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# A New Extracellular Laccase of *Cryphonectria parasitica* Is Revealed by Deletion of *Lac1*

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**The biological function of the extracellular laccase (Laccase 1) of *Cryphonectria parasitica* was investigated by deletion of *Lac1* using recombinant DNA techniques. *Lac1* is one of the genes of this fungus which is down-regulated by the hypovirulence-causing dsRNA virus, CHV1. This virus causes a variety of symptoms of the fungus, including hypovirulence, reduced sporulation, and reduced pigmentation. A transforming vector for gene deletion was constructed by replacing the structural gene of laccase with the hygromycin B resistance gene (*hph*) from *Escherichia coli*. The *Lac1* null mutation was found to have no effect on fungal virulence, pigmentation, conidiation, conidial germination, or sexual crossing capability. A previously unknown inducible extracellular laccase, however, was detected when the mutant strain was grown in tannic acid. The observed lack of phenotype associated with deletion of *Lac1* may be the result of compensation by this newly discovered extracellular laccase.**

*Cryphonectria parasitica* (Murrill) Barr, causal agent of chestnut blight, was responsible for the destruction of the chestnut forests of North America during the early part of this century. Strains of the fungus containing double-stranded (ds)RNA viruses have been isolated from nature and are responsible for a biological control of this disease, particularly in Europe (Van Alfen *et al.* 1975; Day *et al.* 1977; Fulbright *et al.* 1983; Griffin *et al.* 1983). The best studied of these viruses, CHV1, causes hypovirulence, reduced sporulation, and a reduction in colony pigmentation when infected strains are grown in culture, yet there is no detectable effect of the virus on growth of the fungus in culture. These effects of the virus on fungal virulence and sporulation are responsible for the observed biological control of the fungus.

The symptoms of virus infection appear to be the result of reduced expression of a small number of specific fungal genes in viral infected strains (Powell and Van Alfen 1987a, 1987b). Specific deletion of *Vir2*, one of these viral suppressed genes, resulted in a phenotype that mimicked a portion of the viral-induced, sporulation-related symptoms (Zhang *et al.* 1993). Other fungal genes cloned from *C. parasitica* that have been shown to be down-regulated by the virus are *Vir1*, a gene that also appears to be sporulation-related (Powell and Van Alfen 1987a; L. Zhang and N. K. Van Alfen, unpublished), *Crp*, a gene encoding an abundant tissue-specific cell-surface hydrophobin (Zhang *et al.* 1994), and *Lac1*, an extracellular laccase (Rigling and Van Alfen 1991; Choi and Nuss 1992). These genes are down-regulated by the virus at the transcription level and are consequently thought to be coordinately regulated in normal strains of the fungus (P. Kazmierczak, P. Pfeiffer, L. Zhang, and N. K. Van Alfen, unpublished).

Laccase activity, as assessed by the Bavendamm (1928) procedure has been commonly used as a qualitative assay of the effect of CHV1 on *C. parasitica* (Rigling *et al.* 1989; Choi and Nuss 1992). The effect of the virus on laccase activity was first demonstrated by Rigling *et al.* (1989), and on *Lac1* gene expression by Rigling and Van Alfen (1991). It has recently been reported that *C. parasitica* produces two different laccases, LAC1, the extracellular laccase (formerly LacA), and LAC2, an intracellular laccase (formerly LacB), and that the virus affects the activity of both enzymes (Rigling and Van Alfen 1993). Larson *et al.* (1992) postulate that the virus affects expression of the extracellular laccase by affecting signal transduction pathways which regulate *Lac1* expression. The demonstrated value of laccase activity as a marker for CHV1 effects on *C. parasitica* prompted us to further investigate the laccases of this fungus. In particular, we were interested in understanding the role of the extracellular *Lac1* in the biology of this fungus.

Fungal laccases (benzenediol:oxygen oxidoreductase, EC 1.10.3.2) are multi-copper-containing phenol oxidases which oxidize a large variety of phenolics. Fungal laccases have been implicated in the pigmentation of fungal spores (Clutterbuck 1972), development of a fungal fruiting body (Leatham and Stahmann 1981), fungal pathogenicity (Marbach *et al.* 1985; Geiger *et al.* 1986; Rigling *et al.* 1989), lignin degradation (Ander and Eriksson 1976), detoxification of lignin degradation products (Reinhamma 1972), changes leading to sexual differentiation (Aisemberg *et al.* 1989), and rhizomorph formation (Worrall *et al.* 1986). However, with the exception of the conidial laccase of *Aspergillus nidulans* (Clutterbuck 1972; Law and Timberlake 1980; Yelton *et al.* 1985), the biological functions of fungal laccases remain unclear. Since CHV1 affects virulence, sporulation, and pigmentation of *C. parasitica*, we were interested in learning if *Lac1*

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plays an important role in any of these functions of *C. parasitica*, and thus repression of this gene could result in some aspect of viral symptom production. We report here that deletion of *Lac1* resulted in no detectable changes in fungal virulence, sporulation, or pigmentation. Deletion of *Lac1* revealed a second extracellular laccase (LAC3), a redundancy that suggests an important role for the extracellular laccases of this fungus.

