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Altered transcriptional response to nutrient availability in hypovirus-infected chestnut blight fungus

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The gene *lac-1*, encoding the enzyme laccase, is one of several genes of the chestnut blight fungus, *Cryphonectria parasitica*, that are suppressed by virulence-attenuating mycoviruses of the hypovirus group. Two antagonistic regulatory pathways have been shown to govern the activity of the *lac-1* promoter: a positive pathway that stimulates transcription and a negative pathway that represses transcription. We now report that these two regulatory pathways respond independently to specific changes in the nutritional environment. These newly defined conditions were used to confirm that a hypovirus suppresses the activity of the positive regulatory pathway, and to implicate calmodulin and calcineurin as components of the signal transduction cascades regulating *lac-1* transcription. Significantly, *lac-1* transcript accumulation was shown to be affected by amino acid availability. Further analysis revealed that transcriptional repression mediated by the negative regulatory pathway is relieved under conditions of amino acid deprivation. Thus, by blocking the positive pathway, hypovirus infection prevents increased *lac-1* transcript accumulation in response to amino acid deficiency. These observations are consistent with the hypothesis that hypoviruses alter the transcriptional response of the host fungus to changes in nutrient availability.

Key words: calcineurin/calmodulin/*Cryphonectria parasitica*/second messengers/signal transduction

Introduction

Infection of the susceptible American chestnut tree by the chestnut blight fungus, *Cryphonectria parasitica*, is a complex process involving sequential interactions between the invading fungus and several different host tissues. These include the initiation of colonization at a wound site, the penetration of lignified zones and wound periderm formed as a host defense response, and the invasion and destruction of the vascular cambium (Hebard *et al.*, 1984). The presence of endogenous double-stranded (ds) RNA mycoviruses of the hypovirus group can profoundly alter this process, resulting in attenuation of fungal virulence (hypovirulence) (recent reviews include Anagnostakis, 1982; Van Alfen, 1985; MacDonald and Fulbright, 1991; Nuss, 1992). Such hypovirulent fungal strains are impaired in their ability to effectively penetrate host defense barriers and thus produce only superficial cankers that eventually heal. Hypovirulent *C.parasitica* strains also typically exhibit a number of distinguishing characteristics when grown in culture. These hypovirulence-associated traits can include suppressed sporulation (Elliston, 1978; Anagnostakis, 1984a), reduced pigmentation (Anagnostakis, 1982, 1984a; Elliston, 1985) and diminished accumulation of certain metabolites, e.g. oxalate (Havir and Anagnostakis, 1983) and gene products, e.g. the enzyme laccase (Rigling *et al.*, 1989; Hillman *et al.*, 1990; Carpenter *et al.*, 1992). Inherent technical difficulties have hampered efforts to study aspects of virus-mediated fungal hypovirulence *in planta*. However, recent molecular analyses of hypovirulence-associated viruses, and their effects on fungal phenotype and gene expression in culture, are beginning to provide valuable insights into the underlying basis of hypovirulence.

The recent development of an infectious cDNA clone of the prototypic hypovirus, isolate CHV1-713, has firmly established hypovirulence-associated viral dsRNAs as causative agents of fungal hypovirulence (Choi and Nuss, 1992b). Additional studies using deleted forms of the infectious cDNA clone and DNA-mediated transformation with selected portions of the viral coding domains have identified the viral-encoded papain-like protease, p29, as a symptom determinant that contributes to virus-mediated modulation of fungal phenotype (Choi and Nuss, 1992a; Craven *et al.*, 1993). Transformation with an infectious cDNA in which the p29 coding region was deleted resulted in hypovirulent fungal transformants that were much less suppressed in pigmentation and asexual sporulation relative to transformants containing the full-length viral cDNA, illustrating the feasibility of using the infectious cDNA clone to engineer hypovirulent fungal strains with specific phenotypic traits. More recently, virus-mediated hypovirulence was established in additional fungal species by introducing infectious synthetic hypovirus transcripts into fungi not previously shown to harbor such viruses (Chen *et al.*, 1994).

Although the mechanisms underlying virus-mediated attenuation of fungal virulence and the suppression of a diverse array of fungal processes are unclear, progress has been forthcoming in understanding the basis of virus-mediated suppression of laccase accumulation. While a role for laccase in the fungal infection process remains uncertain, the gene encoding this enzyme has served as a useful 'reporter' for studies aimed at understanding the precise details of virus-mediated suppression of fungal gene expression. The structural gene for *C.parasitica* laccase, *lac-1*, has been cloned and characterized (Rigling and Van Alfen, 1991; Choi *et al.*, 1992). Two antagonistic pathways were found to govern the regulation of *lac-1* transcription in virus-free virulent strains (Larson *et al.*, 1992). A positive-acting pathway was found to require both the calcium (Ca^{2+}) and the inositol trisphosphate

(IP_3) second messengers, while a negative-acting pathway was found to require ongoing protein synthesis, i.e. it was sensitive to treatment with low levels of the protein synthesis inhibitor cycloheximide (CHX). A comparison of the effects of a number of pharmacological agents with that of virus infection on *lac-1* transcript accumulation strongly suggested that virus-mediated suppression of *lac-1* transcription results from an interruption of signal transmission through the positive-acting pathway.

In this study, we show that *lac-1* transcription is sensitive to changes in the nutritional environment and describe conditions under which the corresponding regulatory pathways can readily be dissected. These newly defined conditions were used to confirm the hypovirus-mediated perturbation of the Ca^{2+}/IP_3-dependent positive regulatory pathway and to implicate both calmodulin and calcineurin as effectors of *lac-1* transcriptional regulation. The possible consequences of virus-mediated modulation of transcriptional responses to changes in nutrient availability on colonization and penetration of host tissue during fungal infection are discussed.

