The Plant Cell, Vol. 8, 1845–1853, October 1996 © 1996 American Society of Plant Physiologists

Using Hypoviruses to Probe and Perturb Signal Transduction Processes Underlying Fungal Pathogenesis

Donald L. Nuss

Center for Agricultural Biotechnology, University of Maryland Biotechnology Institute, University of Maryland, College Park, Maryland 20742-3351

INTRODUCTION

Imagine the task of designing a reagent to explore regulatory mechanisms that govern fungal pathogenesis. The assignment triggers recollection of a group of cytoplasmically transmissible RNA mycoviruses that have the property of attenuating fungal virulence. Utility of these viruses is significantly enhanced by the construction of an infectious cloned cDNA copy of the viral RNA genome. Subsequent studies reveal that virus infection attenuates fungal virulence by altering cellular signal transduction processes, thus exposing a role for G protein–linked, cAMP-mediated signaling in fungal pathogenesis. Synthetic transcripts derived from the infectious viral cDNA are used to establish virus infection in pathogenic fungi other than the natural host, thereby expanding opportunities for probing and manipulating a broader range of plant–pathogen interactions. As a bonus, the infectious viral cDNA is found to provide enhanced potential for biological control of fungal disease.

Elements of the account presented above represent actual developments derived from recent molecular analyses of mycoviruses, taxonomically grouped within the genus *Hypovirus* of the family Hypoviridae, that attenuate virulence of the chestnut blight fungus *Cryphonectria parasitica*. Details of the intriguing biology and potential applications offered by this system have been the subject of several recent reviews (MacDonald and Fulbright, 1991; Nuss, 1992; Heiniger and Rigling, 1994). This article discusses recent progress on two major fronts. The first involves advances in the development of hypovirus molecular biology and the extended experimental and practical utility provided by these advances. The second focuses on progress in elucidating mechanisms by which hypoviruses alter fungal gene expression/virulence and the insights that these surprising results present for further understanding of signal transduction processes that underlie fungus–plant pathogenic interactions.

HYPOVIRUS MOLECULAR BIOLOGY

It is useful to preface a discussion of hypovirus molecular biology by describing several distinguishing features of these genetic elements relative to other virus groups. Hypovirus infections are persistent and nonlytic, resulting in a variety of stable host phenotypic changes (described below). These viruses do not encode a coat protein, and hypovirus-infected fungal hyphae do not contain discrete virus particles. Rather, hypovirus genetic information is found predominantly in the form of double-stranded RNA (dsRNA) associated with pleomorphic membranous vesicles (Dodds, 1980; Newhouse et al., 1983; Hansen et al., 1985). As has been observed for fungal viruses in general, hypoviruses are not infectious in the classical sense. That is, infections cannot be initiated by exposing uninfected *C. parasitica* to a cell extract prepared from an infected strain. Transmission is limited to vertical dissemination through asexual spores (conidia) or via cytoplasmic mixing after anastomosis (fusion of hyphae). The absence of an extracellular route of infection, combined with the RNA nature of the hypovirus genome, presents obvious obstacles to the genetic manipulation and functional characterization of this virus group. Fortunately, many of these limitations have been overcome after the development of an infectious cDNA clone of the prototypic hypovirus CHV1-713 (Figure 1).

Infectious Hypovirus cDNA Clone

In 1992, Choi and Nuss (1992a) reported that hypovirus infection could be initiated by transformation of virus-free *C. parasitica* spheroplasts with a full-length cDNA copy of hypovirus CHV1-713 dsRNA. Resulting transformants exhibited characteristics indistinguishable from CHV1-713–infected fungal strains and were found to contain both chromosomally integrated viral cDNA and cDNA-derived cytoplasmically replicating virus dsRNA. Using a variety of analytical methods, Chen et al. (1994b) showed subsequently that the cDNA-derived viral RNA present in these "engineered" hypovirulent *C. parasitica* strains originated as large nuclear transcripts that were subsequently trimmed of nonviral nucleotides. These studies also revealed the unexpected presence of a 73-bp deletion within a dispensable portion of the 5′ noncoding leader sequence, the result of a pre-mRNA splicing event. Although

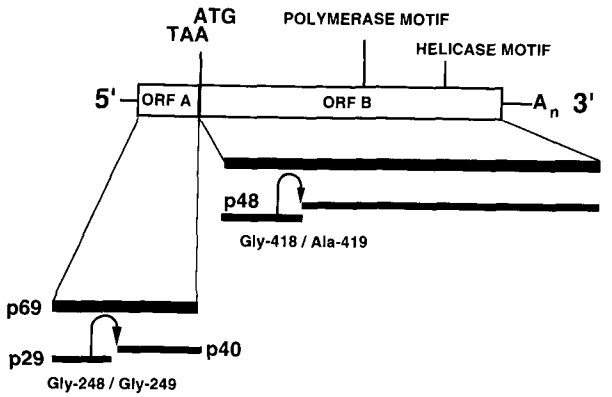

Figure 1. Hypovirus Genetic Organization and Basic Expression Strategy.