## RESULTS

### Deletion of *Lac1* by transformation.

The *Lac1* genomic clone isolated using the *Lac1* cDNA clone (Rigling and Van Alfen 1991) was partially sequenced and found to be identical to the *lac-1* gene isolated by Choi *et al.* (1992). We also found that the transcription initiation site, determined by primer extension, was in agreement with the information reported for the *lac-1* gene. The portion of the *Lac1* gene replaced by *hph* in the transforming vector spanned the reported *lac-1* sequence from 238 to 3,326 bp.

A total of 83 putative transformants were transferred from regeneration medium to PDAmb containing 50 μg/ml hygB. These transformants were single spored and then serially transferred three times on PDAmb medium without hygB. Of these, 52 lost their hygB resistance during the serial transfers. The 31 stable transformants were examined for laccase activity. One transformant, lac84-1, had no detectable laccase activity in its culture filtrate but there were no differences in intracellular laccase (Fig. 1A) and biomass accumulation of this transformant from the wild-type EP155/2 (Rigling and Van Alfen 1993). This transformant was indistinguishable in culture morphology and pigmentation from the wild-type EP155/2.

Southern blot analysis of DNA from EP155/2, UEP1, and lac84-1, are shown in Figure 2A. The DNA was digested with *Eco*RI or *Xho*I and the Southern blot probed with either probe 1 (1.2 kb *Bgl*II-*Hpa*I fragment covering the 3′ region of the laccase gene) and/or probe 2 (3.6-kb *Eco*RI fragment covering *Lac1* gene and the 5′ flanking region of the *Lac1* gene). The resultant blots showed a different banding pattern for the

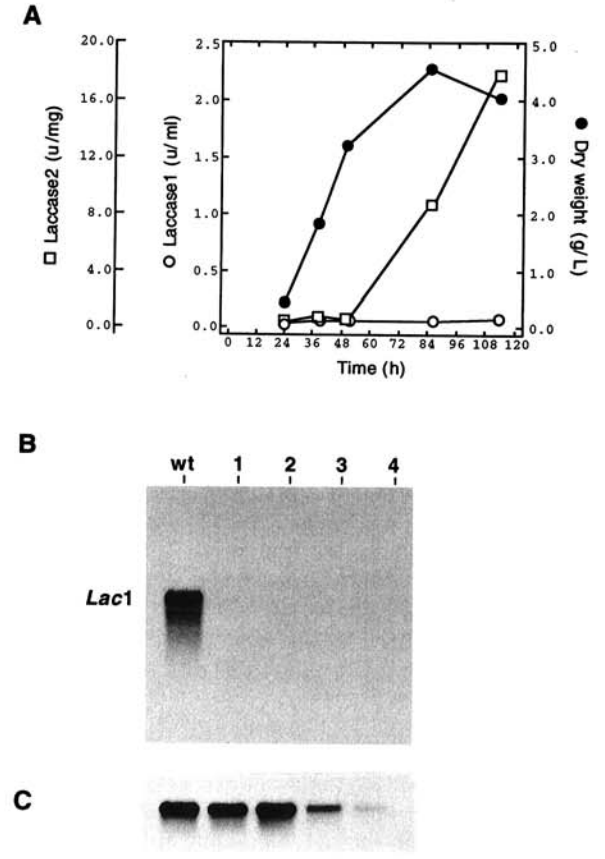


**Fig. 1.** Laccase production of *Lac1* null-mutant (lac84-1). **A,** Biomass (dry weight), and laccase 1 and 2 activity were followed as function of time after inoculating EP complete liquid medium with lac84-1. Each point shows the data collected from a single flask. **B,** Laccase 1 Northern blot analysis. Total RNA was extracted at each time shown above (except for 24 hr) and blotted and hybridized to a *Lac1* specific probe. wt, represents total RNA from the wild-type strain EP155/2. **C,** The *Lac1* probe was stripped from the blot, which was then hybridized to a probe for *Crp* (Zhang *et al.* 1994). Expression of *Crp* mRNA accumulation characteristically decreases after the fungus reaches stationary growth.

