A Tannic Acid–Inducible and Hypoviral-Regulated Laccase3 Contributes to the Virulence of the Chestnut Blight Fungus *Cryphonectria parasitica*

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Submitted 23 June 2008. Accepted 12 August 2008.

A new laccase gene (*lac3*) from the chestnut blight fungus *Cryphonectria parasitica* was induced by the presence of tannic acid, which is abundant in the bark of chestnut trees and is assumed to be one of the major barriers against pathogen infection. However, other commonly known laccase inducers, including ferulic acid, 2,5-xylydine, catechol, and pH, did not induce *lac3* transcription. Moreover, the hypovirus modulated the induction of *lac3* transcription, abolishing the transcriptional induction of the *lac3* gene by tannic acid. A functional analysis of *lac3* using a *lac3*-null mutant indicated that fungal growth and other morphological characteristics, including pigmentation and sporulation, were not affected. However, a virulence assay indicated that the loss of function of a tannic acid-inducible and hypoviral-regulated laccase resulted in reduced virulence without detectable changes in the morphological features. The constitutive expression of *lac3* resulted in no significant differences in the necrotic lesions from those caused by the wild type, but its expression in the presence of the hypovirus led to larger lesions than those caused by the hypovirulent strain. These results suggest that the *lac3* gene product may not be the only determinant of fungal virulence in chestnut trees but is an important factor.

Additional keyword: hypovirulence.

The chestnut blight fungus *Cryphonectria parasitica* virtually eliminated American chestnut (*Castanea dentata*) in the early 20th century (Anagnostakis 1982, 1988). However, strains containing the double-stranded (ds)RNA virus *Cryphonectria* hypovirus1 show characteristic symptoms of reduced virulence, i.e., hypovirulence (Anagnostakis 1982; Milgroom and Cortesi 2004; Nuss 2005; Van Alfen et al. 1975), and display diverse hypovirulence-associated traits such as reduced pigmentation, sporulation, laccase production, and oxalate accumulation (Elliston 1985; Havir and Anagnostakis 1983; Rigling et al. 1989). Because the phenotypic changes in the

fungal host following hypovirus infection are specific, albeit pleiotropic, it has been suggested that the hypovirus specifically disturbs one or several regulatory pathways (Nuss 1996; Shang et al. 2008). Accordingly, several genes have been identified as targets because they are specifically regulated by the presence of hypovirus (Chen et al. 1996; Deng et al. 2007b); Gao and Nuss 1996; Kasahara and Nuss 1997; Park et al. 2004). Recent studies using DNA microarrays have revealed changes in the transcriptional profile of a fungal host as a result of hypovirus infection and have facilitated grouping the genes showing aberrant expression into several metabolic pathways (Allen and Nuss 2004; Allen et al. 2003; Deng et al. 2007a). Among the specific target pathways of the fungal life cycle, the reduced virulence to chestnut appears to be one of the major effects of the fungus-hypovirus interaction.

Measuring the virulence of forest fungal pathogens such as chestnut blight fungus to their native hosts is more difficult than measuring the virulence of grass or annual pathogens, not least because of the unavailability of corresponding host trees in areas such as North America. As a result, inoculation of dormant chestnut stems (Jaynes and Elliston 1980), excised bark (Lee et al. 1992), and other hosts such as Golden Delicious apple (Fulbright 1984) have been considered as alternatives for measuring fungal virulence. In addition, several approaches have been suggested as indirect methods to compare fungal virulence. The two best alternatives are the measurement of extracellular laccase activity in liquid culture and the Baven-damm assay, which measures the degree of oxidizing browning on tannic acid-supplemented plates, because they have a high correlation with virulence (Rigling et al. 1989).

Laccases are multinuclear copper-containing phenol oxidases (*p*-diphenol:oxygen oxidoreductase, EC 1.10.3.2) that catalyze the oxidation of *p*-diphenols with the concurrent reduction of dioxygen to water (Thurston 1994), although the actual substrate specificities of laccases are often broad and vary with the enzyme source. Laccases belong to a small group of enzymes known as large blue copper proteins or blue copper oxidases, characterized by four cupric (Cu²⁺) ions that are coordinated such that each of the ion species (types 1, 2, and 3) is associated with a single peptide chain. Laccase is produced by various sources, including bacteria, fungi, insects, and higher plants (Dean and Eriksson 1994). Many fungi produce multiple laccases that are encoded by gene families (Kim et al. 1995; Kumar et al. 2003; Perry et al. 1993; Yaver and Golightly

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1996). Fungal laccases have been implicated in the pigmentation of fungal spores (Clutterbuck 1972; Smith et al. 1998), the development of fungal fruiting bodies (Leatham and Stahmann 1981), fungal pathogenicity (Geiger et al. 1986; Marbach et al. 1985; Rigling et al. 1989), lignin degradation (Hataka 1998; Ishihara 1980), vegetative growth (Sengupta and Mukherjee 1997), and phytoalexin conversion (Schouten et al. 2002). However, with the exception of the conidial laccase of *Aspergillus nidulans* (Clutterbuck 1972; Law and Timberlake 1980; Yelton et al. 1985) and the secretory laccase of *Botrytis cinerea* (Schouten et al. 2002), very few of these functions have been demonstrated experimentally.

