Papain-Like Protease p29 as a Symptom Determinant Encoded by a Hypovirulence-Associated Virus of the Chestnut Blight Fungus

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Viral double-stranded RNAs (dsRNAs) responsible for virulence attenuation (hypovirulence) of the chestnut blight fungus, *Cryphonectria parasitica*, profoundly influence a range of host functions in addition to virulence. The 5'-proximal open reading frame, A, of the prototypical hypovirulence-associated viral dsRNA, L-dsRNA, present in hypovirulent strain EP713, was recently shown by DNA-mediated transformation analysis to suppress fungal sporulation, pigmentation, and accumulation of the enzyme laccase (G. H. Choi and D. L. Nuss, EMBO J. 11:473–477, 1992). We mapped this suppressive activity to the autocatalytic papain-like protease, p29, present within the amino-terminal portion of open reading frame A-encoded polyprotein p69. Mutational analysis revealed that the ability of p29 to alter fungal phenotype is dependent upon release from the polyprotein precursor but is independent of intrinsic proteolytic activity. Deletion of the p29-coding domain within the context of an infectious L-dsRNA cDNA clone resulted in a replication-competent viral dsRNA that exhibited intermediate suppressive activity while retaining the ability to confer hypovirulence. Thus, p29 is necessary but not sufficient for the level of virus-mediated suppression of fungal pigmentation, sporulation, and laccase accumulation observed for wild-type hypovirulent strain EP713 and is nonessential for viral RNA replication and virulence attenuation. These results also illustrate the feasibility of engineering infectious viral cDNA for construction of hypovirulent fungal strains with specific phenotypic traits.

Viruses and unencapsidated virus-like double-stranded RNAs (dsRNAs) have been identified in fungal isolates representing all major classes of fungi (5, 30, 34). Such elements are generally benign, causing no obvious phenotypic alterations of the fungal host. Notable exceptions include the viral elements associated with La France disease of the cultivated mushroom *Agaricus bisporus* (23, 26, 36), viral elements responsible for the killer phenotype of *Saccharomyces cerevisiae* (45), and the virus-like dsRNA elements responsible for transmissible hypovirulence and resulting biological control of the chestnut blight fungus, *Cryphonectria parasitica* (1, 33). Most identified mycoviruses and fungus-associated virus-like elements have not been well characterized, and their study is complicated by the inability of cell-free preparations to initiate infection (5, 44). Infections are persistent, and transmission is generally limited to strains of the same species following fusion of fungal hyphae (anastomosis). Consequently, evidence for a causal relationship between fungus-associated virus-like agents and symptom expression has necessarily been correlative in nature. In this regard, Choi and Nuss (8) were recently able to establish a direct cause-and-effect relationship for a hypovirulence-associated viral RNA of *C. parasitica* and fungal hypovirulence by transforming virus-free virulent strains with a full-length infectious cDNA clone of the viral dsRNA. Resulting transformants contained chromosomally integrated copies of viral cDNA, as well as cDNA-derived, cytoplasmically replicating viral dsRNA, and were hypovirulent.

Hypovirulent *C. parasitica* strains often exhibit traits (termed hypovirulence-associated traits) in addition to reduced virulence that distinguish them from corresponding isogenic virus-free virulent strains. These can include suppressed pigmentation, reduced asexual sporulation (conidia-

tion), and suppressed transcription of the *lac-1* gene that encodes the fungal enzyme laccase (6, 24, 29, 35). Recent progress in the cloning and molecular characterization of hypovirulence-associated viruses of *C. parasitica*, coupled with the development of a DNA-mediated transformation system for this fungus, has provided experimental approaches for identifying viral gene products involved in fungal phenotype modulation. For example, introduction of a cDNA copy of the 5'-proximal coding domain, open reading frame (ORF) A, of the hypovirulence-associated viral RNA, L-dsRNA, present in hypovirulent *C. parasitica* EP713 into isogenic, virus-free, virulent strain EP155 resulted in suppressed pigmentation, suppressed sporulation, and reduced laccase accumulation (7). Significantly, the ORF A transformants were not reduced in virulence. The finding that associated traits, such as suppressed sporulation, could be uncoupled from hypovirulence suggested that different virus-encoded proteins are responsible for specific traits expressed by individual hypovirulent strains. Additional transformation studies, described in this report, have mapped the ORF A-associated suppressive activity to autocatalytic papain-like protease p29, located within the amino-terminal portion of ORF A-encoded polyprotein p69. Advantage was also taken of the availability of the recently developed infectious cDNA clone of L-dsRNA to test the consequences of p29 deletion for virus RNA replication and fungal phenotype. These results are discussed in terms of virus-mediated disease symptom expression and the feasibility of engineering hypovirulent *C. parasitica* strains with improved potential for biological control.