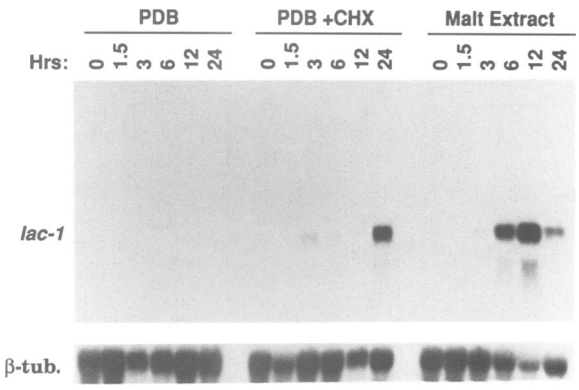

Fig. 1. Accumulation of *lac-1* transcript in strain EP155 following media change. Mycelia were initially grown in PDB as described in the text and changed to either 1.5% (w/v) ME, PDB or PDB + 3 mM CHX. Cultures were harvested at the time indicated following the medium change and RNA was prepared. Total RNA (10 mg per sample) was denatured and separated by electrophoresis using a 1.2% agarose–formaldehyde gel. The RNA was transferred to a nylon membrane, UV irradiated and hybridized with a *lac-1* probe. Following autoradiography, the blot was stripped and rehybridized with a β-tubulin probe.

Results

Initial attempts to examine parameters of *C.parasitica* laccase accumulation were complicated by the sensitivity of *lac-1* regulation to environmental factors such as light intensity, composition of growth medium or age of the culture (Choi *et al.*, 1992). The observation that low levels of the protein synthesis inhibitor CHX consistently effected a marked stimulation of *lac-1* transcript accumulation (Choi *et al.*, 1992; Larson *et al.*, 1992) provided experimental conditions for examining the nature of regulatory pathways governing *lac-1* expression. Subsequent studies revealed that *lac-1* transcription was repressed in rich medium such as potato dextrose broth (PDB) and that the stimulation of laccase accumulation following CHX addition was due to the derepression of *lac-1* transcription. Thus, *lac-1* transcription was found to be under the control of both a positive-acting and a negative-acting regulatory pathway. While these studies were highly informative, the reliance on a protein synthesis inhibitor was considered a complicating factor. Consequently, efforts were initiated to define conditions under which the regulatory pathways governing *lac-1* transcription could reliably be examined in the absence of such inhibitors.

Activity of negative regulatory pathway is modulated by nutrient availability

Cryphonectria parasitica cultures grown on Bavendamm's (1928) medium [1.5% (w/v) malt extract (ME), 0.5% (w/v) tannic acid and 2% agar] produce large quantities of laccase (Rigling *et al.*, 1989). An examination of the effect of various medium components on *lac-1* expression revealed that shifting fungal cultures from PDB to a medium composed solely of ME at the concentration found in Bavendamm's medium resulted in a significant and consistent increase in *lac-1* transcript accumulation (Figure 1). In contrast, very little transcript accumulated in cultures shifted to fresh PDB unless 3 mM CHX was also added (Figure 1). Thus, as with CHX treatment, transfer of a *C.parasitica* culture from the rich PDB to ME reproducibly derepresses *lac-1* transcription. Efforts to examine the effect of tannic acid, alone or in combination with ME, on *lac-1* transcript accumulation failed due to interference by the tannic acid, or a breakdown component, that was carried through the RNA purification process.

As indicated in Figure 1, the magnitude of *lac-1* transcript accumulation was similar whether cultures were transferred to ME or to PDB + CHX. A similar level of *lac-1* transcript accumulation was also observed when cultures were transferred to ME containing CHX (data not shown), indicating that combining the two treatments does not give an additive response. However, the kinetics of *lac-1* transcript accumulation differed significantly under each of these conditions. Transcript accumulation was transient over a 24 h period following transfer to ME, and reached a maximum between 10 and 14 h. In contrast, the CHX-mediated *lac-1* transcript accumulation in PDB peaked at 24 h following the addition of CHX (Figure 1). Moreover, addition of CHX to ME prevented the reduction in *lac-1* transcript accumulation observed at the 24 h time point in unamended ME (data not shown). The similarity in the magnitude of transcript accumulation following transfer of fungal cultures to either ME or PDB + CHX and the absence of an additive effect suggests that both treatments derepress *lac-1* transcription by affecting the same pathway. However, the differences in kinetics of transcript accumulation indicate that derepression proceeds via different mechanisms under the two transfer conditions. Thus, the simple transfer of a culture from PDB to ME provides a physiological, inhibitor-independent, highly reproducible method for relieving the repression of *C.parasitica lac-1* transcription.

In considering the mechanism by which transfer to ME derepresses *lac-1* transcription, it was noted that relative to PDB, ME is a poor source of nitrogen, being particularly limited in amino acids (Bridson and Brecker, 1970). This raised the possibility that the ME-related derepression of *lac-1* transcription may be co-ordinated with the regulation of nitrogen metabolism. A large body of work has shown

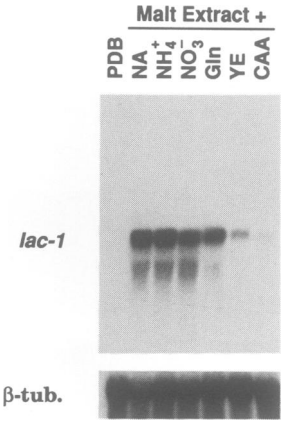

Fig. 2. Effect of various nitrogen sources on the malt-mediated accumulation of *lac-1* transcript in strain EP155. Fungus was prepared as indicated in Figure 1 and the medium was changed to PDB or to ME containing the indicated additions: NA, no addition; NH_4^+, 10 mM NH_4Cl; NO_3^-, 10 mM $NaNO_3$; Gln, 10 mM glutamine; YE, 0.5% (w/v) yeast extract; CAA, 0.5% (w/v) casamino acids. Cultures were harvested 12 h after the media change and total RNA was prepared and analyzed as described in Figure 1.