Cryphonectria parasitica produces at least two laccase isoforms, constitutive extracellular laccase (*lac1*) and intracellular laccase (*lac2*), both of which are modulated by the presence of hypovirus. Previous studies have demonstrated that the *lac1*-null mutant shows no changes in virulence and have suggested the presence of a tannic acid-inducible laccase (*lac3*) (Kim et al. 1995). Tannic acid is a commercial form of a mixture of hydrolyzable galloyl tannins (Yumiko et al. 2003). Tannins, which are abundant in the bark of the chestnut tree, are water-soluble phenolic compounds with molecular weights between 500 and 3,000 Da that have special properties, such as the ability to precipitate alkaloids, gelatin, and other proteins (Griffin 1986). In this study, we characterized the tannic acid-inducible laccase and examined the biological functions of the *lac3* gene.

RESULTS

Characteristics of the *lac3* gene.

Computer-assisted alignment of multiple fungal laccases revealed several conserved regions with more than five consecutive identical amino acid residues. Using degenerate primers designed from conserved regions of fungal laccases (Fig. 1), an 833-bp DNA fragment was amplified, cloned, and sequenced. Sequence analysis of 40 clones led to the identification of two different clone types, one identical to the previous laccase1 sequence (Choi et al. 1992; Rigling and Van Alfen 1991) and another that differs from the previous laccase1 but is similar to other known fungal laccases (Fig. 1). A homology search of this new sequence revealed the highest similarity to a hypothetical gene of *Magnaporthe grisea*, sharing 36 and 59% identity at the nucleotide and amino acid levels, respectively, but only 35 and 51% identity to *lac1*. Screening of a genomic λ library, using the cloned polymerase chain reaction (PCR) product as a probe, resulted in three positive clones out of 40,000. A 4.7-kb *SacI*-digested fragment from the genomic λ clone was subcloned (pG_{lac3}F), sequenced, and found to contain the full-length *C. parasitica* laccase3 gene, *lac3*. Based on the genomic sequence, reverse transcription (RT)-PCR was used to isolate a nearly full-length cDNA fragment, using primers that hybridize at nucleotide positions (nt) -3 to 24 and 2,151 to 2,179, relative to the start codon. Sequence analysis of the cDNA obtained using RT-PCR indicated that the *lac3* gene consists of seven exons, with six intervening sequences ranging in size from 65 to 100 bp (*lac3* GenBank accession number, AY994151). The deduced *lac3* protein product (LAC3) consists of 567 amino acids, including an 18-residue secretory signal peptide typical of extracellular enzymes, which was predicted by the algorithm of Nielsen and associates (1997). The predicted unprocessed and mature protein products have estimated molecular masses of 62 and 60 kDa, with isoelectric points (pI) of 4.14 and 4.12, respectively. The deduced amino-acid sequence contains 11 putative N-glycosylation sites (Asn-Xxx-Ser/Thr) (Gavel and Von Heijne 1990), at nt positions 58, 69, 85, 112, 124, 234, 270, 342, 403, 422, and 545 (Fig. 1).

A primer-extension experiment revealed that the transcription start site of *lac3* is located 214 bp upstream of the putative start codon. Sequence analysis of the first 1,939 bp of the promoter region of the *lac3* gene revealed putative CAAT and TATA boxes at nucleotides -204 and -89, respectively, relative to the transcription start site. In addition, a putative consensus metal-response element, TGCRCNC, and a xenobiotic-response element, TNGCGTG, are present at 602 and 905 bp upstream of the first ATG, respectively. The region also contains the sequence TATCT, a putative binding site for an NIT2-like protein, which could be involved in nitrogen metabolite regulation (Jarai et al. 1992), as well as a consensus binding site, TCT4-6GCTG, for the copper-regulated transcription factor ACE1 (Gralla et al. 1991), at 893 and 470 bp upstream of the first ATG, respectively. The sequence around the first ATG is in good agreement with Kozak's consensus sequence in that the -3 position is the A in CATTATG. A putative consensus polyadenylation signal, AATATA (Kim et al. 2001), is present 240 bp downstream of the stop codon.

All of the residues involved in Cu(II) ligand binding (ten histidine residues and one cysteine residue) that are conserved in fungal laccases (Kumar et al. 2003) are present in the *lac3* coding sequence of *C. parasitica* (Messerschmidt 1997) (Fig. 1). Another residue (leu), 10 amino acids downstream of the conserved cysteine involved in the type-I Cu(II) binding site, is also preserved. However, the absence of the leu-glu-ala tripeptide motif adjacent to the last conserved histidine and the presence of the leucine residue rather than a phenylalanine residue suggests that LAC3 does not possess high ionization potential (Temp and Eggert 1999).

A comparison of the deduced amino-acid sequence of the *lac3* gene product with those of other fungal laccases revealed that LAC3 shows higher similarity to laccases from other species than to LAC1 from the same species. The *lac3* gene is most closely related to *lac2* from *Gaeumannomyces graminis* var. *graminis* (47% amino acid identity), followed by *lac2*

Fig. 1. Alignment of the deduced amino-acid sequences of *Cryphonectria parasitica* *lac1* (top) and *lac3* (bottom). Identical residues are shaded, and dashes represent gaps in the aligned sequences. The putative cleavage site of the signal peptide is indicated by an arrowhead. The amino acid residues proposed to be involved in copper binding are indicated as follows: type 1 (squares), type 2 (asterisks), and type 3 (circles) (Messerschmidt 1997). The last conserved histidine and the leucine residue, 10 amino acids downstream of the conserved cysteine involved in the type I Cu(II) binding site, are indicated by an open square and a triangle, respectively. Conserved regions used for primer design are indicated by arrows.

from *G. graminis* var. *tritici* (46%). The *lac3* shows 41% identity to *lac1* from *C. parasitica*.

