, and Mary Bateson for help with DNA sequencing.

This work was supported in part by the U.S. Army Research Office (grant DAAH04-96-1-01194) and the National Science Foundation (grant MCB9977922).

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