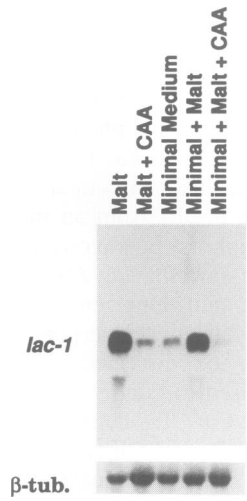

Fig. 3. Stimulation of *lac-1* transcript accumulation in minimal medium supplemented with ME in strain EP155. Cultures were prepared as described in Figure 1 and changed to the indicated media: malt, ME; malt + CAA, ME + 0.5% (w/v) CAA; minimal + malt, MM + 1.5% (w/v) ME; minimal + malt + CAA, MM + 1.5% (w/v) ME + 0.5% (w/v) CAA. Cultures were harvested and total RNA was prepared and analyzed as described in Figure 1.

that when primary sources of nitrogen, e.g. ammonium or glutamine, are limiting, fungal cells derepress the expression of sets of genes that allow the utilization of secondary nitrogen sources such as nitrate, nitrite, purines, amino acids and proteins (Marzluf, 1981). However, as presented in Figure 2, the addition of 10 mM ammonium, 10 mM glutamine or 10 mM nitrate had no effect on ME-related *lac-1* transcript accumulation. These results indicate that *lac-1* transcription is neither under the direct control of nitrogen metabolite repression nor influenced by the addition of a secondary nitrogen source such as nitrate.

In contrast to results obtained with specific primary and secondary nitrogen sources, the addition of 0.5% (w/v) yeast extract (YE), a rich source of several forms of nitrogen (Bridson and Brecker, 1970), repressed the ME-related stimulation of *lac-1* transcript accumulation (Figure 2). The addition of 0.5% (w/v) casamino acids (CAA) to ME also repressed the accumulation of *lac-1* transcript (Figure 2), suggesting that it is the high amino acid content of YE that prevents *lac-1* transcript accumulation. Significantly, *lac-1* transcription was derepressed by the addition of 3 mM CHX to the ME−CAA medium (data not shown). These results suggest that *lac-1* transcription is subject to regulation by the availability of nutrients such as amino acids. That is, *lac-1* transcription is repressed via the CHX-sensitive negative-acting pathway when amino acids are abundant (e.g. in PDB or ME + CAA) and this repression is relieved when amino acids become limiting (e.g. in ME). Interestingly, the *in vitro* transcriptional induction of pisatin demethylase in the pea pathogen *Nectria haematococca* by the isoflavanoid pisatin is also suppressed when the induction medium is supplemented with CAA (Straney and Van Etten, 1994).

Physiological conditions for dissection of lac-1 transcriptional regulation

Examination of other growth conditions revealed that the pathways regulating *lac-1* transcription could be further dissected by transferring cultures to MM, a chemically defined minimal medium. As shown in Figure 3 and indicated in Table I, *lac-1* transcript accumulation was not stimulated when a *C.parasitica* culture was transferred to MM. What makes this result interesting is that the addition of CHX to MM does not stimulate *lac-1* transcription, as occurs when CHX is added to ME + CAA medium (results not shown). We interpret these combined results to indicate that the chemically defined MM fails to activate the positive-acting pathway governing *lac-1* transcription. However, this pathway can be activated by supplementing MM with 1.5% (w/v) ME (Figure 3, Table I). Moreover, as was observed following the transfer of cultures to ME, the addition of CAA (0.5%, w/v) to the MM + ME medium repressed this activation (Figure 3) in a CHX-sensitive manner (data not shown; Table I). Thus, the judicious use of growth conditions allows the manipulation and examination of the pathways regulating

Table I. Predicted activity of the positive and negative regulatory pathways under various conditions and the effect on *lac-1* transcript accumulation

Conditions	Regulatory pathway		Accumulation of *lac-1* transcript
	Positive	Negative	
MM	I	I	−
MM + ME	A	I	+
MM + ME + CAA	A	A	−
MM + ME + CAA + CHX	A	I	+
MM + ME + hypovirus	I	I	−

The media are: minimal medium (MM), minimal medium + malt extract (MM + ME), minimal medium + malt extract + casamino acids (MM + ME + CAA). CHX indicates the addition of 3 mM CHX and hypovirus indicates that the fungus carries hypovirus CHV1-713. For the activity of the positive and negative regulatory pathways, A = active and I = inactive. For the accumulation of *lac-1* transcript, − denotes little transcript accumulation and + denotes significant transcript accumulation.

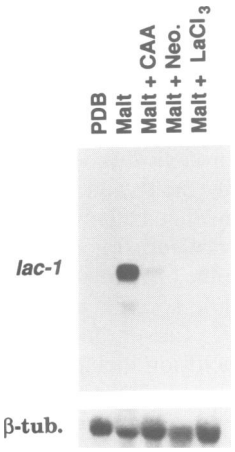

Fig. 4. Effect of compounds that perturb either calcium mobilization or IP_3 generation on malt-mediated accumulation of *lac-1* transcript in strain EP155. Cultures were prepared as described in Figure 1 and the medium was changed to either PDB or ME containing the indicated additions: CAA, 0.5% (w/v) casamino acids; Neo., 0.5 mM meomycin; La_3Cl, 0.5 mM La_3Cl. Cultures were harvested and total RNA was prepared and analyzed as described in Figure 1.

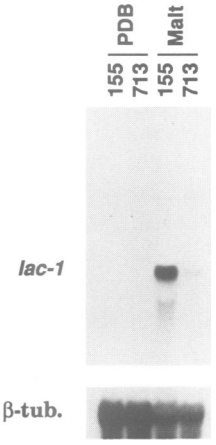

Fig. 5. Viral-mediated suppression of *lac-1* transcript accumulation in ME in strain EP713. Cultures of EP155 and EP713 were prepared, and the medium was changed to either PDB or ME as described in Figure 1. Cultures were harvested and total RNA was prepared and analyzed as described in Figure 1. The hypovirulent strain EP713 contains the hypovirus CHV1-713, but is otherwise isogenic to strain EP155 (Anagnostakis and Day, 1979; Hillman *et al.*, 1990).

lac-1 transcription independent of non-physiological inhibitors, as indicated in Table I.