Regulation of *lac3* gene expression.

Previous studies have shown that the extracellular laccase activity in filtrates from cultures grown in standard growth conditions was due to laccase1, whereas a new laccase activity (laccase3) was detected in culture filtrates grown in the presence of tannic acid and showed different electromobility on native agarose gels (Kim et al. 1995). Similarly, Northern blot analysis showed no accumulation of the *lac3* gene transcript under normal growth conditions, including EP complete liquid medium and solid media such as malt extract agar or potato dextrose agar containing 100 mg of L-methionine and 1 mg of biotin per liter (PDAMB). However, the accumulation of the *lac3* gene transcript was induced in modified Bavendamm's medium, a tannic acid-supplemented malt extract agar (Rigling et al. 1989). Strong induction of the *lac3* transcript on modified Bavendamm's medium was first observed 12 h after induction; the expression peaked at 24 h and then decreased gradually (Fig. 2). The concentration of tannic acid required for the induction was examined by testing 0.7, 0.07, and 0.007% (wt/vol) tannic acid; concentrations as low as 0.007% induced *lac3* expression on modified Bavendamm's medium. Induction of the *lac3* transcript was also observed on minimal medium containing 0.7% tannic acid. However, when the tannic acid in modified Bavendamm's medium was replaced with citric acid, no accumulation of the *lac3* transcript was detected. There-

fore, its induction appears to be specific to the presence of tannic acid rather than other media components or growth conditions such as acidity. The *lac1* expression was also examined in different media containing tannic acid, but its induction was not observed (data not shown).

Because ferulic acid and 2,5-xyldine, aromatic compounds structurally related to lignin, are effective inducers of the production of other fungal laccases, their ability to induce the *lac3* gene was examined. Using Northern blot analysis, the accumulation of the *lac3* gene transcript was measured in both liquid and solid media containing either ferulic acid or 2,5-xyldine. No hybridizing *lac3* band was detected, indicating that the expression of the *lac3* gene was not induced by these lignin-related aromatic compounds (data not shown). Other conditions known to induce other fungal laccases, such as Mg^{2+} and Cu^{2+} supplementation, were also tested, and no inducing effect on the *lac3* expression was observed.

Because the expression of a different *C. parasitica* laccase, *lac1*, is under the tight control of the phospholipase C gene *cplc1* (Chung et al. 2006), we tested whether *cplc1* modulates the induction of the *lac3* gene. When a *cplc1*-null mutant was placed in induction conditions (medium containing tannic acid), the *lac3* gene transcript was induced as in the wild type, indicating that the presence of the *cplc1* gene is not necessary for the induction of the *lac3* gene (Fig. 2).

We also examined whether an osmotic stress-involved mitogen-activated protein (MAP) kinase (*cpmk1*) (Park et al. 2004) and a mycelial growth- and conidiation-related MAP kinase (*cpmk2*) (Choi et al. 2005) affect the induction of the *lac3* gene by placing both *cpmk1*- and *cpmk2*-null mutants in induction conditions. No changes in the induction of the *lac3* transcript were observed in either MAP kinase mutant (data not shown), suggesting that the signaling pathways involving these MAP kinases are not responsible for the induction.

Viral regulation of *lac3* induction.

Among the putative inducers that were tested, tannic acid was able to induce the *lac3* gene. To examine the viral regulation of *lac3* induction, the *lac3* induction pattern in the isogenic hypovirulent strain UEP1 was compared with that in the wild-type strain EP155/2 (Fig. 2). Northern blot analysis of RNA from UEP1 cultured under induction conditions with tannic acid revealed no hybridizing *lac3* band, indicating that the hypoviral infection specifically prevented the induction of the *lac3* transcript by tannic acid.

Construction of a *lac3*-null mutant.

To explore the effects of deletion of the *lac3* gene, a *lac3*-null mutant was constructed by site-directed recombination during integrative transformation. A linearized plasmid DNA containing the disrupted *lac3* gene was used to transform the virus-free *C. parasitica* EP155/2. A total of 750 single-spore transformants were screened by PCR using outer and inner primers corresponding to nt -28 to -11 and 2,190 to 2,210, respectively, relative to the start codon (Fig. 3A). All but one transformant showed both 2.2- and 3.2-kb amplicons, corresponding to the wild-type and disrupted alleles of the *lac3* gene, respectively, suggesting that transformants were ectopic, resulting from random integrations of the transforming vector. However, one transformant did not contain the wild-type 2.2-kb amplicon but showed only the 3.2-kb fragment of the disrupted allele of the *lac3* gene, suggesting that the simple replacement of the wild-type allele with the disrupted allele occurred (data not shown). The putative *lac3*-null mutant (TdLAC3-63-6) was examined by Southern blot analysis (Fig. 3B). The hybridization pattern of *Bam*HI- and *Xho*I-digested genomic TdLAC3-63-6 DNA with a probe corresponding to a

Fig. 2. Northern blot analysis of *lac3* using the nearly full-length cDNA fragment as a probe. Total RNA was extracted from the strains grown on tannic acid (TA)- and citric acid (CA)-containing plates. Numbers indicate the hours after induction. Equal loading of RNA samples is demonstrated in the bottom panel, with a parallel blot that was hybridized with the glyceraldehyde-3-phosphate dehydrogenase gene (*Gpd*) probe as an internal control, and an ethidium bromide-stained gel (rRNA). The strains used, indicated above the panel, were the virus-free wild type (EP155/2), its isogenic virus-containing hypovirulent strain (UEP1), and the phospholipase C gene *cplc1*-null mutant (TdPLC1).