The coding strand RNA of the prototypic hypovirus CHV1-713 consists of 12,712 nucleotides, excluding the poly(A) tail (A_n) (Shapira et al., 1991b). The 5′ proximal coding domain, ORFA (ORF A; 622 codons), encodes two polypeptides, p29 and p40 (thin black bars), that are released from a polyprotein, p69 (thick black bar), by an autoproteolytic event mediated by p29. Cleavage (curved arrow) occurs between Gly-248 and Gly-249 during translation and is dependent on Cys-162 and His-215 within the putative catalytic site of p29 (Choi et al., 1991a, 1991b). Expression of ORFB (ORF B; 3165 codons) also involves an autoproteolytic event in which a 48-kD polypeptide, p48 (thin black bars), is released from the N-terminal portion of the encoded polyprotein (thick black bar). In this case, cleavage (curved arrow) occurs between Gly-418 and Ala-419 and is dependent on essential residues Cys-341 and His-388 in p48 (Shapira and Nuss, 1991). The junction between ORFA and ORFB consists of the sequence 5′-UAAUG-3′. Translational mapping studies indicated that the UAA portion of the pentanucleotide serves as a termination codon for ORFA, whereas the AUG portion is the 5′ proximal initiation codon for ORFB, as indicated by overlapping TAA/ATG codons. Computer-assisted analysis revealed five distinct domains within the CHV1-713 RNA coding regions that show significant sequence similarity to previously described domains within plant potyvirus-encoded polyproteins (Koonin et al., 1991). These include putative RNA-dependent RNA polymerase and RNA helicase motifs located approximately in the portions of ORFB indicated in the figure. This figure has been adapted from Shapira et al. (1991b) with permission.

a total of five potential splice sites were identified by inspection of the CHV1-713 RNA sequence, only the 5′ proximal site was found to undergo splicing in vivo. Although the basis for the apparent differential splicing within the cDNA-derived CHV1-713 pre-mRNA transcript is unclear, the presence of cryptic splicing signals must be taken into consideration when constructing infectious cDNA copies of other mycovirus RNAs or when using infectious hypovirus cDNA for extending hypovirus host range.

The construction of a biologically active infectious hypovirus cDNA presented a number of interesting implications, several of which have now been assessed. An immediate prediction of practical significance was that engineered hypovirulent strains would exhibit expanded capacity for hypovirus transmission. This prediction was verified by Chen et al. (1993), who demonstrated that the integrated viral cDNA copy is stably maintained through repeated rounds of conidiation. Furthermore, unlike natural cytoplasmically replicating hypovirus RNA, the viral cDNA is faithfully and efficiently transmitted to ascospore progeny. The meiotic transmission to ascospores represents an entirely novel mode of hypovirus dissemination that is expected to circumvent barriers to anastomosis-mediated cytoplasmic virus transmission imposed by the fungal vegetative incompatibility system (a genetic system that controls the ability of different *C. parasitica* strains to fuse). These unique transmission properties, in turn, are predicted to confer substantially enhanced biological control potential. Although a detailed discussion of the practical application of engineered hypovirulent strains for effective biological control of chestnut blight is outside the focus of this review, it is noteworthy that studies designed to test virus dissemination and persistence properties for engineered hypovirulent *C. parasitica* strains under actual field conditions are currently in progress in Connecticut and West Virginia (see Handelsman and Stabb, 1996, in this issue, for additional discussion on biocontrol strategies).

Genetic Manipulation of a Hypovirus Genome in the Context of an Infectious cDNA Clone

A second prediction, more directly related to hypovirus molecular biology, was that an infectious cDNA clone would provide significant new opportunities for manipulation and functional analysis of a hypovirus genome. This utility was effectively demonstrated by Craven et al. (1993), with the identification of a hypovirus-encoded protease, p29, as a symptom determinant. Choi and Nuss (1992b) had shown previously that transformation of a virus-free *C. parasitica* strain with a cDNA copy of the CHV1-713 RNA 5′ proximal coding domain (ORFA) resulted in loss of orange pigmentation, a significant reduction in asexual sporulation, and a moderate reduction in production of the enzyme laccase, all qualitative changes associated with CHV1-713 infection. Transformation with ORFA cDNA, however, did not cause virulence attenuation. Craven et al. (1993) were able subsequently to map the activity responsible for these phenotypic changes to the autocatalytic papain-like protease p29 found within the N-terminal portion of the ORFA-encoded polyprotein p69 (Figure 1). These investigators further tested the consequences of p29 deletion on both viral replication and fungal host phenotype. A deletion mutant of the CHV1-713 infectious cDNA clone lacking 88% of the p29 coding domain retained full infectivity. However, in comparison to wild-type–infected fungal colonies, isolates infected with the Δp29 mutant RNA exhibited a near restoration of orange pigment production, a moderate increase in conidiation, and a very slight increase in laccase production, with no reduction in the level of hypovirulence. Thus, although it is not essential for either viral replication or virulence attenua-