Using defined, CHX-independent culture conditions to verify roles for IP_3 and Ca^{2+} mobilization in the regulation of lac-1 transcription and the site of action of virus-mediated suppression

Previous studies provided evidence that the positive-acting pathway regulating *lac-1* transcription was dependent on intact IP_3 and Ca^{2+} second messenger systems, and that the suppression of *lac-1* transcription observed for hypovirulent *C.parasitica* strains was due to virus-mediated interruption of signal transmission through this pathway (Larson *et al.*, 1992). Those studies required inactivation of the negative-acting pathway by treatment with 3 mM CHX. The availability of defined, physiological, inhibitor-independent conditions for derepressing *lac-1* transcription provided an opportunity to verify those results in the absence of interference by the negative-acting regulatory pathway (Table I).

One indication of a role for IP_3 as a component of the positive-acting pathway was the observation that neomycin, an antagonist of IP_3 generation (Downes and Mitchell, 1981; Carney *et al.*, 1985; Cockcroft *et al.*, 1987; Slivka and Insel, 1987), prevented the CHX-mediated derepression of *lac-1* transcription in cultures grown in PDB (Larson *et al.*, 1992). As indicated in Figure 4, neomycin also prevented the PDB to ME shift-related derepression of *lac-1* transcription when added at concentrations similar to those used in the previous studies. Evidence that Ca^{2+} mobilization was also required for the CHX-mediated derepression included the ability of $LaCl_3$, a calcium-channel blocker (Nathan *et al.*, 1988), to prevent derepression (Larson *et al.*, 1992). $LaCl_3$ was also shown to prevent derepression of *lac-1* transcription following transfer to ME (Figure 4). These combined results clearly corroborate previous findings that IP_3 generation and calcium mobilization are required components of the positive-acting pathway regulating *lac-1* transcription. As shown in Figure 5, *lac-1* transcript does not accumulate when an isogenic hypovirus-infected strain is transferred to ME. Thus, the hypovirus prevents the stimulation of *lac-1* transcription that results in the virus-free strain under conditions where the transcriptional repression mediated by the negative regulatory pathway is relieved (Table I). This result is consistent with our earlier conclusion that hypovirus CHV1-713 prevents the stimulation of *lac-1* transcription by squelching the activity of the positive regulatory pathway (Larson *et al.*, 1992).

Accumulation of lac-1 transcript requires calmodulin activity

The calcium-mediated regulation of *lac-1* promoter activity (Larson *et al.*, 1992; Figure 4) suggested that calmodulin may play a role in the positive regulatory pathway governing the accumulation of *lac-1* transcript. Two different antagonists of calmodulin were used to test this hypothesis. The first inhibitor, ophiobolin A, is a phytotoxin produced by the fungus *Helminthosporium maydis* that irreversibly inactivates calmodulin by covalently bonding to it (Leung *et al.*, 1988). The second inhibitor, trifluoperazine, is a phenothiazine derivative with neuroleptic properties that appears to inhibit calmodulin by reversibly binding to the surface that interacts with the enzymes that it regulates (Cheung, 1982). Both of these inhibitors reduced the increase in *lac-1* transcript levels observed in ME in a dose-dependent manner (Figure 6). Doses of ophiobolin A > 10 μM were toxic, as judged by the effect on the β-tubulin transcript levels (data not shown). This concentration of ophiobolin A has been shown to produce half-maximal inhibition of bovine brain calmodulin *in vitro* (Leung *et al.*, 1988). The dose range of trifluoperazine that inhibited *lac-1* transcription was similar to the concentrations that inhibited the yeast to hyphal phase transition in both *Ophiostoma ulmi* (Muthukumar and Nickerson, 1984) and *Candida albicans* (Roy and Datta, 1987; Sabie and Gadd, 1989; Paranjape

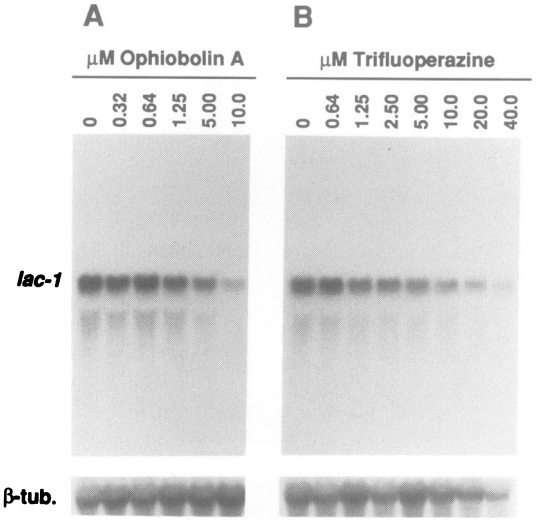

Fig. 6. Inhibition of *lac-1* transcript accumulation in ME by inhibitors of calmodulin. Cultures of EP155 were prepared as for Figure 1 and the medium was changed to ME with the indicated concentrations of (**A**) ophiobolin A or (**B**) trifluoperazine. Cultures were harvested and total RNA was prepared 12 h after the medium was changed. Total RNA was analyzed as described in Figure 1.

et al., 1990). The inhibition of *lac-1* transcript accumulation by two compounds that inhibit calmodulin through independent mechanisms provides strong evidence of a role for calmodulin in the Ca^{2+}/IP_3-dependent regulatory pathway that governs this process.

Repression of lac-1 transcription requires calcineurin activity

Cyclosporin A relieves the repression of *lac*-1 transcription in PDB (Larson and Nuss, 1993). The stimulation of *lac-1* transcription by cyclosporin is cyclophilin dependent (Larson and Nuss, 1993), indicating that calcineurin is the target that mediates the effect of the compound (Schreiber, 1992; Schreiber and Crabtree, 1992). To reduce the possibility that cyclosporin might be stimulating *lac-1* transcription by acting at a site other than calcineurin, we tested a second calcineurin agonist, FK506 (Schreiber, 1992; Schreiber and Crabtree, 1992). Both cyclosporin A and FK506 derepressed *lac-1* transcription in rich medium (PDB) at doses comparable with those observed to inhibit the calcineurin-dependent nuclear translocation of the cytoplasmic factor of the NF-AT transcription factor in mammalian systems (Flanagan *et al.*, 1991). Since the two compounds are structurally unrelated and inhibit calcineurin through different receptors (Schreiber, 1991; Schreiber and Crabtree, 1992), the observation that both derepress *lac-1* transcription in rich medium (Figure 7) provides compelling evidence that calcineurin plays a role in the regulatory pathway that is responsible for the repression of *lac-1* transcription in amino acid-rich environments.