1.1-kb *Xho*I fragment differed from that of the wild type, suggesting that the transforming vector integrated at the *lac3* locus by site-directed homologous recombination (Fig. 3). Moreover, *Xho*I-digested TdLAC3-63-6 genomic DNA showed a hybridizing band of 4.6 kb, corresponding to the expected size of the gene-replacement vector pDlac3 (Fig. 3A), and the 4.6-kb band also hybridized to the 0.8-kb *Eco*RI/*Bam*HI-*hph* fragment, indicating that the *lac3* gene was replaced with part of the transforming vector rather than having been disrupted, as expected.

Phenotypic characteristics of the *lac3*-null mutant.

The mycelial growth rate of the *lac3*-null mutant was not changed on PDAMB in standard growth conditions (constant low-level light at 25°C; Fig. 4). No difference in the growth rate as compared with that of the wild type was observed on minimal medium. However, when the *lac3*-null mutant was grown on minimal medium supplemented with tannic acid, the mycelial growth rate was significantly reduced to less than 55% of the radial growth of the wild type (Fig. 4). Surprisingly, even though the *lac3* gene was strongly induced in the presence of tannic acid, no change in the growth rate was observed on tannic acid-containing modified Bavendamm's medium. In

addition, no significant change in the size or darkness of the brown coloration was observed in the *lac3*-null mutant. Therefore, these results suggest that, although the protein product of the *lac3* gene is likely to be most important for *C. parasitica* to metabolize tannic acid, some other laccases or enzymes, such as laccase1 or peroxidases, respectively, may also be responsible for the brown coloration on modified Bavendamm's medium.

To ensure that the phenotypic changes attributed to the *lac3*-null mutant were due to *lac3* gene replacement, the *lac3*-null mutant TdLAC3-63-6 was complemented in trans with a wild-type allele of the *lac3* gene. When TdLAC3-63-6 was transformed with the *lac3* genomic clone, the defective growth on minimal medium containing tannic acid was corrected, confirming that the phenotypic changes in the *lac3*-null mutant were due to the specific deletion of the *lac3* gene.

The asexual sporulation of TdLAC3-63-6 was compared with that of the virus-free EP155/2 and virus-containing UEP1 strains. There was no significant difference in the asexual sporulation of TdLAC3-63-6 and EP155/2. The number of conidia produced by the hypovirulent UEP1 was significantly lower than that of the other two strains, as expected. No changes in pigmentation were caused by the loss of function of the *lac3* gene (Fig. 4).

Osmotic and temperature sensitivity of the *lac3*-null mutant.

During cultivation under hypertonic conditions (PDAMB supplemented with 2 M sorbitol) (Park et al. 2004), the *lac3*-

Fig. 3. Restriction and Southern blot analyses of the *lac3*-null mutant (TdLAC3-63-6) and the wild-type strain EP155/2. A, Restriction map of the *lac3* genomic region and the gene-replacement vector pDlac3, which contains 1,890- and 1,370-bp fragments from the 5' and 3' flanking regions, respectively. B, *Bam*HI; E, *Eco*RI; S, *Sal*I; ScI, *Sac*I; and X, *Xho*I. B, Southern blot analysis of the wild-type EP155/2 strain (lane 1) and the *cpl*1-replaced transformant TdLAC3 (lane 2). All DNA samples were digested with *Bam*HI or *Xho*I. The blots were probed with the 1.1-kb *Xho*I fragment (probe 1) and the 0.8-kb *Eco*RI/*Bam*HI-*hph* fragment (probe 2). The TdLAC3-63-6 transformant has undergone the desired replacement at *lac3*, as evidenced by expected size changes in fragments that hybridized with probe 1 and the hybridization of the corresponding band with probe 2. The probes are indicated in the restriction map of the *lac3* gene in A.

Fig. 4. Colony morphologies on different media. The colony morphologies of four strains after 14 days of cultivation on different media are shown. The growth media, indicated at the left, are potato dextrose agar containing 100 mg of L-methionine and 1 mg of biotin per liter (PDAMB), minimal medium (MM), and malt extract medium (ME); (+) indicates the inclusion of 0.7% tannic acid. The strains used, indicated above the panel, were the virus-free wild type (EP155/2), its isogenic virus-containing hypovirulent strain (UEP1), the *lac3*-null mutant (TdLAC3-63-3), and the *lac3*-complemented strain (TcLAC3).

null mutant and the wild-type strain EP155/2 showed similar hyphal growth patterns. These results agree well with those of Northern blot analysis of the osmosensitive *cpmk1*-null mutant, which showed no changes in the induction of the *lac3* gene. These facts strongly suggest that the loss of function of the *lac3* gene caused no changes in the osmotic sensitivity.

The temperature sensitivity of the *lac3*-null mutant was examined at various temperatures. No significant changes in relative growth rate were observed, suggesting that the mutant is not temperature sensitive.

Virulence assay of the *lac3*-null mutant.

