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Regulation of Laccase Biosynthesis in the Plant-Pathogenic Fungus *Cryphonectria parasitica* by Double-Stranded RNA

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Transmissible hypovirulence of the chestnut blight fungus, *Cryphonectria parasitica*, is associated with cytoplasmic double-stranded-RNA (dsRNA) viruses. The fungal laccase has attracted interest because its activity is reduced in hypovirulent dsRNA-containing strains. A laccase cDNA clone was isolated by screening a cDNA expression library with antibodies against the purified extracellular laccase. The amino acid sequence deduced from part of the cDNA clone revealed high homology to other fungal laccases, especially to the *Neurospora crassa* laccase. A major laccase transcript 2.3 kb in length was detected in Northern (RNA) blots. In liquid culture, extracellular laccase activity was reduced by about 75% in the hypovirulent (dsRNA-free) strain EP155/2. In contrast, production of biomass was not affected by the dsRNA. Northern blot analysis indicated that dsRNA down regulates laccase biosynthesis by reducing laccase mRNA accumulation. The laccase gene is one of several developmentally regulated genes affected by the presence of dsRNA.

Naturally occurring hypovirulent strains of the chestnut blight fungus, *Cryphonectria parasitica* (Murr.) Barr., have been associated with biological control of the disease in Europe and in some areas of North America. The hypovirulent phenotype is cytoplasmically controlled and correlated with the presence of double-stranded RNA (dsRNA) of viral origin (for recent reviews, see references 1, 11, 19, and 24).

How the dsRNA viruses regulate virulence expression of the fungus is not clear. Isolates of the fungus containing the European type of dsRNA characteristically have reduced virulence, reduced pigmentation, and reduced sporulation compared with the characteristics of normal strains of the fungus (8). Powell and Van Alfen (21, 22) have demonstrated that specific poly(A)$^+$ RNAs and polypeptides are down regulated in dsRNA-containing strains, which suggests that the dsRNA affects expression of specific fungal genes. From our current evidence it appears that the virus is perturbing specific fungal genes at the mRNA level which are involved in development (24).

Laccase production has been reported to be reduced in hypovirulent strains of *C. parasitica* containing the European type of dsRNA (23). This oxidative enzyme is widely distributed in fungi; however, its biological function is still not clear (17). In *Aspergillus nidulans*, laccase levels are developmentally regulated (15). One laccase enzyme of this fungus appears to be involved in conidial pigmentation (6). The gene encoding the conidial laccase of *A. nidulans* has been isolated and sequenced (1, 25). In other fungi, laccase activity has been suggested to play a role in degradation of lignin (2), pathogenesis (5), and formation of fruiting bodies (16).

In the present study we report the isolation and identification of a laccase cDNA clone of *C. parasitica*. In addition, we present evidence that the European type of dsRNA, when present in the fungus, down regulates laccase biosynthesis by reducing laccase mRNA accumulation.

Laccase cDNA cloning. In order to isolate the *C. parasitica* laccase gene we screened a cDNA expression library with laccase antiserum. As a result of experiments to be reported elsewhere, the extracellular laccase of *C. parasitica* was purified and characterized as a glycoprotein with a molecular mass of approximately 77 kDa (23a). Polyclonal antibodies against the sodium dodecyl sulfate-denatured extracellular laccase of *C. parasitica* were raised in rabbits.

Western blot (immunoblot) analysis revealed that the antisera reacted with the purified laccase as well as with several components of the crude extracellular culture filtrate (Fig. 1). We assume that the nonspecific reactions of the antiserum were due to antibodies directed against the carbohydrate part of the laccase, as was found for other glycoproteins (20).

Poly(A) RNA was purified from laccase-producing mycelium of the virulent strain EP115/2 by using oligo(dT)-cellulose columns (4). The cDNA library was constructed from this poly(A) RNA by using the Lambda ZAP cDNA synthesis kit (Stratagene). The RNA used was shown to contain laccase mRNA by immunological analysis of the in vitro translation product (data not shown).