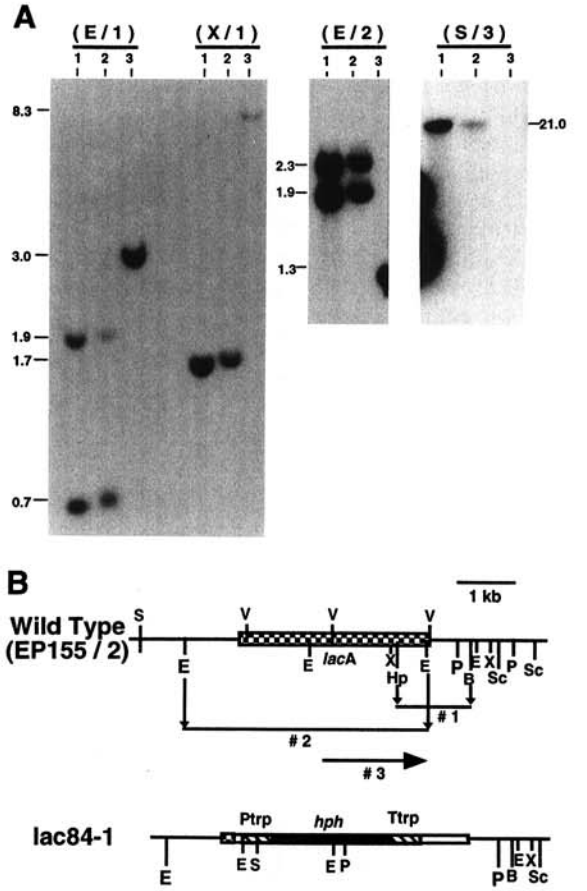


**Fig. 2.** Southern analysis and the restriction map of the *Lac1*- null mutant (lac84-1) compared with wild-type EP155/2. **A,** Restriction enzyme and probe combinations for Southern blots are given in parenthesis; i.e. (E/1) is Southern blot of *Eco*RI digested DNA probed with probe 1. Lane numbers refer to strains of *Cryphonectria parasitica*; 1 (EP155/2), 2 (UEP1), and 3(lac84-1). Numbers on left and right refer to the size of bands in kilobases. Probes are shown in the map in Figure 2B. **B,** Restriction map of (lac84-1) compared with a wild-type EP155/2. Restriction sites refer to Figure 5. Arrow of cDNA probe 3 shows the transcriptional direction.

DNA from lac84-1 compared with that of EP155/2, indicating that the transforming vector integrated at *Lac1* by site-directed homologous recombination. Moreover, when lac84-1 DNA was digested with *Eco*RI or *Xho*I, which have double and a single cutting site, respectively, and the resulting blot probed with either probe 1 and/or probe 2, a single band hybridized to both probes. The results indicate that *Lac1* was replaced with part of the transforming vector, rather than disrupted. Probe 3, which consisted of a 1.6-kb fragment from the 3′ end of the *Lac1* gene, hybridized to a single band on Southern blots of EP155/2, and UEP1 DNA, but did not hybridize to lac84-1 DNA, a result consistent with the deletion of Lac1 from lac84-1. A restriction map of lac84-1, based on the results of the Southern blots, is shown in Figure 2B. Northern blot analysis probed with *Lac1* partial cDNA clone (Rigling and Van Alfen 1991) showed the presence of the 2.3-kb *Lac1* transcript in EP155/2, but not in lac84-1 (Fig. 1B). The same blot rehybridized with the *Crp* gene probe indicates a proper loading of sample RNA and a normal expression of *Crp* mRNA in lac84-1 (Fig. 1C).

**Virulence.**

The *Lac1* null mutant was as virulent as the wild-type EP155/2, while UEP1 produced a significantly smaller canker on the dormant chestnut stem after 5 wk (Table 1).

**Asexual sporulation.**

Asexual sporulation of lac84-1 was compared with that of strains EP155/2 and UEP1 (Table 1). There was no significant difference in asexual sporulation between lac84-1 and EP155/2. The numbers of conidia produced by the hypovirulent strain, UEP1, were significantly lower than those of the other two strains, as expected.

**Germination of conidia.**

The percentage of conidia of lac84-1 that germinated did not differ from those of EP155/2 and UEP1. More than 70% of the conidia from the three strains tested germinated in the methionine and biotin-supplemented water. The percentage of conidia that germinated was compared at different times after the spores were harvested. There were no differences in the germination of lac84-1 and EP155/2 at any of the observed times (Table 1).

**Sexual mating.**

Sexual mating capability of lac84-1 was examined on stems of chestnut trees. This *Lac1* null mutant strain produced fertile perithecia containing characteristic asci and two-celled ascospores (data not shown). No impairment of sexual mating was observed as a result of the mutation.