In a virulence assay on excised chestnut tree bark (Lee et al. 1992), the size of the necrotic area induced on excised bark by the *lac3*-null mutant was smaller than that of the wild-type strain EP155/2, indicating that the *lac3*-null mutant is less virulent than EP155/2 (Table 1). The *lac3*-complemented strain of the mutant caused a necrotic area of size similar to that of the wild type, suggesting that the *lac3* gene is involved in determining the virulence of this fungus. However, the *lac3*-null mutant produced a larger necrotic area than the hypovirulent strain UEP1, indicating that although the *lac3* gene appears to be a pathogenic determinant, clearly other factors contribute to fungal pathogenicity as well.

Characteristics and altered virulence of the *lac3* transformants.

As an approach to modulate the expression of the *lac3* gene, a fusion construct replacing the *lac3* promoter with a constitutive and hypoviral-independent promoter (Kim et al. in press) was used to transform the wild-type strain EP155/2. The resulting transformant, T188::*lac3*-1, was then single-spored and used for coculture with the hypovirulent strain UEP1 to obtain a hypovirus-containing transformant, T188::*lac3*-1/CHV1. As shown in Figure 5, the transformant T188::*lac3*-1 was indistinguishable from the parental wild-type EP155/2 in conidiation, pigmentation, and colony morphology on PDAMB medium under standard growth conditions. Upon transfer of CHV1 through anastomosis, T188::*lac3*-1/CHV1 exhibited the cultural characteristics of UEP1. However, Northern blot analysis revealed the accumulation of the *lac3* transcript in the transformant T188::*lac3*-1 grown in EP complete medium, whereas no transcript was observed in the wild-type EP155/2, suggesting that the transformant T188::*lac3*-1 does not require tannic-acid induction for the expression of the *lac3* gene. Moreover, the hypovirus-containing transformant T188::*lac3*-1/CHV1 showed considerable accumulation of the *lac3* transcript, confirming that the *lac3* expression derived from the cryparin core promoter became constitutive rather than inducible in this transformant,

and its expression was not affected by the presence of CHV1. Examination of the laccase activities in culture filtrates showed that the enzyme activity of the transformant T188::*lac3*-1 was higher than that of EP155/2. In addition, the hypovirus-containing transformant T188::*lac3*-1/CHV1 showed higher laccase activities than UEP1, in good agreement with the Northern blot results. These results indicate that the modulation of *lac3* gene expression resulted in no detectable changes in the morphological characteristics of the fungus. In addition, the *lac3* induction in response to tannic-acid supplementation appeared to be determined by the promoter activity at the transcription

Table 1. Role of the *Cryphonectria parasitica* *lac3* gene in virulence^w

Strain or mutant	<i>lac3</i> genotype ^x	CHV1 ^y	Canker area ^z
EP155/2	+	–	123.2 ± 5.7 a
UEP1	+	+	76.3 ± 2.2 b
TdLAC3-63-3	–	–	98.3 ± 1.9 c
TcLAC3	+	–	120.1 ± 11.3 a
T188:: <i>lac3</i> -1	C	–	131.4 ± 21.7 a
T188:: <i>lac3</i> -1/CHV1	C	+	103.0 ± 4.1 c

^w Virulence was measured using excised chestnut tree bark, as described previously (Lee et al. 1992). Three replicates for each strain were used, and each experiment was repeated three times. Lesion measurements are shown as mean ± standard deviation (mm²).

^x + and – indicate the presence or absence of the *lac3* gene, respectively. C indicates the constitutive expression of the *lac3* gene.

^y + and – indicate the presence or absence of CHV1, respectively.

^z Values followed by the same letter are not significantly different ($P < 0.05$), as determined by Duncan's multiple-range test.

Fig. 5. Characteristics of transformants showing modulated expression of the *lac3* gene. **A**, Colony morphology on potato dextrose agar containing 100 mg of L-methionine and 1 mg of biotin per liter (PDAMB). **B**, Ethidium bromide-stained gel showing the characteristic 12.7-kb genome of CHV1. The arrow indicates the viral genome of CHV1. **C**, Northern blot analysis of *lac3* expression. Total RNA was extracted at the times indicated in hours after inoculation. Equal loading of RNA samples is shown with a parallel blot that was hybridized with the glyceraldehyde-3-phosphate dehydrogenase gene (*Gpd*) probe as an internal control. An ethidium bromide-stained gel (rRNA) is also shown. **D**, Laccase activities of culture filtrates of transformants. The laccase activity was determined in filtered media harvested 3 days after inoculation in EP complete medium. The strains used, indicated above the panel, were the virus-free wild type (EP155/2), its isogenic hypovirus-containing hypovirulent strain (UEP1), the constitutive *lac3*-expressing transformant (T188::*lac3*-1), and the hypovirus-containing *lac3* transformant (T188::*lac3*-1/CHV1).

level, and the increased laccase activities in culture filtrates of the transformants suggested that the protein product of the *lac3* gene is secreted as an extracellular enzyme.

In the virulence assay, no significant difference in lesion size was observed between the transformant T188::*lac3*-1 and its parental wild-type strain EP155/2. However, the hypovirus-containing transformant T188::*lac3*-1/CHV1 did cause a larger lesion than the hypovirulent strain UEP1 but not as large as that caused by the wild-type (Table 1). These results suggest that the constitutive expression of the *lac3* gene alone did not further increase the fungal virulence of the wild-type EP155/2. However, when the expression of the *lac3* gene was severely hampered by the presence of CHV1, such as in the hypovirulent strain UEP1, the restoration of *lac3* expression made it possible to enhance the fungal virulence. Therefore, it appears that the expression of the *lac3* gene may not be necessary and sufficient as a single determinant of virulence but its expression at a certain level contributes to the maintenance of wild-type fungal virulence.