Discussion

Although fungal pathogenicity is recognized as a complex process, several lines of evidence have supported the concept that the level of virulence exhibited by a particular phytopathogen is primarily dependent on the expression of one, or a limited number, of specific pathogenesis determinants (e.g. Kolattukudy, 1985; Schafer *et al.*, 1989; van Etten *et al.*, 1989). However, the results of several recent studies in which the disruption of genes encoding putative pathogenesis determinants resulted in no change in virulence have brought this view into question (Scott-Craig *et al.*, 1990; Stahl and Schafer, 1992). In this context, the virus-mediated attenuation of fungal virulence observed for hypovirulent strains of *C.parasitica* appears to involve modulation of regulatory cascades rather than the suppression of a specific pathogenesis determinant (Larson *et al.*, 1992; Larson and Nuss, 1993). Evidence supporting this conclusion includes reports of differential accumulation of poly(A)$^+$ RNA and polypeptides between isogenic virulent and hypovirulent strains (Powell and Van Alfen, 1987a,b), suppressed accumulation of a number of specific metabolites, enzymatic activities and mRNA transcripts in hypovirulent strains (Dodds, 1980; Havir and Anagnostakis, 1983; Rigling *et al.*, 1989; Rigling and Van Alfen, 1991; Larson *et al.*, 1992; Varley *et al.*, 1992) and virus-mediated alterations of certain processes such as pigment production and asexual sporulation (Anagnostakis, 1982, 1984b; Elliston, 1978, 1985). While none of these alterations has been shown to be directly linked to virulence attenuation, the fact that virus infection has such varied effects on fungal phenotype is consistent with the suggestion that hypovirulence and associated traits are a consequence of alterations of the regulatory pathways that control expression of sets of host genes. Our recent studies on virus-mediated suppression of *C.parasitica* laccase accumulation have provided direct evidence that hypoviruses can affect the expression of a fungal gene at the level of promoter activity and that the mechanism involves the perturbation of signal transduction pathways that govern *lac-1* transcription in virus-free virulent fungal strains (Larson *et al.*, 1992). The results presented in this report confirm and extend those observations under better defined physiological conditions and provide new evidence that hypovirulent *C.parasitica* strains are altered in the ability to modulate nuclear gene expression in response to nutrient availability.

Based on the results summarized in Table I, the regulatory pathways previously identified as governing *lac-1* transcription can be reproducibly manipulated by changing the nutritional environment. Both regulatory pathways appear to be inactive in MM (Table I), furnishing an experimental system for examining the mechanism(s) of action of compounds that stimulate the positive regulatory pathway. This pathway is activated by components present in ME, providing conditions (MM + ME) where the effects of agents that either interfere with the activity of the positive pathway or stimulate the activity of the negative regulatory pathway can be investigated (Figure 3, Table I). Alternatively, the positive regulatory pathway can be examined independently of the negative regulatory pathway by transferring fungus from rich PDB medium to ME, resulting in inhibitor-independent derepression of *lac-1* transcription (Figures 1–4). Finally, antagonists of the negative regulatory pathway can be examined in MM + ME supplemented with CAA or in PDB (Figure 7, Table I), conditions under which both pathways are active (Figures 2–4). These conditions were used to confirm that the positive regulatory pathway requires intact IP_3 and

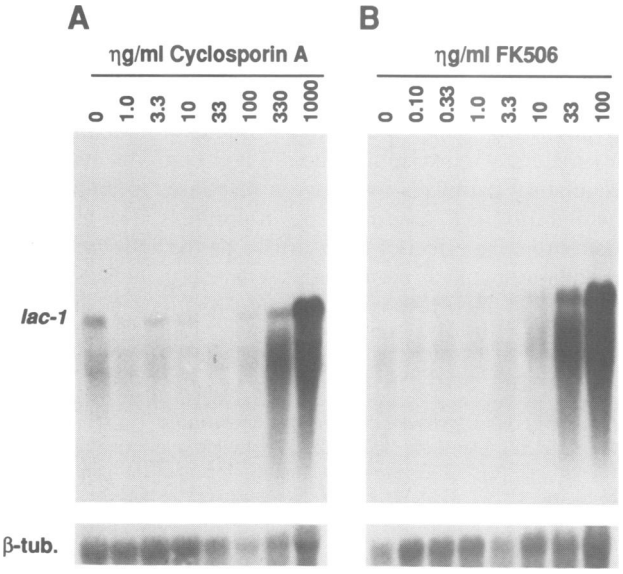

Fig. 7. Derepression of *lac-1* transcript accumulation in PDB by inhibitors of calcineurin. Cultures of EP155 were prepared as for Figure 1 and the medium was changed to fresh PDB containing the indicated concentrations of (**A**) cyclosporin A or (**B**) FK506. Cultures were harvested and total RNA was prepared 24 h after the medium was changed. Total RNA was analyzed as described in Figure 1.

Ca^{2+} second messenger systems (Figure 4). Results obtained using these conditions (Figure 5, Table I) also support the hypothesis that hypoviruses suppress *lac-1* transcription by interfering with signal transmission through the positive regulatory pathway (Larson *et al.*, 1992). The development of defined, inhibitor-independent conditions for examining regulation of *lac-1* transcription will be of considerable utility in future attempts to dissect the regulatory pathways and to determine the precise nature of virus-mediated suppression of transcription.