**Induced extracellular laccase.**

Because one of the postulated roles for LAC1 is detoxification of tannic acid present in the bark of infected trees, we examined the effect of tannic acid in the growth medium on laccase activity. EP155/2, UEP1, and lac84-1 were grown in EP complete medium containing three different concentrations of tannic acid, 4, 8, and 16%. Extracellular laccase activity was determined after 2.5, 4.5, and 7 days of growth. Laccase activity was detectable in the culture filtrates of all three strains in the presence of tannic acid; however, very little activity was detectable in lac84-1 or UEP1 culture filtrates when these strains were grown without tannic acid (Fig. 3C). Figure 3A shows the effect of tannic acid concentration on extracellular laccase activity of the three strains. At all concentrations of tannic acid, the *Lac1* null-mutant strain lac84-1 has a laccase activity which is considerably higher than that of hypovirulent strain UEP1, but either equivalent to, or lower than, that of the wild-type strain EP155/2. All three strains showed an increase in laccase activity in response to increasing concentrations of tannic acid in media that contained up to 8% tannic acid. The laccase activity decreased in 16% tannic acid. The activity of extracellular laccase of the three strains grown without tannic acid is consistent with previous results, i.e., viral suppression of *Lac1* of UEP1 results in less extracellular laccase for this strain than for EP155/2 (Rigling and Van Alfen 1991). No extracellular laccase activity was detected in strain lac84-1 when the growth medium lacked tannic acid. The significant laccase activity of lac84-1 in the presence of tannic acid demonstrates that there is an extracellular laccase gene in *C. parasitica* redundant to *Lac1*. The tannic acid-induced laccase activity of UEP1 is about 50% less than that of EP155/2, and reproducibly lower than that of lac84-1, suggesting that the virus also reduces expression of this new extracellular laccase. The new extracellular laccase showed a peak in activity in all strains at 2.5 days, with a decrease in activity after that (Fig. 3B). The loss of laccase activity between 4.5 and 7 days in the presence of tannic acid suggests that tannic acid may be toxic to the enzyme. These results show that a previously unknown extracellular laccase, which we have named LAC3, is induced by tannic acid. A comparison of the extracellular laccase activities expressed by EP155/2 and lac84-1 suggests that there is an inhibition to simultaneous optimum expression of LAC1 and LAC3 in EP155/2. The amount of extracellular laccase activity expressed by EP155/2 would be much greater than that of lac84-1 if both laccases were expressed at optimum levels.

To confirm that a third laccase is produced by this fungus, the extracellular laccases were partially purified and then the different laccases separated using a non-denaturing activity

**Table. 1.** Characteristics of *Lac1* null mutant compared with the wild-type and hypovirulent strains

| Strains | Canker area | Number of conidia | Conidia germination[a] (%) |
|---|---|---|---|
| EP 155/2 | 30.88 ± 9.95[b] | $4.38\ 10^9 \pm 3.46\ 10^7$ | 76 ± 12 |
| UEP 1 | 1.04 ± 0.85[c] | $2.22\ 10^{7c} \pm 0.71\ 10^7$ | 72 ± 09 |
| *lac*84-1 | 28.00 ± 14.26 | $3.58\ 10^9 \pm 4.52\ 10^7$ | 71 ± 11 |

[a] Values are percentage of germinated conidia from total conidia observed at 48 hr after the spores were harvested.
[b] Values are means ± standard deviation ($cm^2$).
[c] Significant at $P = 0.01$.