MATERIALS AND METHODS

Construction of transformation plasmids. Transformation vector pCPXHY1, presented in Fig. 1B and described in Results, was used for construction of all of the transformation

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FIG. 1. Genetic organization of EP713 L-dsRNA, predicted amino acid sequence of the p29-coding domain, and diagram of transformation vector pCPXHY1. The basic organization of L-dsRNA present in hypovirulent strain EP713 (39) and the current view of ORF A expression (9, 10) are presented in panel A and described in the text. A diagram illustrating the relevant properties of transformation vector pCPXHY1 is presented in panel B. pCPXHY1 is a derivative of transformation plasmid pAXHY2, previously used for transformation of *C. parasitica* with ORF A (7), and consists of unique cloning sites flanked by the *C. parasitica* glyceraldehyde-3-phosphate dehydrogenase gene promoter (*PgpD*) and terminator (*Tgpd*). The vector also contains the *E. coli* hygromycin B phosphotransferase gene (*hygB*) flanked by the *A. nidulans* *trpC* promoter (*PtrpC*) and terminator (*TtrpC*) (14) in a pUC19 background. nt, nucleotides; RIG/GRL, the amino acid sequence flanking the ORF A autocatalytic cleavage dipeptide Gly-248/Gly-249.

plasmids used, and virulent *C. parasitica* EP155 served as the recipient for all of the transformation experiments described in this report (see the next section for details). The basic organization of viral L-dsRNA from hypovirulent *C. parasitica* EP713 and pertinent amino acid sequence information for the ORF A coding domain are presented in Fig. 1A. Plasmids pXH101 and pXH102 contained intact cDNA copies of the L-dsRNA ORF A coding region in the sense and antisense orientations, respectively. These plasmids were generated by subcloning the T4 DNA polymerase-treated *KpnI*-*XbaI* fragment from plasmid pORFUS1 (10) into the T4 DNA polymerase-treated *KpnI* site of pCPXHY1. Fungal transformants containing pXH101 and pXH102 were designated p69(+) and p69(-), respectively, since the transforming plasmids contained the intact region specifying ORF A-encoded polyprotein p69 (Fig. 1A) in either orientation. Plasmids pXH7 and pXH8 contained a cDNA copy of ORF A that was mutated to prevent processing of polyprotein p69. The mutation involved alteration of the codon that specifies Gly-248 at the autocatalytic cleavage site to encode an arginine residue. These plasmids were con-

structed by subcloning both orientations of the *KpnI*-*XbaI* fragment of the corresponding pORFUS1 substitution mutation (9) into pCPXHY1. Transformants recovered after transformation with plasmids pXH7 and pXH8 were designated p69-G²⁴⁸(+) and p69-G²⁴⁸(-), respectively. Transformation plasmids containing both orientations of the p29-coding portion of ORF A (pXH1 and pXH2), both orientations of the p40-coding portion of ORF A (pXH3 and pXH4), and the sense orientation of the N-terminal (codons 1 to 160) and C-terminal (codons 135 to 248) portions of p29 (pXH13 and pXH15, respectively) were all generated with the aid of the polymerase chain reaction (PCR) (37) from plasmid 1115. This plasmid contained a partial cDNA copy of L-dsRNA corresponding to 2,798 bp of the L-dsRNA 5' (positive-strand) terminus (39). Primers were designed such that the 5'-terminal portions specified either *KpnI* or *SphI* restriction sites to allow directional cloning of the resulting amplicon into pCPXHY1. A plasmid containing p29 in which His-215 was replaced with a serine residue (pXH11) was also generated with the aid of PCR but from the appropriately mutated pORFUS construct (9). The designations given transformants containing these plasmids were as follows: pXH1, p29(+); pXH2, p29(-); pXH3, p40(+); pXH4, p40(-); pXH11, p29-H²¹⁵; pXH13, p29¹⁻¹⁶⁰; pXH15, p29¹³⁵⁻²⁴⁸.

Construction of plasmids pXHΔ1 and pXHΔ2, in which most of the p29-coding region was deleted within the context of the otherwise full-length L-dsRNA cDNA clone, required a multistep approach. The 5.3-kb *XbaI*-*KpnI* fragment corresponding to the 5' (positive-strand) terminus of the L-dsRNA sequence was isolated from the L-dsRNA cDNA copy present in plasmid pLDST (Fig. 1 in reference 8) and subcloned into pUC18 to yield plasmid pLC1. This plasmid contained two *Bam*HI restriction sites, both of which resided within the p29-coding domain. Deletion of the 657-bp *Bam*HI fragment and religation gave rise to plasmid pLC1-ml, which then served as the source of a 4.6-kb *SspI*-*KpnI* fragment containing the deleted region that was substituted for the intact 5.3-kb *SspI*-*KpnI* fragment in pLDST to give plasmid pLDST3. Deletion of the *Bam*HI fragment generated an in-frame mutation of p29 that fused Val-22 to Asp-242. In the final step, the 12.7-kb *SspI*-*SpeI* fragment from pLDST3 containing the mutated L-dsRNA cDNA was isolated, treated with the Klenow fragment, and ligated into the *StuI* site of pCPXHY1, yielding plasmids pXHΔ1 (sense orientation) and pXHΔ2 (antisense orientation). Fungal transformants containing pXHΔ1 and pXHΔ2 were designated HAVΔ^{p29}(+) and HAVΔ^{p29}(-), respectively. All mutations, cloning steps, and PCR amplicons were confirmed by nucleotide sequence analysis. The transformation plasmids and transformant designations used in this study are listed in Table 1.