DISCUSSION

At least three laccases were predicted to be present in *C. parasitica* (Kim et al. 1995). Two are extracellular laccases, one constitutive (*lac1*) and the other tannic acid-inducible (*lac3*), and one is an intracellular laccase (*lac2*) (Kim et al. 1995). In this study, a new laccase-encoding gene and its corresponding cDNA were isolated from *C. parasitica*. Based on the sequence divergence from the *lac1* gene structure having a canonical secretory signal peptide and tannic acid induction, the new laccase gene appeared to be the *lac3* gene. In *lac3*, the single cysteine residue and 10 histidine residues that are required to bind the four catalytic cupric ions in all blue copper oxidases are conserved. Studies of type-I copper centers have shown that an additional residue 10 amino acids downstream of the conserved cysteine residue has a major effect on the redox potential of the cupric ion (Temp and Eggert 1999). This residue is a leucine residue in the *lac3* gene, but phenylalanine and methionine residues are present in the laccase sequences of other fungi and plants (Jarai et al. 1992). Based on its postulated redox potential (Eggert et al. 1998), the protein product of the *lac3* gene corresponds to a class 2 laccase. The *Aspergillus nidulans* laccase, which contains a methionine residue at the site discussed above, belongs to class 1 and exhibits a very low similarity to all other known laccases, reflecting the very specialized function of spore morphogenesis in this fungus. In addition, the *Pycnoporus cinnabarinus* laccase, with a phenylalanine residue at the crucial site, belongs to class 3 and appears to be the only phenoloxidase but not a lignin peroxidase or manganese peroxidase; a single isoform is produced under conditions of lignin degradation. These results demonstrate the variety of specialized physiological functions of laccases. Interestingly, the deduced sequence of the previously characterized *C. parasitica* laccase1 revealed only 56% similarity to laccase3 at the amino-acid level but both are class 2 laccases, suggesting that the enzymes have a similar enzymatic function in different environments, whether or not tannic acid is present.

Tannic acid is a strong laccase3 inducer. Fungi are diverse in their responses to putative laccase inducers, and differences in the regulation of gene transcription may reflect different physiological functions for fungal laccases. Most well-known inducers are toxic compounds that could resemble either breakdown products of lignin or antimicrobial agents secreted by hosts or competing fungi. Thus, the elicitation effect most likely represents a response to eliminate such toxic compounds by enzymatic transformation. However, some fungi may not require such an enhanced defense reaction, e.g., the ligninolytic

basidiomycete PM1, in which laccase activity is not stimulated by several putative laccase inducers (Coll et al. 1993). Likewise, in *C. parasitica*, elicitation was not observed with other well-known lignin-related inducers but instead by tannic acid, which is abundant in the bark of the chestnut tree (Griffin 1986). These results are in good agreement with the fact that *C. parasitica* is not a wood-decaying fungus but a canker-causing fungus, which is not involved in lignin degradation but must deal with the natural host barrier of tannic acid.

At least three laccases are present in *C. parasitica* and all appear to be under the control of complex but specific regulation (Kim et al. 1995). Previous studies have shown that both positive and negative pathways are involved in the modulation of *lac1* expression (Larson et al. 1992). Moreover, the IP_3/Ca^{2+} second-messenger system regulates the viral-specific stimulatory pathway of *lac1* expression, and the negative pathway appears to depend on ongoing protein synthesis for the proteinaceous inhibitory factor (Larson et al. 1992). In addition, the presence of the wild-type *cplc1* is a prerequisite for both proper expression and modulation of the *lac1* transcript (Chung et al. 2006). Our results indicate that the expression of the *lac1* gene is unaffected by the presence of tannic acid, and *cplc1*, which is necessary to mediate the appropriate expression of the *lac1* gene, is not required for the induction of the *lac3* gene by tannic acid. Considering the different responsiveness to tannic acid and differences in the requirement for the *PLC* gene, we propose the following model for the regulatory pathways that govern the expression of *C. parasitica* laccases (Fig. 6). There are at least two regulatory pathways that regulate the expression of laccases, and the expression of each laccase is governed independently by the corresponding specific pathway. Given that laccase1 and laccase3 each has its own specific regulatory pathway, it is of interest to see how the hypovirus can regulate both specific regulatory pathways. The small genome size of CHV1-713 and the efficient use of the viral genome suggest that, although the regulatory pathways for laccase expression are independent, there may be a common denominator in appropriate laccase expression, which could be the primary target of the viral regulation of fungal laccase expression. It is possible that this common denominator is the beginning component in a regulatory hierarchy, although the opposite regulatory cascade is still possible. This hypothesis of distinct regulation mechanisms for laccase expression suggests different physiological roles for these enzymes. However, complicating the general gene regulation-function relationship, the induction of a specific laccase gene does not necessarily have a beneficial effect on fungal fitness. In *Botrytis cinerea*, the laccase *Bclcc2* is strongly expressed in the presence of either tannin or resveratrol, a phytoalexin from grapevine;

Fig. 6. A possible model of the regulatory pathway governing the modulation of hypoviral-specific laccases. Arrows indicate stimulatory or essential pathways, and lines with a bar at the end indicate inhibitory pathways for laccase expression. RPS = proteinaceous inhibitory factors, and CHX = cycloheximide.

however, the protein product of *Bclcc2* did not detoxify resveratrol, but rather, converted it into compounds that are more toxic to the fungus, resulting in self-intoxication (Schouten et al. 2002).