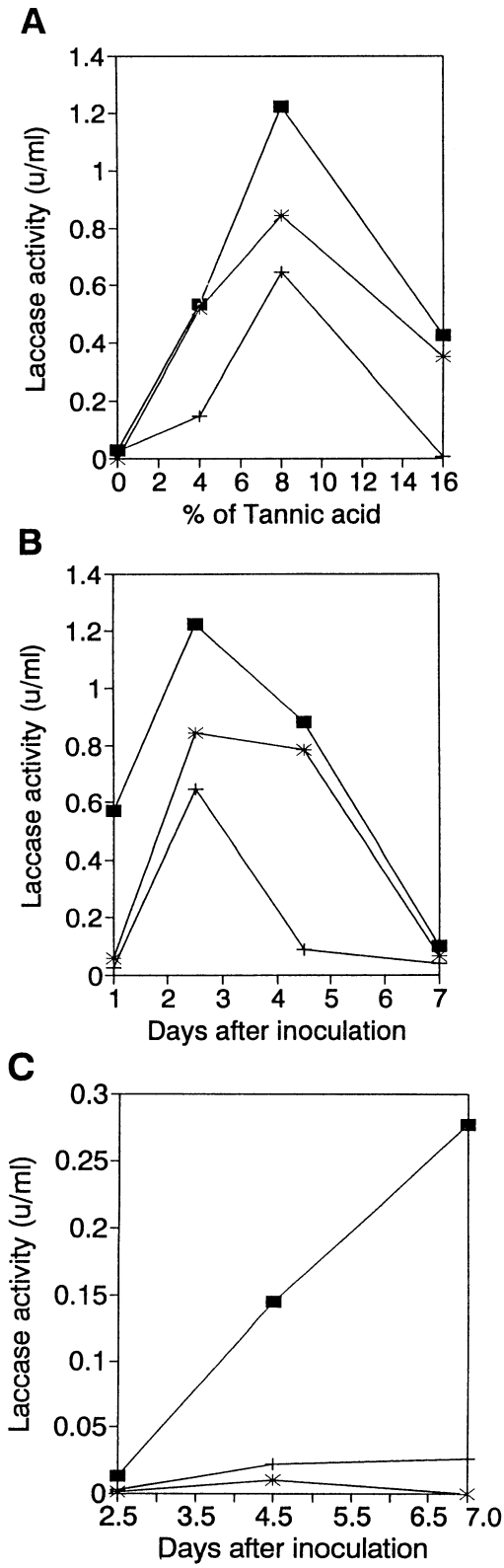


**Fig. 3.** Laccase activity from tannic acid containing culture filtrates of EP 155/2(■), UEP1(+), and lac84-1(*). **A,** Culture filtrates of cultures grown on various concentrations of tannic acid were collected at 2.5 days after inoculation and the laccase activities measured. **B,** Laccase activities measured at different times after innoculation from cultures grown in 8% tannic acid containing media. **C,** Laccase activities determined at various times from cultures grown without tannic acid.

gel. At pH 5.2, all three laccases migrated towards the anode in diffuse bands. The activity staining of the gel resulted in three different bands being visible: LAC1 from EP155/2 grown in EP complete medium is the slowest moving band, LAC2, the intracellular laccase from EP155/2 grown in EP complete medium, is intermediate in mobility, and the new laccase, LAC3, from lac84-1 grown in 4% tannic acid-EP complete medium is the fastest running of the three bands (Fig. 4). Activity gel staining was also used to demonstrate that LAC2 is produced by the *Lac1* null mutation strain lac84-1, confirming that LAC2 is not a precursor of LAC1 (data not shown).

## DISCUSSION

Although laccases are widely distributed in higher plants and fungi (Mayer 1987) relatively little is known of their biological functions. There has been much speculation concerning the role of fungal laccases, but in only one case has the role of a fungal laccase been clearly demonstrated (Clutterbuck 1972; Law and Timberlake 1980; Yelton *et al.* 1985). Fungal laccases have been found both as intracellular and extracellular enzymes. Linden *et al.* (1991) reported the presence of two different laccase activities in *N. crassa*, one being extracellular and the other intracellular, with the intracellular enzyme being the precursor of the extracellular one. The wood rot fungi, *Fomes annosus* and *Pleurotus ostreatus*, produce laccases, with nine different isozymes, possibly glycoforms, being reported from *P. ostreatus* (Harrs and Huttermann 1983; Prillinger and Molitoris 1979). *F. annosus* produces two forms of laccase; one is a constitutive intracellular form and the other is an inducible exoenzyme (Harrs and Huttermann 1983). The ascomycete *Podospora anserina* has three laccases, laccase I, II, and III, with multiple forms of laccase II being detectable as a result of difference in the carbohydrate moiety of the glycopeptide (Esser and Minuth 1971). Bollag and Leonowicz (1984) studied the induction of extracellular laccases in a number of different fungi, and reported that 2,5-xylidine could induce laccases in some fungi, but not others, such as *Botrytis cinerea* and *P. anserina.* Marbach *et al.* (1984), however, reported the induction of one extracellular laccase of *B. cinerea* by grape juice and another one by gallic acid, and they suggested that a third laccase may be induced by *p*-courmaric acid. The large number of fungi reported to produce laccases, and the apparent diverse isoforms of the enzymes that have been detected in some fungi, suggest that the laccases play an important role in fungal biology. While the most commonly postulated role is the detoxification of phenolics encountered by fungi in the environment, careful genetic studies are needed to clearly elucidate the roles of these enzymes.

*C. parasitica* is a bark canker pathogen of tannic acid rich chestnut and oak trees (Griffin and Elkins 1986), and it is assumed that the laccases produced by this pathogen are important in the adaptation of the fungus to this special niche. The discovery that the extracellular laccase of this fungus is expressed in much smaller amounts in viral-infected hypovirulent strains of the fungus compared with normal virulent strains (Rigling *et al.* 1989) sparked interest in this enzyme as a molecular marker for viral infection, as well as possibly explaining the reduced virulence of viral-infected

strains. *C. parasitica* produces an extracellular (LAC1) and an intracellular (LAC2) laccase (Rigling and Van Alfen 1993). In this study we report that a tannic acid-inducible extracellular laccase (LAC3) is also produced by the fungus. LAC1 and LAC3 are different enzymes encoded by separate genes since LAC3 was produced by the *Lac1* null mutant strain lac84-1. The synthesis of LAC2 by the *Lac1* null mutant strain also demonstrates that LAC2 is encoded by a different gene than is LAC1, and thus LAC1 is not processed from LAC2. The relationship between LAC2 and LAC3 is not yet clear, although they have different mobilities on native activity gels (Fig. 4).