Fungal strains and manipulations. Isogenic *C. parasitica* strains EP155 (virulent, dsRNA free) and EP713 (hypovirulent, contains L-dsRNA) were maintained as described previously (24). Transformation of EP155 spheroplasts was performed essentially as described by Churchill et al. (11) and was followed by selection on hygromycin B-containing medium (40 μg/ml). Transformed strains were grown on potato dextrose agar (PDA; Difco) on a laboratory bench (light, <1,000 lx; temperature, 22 to 24°C) as described previously (7, 24), and levels of pigmentation and conidiation were observed under these conditions. For pXHΔ1 transformants, the relative level of conidiation was also observed when they were grown in an environmentally controlled growth chamber under fluorescent and incandescent light at 2,000 lx with a 12-h photoperiod at 24°C. Conidia were harvested at either 10 or 13 days by addition of 10 ml of 0.15% Tween 80 to the culture plate and

TABLE 1. Transformation plasmids and fungal transformants used in this study

Transformation plasmid	Transformant designation	Description
pXH101	p69(+)	Contains ORF A coding region
pXH102	p69(-)	Antisense construct of pXH101
pXH1	p29(+)	Contains p29-coding region
pXH2	p29(-)	Antisense construct of pXH1
pXH3	p40(+)	Contains p40-coding region
pXH4	p40(-)	Antisense construct of pXH3
pXH7	p69-G ²⁴⁸ (+)	Contains mutated ORF A (G-248→R), cleavage defective
pXH8	p69-G ²⁴⁸ (-)	Antisense construct of pXH7
pXH11	p29-H ²¹⁵	Contains mutated p29 (H-215→S), protease defective
pXH13	p29 ¹⁻¹⁶⁰	Contains p29-coding region, aa ^a 1-160.
pXH15	p29 ¹³⁵⁻²⁴⁸	Contains p29-coding region, aa 135-248
pXHΔ1	HAVΔ ^{p29} (+)	Contains 657-nt ^b deletion of p29-coding region in context of infectious L-dsRNA cDNA clone
pXHΔ2	HAVΔ ^{p29} (-)	Antisense construct of pXHΔ1

^a aa, amino acids.^b nt, nucleotide.

liberation of the conidia with a glass rod. The conidial suspensions were strained through Miracloth (Calbiochem), counted under a microscope with a hemacytometer, and tested for viability by spreading aliquots of serial dilutions on PDA plates and scoring them for colonies derived from germinating spores. To test for laccase activity, colonies were grown in the dark at 22 to 24°C on agar plates containing Bavendamm's medium (3). Virulence assays were performed on dormant chestnut stems, and measurements were taken 21 days post-inoculation, as previously described (8, 27). L-dsRNA was extracted from fungal transformants and characterized by reverse transcriptase (RT)-PCR (8).

RESULTS

To understand ORF A-mediated modulation of host functions better, mutated forms and individual coding portions of ORF A were tested for the ability to confer hypovirulence-associated traits following DNA-mediated transformation. Relevant information regarding the basic organization and expression of ORF A is illustrated in Fig. 1A. Available evidence indicates that ORF A-encoded polypeptide p69 is cotranslationally and autocatalytically cleaved to yield polypeptide products p29 and p40 (9, 10). Cleavage occurs between Gly-248 and Gly-249, and the catalytic activity is located within the carboxy-terminal portion of p29. Consistent with these results, mutational analysis revealed that residues Cys-162 and His-215 are essential for autocatalytic cleavage (9). To compare directly the phenotypic consequences of transformation with normal and mutated forms of ORF A, it was necessary to develop a common transformation vector. Thus, vector pCPXHY1 was constructed (8) (Fig. 1B). This vector, a derivative of vector pAXHY2, used in previous transformation studies (7), consists of unique cloning sites inserted between the *C. parasitica* glyceraldehyde-3-phosphate dehydrogenase (*gpd-1*) promoter and the *gpd-1* terminator. pCPXHY1 also contains the *Escherichia coli* hygromycin B phosphotransferase gene as a selectable marker flanked by the *Aspergillus nidulans trpC* promoter and terminator domains (14), all in the genetic background of pUC19.