Although fungal laccases participate in a variety of physiological processes, they can be grouped into three major biological processes, i.e., lignin metabolism, fungal morphogenesis, and defense reactions. Defense reactions in a living host can be categorized as protection against host oxidative responses, detoxification of adverse phenolic compounds, and pigmentation (Hoegger et al. 2004). Based on the strong induction of the *lac3* transcript by tannic acid, which is abundant and assumed to be one of the major barriers against pathogen infection, it was unexpected that the *lac3*-null mutant showed no significant changes in colony morphology or brown coloration when cultured on a tannic acid-containing malt extract medium, Baven-damm's medium. However, deletion analysis of *lac3* indicated that LAC3 is involved in mycelial growth under stressed conditions, such as on minimal medium containing supplemented tannic acid. Because the *lac3* gene is induced by tannic acid and is required for active growth in nutrient-poor conditions, which may be reminiscent of chestnut bark, the biological function of the *lac3* gene seems to be defense against the host barrier and facilitates the fitness of the pathogenic *C. parasitica* in the hostile tannin-rich environment of chestnut bark. This speculation agrees well with the fact that the *lac3*-null mutant caused a smaller necrotic area on excised chestnut bark than did the wild type. In addition, the virulence assay indicated that, although the constitutive expression of *lac3* by itself did not increase fungal virulence, its expression in the presence of the hypovirus was able to restore fungal virulence to an intermediate level. Therefore, *lac3* may not be necessary and sufficient for virulence, but it is an essential factor for *C. parasitica* to maintain an appropriate level of virulence in response to the host defensive barrier.

MATERIALS AND METHODS

Fungal strains and growth conditions.

The CHV1-713-containing hypovirulent *C. parasitica* UEP1 and its isogenic virus-free strain EP155/2 (ATCC 38755) were maintained on PDAMB at 25°C under constant low-level (< 2,000 lx) light (Kim et al. 1995). The preparation of the primary inoculum for liquid and plate cultures and the culture conditions were as described (Kim et al. 1995).

Radial growth on plates was assessed by measuring colony diameters. To determine biomass, mycelia were filtered through Miracloth (Calbiochem, La Jolla, CA, U.S.A.), were washed with sterile distilled water, were blotted between dry paper towels until no excess water emerged, were weighed to obtain the fresh weight, and were further dried at 80°C for 2 days, as described previously, to obtain the dry weight (Kim et al. 1995).

The acute induction of the *lac3* gene was performed as follows. Ten agar plugs (0.5 cm diameter) containing actively growing young hyphae were inoculated on cellophane that was layered on the top of PDAMB medium and were incubated until the mycelia reached the end of the petri plate. The cellophane and the actively growing *C. parasitica* were then transferred to a plate containing an appropriate concentration of inducing materials. To determine the effect of tannin on *lac3* induction, tannic acid (Sigma, St. Louis) was supplemented as described previously (Rigling and Van Alfen 1993). Likewise, the induction of the *lac3* gene by aromatic compounds and copper was measured using 2,5-xyldine and ferulic acid (Bollag and Leonowicz 1984; Collins and Dobson 1997; Soden and Dobson 2001; Yaver and Golightly 1996) and CuSO₄ (Giardina et al. 1999; Palmieri et al. 2000), respectively, as described. Citric acid was used to determine the effect of acidity on *lac3* expres-

sion (Kim et al. 2001). For chronic induction of the *lac3* gene, growth media containing corresponding components were inoculated with an appropriate strain and were incubated under standard culture conditions.

Isolation and characterization of an inducible laccase gene, *lac3*.

Degenerate primers specific for consensus nucleotide sequences corresponding to the most conserved amino acids in the inducible laccases were designed. The primers used were LAC3 (forward) 5'-GGNCARAGRTAYGAYGTN-3' and LAC3 (reverse) 5'-RTGCCAKGCRATRTGGCA-3'. PCR was conducted as described previously (Kim et al. 2002). The 833-bp PCR amplicon was inserted into the pGEM-T vector (Promega, Madison, WI, U.S.A.). The inserted DNA fragments of positive bacterial clones were sequenced using the dideoxynucleotide method and were analyzed before being used as hybridization probes for genomic λ library screening according to a previously described procedure (Kim et al. 2002).

To obtain the *lac3* cDNA clone, PCR using reverse transcriptase was performed with the cLAC3-F1 (forward) 5'-TCTCGTCATTATGGCCCT-3' and cLAC3-R1 (reverse) 5'-TATGCCCGAATCGTCCTGCAC-3' primers. The cDNA was sequenced using the dideoxynucleotide method and synthetic oligonucleotide primers.

A primer extension experiment was performed to identify the transcriptional initiation site. The reverse primer 5'-CTGCGGTCATCTGCCGAGTTGAGA-3' was used along with standard methods (Kim et al. 2002).

Southern and Northern blot analysis.

Genomic DNA from *C. parasitica* was extracted as described (Churchill et al. 1990). DNA (10 μ g) was digested with restriction enzymes, was blotted onto a nylon membrane, and was hybridized with radioactively labeled probes.