There is some evidence that either LAC3 or tannic acid may suppress expression of *Lac1* since the total extracellular laccase activity of EP155/2, while reproducibly greater than that of lac84-1, is much lower than would be expected if both LAC1 and LAC3 were produced at optimum levels. It is particularly interesting that the extracellular laccase activity of the viral-infected strain UEP1 is considerably lower than that of lac84-1 (Fig.3). If LAC1 activity in the tannic acid media were typical of that without the tannic acid, the total activity of LAC1 and LAC3 in culture filtrates of UEP1 would be greater than that of lac84-1, since only LAC3 is expressed by lac84-1. The fact that the extracellular laccase activity of UEP1 was much lower than that of lac84-1 suggests that LAC3 expression is reduced in the viral-containing strain, as are LAC1 and LAC2. The precipitated tannic acid in the liquid medium interfered with measurement of relative fungal growth during these experiments. On Bavendamm medium, which contains 1% tannic acid, however, lac84-1 grows normally and gives a positive laccase reaction. UEP1 grows normally, but does not give a positive reaction, supporting our conclusions from the liquid media experiments that LAC3 expression is reduced by the virus. Since both LAC1 and LAC3 can be responsible for a positive Bavendamm reaction, care must be taken by those using this assay that it not be interpreted as an assay for just LAC1.

The null mutation of *Lac1* did not affect any detectable phenotype of the fungus, thus the biological function of this extracellular laccase in *C. parasitica* is still unknown. The discovery of a new extracellular laccase in the mutant strain may prove to be another example of redundancy of biologically critical enzymes (Bulawa and Osmond 1990; Bulawa 1992; Kotoujansky 1987). The induction of LAC3 by tannic acid suggests a close relationship between laccase and tannic acid, a substance thought to be important for tree defense (Nienstaedt 1955), and thus presumably important for virulence of this fungus. The cloning and deletion of *Lac3* will be necessary before the role of extracellular laccases in the biology of this fungus can be resolved.

The symptoms of virus infection suggest that the effects of the virus are primarily the result of specific perturbation of host developmental processes. The viral-regulated host genes that have been thoroughly characterized to date generally support the hypothesis that developmental processes are being perturbed. *Vir1* and *Vir2* appear to encode a mating-type-specific pheromone (L. Zhang and N. K. Van Alfen, unpublished) and *Crp* encodes a cell-surface hydrophobin that is detectable in fungal fruiting bodies (Carpenter *et al.* 1992). These results suggest that we should be cautious in our assumptions concerning the role of laccases in the biology of this fungus. While it is easy to assume that detoxification of tannic acids is the primary role, other roles for laccases that are more closely related to developmental events are also possible. The only laccase with a known function is that of *A. nidulans*, a laccase that is developmentally regulated and involved in spore pigment biosynthesis (Clutterbuck 1972; Law and Timberlake 1980; Yelton *et al.* 1985). Extracellular laccases may as easily be involved in fungal cell wall synthesis as in degradation of host cell wall components. Recent studies

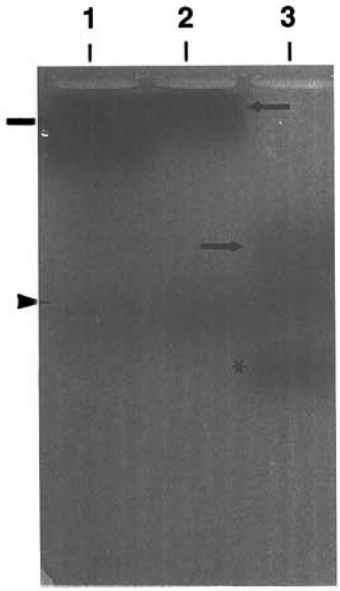


**Fig. 4.** Laccase activity-stained agarose gel. Numbers on top represent different types of laccases of *Cryphonectria parasitica*; 1, intracellular laccase LAC2; 2, extracellular laccase LAC1; and 3, inducible extracellular laccase LAC3. Arrows indicated laccase bands. Blue dye across the lanes and dark brown tannic acid precipitate in lane 3 are indicated by arrowhead and asterisk, respectively.