tion, p29 clearly contributes to the reduction in fungal pigmentation, asexual sporulation, and laccase accumulation observed in CHV1-713–infected fungal strains. One can easily imagine an extension of these studies to determine functional roles for other hypovirus-encoded protein products.

The results of Craven et al. (1993) also illustrate the feasibility of engineering infectious viral cDNA for construction of hypovirulent fungal strains with specific phenotypic traits, a capability that can have significant practical implications. The effectiveness of hypovirulent *C. parasitica* strains as biological control agents can be compromised by hypovirulence-associated traits, such as reduced conidiation, that negatively affect the dissemination of introduced hypovirulent strains and consequently the predicted effectiveness of disease control (Anagnostakis, 1982; MacDonald and Fulbright, 1991). In this regard, the transformation of *C. parasitica* with an infectious cDNA clone containing the p29 deletion provides a second-generation engineered hypovirulent *C. parasitica* strain with increased asexual sporulation capacity that should provide increased ecological fitness and thus even further enhanced biocontrol potential. The ability to identify and functionally delete a viral determinant responsible for an undesirable phenotype, while retaining hypovirulence, represents a significant practical advancement for the genetic manipulation of this virus group.

Extending Hypovirus Host Range

A more recent prediction concerns expansion of the hypovirus fungal host range. Given the demonstration that cDNA-derived virus dsRNA present in engineered hypovirulent strains originates as a processed nuclear transcript (Chen et al., 1994b), it was reasoned that it should be possible to establish virus infection by introduction of a synthetic copy of a hypovirus RNA coding strand into fungal spheroplasts. It was further considered that if this approach were successful for *C. parasitica*, it might also work to establish hypovirus infections in additional fungal species. Chen et al. (1994a) tested these predictions by electroporating full-length in vitro–synthesized CHV1-713 transcripts into spheroplasts prepared from virus-free *C. parasitica*. Electroporation of the complete transcription reaction or of DNase-treated reaction products yielded regenerated mycelia that were found consistently to harbor cytoplasmically replicating CHV1-713 dsRNA.

Extending the hypovirus transfection system, Chen et al. (1994a) were able to establish virus infection in several fungal species taxonomically related to *C. parasitica*. These included a nonpathogenic species, *Cryphonectria radicalis*, two pathogens of eucalyptus, *C. havanensis* and *C. cubensis*, and a pathogen of oak, *Endothia gyrosa*. Similar to the effects of virus infection of *C. parasitica*, infection of these species also resulted in a variety of very pronounced phenotypic changes (Figure 2), including the attenuation of fungus–plant pathogenic interactions, that is, hypovirulence. By comparing virus-mediated phenotypic changes in different fungal hosts transfected with wild-type and mutated viral transcripts, Chen et al. (1996a) examined the relative contribution of viral and fungal genetic backgrounds for some traits. For example, virus infection reduced asexual sporulation in all fungal species tested, indicating that the viral genome is the primary determinant for this phenotypic trait. In contrast, the efficiency of