We previously demonstrated that cyclosporin A can stimulate *lac-1* transcription (Larson and Nuss, 1993). This immunosuppressant/antifungal agent is a potent inhibitor of calcineurin (Phosphatase 2B) (Schreiber, 1991, 1992). Like cyclosporin A, FK506 also inhibits calcineurin (Schreiber, 1991, 1992). Although cyclosporin and FK506 inhibit calcineurin through a common mechanism, they belong to different classes of chemical compounds (cyclic peptide and macrolide) and bind to different initial targets (cyclophilin versus FK Binding Protein), making it unlikely that the stimulation of *lac-1* transcription by both compounds would be due to action on a target other than calcineurin. The observation that both inhibitors derepress *lac-1* transcription in rich medium (Figure 7) provides compelling evidence that this Ca^{2+}/calmodulin-dependent protein phosphatase is an effector of the negative pathway involved in repression of *lac-1* transcription when amino acids are in abundant supply. The involvement of calcineurin in the negative pathway suggests that protein dephosphorylation is likely to play a role in maintaining the repression of *lac-1* transcription under conditions of amino acid sufficiency.

The inhibition of the Ca^{2+}/IP_3-dependent increase in *lac-1* transcript accumulation by two independent inhibitors of calmodulin activity (Figure 6) strongly indicates a role for this Ca^{2+}-dependent regulatory protein in the positive regulatory pathway governing *lac-1* transcription. Possible roles for calmodulin in fungal pathogenesis have been suggested by several recent studies. For example, calmodulin levels were found to be 5-fold higher in *Phytophthora infestans* when grown *in planta* than when grown on synthetic medium (Pieterse *et al.*, 1993). Inhibition of calmodulin activity has also been shown to prevent transition from the yeast phase to the invasive hyphal form for both the phytopathogenic fungus *O.ulmi* and the human pathogen *C.albicans* (Muthukumar and Nickerson, 1984; Roy and Datta, 1987; Sabie and Gadd, 1989; Paranjape *et al.*, 1990; Brunton and Gadd, 1991). The observation that hypovirus infection suppresses the calmodulin-dependent *lac-1* positive regulatory pathway raises the possibility that the virus-mediated suppression of other calmodulin-dependent regulatory cascades, including some involved in transcriptional regulation of fungal genes required for pathogenesis, may contribute to virulence attenuation in hypovirulent *C.parasitica* strains.

The identification of both calmodulin and calcineurin as effectors of *lac-1* transcriptional regulation has several interesting implications. As indicated above, calmodulin also serves as a regulatory subunit of calcineurin. Thus, it is likely that calmodulin antagonists block the action of both the positive and negative pathways, thereby preventing the stimulation of *lac-1* transcription mediated by the positive regulatory pathway (Figure 6). In contrast, calcineurin antagonists only block the action of the negative regulatory pathway, thereby preventing the repression of *lac-1* transcription mediated by this pathway. Calmodulin has been shown to regulate the activity of both protein kinases, such as kinase II (Cheung, 1982; Hanson and Schulman, 1992), and phosphatases, such as calcineurin (Cheung, 1982; Cohen, 1989). These observations, coupled with the results presented in Figures 6 and 7, suggest that a calmodulin-dependent kinase may play a role in the positive-acting pathway. Thus, *lac-1* transcription may be regulated by the co-ordinated actions of a calmodulin-dependent kinase and a calmodulin-dependent phosphatase on a single transcription factor or on a combination of targets. The kinase and phosphatase may act directly on these targets, or they may act indirectly through a cascade of additional kinases and phosphatases. Again, the development of defined, CHX-independent conditions for examining *lac-1* transcriptional regulation will be invaluable in efforts to determine the precise role of protein phosphorylation in this nutritionally regulated transcriptional pathway.

The apparent nutritional regulation of *lac-1* transcription described in this report (Figures 1–3, and Table I) is particularly relevant to recent genetic studies of *lacc*, the *Neurospora crassa* laccase gene. The accumulation of *lacc* transcript, which is repressed under normal vegetative growth, is also induced by treatment with low concentrations of CHX (Froehner and Eriksson, 1974). Thus, as with *lac-1*, the transcription of *lacc* appears to be repressed by a CHX-sensitive negative regulatory pathway. Mutations at the *N.crassa lah-1* locus result in constitutive, CHX-independent derepression of *lacc* expression, suggesting that this locus encodes a *trans*-acting factor necessary for the action of such a negative regulatory pathway (Tamaru and Inoue, 1991). Mutations at a second

locus, *lni-1*, render *lacc* expression resistant to CHX induction, suggesting that this locus encodes a component required for the maintenance of a positive regulatory pathway (Zamma *et al.*, 1993) that may be comparable with the pathway predicted by pharmacological analysis to stimulate *lac-1* transcription (Larson *et al.*, 1992). Genetic analysis revealed *lni-1* to be allelic with *cpc-1* (Zamma *et al.*, 1991; Tamaru and Inoue, 1993), the gene encoding the transcription factor that co-ordinates global transcriptional responses to amino acid starvation (general amino acid control). Thus, in *N.crassa* the key transcription factor involved in the global response to amino acid starvation also appears to be required for the regulation of *lacc*, a gene not thought to play a role in responding to nutritional availability. These observations warrant a determination of whether the general amino acid control response also plays a role in the positive regulation of *lac-1* transcription.

One of the key findings of this study is that hypovirus CHV1-713 impairs the ability of *C.parasitica* to modulate *lac-1* mRNA accumulation in response to changes in nutrient availability. When considered in a broader context, hypovirus-mediated alterations in host transcriptional responses to changes in the nutritional environment would be predicted to have an important impact on fungal virulence. In this regard, Hebard *et al.* (1984) suggested that one of the most important determining factors of canker development by *C.parasitica* on American chestnut is the ability of the invading pathogen to form a structure consisting of densely packed parallel hyphal aggregates called a mycelial fan. This structure appears to be essential for penetration through lignified zones and wound periderm deposited by the infected tree in an attempt to isolate the invading fungus. The formation of superficial non-lethal cankers by virulent *C.parasitica* strains on blight-resistant Chinese chestnut trees and by hypovirulent strains on blight-susceptible American chestnut trees appeared to be related to the rate and extent of mycelial fan formation (Hebard *et al.*, 1984). These authors speculated further that a key to blight susceptibility was the ability of *C.parasitica* to obtain nutrients during the infection process, particularly during the critical stage of mycelial fan formation. Thus, the efficient formation of and penetration by mycelial fans, events that undoubtedly contribute to virulence, may be dependent on the availability of nutrients following initial colonization.