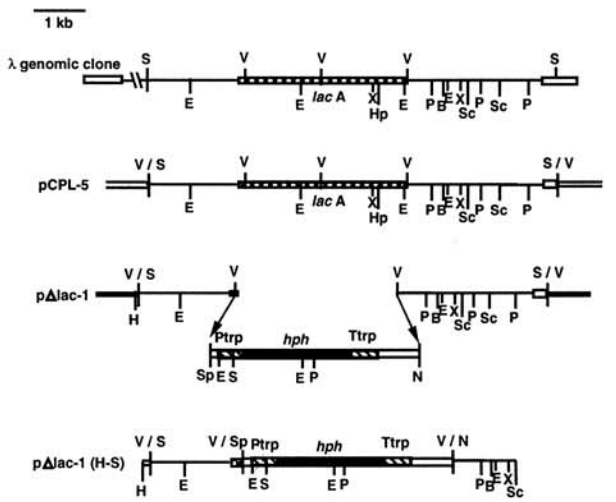


**Fig. 5.** Construction of transforming vector for *Lac1* deletion. Laccase gene (*Lac1*), and hygomycin resistant gene (*hph*) flanked by *Aspergillus nidulans trp*C promotor (Ptrp) and terminator (Ttrp) are represented by dotted box and cross-hatched box respectively. Genomic DNA from *Cryphonectria parasitica*, bacteriophage vector and plasmid DNA are represented by single line, open box and double line respectively. Each bar represents restriction sites; S (*Sma*I), E (*Eco*RI), V (*Eco*RV), X (*Xho*I), Hp (*Hpa*I), P (*Pst*I), Sc (*Sac*I), H (*Hin*dIII), and B (*Bgl*II)

have suggested a role for plant laccases in cell wall polymerization, particularly in association with some of the hydroxyproline and glycine rich cell wall proteins (Bradley *et al.* 1992; Bao *et al.* 1993). This possibility in fungi is supported by mutational evidence in *C. parasitica* which suggests that *Lac1* and *Crp* are normally coordinately regulated. CRP, a protein containing a region rich in hydroxylated amino acids (Zhang *et al.* 1994) could play a similar role in certain fungal cell walls as do the hydroxyproline rich proteins in plant cell walls (Bradley *et al.* 1992).

Recent studies on *Lac1* regulation suggest that normally *Lac1* synthesis is under two different opposing regulatory pathways, and that induction removes the suppression (Larson *et al.* 1992; Larson and Nuss 1993). These studies suggested that the virus acts to interfere with the stimulatory signal and also suppress the cycloheximide (CHX) and cyclosporinA (CspA)-mediated induction. The only evidence that we have relative to these experiments is that we have not found it necessary to induce *Lac1* expression in EP155/2 by the use of cycloheximide. In the study reported here, *Lac1* expression was not knowingly induced; LAC3 was the induced enzyme. The three laccases produced by this fungus appear to be independently regulated, yet their common perturbation by the virus indicates some interaction in their regulation. It remains to be demonstrated that interference with induction is the common mechanism.

## MATERIALS AND METHODS

**Fungal strains and culture conditions.**

The CHV1-713 containing strain UEP1 and the isogenic virus-free strain EP155 (Powell and Van Alfen 1987a) were used in these studies. EP155/2 is a single spore isolate of EP155. Culture conditions and methods for preparation of inoculum for liquid cultures were previously described except that in these studies the fungus was grown on agar plates under constant low light (4.43 μmole $m^2$ $s^{-1}$) at 25 C. (Rigling and Van Alfen 1993). Seven-day-old cultures of *C. parasitica* growing on plates were homogenized in 100 ml of sterile water using a Waring blender, and the resulting slurry was used to inoculate 1 L of EP complete liquid media (Puhalla and Anagnostakis 1971). Liquid cultures were grown at 25° C with shaking at 100 rpm under continuous light (86.5 μmole $m^2$ $s^{-1}$).

**Construction of transforming vector and transformation.**

A cDNA clone of *Lac1* (Rigling and Van Alfen 1991) was used to screen a genomic library of EP155 (Powell and Van Alfen 1987a). A 17-kb genomic clone containing the *Lac1* gene was identified from this library. The 8.0-kb *Sma*I-digested fragment containing the *Lac1* genomic DNA was subcloned into the *Eco*RV site of pBluescriptII SK(–)(pCPL-5). A vector (pΔlac-1) was constructed by replacing two 3.1-kb *Eco*RV fragments of the clone pCPL-5 encoding the laccase gene with a *Ssp*I and *Nru*I double-digested 3.8-kb cassette from pDH25 (Cullen *et al.* 1987) containing the hygromycin resistance gene (*hph*) with the *Aspergillus nidulans Trp*C promoter and terminator. The transforming vector (pΔlac-1) was then linearized by digestion with *Hin*dIII and *Sac*I. The linearized insert [pΔlac-1(H-S)] containing the *hph* gene, flanked by a 1.9-kb 5′ and a 2.6-kb 3′ region of the *Lac1* gene, was gel purified and used for *C. parasitica* transformation. The final and intermediate constructs of the vector constructed for recombination with, and deletion of, the *Lac1* gene are shown in Figure 5.