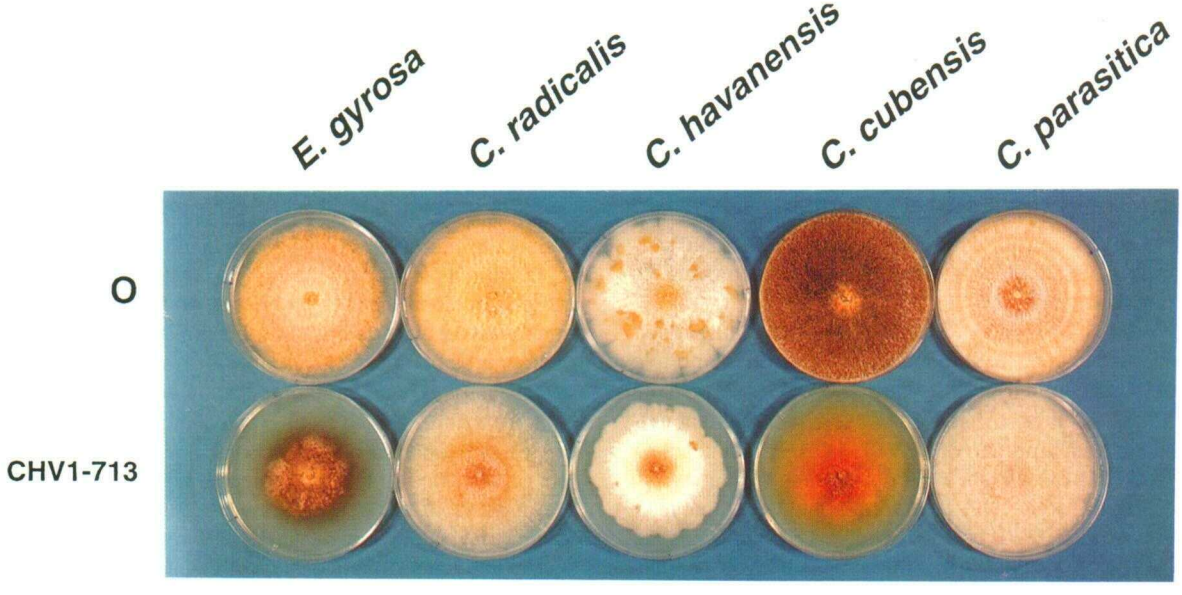

Figure 2. Morphology of Hypovirus-Transfected Fungi.

Each pair compares the morphology of virus-free (0) and hypovirus-transfected (CHV1-713) colonies. Fungal species from the left include *E. gyrosa*, *C. radicalis*, *C. havanensis*, *C. cubensis*, and *C. parasitica*. A detailed comparative analysis of hypovirus-mediated phenotypic changes, including the effects on fungal virulence, was recently reported by Chen et al. (1996a).

virus transmission through asexual spores was found to be dependent on the fungal host, ranging from 100% for *C. parasitica* to 0% for *C. cubensis*. The observation that fungal host genotype affects virus transmission has obvious practical implications for the use of these transfected species for biological control.

The successful development of an infectious hypovirus cDNA clone and synthetic transcripts to establish hypovirus infection in four fungal species related to *C. parasitica* raises the question of just how far hypovirus host range can be extended.

Future Directions: Hypovirus Molecular Biology

Progress in elucidating the fundamentals of hypovirus genomic organization and expression strategies has been rapid (Hillman et al., 1992, 1994; Nuss, 1992). As indicated above, this information, coupled with the development of facile infectious cDNA transformation and transfection systems, has resulted in engineered fungal strains with enhanced biocontrol potential, expansion of hypovirus host range, and identification of a hypovirus-encoded symptom determinant. However, gaps in our current understanding of some very basic aspects of hypovirus replication and gene expression remain, hampering the development of rational approaches for further optimizing the utility of this virus group.

In considering future research directions, studies on hypovirus polyprotein processing, hypovirus replication, and comparative molecular virology stand to provide the greatest short-term returns. Known details of hypovirus protein processing are limited to two autocatalytic cleavage events, one within each of the two virus-encoded open reading frames (ORFs; Choi et al., 1991a, 1991b; Shapira and Nuss, 1991). Knowledge of ORFA proteolytic processing was essential for the design of transformation and mutagenesis experiments that successfully identified p29 as a determinant of virus-mediated reduction of fungal conidiation (Choi and Nuss, 1992b; Craven et al., 1993). In a similar manner, it is likely that a detailed understanding of ORFB polyprotein processing will greatly facilitate efforts to define functions for other hypovirus-encoded proteins and to identify the viral determinate(s) responsible for virulence attenuation.

Hypovirus RNA replication and transcription have been demonstrated to occur in vitro in association with membrane vesicles isolated from hypovirus-infected *C. parasitica* (Fahima et al., 1993). By using antisera raised to a recombinant protein derived from a portion of CHV1-713 ORFB that contains conserved RNA-dependent RNA polymerase sequence domains, Fahima et al. (1994) reported the detection of a vesicle-associated, 87-kD putative hypovirus RNA polymerase. Characterization of defective hypovirus RNAs derived from full-length CHV1-713 RNA has also localized the *cis*-acting RNA domains required for hypovirus replication to positions within the 5′ and 3′ noncoding domains (Shapira et al., 1991a). These preliminary findings provide a sound basis for detailed future studies aimed at the identification of both viral and host proteins required for hypovirus replication and transcription as well as a precise determination of the viral RNA signals that regulate these processes. This information, in turn, will significantly benefit current efforts to develop hypoviruses as transmissible fungal cytoplasmic expression vectors and will contribute to a broader understanding of the factors that influence hypovirus host range.