Laccase-specific clones of the cDNA library were detected by a double-antibody enzyme immunoassay system (Bio-Rad) using alkaline phosphatase-labeled second antibody. From about 10 plaques, seven positive clones were detected by screening with laccase antiserum. One clone was lost during the second screening at a low plaque density. All six remaining clones showed similar restriction enzyme patterns, indicating that they are probably derived from the same gene. The largest cDNA clone, 1.8 kbp in length, lacks about 500 bp from the 5′-terminal end and so does not represent a full-length copy of the laccase mRNA.

The 5′ end of the clone was sequenced by using the Sequenase chain termination technique (United States Biochemical Corp.). The amino acid sequence deduced from the analyzed region showed high homology to that of the laccase of *Neurospora crassa* (12) and to a lesser extent to those of the laccases of *Coriolus hirsutus* (14) and *A. nidulans* (3) (Fig. 2). A sequence of 18 amino acid residues of the *C. parasitica* laccase is identical to an amino acid sequence of the *N. crassa* laccase. This sequence includes two histidine residues, which are proposed to be involved in copper

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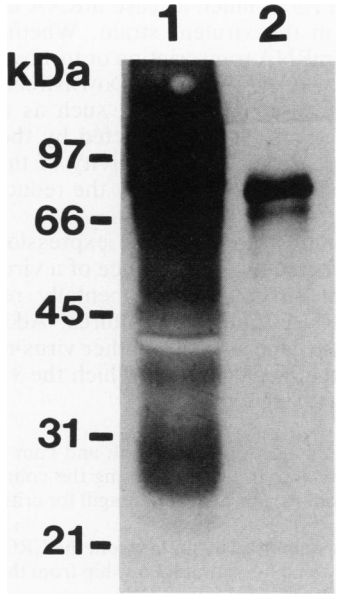

FIG. 1. Western blot analysis of crude culture filtrate (lane 1) and purified extracellular laccase (lane 2) of *C. parasitica*. An amount corresponding to 0.1 laccase unit (approximately 200 ng of laccase) was applied to each lane, fractionated by electrophoresis, and probed with laccase antiserum. Molecular mass markers are indicated on the left.

binding by the blue oxidases (18). It will be interesting to see whether the high homology between these two laccases reflects functional similarity of the enzymes or close evolutionary relatedness of the species. The biological function of both laccases is unknown.

Regulation of laccase expression. The virulent strain (EP155/2) and the hypovirulent strain (UEP1) of *C. parasitica* used in this study were isogenic except for the presence of the European type of dsRNA in the hypovirulent strain (21). The strains were grown in complete liquid medium (7) at a pH of 8. To produce inoculum, the fungus was grown on

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Ch-po    91  Q K G T N W A D G P A F V N Q C P I S S - G H S F L Y
An-l     85  M R E T P E A D G V P G L T Q T P I E P - G A T F T Y
Nc-lac  101  Q R N S N I Q D G V N G V T E C P I P P R G G S K V Y
Cp-lac    1  Q L N T N L Q D G V N G I T E C P I P P N G G S K T Y

Ch-po   117  D F Q V P D Q A G T F W Y H S H L S T Q Y C D G
An-lac  111  R F R A Y - P A G T F W Y H S H Y K G L M Q D G
Nc-lac  128  R W R A T - Q Y G T S W Y H S H F S A Q Y G N G
Cp-lac   28  T F I A H - Q Y G T S W Y H S H F S A Q Y G N G
                                    *       *
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FIG. 2. Amino acid sequence comparison between part of the *C. parasitica* laccase (Cp-lac) and homologous parts of other fungal laccases. Ch-po, phenoloxidase (laccase) of *Coriolus hirsutus* (17); An-lac, *A. nidulans* laccase (3); Nc-lac, *N. crassa* laccase (14). The number on the left of each sequence represents the position of the first amino acid residue in the sequence of these enzymes. For Cp-lac, the number refers to the first position shown as 1. Dashes represent gaps introduced for alignments. Amino acid residues identical to those found in Cp-lac are boxed. Amino acid residues proposed to be involved in copper binding are marked with asterisks.