Lewis (1953), in his 'Balance Hypothesis', and Garber (1956), in his 'Nutrition-Inhibition Hypothesis', have argued that the nutritional environment provided by the host must be adequate to support the metabolism of the parasite if it is to be virulent. The data presented in this report would be consistent with a view that hypoviruses alter the nutritional requirements of *C.parasitica* such that the nutritional environment provided by the susceptible host is no longer sufficient to support the metabolism of the fungus as a parasite. From the perspective of the fungus, this problem becomes more acute as the tree mounts defense mechanisms that have evolved to physically isolate the invading parasite. In this regard, the inability of the fungus to adequately adapt to changes in amino acid availability as it attempts to overcome host defense barriers could conceivably contribute to the underlying basis of hypovirulence. Puhalla and Anagnostakis (1971) have reported that the *arg-1* (arginine-requiring) and *rf-1* (riboflavin-requiring) mutants of *C.parasitica* are non-pathogenic, lending support to a connection between a reduced ability to respond to nutrient availability and hypovirulence in *C.parasitica*. Studies to identify and characterize transcription factors and components of the regulatory pathways that govern responses to amino acid availability in *C.parasitica* are in progress. The ability to determine the effect of hypoviruses on the activity of such regulatory pathway components will be crucial in assessing the role of such responses in fungal virulence.

Materials and methods

Fungal strains, growth conditions and media

Cryphonectria parasitica strains EP155 (virulent and virus-free, ATCC 38755) and EP713 (isogenic to EP155, hypovirulent and contains hypovirus CHV1-713, ATCC 52571) were maintained as described by Hillman *et al.* (1990). Stationary liquid cultures were prepared and maintained as described previously (Choi *et al.*, 1992). CHX, $LaCl_3$, neomycin (Larson *et al.*, 1992) and cyclosporin A (Larson and Nuss, 1993) were added as previously described. Trifluoperazine was added to cultures from a 50 mM stock in dimethyl sulfoxide. Ophiobolin A was added from a 25 mM stock in ethanol. FK506 was a generous gift of Drs George Livi and Randall Johnson of SmithKline Beecham Pharmaceuticals, and was added to cultures from a 10 mg/ml stock dissolved in ethanol. To change the growth medium, mycelia were collected by centrifugation at 2000 r.p.m. for 10 min at 4°C and the old medium was removed by decanting. The pellet was then resuspended in an equal volume of new medium by vortexing.

Potato dextrose agar (PDA) and PDB were obtained from Difco Laboratories, and were prepared as directed. ME (Difco Laboratories) was prepared at 1.5% (w/v) in distilled H_2O and autoclaved. Chemically defined minimal medium (MM) was prepared using the recipe described by Puhalla and Anagnostakis (1971). Minimal medium with 1.5% ME (MM + ME) was prepared by combining double-strength stocks of MM and ME. All synthetic media were adjusted to pH 4.5 using NaOH or HCl as needed, and sterilized by filtration. Yeast extract (Difco Laboratories) and CAA (Difco Laboratories) were added from filter-sterilized 20% (w/v) stocks. For solid media, Nobel Agar (Difco Laboratories) was used at a final concentration of 2%.

RNA preparation and analysis

Total RNA was prepared as previously described (Larson *et al.*, 1992). Cultures grown in media containing ME tended to produce large amounts of polysaccharide that carried through the procedure. This problem was circumvented by incubating the phenol–chloroform emulsions on ice for 10 min prior to centrifugation with the centrifugation step being carried out at room temperature as originally described. Total RNA (10 mg) was separated by formaldehyde–agarose electrophoresis and transferred to nylon membranes (Gene Screen Plus, DuPont, Wilmington, DE) using standard procedures (Sambrook *et al.*, 1989) under the conditions described elsewhere (Choi *et al.*, 1992; Larson *et al.*, 1992). Hybridization, washing and stripping procedures were performed using conditions recommended by the manufacturer of the membrane. The preparation and labeling of the *lac-1* and β-tubulin probes have been described elsewhere (Choi *et al.*, 1992).

References

Anagnostakis,S.L. (1982) *Science*, **215**, 466–471.

Anagnostakis,S.L. (1984a) In Jennings,D.H. and Rayner,A.D.M. (eds), *The Ecology and Physiology of the Fungal Mycelium*. Cambridge University Press, Cambridge, MA, pp. 353–366.

Anagnostakis,S.L. (1984b) In Jennings,D.H. and Rayner,A.D.M. (eds), *The Ecology and Physiology of the Fungal Mycelium*. Cambridge University Press, Cambridge, MA, pp. 499–507.

Anagnostakis,S.L. and Day,P.R. (1979) *Phytopathology*, **69**, 1226–1229.

Bavendamm,W. (1928) *Z. Pflanzenkrank. Pflanzenschutz*, **38**, 257–276.

Bridson,Y.E. and Brecker,A. (1970) In Norris,J.R. and Ribbons,D.W. (eds), *Methods in Microbiology*. Academic Press, New York, Vol. 3A, pp. 229–295.

Brunton,A.H. and Gadd,G.M. (1991) *Mycol. Res.*, **95**, 484–491.