There appears to be a considerable level of diversity among viral elements found to infect *C. parasitica*. Although viral RNAs related to the prototypic hypovirus CHV1-713 predominate, a reo-like dsRNA virus consisting of 11 dsRNA segments (Enebak et al., 1994) and a mitochondrial dsRNA ancestrally related to yeast cytoplasmic T and W viral dsRNAs (Polashoch and Hillman, 1994) have also been described and characterized. Even among viral isolates currently assigned tentatively to the genus *Hypovirus*, considerable diversity has been detected in sequence similarity, degree of virulence attenuation, and the constellation of hypovirulence-associated traits (Elliston, 1978, 1985; L'Hostis et al., 1985; Paul and Fulbright, 1988; Hillman et al., 1992, 1994; Chung et al., 1994; Enebak et al., 1994). For example, Hillman et al. (1994) showed that a second sequenced hypovirus, CHV2-NB58, does not encode an equivalent to the CHV1-713–encoded p29 protease. Consistent with this observation, *C. parasitica* strains infected with CHV2-NB58 exhibit no reduction in pigment production or asexual sporulation. By taking advantage of such diversity, future systematic comparative studies of natural hypovirus isolates should reveal fine details of hypovirus genome structure/function relationships and protein function.

MOLECULAR MECHANISMS UNDERLYING HYPOVIRUS-MEDIATED ALTERATION OF PLANT–FUNGUS PATHOGENIC INTERACTION

Canker formation by *C. parasitica* on the susceptible American chestnut *Castanea dentata* is a complex process involving sequential interactions between the invading fungus and several different plant host tissues. The fungal infection process starts with colonization at a preexisting wound site. Subsequent penetration of plant defense barriers is aided by the formation of structures composed of parallel arrays of hyphae, termed mycelial fans. These structures invade and destroy the vascular cambium, causing girdling and death of the distal portion of the tree (Hebard et al., 1984). Hypovirus-infected hypovirulent *C. parasitica* strains also colonize wound sites. However, they are unable to effectively penetrate plant defense barriers, forming only superficial cankers that eventually heal. In addition to reduced virulence, hypovirus-infected *C. parasitica* isolates usually exhibit a number of stable phenotypic changes, designated hypovirulence-associated traits, that can include reduced asexual sporulation, altered sexual sporulation (female infertility), reduced pigmentation, and diminished

accumulation of certain metabolites (Elliston, 1978, 1985; Anagnostakis and Day, 1979; Anagnostakis, 1982, 1984). The basis for virus-mediated disruption of the fungal infection process and hypovirulence-associated traits remains incompletely understood. However, recent progress in understanding the mechanisms by which hypovirus infection persistently alters fungal gene expression provides clues to the mechanisms that underlie virus-mediated alterations of complex cellular and developmental processes.

The first molecular evidence demonstrating an effect of hypovirus infection on fungal gene expression appeared in related publications by Powell and Van Alfen (1987a, 1987b). Two-dimensional PAGE and differential hybridization techniques were employed to demonstrate differential accumulation of a number of fungal proteins and poly(A)$^+$ RNAs, respectively, in isogenic virus-free and hypovirus-infected *C. parasitica* strains. These early studies preceded numerous reports of hypovirus-mediated decreases in the accumulation of specific fungal gene products. Examples include a laccase (Rigling and Van Alfen, 1991), cutinase (Varley et al., 1992), a hydrophobin-designated cryparin encoded by the gene *Crp* (Zhang et al., 1994), putative pheromones encoded by *Vir1* and *Vir2* (Zhang et al., 1993), a cellobiohydrolase I encoded by *cbh-1* (Wang and Nuss, 1995), and a polygalacturonase encoded by *enpg-1* (Gao and Shain, 1995; Gao et al., 1996).

The subsequent characterization of cloned genes encoding several of the hypovirus-regulated fungal proteins has begun to provide insight into the mechanisms involved in hypovirus-mediated alteration of fungal gene expression. In this regard, the gene *lac-1*, encoding a *C. parasitica* laccase, has been studied in greatest detail. Two independent reports describing the initial cloning of the *lac-1* gene (Rigling and Van Alfen, 1991; Choi et al., 1992) concluded that the hypovirus-mediated reduction in laccase accumulation is due to reduced *lac-1* transcript accumulation. This finding was confirmed by Larson et al. (1992), who also provided evidence that the reduction in *lac-1* accumulation results from a reduced level of promoter-dependent *lac-1* transcription. Evidence for reduced levels of transcription following hypovirus infection has also been reported for *Vir1*, *Vir2*, *Crp* (Kazmierczak et al., 1996), and *cbh-1* (Wang and Nuss, 1995).