Mapping and characterization of ORF A-mediated suppressive activity. Since ORF A expression involves processing of an encoded polypeptide, a logical first step was to determine whether disruption of processing affected the ability of ORF A to modulate host functions. Autocatalytic cleavage was shown

previously to be abolished when the cleavage site was mutated by substituting an arginine residue for Gly-248 (9). As indicated in Fig. 2, transformation of strain EP155 with a positive-sense cDNA copy of ORF A in vector pCPXHY1 [p69(+)] resulted in a significant reduction in orange pigment production, an indicator of suppressive activity, while the antisense copy of ORF A [p69(-)] failed to alter host phenotype. These results are consistent with those previously reported for sense and antisense ORF A vectors pAXHY2 and pAXHY5, respectively (7). As indicated by colonies designated p69-G²⁴⁸(+) and p69-G²⁴⁸(-), no alteration in fungal pigmentation resulted from transformation with either orientation of the ORF A cleavage mutant (Gly-248 to Arg).

The apparent requirement of p69 processing for alteration of host functions by ORF A suggested that the cleavage products, independently or in combination, may be responsible for conferring the observed hypovirulence-associated traits. To test this possibility, the p29- and p40-coding domains were introduced independently in both the sense and antisense orientations into vector pCPXHY1 as described in Materials and Methods. As indicated in Fig. 3, colonies transformed with p40 in either orientation [p40(+)] or p40(-)] and p29 in the antisense orientation [p29(-)] resembled untransformed strain EP155. In contrast, transformants containing p29 in the sense orientation [p29(+)] resembled hypovirulent strain EP713, suggesting that this cleavage product is responsible for the reduction in orange pigmentation observed for the ORF A transformants.

Since several viral proteases have been implicated in symptom expression (12, 16, 18, 28, 31) and release of p29 from the p69 precursor appeared to be required for suppressive activity [i.e., p69-G²⁴⁸(+)] conferred no observable phenotype (Fig. 2)], we next determined whether the p29-associated papain-like autoproteolytic activity involved in p69 processing was also necessary for p29-mediated modulation of fungal phenotype. Mutational analysis of p29 had earlier demonstrated that amino acid residues Cys-162 and His-215 are essential for autoproteolysis (9). As indicated in Fig. 4, conversion of His-215 to Ser, a mutation previously shown to eliminate autoproteolytic activity (9), did not affect the suppressive activity of p29 in transformed fungal isolates. Deletion mutagenesis had previously shown that autoproteolytic cleavage at Gly-248/Gly-249 was unaffected by deletion of the N-terminal 134 amino acid residues of p29 (9). In contrast, deletion of 134 amino acids from the N terminus or of 88 amino acids from

FIG. 2. Effect of a processing mutation on ORF A-mediated suppression of fungal pigmentation. Transformants designated p69(+) and p69(-) were transformed with plasmids pXH101 and pXH102, which contained the intact ORF A coding region in the sense and antisense orientations, respectively. Transformants designated p69-G²⁴⁸(+) and p69-G²⁴⁸(-) were transformed with plasmids pXH7 and pXH8, respectively, which contain both orientations of a cDNA copy of ORF A that specifies a cleavage-defective mutated form of p69. The mutation involved alteration of the codon for one of the cleavage dipeptide residues, Gly-248, to specify an arginine residue (9). Transformants were grown in parallel with untransformed EP155 and isogenic, hypovirulent strain EP713 for 8 days on PDA (Difco) on a laboratory bench as described previously (7).

FIG. 4. Effect of His-215 substitution and N-terminal or C-terminal deletion mutations on p29-mediated suppression of fungal pigmentation. The transformant designated p29-His²¹⁵ was transformed with plasmid pXH11, which contained a mutation within the p29-coding portion of ORF A in which the codon for His-215, a residue shown to be essential for autocatalytic cleavage of polyprotein p69 (9), was altered to specify a serine residue, thus abolishing proteolytic activity. Transformants designated p29¹⁻¹⁶⁰ and p29¹³⁵⁻²⁴⁸ were transformed with plasmids pXH13 and pXH15, which contained p29 codons 1 to 160 and 135 to 248, respectively. That is, the former represents a C-terminal deletion and the latter represents an N-terminal deletion of the p29-coding domain.

FIG. 3. Independent contribution of p29 and p40 to ORF A-mediated suppression of fungal pigmentation. Transformants designated p29(+) and p29(-) were transformed with plasmids pXH1 and pXH2, which contained the p29-coding portion of ORF A in the sense and antisense orientations, respectively. Transformants designated p40(+) and p40(-) were transformed with plasmids pXH3 and pXH4, respectively, which contained both orientations of the p40-coding portion of ORF A.