A total of 2 μg of linearized insert was used to transform spheroplasts of EP155/2 following the procedures of Churchill *et al.* (1991). Selected transformants were single-spored and serially transferred, alternating between hygromycin selective and nonselective media to determine the mitotic stability of hygromycin resistance. The media used were potato-dextrose agar containing L-methionine (100 mg/L) and biotin (1 mg/L) (PDAmb) with or without 50 μg/ml hygromycin B. Extracellular laccase activity of the stable transformants was examined by both growing them on a modified Bavendamm medium and by the spectrophotometric method (Rigling *et al.* 1989).

DNA from *C. parasitica* was extracted using the method described by Churchill *et al.* (1991) and restriction maps of selected transformants were constructed. RNA was extracted from cultures between 1 and 5 days after inoculation and Northern blot analysis of the transformants conducted as previously described by Rigling and Van Alfen (1991).

**Laccase activity.**

The culture fluid was filtered through Miracloth (Calbiochem, La Jolla, CA), and extracellular laccase activity in the culture fluid was determined by a spectrophotometric assay using 2,6 dimethoxyphenol as substrate (Rigling *et al.* 1989). A laccase unit was defined as a 1.0/min $A_{468}$ increase at 25° C. To determine intracellular laccase activity, 0.5 g of lyophilized mycelium was ground in liquid nitrogen, then added to 10 ml of cold 0.1 M sodium phosphate, pH 6.0, and homogenized at 8,000 rpm for 1 min using a Polytron (Brinkman Instruments, Inc., Westbury, NY). The homogenate was centrifuged for 10 min at 4,000 *g* and laccase activity determined in the supernatant (Rigling and Van Alfen 1993). To determine effects of growth in tannic acid on laccase activity, tannic acid (Gallotannin, Fluka, Buchs, Switzerland) was added in various concentrations to 50 ml of 0.5× EP complete liquid media, pH 6.2, contained in 250 ml of tissue culture flasks and inoculated with 5 ml of an inoculum slurry prepared as described above. Duplicate flasks were harvested after 2.5, 4.5, and 7 days of growth and used to determine extracellular laccase activity.

**Native gel electrophoresis.**

Culture filtrate (150 ml) containing tannic acid was centrifuged at 4,000 *g* for 15 min at 4° C, and the clarified supernatant was loaded onto a 20-ml column of DEAE-Sepharose CL-6B column (1.6 × 12 cm) (Pharmacia LKB, Piscataway, NJ) equilibrated with 0.01 M of sodium phosphate (pH 7.4). The column was washed with 400 ml of 0.01 M sodium phosphate at a flow rate of 50 ml/hr. Fractions showing the laccase activity were pooled, concentrated to one third of the original volume in a Centriprep-30 Concentrator (Amicon, Danvers, MA). The 1% agarose native gel was performed using Tris-citrate buffer (pH 5.2) at a constant 60 V (Dubernet *et al.* 1977) and 50 μl of samples was loaded in 15% glycerol containing 0.05% bromophenol blue. The gel was stained with a substrate solution to locate protein bands with laccase enzyme activity (Rigling and Van Alfen 1993).

**Virulence test.**

To assay virulence of the transformants, dormant stems of chestnut trees were inoculated with a plug of mycelium from the leading edge of the colony. The cankers were measured at 1-wk intervals for 5 wk and canker areas determined using the formula for an ellipse. The wild-type virulent strain EP155/2 (Powell and Van Alfen 1987) as well as its isogenic hypovirulent strain UEP1 were used as controls.

**Sporulation.**

Strains to be tested were grown on PDAmb agar under continuous low intensity light (4.43 μmole $m^2$ $s^{-1}$) at 25° C for 3 wk. The conidia from each plate were harvested with 10 ml of sterile water and the number of conidia per plate was determined using a hemacytometer. Five replicates of each strain were used and the experiment was repeated three times.

**Spore germination.**

Conidia were diluted in $H_20$ containing L-methionine (100 mg/L) and biotin (1 mg/L) and pipetted onto a glass slide, a coverslip was added and the slide placed in a humid petri dish. At least 50 conidia were examined on each slide. Conidia were considered germinated when the germ tube was at least twice the spore length.

**Sexual mating.**

Sexual mating capability of transformants was determined on chestnut stem pieces using the procedure of Anagnostakis (1979). EP155/2 and the transformants (Mat*A*) were crossed with EP6 (ATCC 22508) (Mat*a*, *met–*).

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