The *lac-1* gene has also served as a useful reporter gene with which to examine the effect of hypovirus infection on fungal gene regulatory pathways. Larson et al. (1992) provided evidence for two antagonistic pathways that govern *lac-1* transcription in virus-free *C. parasitica*. The first consists of a Ca^{2+}-, inositol trisphosphate–, and calmodulin-dependent stimulatory pathway. A second, negative regulatory pathway was first identified by a requirement for ongoing protein synthesis, that is, repression of *lac-1* transcription was found to be sensitive to treatment with low levels of protein synthesis inhibitors such as cycloheximide (Choi et al., 1992). By comparing the effect of a number of pharmacological agents with the effect of virus infection on *lac-1* transcript accumulation, researchers concluded that virus-mediated suppression of *lac-1* transcription results from perturbation of the positive regulatory pathway. These results, coupled with the pleiotrophic nature of the hypovirulence-associated traits, provided justification for expanded examination of the effect of hypovirus infection on cellular signal transduction processes.

Virus-Mediated Disruption of G Protein–Linked Signal Transduction and Alteration of Fungus–Plant Host Interactions

Heterotrimeric guanine nucleotide binding proteins (G proteins) are a family of regulatory proteins that play an essential role in the response of eukaryotic cells to a variety of environmental stimuli (Gilman, 1987). Reasoning that G proteins are also likely candidates as transducers of molecular cues that drive the fungal infection process, Choi et al. (1995) cloned two *C. parasitica* G protein α subunits, designated *cpg-1* and *cpg-2*. The deduced amino acid sequence for the *cpg-1* protein product, CPG-1, was found to be surprisingly similar to a G protein α subunit of the G_i family previously cloned from *Neurospora crassa* (Turner and Borkovich, 1993), showing 98% identity. The predicted *cpg-2* amino acid sequence shares 49% identity with the CPG-1 sequence but fails to conform to any currently defined α-subunit subgroup.

Using monospecific anti–CPG-1 antisera, Choi et al. (1995) showed that CPG-1 accumulation is reduced to nearly undetectable levels in virus-infected *C. parasitica*. A second, completely unexpected result followed when a transgenic approach was employed to reduce CPG-1 levels in the absence of virus infection. Transformation of the virus-free strain EP155 with an antisense orientation of *cpg-1* resulted in no changes in fungal phenotype or alteration in CPG-1 levels. In contrast, altered colony morphology and a corresponding reduction in CPG-1 accumulation were observed in >60% of transformants containing ectopic *cpg-1* sense orientation copies. Moreover, the sense transformants were severely reduced in virulence. Thus, reduced CPG-1 accumulation, either in hypovirus-infected strains or in virus-free *cpg-1* sense transformants, correlates with attenuation of fungal virulence. Consistent with these observations, Wang and Nuss (1995) recently reported that the induction of *C. parasitica*–encoded cellulases, enzymes with the potential for degrading plant host cell wall components during the infection process, is both regulated through the CPG-1 pathway and prevented by virus infection. Together, these results provide compelling evidence for the role of G protein–linked signal transduction in fungal virulence. Additionally, they indicate that one important way in which a hypovirus alters the interaction between *C. parasitica* and its plant host is by reducing the accumulation of a key fungal molecular signaling component.

The unexpected suppression of CPG-1 accumulation in *cpg-1* sense transformants requires further comment. It is now well established that the introduction into plants of sense orientation transgenes can result in suppression of both the

endogenous gene homolog and the introduced transgene (Napoli et al., 1990; Lindbo and Dougherty, 1992; Flavell, 1994; Baulcombe, 1996, in this issue), a phenomenon known as transgenic cosuppression. A discussion of the parallels between cosuppression in plants and *cpg-1* transgenic sense suppression is provided by Choi et al. (1995). However, it is important to note that cosuppression has not been observed following transformation with sense orientation copies of any other *C. parasitica* genes, for example, endothiapepsin (Choi et al., 1993). It is intriguing that a *C. parasitica* gene encoding a signaling component involved in rapid responses to changes in the extracellular environment was found to be susceptible to the phenomenon of cosuppression. In this regard, the mechanisms underlying both virus-mediated and transgenic suppression of CPG-1 accumulation are likely to be the subject of intense future investigations.