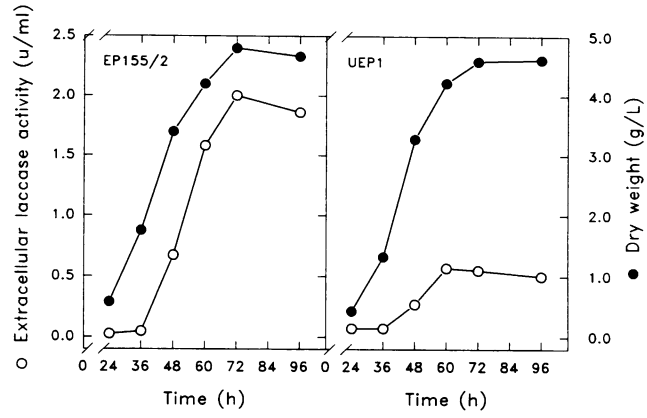

FIG. 3. Biomass production and extracellular laccase activities of the dsRNA-free strain EP115/2 and the isogenic dsRNA-infected strain UEP1 of *C. parasitica*. Biomass (dry weight) and laccase activity were monitored as a function of time after inoculating liquid media. Each point represents the data collected from a single flask. Representative results from one out of three similar experiments are shown.

plates of potato dextrose agar supplemented with L-methionine (100 mg/liter) and biotin (1 mg/liter) (PDAmb) in the dark at 25°C until the mycelial growth almost covered the plates (6 to 7 days). The total contents of the plates were then homogenized in distilled water (100 ml per PDAmb culture plate) in a Waring blender, and 100 ml of the resulting slurry was used to inoculate 1 liter of medium contained in Fernbach flasks. The cultures were incubated under constant light (2,500 lx) at 26°C on a rotary shaker (105 rpm) for the times indicated. Laccase activity in the culture filtrate was determined by using 2,6-dimethoxyphenol as the substrate (23). One laccase unit was defined as an increase in the A_{460} of 1.0/min at 25°C. Total RNA was isolated by selective precipitation by using lithium chloride as previously described by Powell and Van Alfen (21). Total-RNA samples (15 μg) were treated with glyoxal, fractionated on a 1% agarose gel, blotted onto GeneScreen Plus nylon membranes (DuPont), and probed according to the instructions of the manufacturer. The probe, a laccase-specific restriction fragment, was purified from an agarose gel and labeled with [^{32}P]dCTP by using a randomly primed DNA labeling kit (Boehringer Mannheim). A DNA probe from *N. crassa* (9) was used to show the integrity and amount of RNA in each lane. The laccase mRNA was quantitated by scanning densitometry by using an UltraScan XL laser densitometer (Pharmacia LKB). The blots were exposed for different time periods to enable quantitation of each sample in the linear range of film response.

Dry weight, laccase activity, and laccase mRNA accumulation of both strains were monitored as a function of time (Fig. 3 and 4). Each strain reached stationary growth phase about 72 h after inoculation, and they produced comparable final dry weights. Extracellular laccase activity increased more rapidly in the virulent cultures that in the hypovirulent cultures. Major differences in activity between the two strains were detected 72 h after inoculation. The maximum enzyme activity was reduced by approximately 75% in hypovirulent cultures compared with that of virulent cultures.