FIG. 5. Phenotypic consequences of deleting p29 from an infectious full-length L-dsRNA cDNA clone. EP155 spheroplasts were transformed with pCPXHY1 derivatives containing either the full-length L-dsRNA cDNA clone (plasmid pXH9, yielding the transformant designated HAV) or the L-dsRNA cDNA clone from which 657 bp of the p29-coding region had been deleted [plasmids pXHΔ1 and pXHΔ2, yielding transformants HAVΔ^{p29}(+) and HAVΔ^{p29}(-), respectively]. Two independent HAVΔ^{p29}(+) transformants are shown: transformant HAVΔ^{p29}(+)-1-4 is shown at the upper right, while transformant HAVΔ^{p29}(+)-1-1 is shown at the lower right. The HAV and HAVΔ^{p29}(+) transformants were each shown to contain cDNA-derived cytoplasmically replicating L-dsRNA (see Fig. 6; data not shown for HAV).

the C terminus of p29 abolished suppressive activity (Fig. 4, colonies designated p29¹³⁵⁻²⁴⁸ and p29¹⁻¹⁶⁰). These results indicate that p29-mediated modulation of fungal phenotype, while dependent on release from the p69 precursor protein, is independent of the intrinsic papain-like protease activity involved in autoproteolysis and that gross deletions from either terminus of p29 abolish suppressive activity.

In addition to reduced orange pigmentation, EP155 cultures transformed with intact ORF A were previously shown to be suppressed in conidiation and in production of the enzyme laccase (7), although to a lesser extent than observed for L-dsRNA-containing hypovirulent strain EP713 (Fig. 3 in reference 7). As indicated in Table 2, transformants containing the p29(+) construct were also suppressed in conidiation. Although a considerable range of suppression was observed (data not shown), several of the p29(+) transformants exhibited reduced levels of conidiation, approaching that observed for hypovirulent strain EP713 (Table 2). A very slight reduction (about two- to ninefold) in conidiation was routinely observed for some p40(+) transformants (Table 2) and for several p40(-) transformants. The significance of these findings remains unclear. In contrast, transformation with the p29(-) or p69-G²⁴⁸ constructs resulted in no consistent reduction in conidiation levels (Table 2). Laccase enzyme activity, as judged by color formation on Bavendamm's medium, was also reduced only in transformants containing the p69(+), p29(+), and p29-His²¹⁵ constructs (data not shown) and to a level similar to that previously reported for transformants containing intact ORF A (Fig. 3 in reference 7). Thus, the activity responsible for ORF A-mediated suppression of fungal functions appears to reside predominantly, if not exclusively, within the p29-coding domain.

Deletion of p29 in the context of an infectious L-dsRNA cDNA clone. Choi and Nuss (8) recently reported the development of a full-length infectious cDNA clone of the L-dsRNA present in strain EP713. The transformation plasmid used to deliver the viral cDNA, plasmid pXH9, consisted of the viral cDNA cloned into the *Stu*I site of vector pCPXHY1 (Fig. 1B). Resulting transformants were shown to be hypovirulent,

to express the entire range of traits exhibited by wild-type hypovirulent strain EP713, and to contain chromosomally integrated copies of the transformation plasmid, as well as cDNA-derived (resurrected), cytoplasmically replicating L-dsRNA (8). The ability to engineer hypovirulent fungal strains with the aid of this infectious cDNA clone provided the opportunity to test the consequences of p29 deletion on viral replication and fungal phenotype. Advantage was taken of the presence of a pair of *Bam*HI sites located within the p29-coding domain to engineer a mutated form of pXH9, designated pXHΔ1. Removal of the 657-bp *Bam*HI fragment resulted in a 219-codon in-frame deletion that fused the Val-22 codon with that which encodes Asp-242 (Fig. 1A). Deletion of the region containing the residues required for p29 autoproteolytic activity, residues Cys-162 and His-215 (9), prevents cleavage at Gly-248/Gly-249, resulting in production of a p40 protein modified by addition of 29 heterologous N-terminal amino acid residues.

As indicated in Fig. 5, isolates obtained following transformation with plasmid pXHΔ1 [designated HAVΔ^{p29}(+)], unlike pXH9 transformants, produced orange pigments. However, the colony morphology of the HAVΔ^{p29}(+) transformants, aside from pigmentation, more closely resembled that of EP713 and pXH9 transformants. As anticipated, transformation with related plasmid pXHΔ2, which contained the p29-deleted viral cDNA copy in the antisense orientation, resulted in no phenotypic alterations [compare the colonies designated HAVΔ^{p29}(+) and HAVΔ^{p29}(-) in Fig. 5]. Consistent with the differences in phenotypic traits, extracts prepared from HAVΔ^{p29}(+) transformants were found to contain viral dsRNA (Fig. 6A), while extracts prepared in a similar manner from HAVΔ^{p29}(-) transformants were free of viral dsRNA (data not shown). Verification that the L-dsRNA present in HAVΔ^{p29}(+) transformants was derived from transformation plasmid pXHΔ1 and contained the *Bam*HI deletion within the p29 coding domain was provided by RT-PCR analysis (Fig. 6B). Thus, while p29 is not required for viral RNA replication, its deletion has an observable impact on virus-mediated modulation of the fungal phenotype.