Extent of Virus-Mediated Alteration of Fungal Gene Expression

Although there is now considerable evidence that hypovirus infections alter fungal gene expression and compromise signal transduction pathways, questions remain concerning the magnitude of changes and the linkages between alterations in signaling pathways and altered gene expression. Additional insights into these issues were recently supplied by applying the technique of mRNA differential display on three near-isogenic *C. parasitica* strains: virus-free strain EP155, isogenic hypovirus CHV1-713–infected strain EP713, and a CPG-1 cosuppressed transformant derived from strain EP155 (Chen et al., 1996b). As indicated in Figure 3, inspection of even a relatively small portion of the total display revealed a surprisingly large number of differences in polymerase chain reaction (PCR) product accumulation between strains EP155 and EP713. A thorough examination of display gels generated with 80 primer pair combinations revealed ~139 PCR products that changed in intensity by more than fourfold as a result of virus infection (108 products increased in accumulation, whereas 31 products decreased). Such a pronounced alteration in the transcript accumulation profile is consistent with the broad range of phenotypic changes exhibited by hypovirus-infected *C. parasitica* strains. However, the observation that virus infection resulted in enhanced accumulation of a large number of differentially diplayed PCR products came as a surprise in view of the preponderance of reports indicating hypovirus-mediated suppression of fungal gene expression. This result prompted the cloning of several differential PCR products followed by RNA gel blot analysis to confirm the virus-mediated elevation in the accumulation of the corresponding mRNAs. The combined results challenge a currently held view that hypovirus infection is associated with reduced accumulation of a small number of host mRNAs and proteins (Powell and Van Alfen, 1987a, 1987b; Kazmierczak et al., 1996).

Inspection of the display patterns for the CPG-1 transgenic cosuppressed strain revealed that >65% of the changes in PCR product intensity caused by virus infection were also conferred by transgenic cosuppression of CPG-1 accumulation in the absence of virus infection. These results confirmed that virus-mediated alterations in fungal gene expression are mediated primarily through modification of the CPG-1 signaling pathway. The magnitude of changes revealed by the differential display technique is likely to force a general shift in research emphasis. Questions of how hypovirus infection alters the expression of specific fungal genes are likely to be replaced by broader mechanistic considerations of how hypoviruses modify regulatory circuits that coordinately govern the expression of many fungal genes.

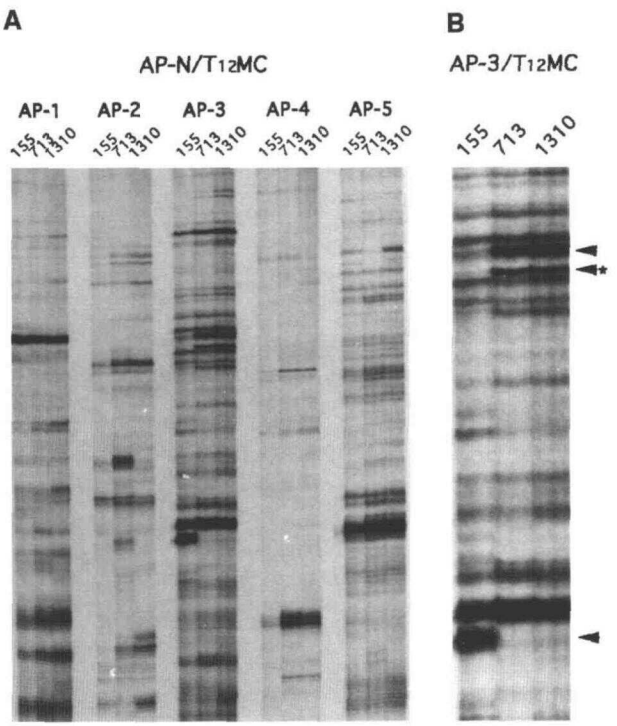

Figure 3. Differential Display of mRNAs Resulting from Hypovirus Infection and CPG-1 Cosuppression.

(A) Shown is a portion of a sequencing gel autoradiograph displaying PCR products generated with the same anchored primer (AP-N/T_{12}MC) and five different arbitrary primers (AP-1, AP-2, AP-3, AP-4, and AP-5). Differentially accumulating PCR products are abundant and easily observable without marking.

(B) An enlargement of a portion of **(A)** that contains examples of PCR products that exhibit a similar increase or decrease in accumulation (arrowheads) as a result of hypovirus infection and CPG-1 transgenic cosuppression in the absence of hypovirus infection. The asterisk indicates a PCR product selected for cloning.

This figure is reproduced from Chen et al. (1996b) with permission.

A Role for G Protein–Regulated cAMP Accumulation in Virus-Mediated Alteration of Fungal Gene Expression

Taking a cue from reports that mammalian $G_i\alpha$ subunits negatively regulate adenylyl cyclase activity, Chen et al. (1996b) were

also able to show that both virus infection and CPG-1 transgenic cosuppression result in a three- to fivefold elevation of cAMP levels. It was also possible to simulate the effect of virus infection and CPG-1 cosuppression on transcript accumulation for representative fungal genes by drug-induced elevation of cAMP levels. The combined observations suggested that *C. parasitica* CPG-1, like mammalian $G_i\alpha$ subunits, downregulates cAMP levels, presumably by inhibiting adenylyl cyclase, and that virus-mediated suppression of CPG-1 accumulation results in elevated intracellular cAMP levels. Moreover, the demonstration that one can mimic the effect of virus infection on transcript accumulation for representative genes by artificially elevating cAMP levels with phosphodiesterase inhibitors firmly establishs a role for G protein–regulated cAMP accumulation in virus-mediated alteration of fungal gene expression.