Carney,D.H., Scott,D.L., Gordon,E.A. and LaBelle,E.F. (1985) *Cell*, **42**, 479–488.
Carpenter,C.E., Mueller,R.J., Kazmierczak,P., Zhang,L., Villalon,D.K. and Van Alfen,N.K. (1992) *Mol. Plant–Microbe Interact.*, **4**, 55–61.
Chen,B., Choi,G.H. and Nuss,D.L. (1994) *Science*, **264**, 1762–1764.
Cheung,W.Y. (1982) *Fed. Proc.*, **41**, 2253–2257.
Choi,G.H. and Nuss,D.L. (1992a) *EMBO J.*, **11**, 473–477.
Choi,G.H. and Nuss,D.L. (1992b) *Science*, **257**, 800–803.
Choi,G.H., Larson,T.G. and Nuss,D.L. (1992) *Mol. Plant–Microbe Interact.*, **5**, 119–128.
Cockcroft,S., Howell,T.W. and Gomperts,B.D. (1987) *Biochem. Biophys. Res. Commun.*, **105**, 2745–2750.
Cohen,P. (1989) *Annu. Rev. Biochem.*, **58**, 453–508.
Craven,M.G., Pawlyk,D.M., Choi,G.H. and Nuss,D.L. (1993) *J. Virol.*, **67**, 6513–6521.
Dodds,J.A. (1980) *Phytopathology*, **70**, 1217–1220.
Downes,C.P. and Mitchell,R.H. (1981) *Biochemistry*, **198**, 133–140.
Elliston,J.E. (1978) In MacDonald,W.L., Cech,F.W., Luchok,J. and Smith,C. (eds), *Proceedings of the American Chestnut Symposium*. West Virginia University, Morgantown, WV, pp. 95–100.
Elliston,J.E. (1985) *Phytopathology*, **75**, 151–158.
Flanagan,W.M., Corthesy,B., Bram,R.J. and Crabtree,G.R. (1991) *Nature*, **352**, 803–807.
Froehner,S.C. and Eriksson,K.-E. (1974) *J. Bacteriol.*, **120**, 450–457.
Garber,E.D. (1956) *Am. Nat.*, **90**, 183–194.
Hanson,P.I. and Schulman,H. (1992) *Annu. Rev. Biochem.*, **61**, 559–601.
Havir,E.A. and Anagnostakis,S.L. (1983) *Physiol. Plant Pathol.*, **23**, 369–376.
Hebard,F.V., Griffin,G.J. and Elkins,J.R. (1984) *Phytopathology*, **74**, 140–149.
Hillman,B.I., Shapira,R. and Nuss,D.L. (1990) *Phytopathology*, **80**, 950–956.
Kolattukudy,P.E. (1985) *Annu. Rev. Phytopathol.*, **23**, 223–250.
Larson,T.G. and Nuss,D.L. (1993) *Proc. Natl Acad. Sci. USA*, **90**, 148–152.
Larson,T.G., Choi,G.H. and Nuss,D.L. (1992) *EMBO J.*, **11**, 4539–4548.
Leung,P.C., Graves,L.M. and Tipton,C.L. (1988) *Int. J. Biochem.*, **12**, 1351–1359.
Lewis,R.W. (1953) *Am. Nat.*, **87**, 273–281.
MacDonald,W.L. and Fulbright,D.W. (1991) *Plant Dis.*, **75**, 656–661.
Marzluf,G.A. (1981) *Microbiol. Rev.*, **45**, 437–461.
Muthukumar,G. and Nickerson,K.W. (1984) *J. Bacteriol.*, **159**, 390–392.
Nathan,R.D., Kanai,K., Clark,R.B. and Giles,W. (1988) *J. Gen. Physiol.*, **91**, 549–572.
Nuss,D.L. (1992) *Microbiol. Rev.*, **56**, 561–576.
Paranjape,V., Roy,B.G. and Datta,A. (1990) *J. Gen. Microbiol.*, **136**, 2149–2154.
Pieterse,C.M.J., Verbakel,H.M., Spaans,J.H., Davidse,L.C. and Govers,F. (1993) *Mol. Plant–Microbe Interact.*, **6**, 164–172.
Powell,W.A. and Van Alfen,N.K. (1987a) *J. Bacteriol.*, **169**, 5324–5326.
Powell,W.A. and Van Alfen,N.K. (1987b) *Mol. Cell. Biol.*, **7**, 3688–3693.
Puhalla,J.E. and Anagnostakis,S.L. (1971) *Phytopathology*, **61**, 169–173.
Rigling,D. and Van Alfen,N.K. (1991) *J. Bacteriol.*, **173**, 8000–8003.
Rigling,D., Heiniger,U. and Hohl,H.R. (1989) *Phytopathology*, **79**, 219–223.
Roy,B.G. and Datta,A. (1987) *FEMS Microbiol. Lett.*, **41**, 327–329.
Sabie,F.T. and Gadd,G.M. (1989) *Mycopathologia*, **108**, 47–54.
Sambrook,J., Fritsch,E.F. and Maniatis,T. (1989) *Molecular Cloning: A Laboratory Manual*, 2nd edn. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
Schafer,W., Straney,D., Ciuffetti,L., van Etten,H.D. and Yoder,O.C. (1989) *Science*, **247**, 247–249.
Schreiber,S.L. (1991) *Science*, **251**, 283–287.
Schreiber,S.L. (1992) *Cell*, **70**, 365–368.
Schreiber,S.L. and Crabtree,G.R. (1992) *Immunol. Today*, **13**, 136–142.
Scott-Craig,J.S., Panacclone,D.G., Cervone,F. and Walton,J.D. (1990) *Plant Cell*, **2**, 1191–1200.
Slivka,S.R. and Insel,P.A. (1987) *J. Biol. Chem.*, **262**, 4200–4207.
Stahl,D.J. and Schafer,W. (1992) *Plant Cell*, **4**, 621–629.
Straney,D.C. and Van Etten,H. D. (1994) *Mol. Plant–Microbe Interact.*, **7**, 256–266.
Tamaru,H. and Inoue,H. (1991) *Jpn J. Genet.*, **66**, 767.
Van Alfen,N.K. (1985) In Buck,K. (ed.), *Fungal Virology*. CRC Press, Boca Raton, FL, pp. 143–162.
Van Etten,H.D., Matthews,D.E. and Matthews,P.S. (1989) *Annu. Rev. Phytopathol.*, **27**, 143–164.
Varley,D.A., Podila,G.K. and Hiremath,S.T. (1992) *Mol. Cell. Biol.*, **12**, 4539–4544.
Zamma,A., Tamaru,H., Harashima,T. and Inoue,H. (1993) *Mol. Gen. Genet.*, **240**, 231–237.

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