Although suppression of pigmentation was significantly relieved by deletion of p29, other hypovirulence-associated traits were considerably less affected by this mutation. When cultured on a laboratory bench, HAVΔ^{p29}(+) transformants produced as much as 30-fold more conidia than did pXH9 transformants (data not shown). Interestingly, however, conidiation by the former transformants was much more sensitive to environmental conditions. For example, when cultured under moderate levels of light intensity (~2,000 versus 600 lx at the bench top), HAVΔ^{p29}(+) transformants produced roughly 1,000 times more conidia than either wild-type hypovirulent strain EP713 or engineered strains transformed with the full-length L-dsRNA cDNA (pXH9) and only 100-fold less than untransformed virulent strain EP155 (Table 3). HAVΔ^{p29}(+) transformants also produced stromata containing pycnidia (asexual fruiting bodies) protruding through the bark of inoculated dormant chestnut tree stems much more readily than did EP713 or pXH9 transformants (data not shown). As indicated in Fig. 7, suppression of laccase activity was only moderately relieved as a result of p29 deletion. Thus, deletion of p29 appears to have a differential effect on the suppression of different host functions. Its deletion certainly failed to restore normal host functions, as would be predicted by the finding that p29 confers significant suppressive activity

TABLE 2. Effects of transformation with complete and partial cDNA copies of the ORF A coding region on fungal conidiation^a

Strain or transformant	No. of conidia/ml	
	Data set 1	Data set 2
EP155	1.4×10^7	2.1×10^6
EP713	1.2×10^3	5.1×10^3
p69(+)	6.3×10^3	2.7×10^3
p69(-)	9.6×10^6	2.3×10^6
p69-G ²⁴⁸ (+)	8.5×10^6	1.3×10^6
p69-G ²⁴⁸ (-)	ND	4.5×10^6
p29(+)	1.0×10^3	3.0×10^3
p29(-)	ND	1.3×10^6
p40(+)	1.5×10^6	6.4×10^5
p40(-)	9.0×10^6	2.8×10^6

^a Cultures were grown on PDA plates on a laboratory bench (light, ~600 lx; temperature, 22 to 24°C) for 10 days. Conidia were collected and quantified as described in Materials and Methods. The two data sets were generated with the same set of transformants on different dates by using different lots of PDA growth medium. Results are presented as average numbers of conidia per milliliter of an original 10-ml suspension from single plates and are representative of results obtained from independent experiments involving subsets of the transformants listed. Strain and transformant designations are as described in Fig. 2 and 3. ND, not determined.

FIG. 6. Characterization of dsRNA recovered from fungal isolates transformed with L-dsRNA cDNA containing a p29 deletion mutation [plasmid pXHΔ1; transformant designation, HAVΔ^{p29}(+)]. (A) Double-stranded RNAs were extracted from fungal mycelia (24), separated on a 0.8% agarose gel, and detected under UV light after staining with ethidium bromide. The fungal isolates analyzed included wild-type hypovirulent strain EP713, a transformant generated by transformation of EP155 by plasmid pXH9 (designated pXH9-3), and two single-spored isolates of pXHΔ1 designated HAVΔ^{p29}(+)-1-1 and HAVΔ^{p29}(+)-1-4. Lane M contained a 1-kb DNA ladder (Bethesda Research Laboratories). (B) The origin of L-dsRNAs recovered from HAVΔ^{p29}(+) transformants was confirmed by combined RT-PCR amplification of L-dsRNA map positions 364 to 1,758 and agarose gel analysis (38). The ORF A coding domain spans L-dsRNA map positions 496 to 2,364, and the p29-coding domain extends from map position 496 through position 1,237 (39). The relative migration positions of amplicons generated from plasmids pXH9 and pXHΔ1 were consistent with the expected sizes of 1,395 and 738 bp, respectively. Amplicons generated by RT-PCR from dsRNA isolated from single-spored transformants HAVΔ^{p29}(+)-1-1 and HAVΔ^{p29}(+)-1-4 comigrated with the 738-bp band generated from plasmid pXHΔ1, confirming deletion of the p29-coding domain. No amplicons were observed for dsRNA reactions that lacked RT (data not shown).

TABLE 3. Conidiation by engineered *C. parasitica* strains transformed with L-dsRNA cDNA containing a p29 deletion mutation^a

Strain or transformant	No. of conidia/ml	
	Day 10	Day 13
EP155	2.4×10^8	4.0×10^8
EP713	6.7×10^3	4.8×10^3
CN2	2.9×10^3	3.4×10^3
EP155/pCPXHY1	1.9×10^8	3.8×10^8
HAVΔ ^{p29} (+)-1-1	3.4×10^5	1.7×10^6
HAVΔ ^{p29} (+)-4-4	6.5×10^5	3.0×10^6
HAVΔ ^{p29} (+)-9-3	5.7×10^5	2.9×10^6