Regulation of laccase mRNA accumulation was investigated by Northern (RNA) blot analysis. A major transcript

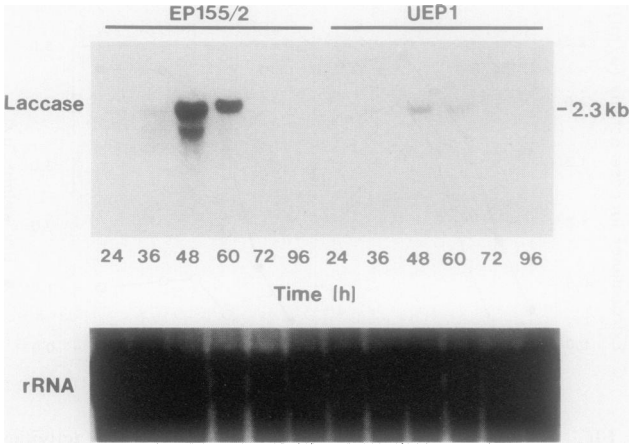

FIG. 4. Time course of laccase mRNA expression in liquid cultures of the dsRNA-free strain EP155/2 and the isogenic dsRNA-infected strain UEP1 of *C. parasitica*. Total RNA was extracted from each strain at the times indicated. Equal amounts (15 μg) were then subjected to Northern blot analysis using a laccase-specific DNA probe. The molecular weight of the laccase mRNA was estimated by using glyoxylated *Hin*dIII fragments of lambda DNA. The laccase probe was stripped from the blot, which was then hybridized to a probe for rRNAs to show the integrity and amount of RNA subjected to Northern blot analysis.

with an estimated length of 2.3 kb was detected by the probe in both the virulent and hypovirulent strains. Laccase mRNA was detected at 24 h and 36 h in both strains but at much lower levels in the hypovirulent strain than in the virulent one. As shown in Fig. 4, expression of the laccase gene increases between 36 and 48 h after inoculation in both strains. However, less laccase mRNA accumulated in the hypovirulent strain. The reductions of laccase mRNA in the hypovirulent strain were estimated by densitometric analysis to be 85 and 87% at 48 and 60 h, respectively. By 72 h after inoculation, laccase mRNA was no longer detectable in either strain.

In this report we described the cloning of the laccase gene of *C. parasitica* and its use to study regulation of laccase expression in liquid cultures of the fungus. An increase in laccase mRNA was observed during early growth, resulting in production of increasing amounts of extracellular laccase. At the time when laccase enzyme activity peaked, however, laccase mRNA was no longer detectable. These data suggest that laccase transcripts are degraded and that transcription of the laccase gene is terminated by 72 h after inoculation. Clearly, the laccase is not constitutively expressed. Our study suggests that laccase biosynthesis by *C. parasitica* is primarily regulated at the level of mRNA. Both transcriptional regulation and mRNA turnover appear to be involved in laccase regulation. In contrast to *C. parasitica*, vegetative liquid cultures of two other ascomycetes, *N. crassa* and *A. nidulans*, do not produce significant amounts of laccase enzyme activity (10, 15).

Previous studies (13, 23) have shown that dsRNA of *C. parasitica* reduces the level of laccase in crude culture filtrates of the fungus. We show here that regulation of laccase by the dsRNA is at the level of mRNA accumulation. Temporal control of laccase expression is comparable in the virulent (dsRNA-free) and hypovirulent (dsRNA-infected) strains. In the hypovirulent strain, laccase mRNA does not accumulate to the same levels as in the virulent strain. We detected only 15% as much laccase mRNA in the hypovirulent strain as in the virulent strain. Whether the dsRNA affects laccase mRNA transcription or turnover is not known and needs to be further studied. Downstream processes of extracellular laccase biosynthesis, such as translation and secretion, apparently are not affected by the dsRNA. The detectable decrease in laccase activity of the hypovirulent strain can be explained entirely by the reduction of mRNA accumulation.

We have demonstrated that the expression of a specific host gene is affected by the presence of a virus. The laccase gene is one of several developmentally regulated genes affected in dsRNA-containing cultures. Additional studies using the laccase gene as well as other virus-regulated genes should reveal the mechanism by which the virus is perturbing specific host gene expression.

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