RNA from liquid cultures was extracted as described (Kim et al. 1995). RNA from solid cultures was prepared from mycelial mats grown on cellophane that was layered on top of appropriate medium as described previously (Park et al. 2004). Northern blot analysis was conducted as described (Kim et al. 1995). The levels of *lac3* transcript in *C. parasitica* were assessed using the glyceraldehyde-3-phosphate dehydrogenase gene as the internal control (Choi and Nuss 1990).

Construction of a replacement vector and fungal transformation.

The replacement vector pDlac3, which was designed to favor double-crossover integration events, was constructed as follows. The 5.0-kb *SacI* fragment containing the 5' and 3' flanking regions of *lac3* was ligated into *SalI*-inactivated pBluescriptII SK (+), and the resulting plasmid was used as a template for inverse PCR, using the primers 5'-GGTCCGACCACCGTAGACGCCGTCCCA-3' and 5'-GGTCCGACCAACGGAGAGAGAAGAG-3'. The primers were modified to incorporate the restriction site for *SalI* (underlined). The PCR amplicon was digested with *SalI* and religated before transformation into *Escherichia coli*. The resulting plasmid was digested further with *SalI* and was inserted along with the 2.4-kb *SalI* fragment of pDH25 (Cullen et al. 1987), which carries the hygromycin phosphotransferase gene (*hph*) cassette. In the replacement vector pDlac3, the *hph* cassette was inserted between nt 759 and 2,208 of the *lac3* gene, relative to the start codon, and was flanked by approximately 1,890 and 1,370 bp of the 5' and 3' end sequences, respectively. The *SacI*-digested insert fragment of pDlac3 was then used to transform the virus-free EP155/2 strain.

Functional complementation of the *lac3*-null mutant was carried out using the wild-type allele. The complementing vec-

tor pRlac3 was constructed by insertion of the 2.6-kb *Hind*III fragment of pBen⁺ (Chung et al. 2006) into the *Hind*III-digested pLac3, which contains the full-length *lac3* gene, in pBluescriptII KS (+). The resulting vector was then used to transform the *lac3*-null mutant.

Protoplast preparation and transformation were performed as described previously (Churchill et al. 1990; Kim et al. 1995, 2002). PCR and Southern blot analyses were conducted on the genomic DNA of the transformants to screen for gene replacement and *trans*-complementation of the *lac3* gene.

Construction of a vector for constitutive and hypoviral-independent expression of the *lac3* gene.

To modulate the expression pattern of the *lac3* gene, the core promoter of the cryparin gene was selected, because it is a strong constitutive promoter and is not affected by the presence of hypovirus (Kim et al. in press). The strong core promoter of the cryparin gene, consisting of the fragment between nt -188 and the translation start codon, was used to replace the corresponding endogenous *lac3* promoter. The expression vector pCrylac3 was constructed using an overlap extension PCR method (Ge and Rudolph 1997). Briefly, the 188-bp PCR fragment of the core promoter was obtained from pCEB7 with the primers pCry-F (forward: 5'-GGGAGCTCTCGAGACAGGA C-3') and pCry-R (reverse: 5'-GAAGGGCCATTTTGATTGA AGTTTGGAGGG-3'). The primers plac3-F (forward: 5'-AAT CAAATGGCCCTTCGAGCCTTT-3') and plac3-R (reverse: 5'-TTGAGCTCTTATATGCCCGAATCGTC-3') were used to amplify the *lac3* gene. The underlined sequences indicate the junction between the core promoter and the *lac3* gene.

Transmission of the dsRNA virus.

Virus transmission was performed as previously described (Cortesi et al. 2001). Briefly, mycelial plugs of the virus-containing strain UEP1 were placed on PDAMB medium adjacent to mycelial plugs of the virus-free recipient transformants. After 10 days of coculture, putatively fused mycelia at the border between each pair of strains were transferred to fresh PDAMB and were incubated until they presented a sector that showed viral symptoms, such as reduced pigmentation. Mycelia in the low-pigmented region were transferred to benomyl-containing medium to select for the virus-infected recipient transformants. Strains were single-spored, and the presence of hypovirus was confirmed by the purification of dsRNA from the single-spored isolates.

Characterization of the *lac3*-null mutant.

The phenotypic and molecular characteristics of the *cplc1*-null mutant were compared with those of the wild-type EP155/2 and hypovirulent UEP1 strains.

Phenotypic changes in pigmentation, conidiation, and mating capability were measured as described (Kim et al. 1995). Responses to chronic hyperosmotic stress and temperature sensitivity were also measured as described (Chung et al. 2006; Park et al. 2004). A virulence test using excised chestnut tree bark was conducted as described (Lee et al. 1992). Laccase activity was judged based on the coloring of the modified Bavendamm's medium (0.7% tannic acid, 1.5% malt extract, and 2.0% agar) on which the strains were grown (Rigling et al. 1989), and activity in the culture fluid was determined by a spectrophotometric assay using 2,6-dimethoxyphenol as the substrate (Rigling et al. 1989).

ACKNOWLEDGMENTS

This work was supported by a Korea Science and Engineering Foundation (KOSEF) grant funded by the Korean Government (MOST) (R01-

2007-000-10369-0). We thank the Research Center of Bioactive Materials and the Research Center for Industrial Development of BioFood Materials at Chonbuk National University for kindly providing the facilities for this research.

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