^a Cultures were grown on PDA plates in an environmental growth chamber under fluorescent and incandescent lights set at a level of 2,000 lx with a 12-h photoperiod at 24°C. Conidia were harvested and quantified as described in Materials and Methods. Transformant CN2 was generated from strain EP155 by transformation with the full-length L-dsRNA cDNA clone carried on transformant plasmid pXH9 (8). Control transformant EP155/pCPXHY1 was generated from strain EP155 by transformation with vector plasmid pCPXHY1 (Fig. 1B). Transformants HAVΔ^{p29}(+)-1-1, HAVΔ^{p29}(+)-4-4, and HAVΔ^{p29}(+)-9-3 represent single-spored isolates of the independent transformants generated by transformation of EP155 with plasmid pXHΔ1, containing a 657-bp deletion in the p29-coding domain. Duplicate cultures of each transformant were inoculated. One culture of each set was subsequently analyzed on day 10, while the second culture was analyzed on day 13 following inoculation.

FIG. 7. Effect of p29 deletion on virus-mediated suppression of laccase accumulation. The same fungal strains and transformants presented in Fig. 5 were grown for 8 days in the dark at 22 to 24°C on agar plates containing Bavendamm's medium (3). The level of brown color produced provides an indication of the relative level of laccase enzymatic activity produced by the different transformants. Note that laccase activity was still significantly suppressed in transformants that contained the p29 deletion mutation in L-dsRNA [transformant HAVΔ^{p29}(+)].

when expressed independently of virus infection (Fig. 3 and 4). We conclude from these results that p29 contributes to, but is not entirely responsible for, the level of suppressive activity exhibited by strain EP713 or pXH9 transformants, suggesting the involvement of other virus-encoded proteins in L-dsRNA-mediated suppressive activity.

Previous studies have shown that although transformation with ORF A resulted in suppression of fungal pigmentation, sporulation, and laccase accumulation, it caused no reduction in fungal virulence (7). Thus, it was of considerable interest to determine whether virus-mediated hypovirulence was modified as a result of the deletion of p29. As indicated in Table 4, cankers incited on dormant chestnut tree stems by HAVΔ^{p29}(+) transformants were not significantly different in size from those produced by wild-type hypovirulent strain EP713 and engineered hypovirulent strain CN2 generated by transformation of virulent strain EP155 with plasmid pXH9. Thus, the deletion of p29 had no measurable effect on virus-mediated hypovirulence.

TABLE 4. Results of virulence assay for engineered hypovirulent strains transformed with L-dsRNA cDNA containing a p29 deletion mutation^a

Strain or transformant	Mean canker area (cm ²) ± SEM
EP155	29.5 ± 3.9
EP713	4.0 ± 2.5
CN2	5.2 ± 2.3
HAVΔ ^{p29} (+)-1-1	3.5 ± 2.4
HAVΔ ^{p29} (+)-4-4	2.9 ± 1.9

^a Virulence assays were performed on dormant chestnut tree stems (8, 27), and measurements were taken on day 21 after inoculation. Data are presented as mean canker areas based on seven replicates, except for strain EP713 and transformant HAVΔ^{p29}(+)-4-4, for which values were calculated from six replicates. Strain and transformant designations are as described in Table 2.

DISCUSSION

An understanding of the molecular basis of virus-mediated symptom expression and the identification of virus-encoded disease symptom determinants represent major goals in contemporary virology. The hypovirulence-associated traits of reduced pigmentation, suppressed sporulation, and reduced laccase activity exhibited by hypovirulent *C. parasitica* strains can be viewed as the equivalent of disease symptoms observed for other virus-host systems. The fungal transformation results presented in this report demonstrate that the expression of viral papain-like protease p29 elicits similar symptoms in the absence of viral RNA replication. Thus, within this context, p29 can be considered the equivalent of a viral symptom determinant.

Several virus-encoded proteases have been implicated in the modification of host factors, leading to modulation of host functions. The 2A protease encoded by enteroviruses and rhinoviruses and the L/L' protease of foot-and-mouth disease virus have been reported to mediate cleavage of the p220 component of the cap-binding protein complex, thereby causing inhibition of cap-dependent translation of host mRNA (4, 16, 28, 31, 40). The poliovirus 3C protease has been reported to convert an active form of transcription factor IIIC to an inactive form, resulting in inhibition of host transcription (12, 13). Also implicated in shutoff of cell RNA synthesis, foot-and-mouth disease virus protease 3C has been reported to induce specific cleavage of host cell histone H3 (18). Mutational analysis of 2A^{Pro} and 3C^{Pro} has revealed that the residues required for autocatalytic activity are also essential for induction of p220 and TFIIC cleavage (13, 22, 46). As indicated in Fig. 2, impairment of p29 autocatalytic processing from ORF A-encoded polyprotein p69 by modification of the cleavage site also interferes with the ability of p29 to modulate host functions. However, substitution of a residue required for autocatalytic activity (e.g., His-215; Fig. 4) does not impair the ability of p29 to modulate the fungal phenotype when expressed independently of the polyprotein precursor. Thus, the proteolytic activity associated with p29 appears to be of secondary importance in modifying the fungal phenotype, being required only for p29 release from the polyprotein precursor. It is also noteworthy that, with the exception of viral transforming proteins, p29 represents one of the few reported examples (15) in which the expression of a virus-encoded protein confers overt symptoms when expressed in the absence of virus replication.

The availability of a full-length infectious cDNA clone of L-dsRNA provided an opportunity to test the consequences of p29 deletion for both viral replication and fungal phenotype. As demonstrated in Fig. 6, p29 is nonessential for viral replication. L-dsRNA cDNA modified by deletion of over 88% of the p29-coding domain retained infectivity, yielding transformants that contained a resurrected, mutated form of L-dsRNA. In view of the very dramatic changes in fungal pigmentation (Fig. 3 and 4), sporulation (Table 2), and laccase accumulation (data not shown but equivalent to Fig. 3 in reference 7) observed as a result of DNA-mediated transformation by the p29-coding domain in the absence of virus infection, it was anticipated that deletion of p29 in the context of the infectious cDNA clone would result in significant relief from, perhaps elimination of, virus-mediated suppressive activity. Surprisingly, suppressive activity was only partially relieved in Δ p29 transformants (Fig. 5 and 7 and Table 3), and different suppressive activities were relieved to different extents. Suppression of laccase accumulation was minimally relieved (Fig. 7), while pigmentation was restored to levels

approaching that of untransformed strain EP155 (Fig. 5). As indicated in Table 3, deletion of p29 resulted in a moderate but significant increase in the level of asexual sporulation. Interestingly, the range of suppressive activities observed as a result of p29 deletion closely resembles that exhibited by the related hypovirulence-associated virus present in hypovirulent *C. parasitica* isolate NB58 (25). Fungal colonies containing this virus are only slightly suppressed in orange pigmentation and conidiation but are significantly suppressed in the accumulation of laccase activity. Significantly, ORF A of the NB58 L-dsRNA, while showing sequence similarity to the N-terminal cysteine-rich portion of p29 and to the p40 region of ORF A of EP713 L-dsRNA, lacks a papain-like catalytic domain or a p29 cleavage domain (23a). This correlation and the finding that deletion of p29 in the context of the EP713 L-dsRNA fails to eliminate suppressive activity strongly suggest the involvement of additional virus-encoded proteins in virus-mediated suppressive activity. Efforts to define additional symptom determinants are in progress.

Although the mechanism by which a hypovirulence-associated virus can mediate a diverse array of suppressive activities remains unclear, some progress has been made in understanding the basis of L-dsRNA-mediated suppression of laccase accumulation. The gene that encodes *C. parasitica* laccase, *lac-1*, has been cloned and characterized (6, 35). Larson et al. (29) recently provided evidence to suggest that L-dsRNA mediates suppression of laccase accumulation at the level of transcription initiation by interfering with an intracellular inositol triphosphate/calcium-dependent signal transduction pathway. It is tempting to extrapolate the results obtained for suppression of *lac-1* transcription to suggest that perturbation of such fundamental regulatory pathways may also explain the ability of p29 to produce manifold effects on host metabolism. In this regard, it is likely that virus-mediated attenuation of fungal virulence will also be shown to involve modulation of regulatory cascades rather than down-regulation of a specific virulence factor.

Initial interest in the viral dsRNA elements associated with hypovirulence of *C. parasitica* stemmed primarily from their potential as biological control agents (1, 21, 32, 42). Strains harboring these elements, while still able to initiate an infection at an appropriate wound site, are unable to overcome host defense measures effectively and generally cause only superficial damage. More importantly, from the perspective of disease control, application of a hypovirulent strain can result in conversion of vegetatively compatible resident virulent strains to the hypovirulence phenotype following transmission of the hypovirulence-associated viral element via anastomosis (1, 19, 20, 43). Successful control of chestnut blight following natural or artificial introduction of hypovirulent *C. parasitica* strains has been observed in Europe and to a limited extent in North America (1, 2, 32, 41). However, the effectiveness of hypovirulent *C. parasitica* strains as biocontrol agents can be compromised by hypovirulence-associated traits, such as suppressed conidiation, that negatively affect the dissemination of introduced natural hypovirulent strains and, consequently, the effectiveness of disease control (17). Consequently, the ability to identify a viral determinant responsible for modulation of host functions and then to introduce corresponding modifications into an infectious viral cDNA clone to generate fungal strains altered in specific traits while retaining hypovirulence, as demonstrated in this report for Δ p29 transformants, represents a significant practical advancement. This capability, coupled with the recent demonstration of mitotic and meiotic stability of integrated viral cDNA in engineered hypovirulent strains (5a), has very positive implications for future prospects

of engineering hypovirulent *C. parasitica* strains with improved biocontrol potential.

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