FUTURE DIRECTIONS: HYPOVIRUS-MEDIATED ALTERATION OF PLANT-FUNGUS PATHOGENIC INTERACTIONS

The largely unanticipated results discussed above provide the first testable mechanistic model for hypovirus-mediated attenuation of fungal virulence. As described below, one can broadly envision the role of G protein signaling in mediating interactions between *C. parasitica* and its plant host and the impact of hypovirus infection on those interactions. Virus-free, virulent *C. parasitica* strains are able to respond to extracellular cues via a number of mechanisms, including signal transduction through the CPG-1 pathway. Activation of undefined G protein–linked receptors by as yet unidentified ligands results in dissociation of CPG-1 from its β and γ partners. By negatively regulating adenylyl cyclase, activated CPG-1 modulates the level of intracellular cAMP, which in turn modulates gene expression to elicit appropriate adaptive responses, such as cellulase induction. By suppressing CPG-1 accumulation, virus infection constitutively elevates cAMP levels by relieving the CPG-1–mediated negative regulation of adenylyl cyclase. This effectively compromises the ability of the invading fungus to respond appropriately to events at the fungus–plant interface, thereby impeding penetration and canker formation.

As with any model, this emerging view of G protein–linked signaling in virus-free and hypovirus-infected *C. parasitica* raises a multitude of additional questions. What ligands or extracellular cues activate the CPG-1 signaling pathway? What role does the $\beta\gamma$ dimer play in *C. parasitica* signaling processes? What other components comprise the CPG-1 signaling pathway? Does CPG-2, a second characterized *C. parasitica* G_α subunit, play a role in elaborating fungal virulence? Does virus infection impact other fungal signaling processes in addition to the CPG-1 and inositol trisphosphate pathways? What is the level of cross-talk between the different virus-affected signaling pathways? What mechanisms regulate CPG-1 accumulation in virus-free and virus-infected strains? Which hypovirus proteins are responsible for reducing CPG-1 accumulation? What is the molecular basis of CPG-1 transgenic cosuppression? Fortunately, many of the genetic and molecular tools, including an infectious hypovirus cDNA clone and an efficient *C. parasitica* transformation system, are readily available to address these and related questions. For example, the recent targeted disruption of the *cpg-1* and *cpg-2* genes resulted in complete loss of virulence for the former disruptant and no statistically significant reduction in virulence for the latter (Gao and Nuss, 1996). Thus, an intact CPG-1 pathway is required for *C. parasitica* virulence, whereas an intact CPG-2 pathway is dispensable.

Much of the literature on fungal virulence focuses on the role of putative virulence determinants. The recent insights provided by the molecular analysis of the hypovirus–*C. parasitica* system force a deeper consideration of the larger picture involving signaling pathways that coordinate regulation of an array of putative virulence determinants in response to extracellular cues derived from the dynamic interaction between a fungal pathogen and a plant host. Put more simply, the role of putative fungal virulence determinants must be considered in the context of the regulatory pathways that govern their elaboration at the fungus–host interface. Given the conservative nature of signal transduction processes among eukaryotes, there is a high probability that the information gained from an investigation of G protein–linked signal transduction in *C. parasitica* virulence will add significantly to a general understanding of the signal transduction processes that drive fungal infection in many other host–pathogen systems.

ACKNOWLEDGMENTS

Portions of this review were adapted in part from Choi et al. (1995) and Chen et al. (1996a, 1996b). I thank the following members of our laboratory for their critical reading of this review and their many useful suggestions: Baoshan Chen, Shaojian Gao, Lynn Geletka, Shin Kasahara, and Ping Wang.

REFERENCES

Anagnostakis, S.L. (1982). Biological control of chestnut blight. Science **215,** 466–471.

Anagnostakis, S.L. (1984). The mycelial biology of *Endothia parasitica*. I. Nuclear and cytoplasmic genes that determine morphology and virulence. In The Ecology and Physiology of the Fungal Mycelium, D.H. Jennings and A.D.M. Rayner, eds (Cambridge, UK: Cambridge University Press), pp. 353–366.

Anagnostakis, S.L., and Day, P.R. (1979). Hypovirulence conversion in *Endothia parasitica*. Phytopathology **69,** 1226–1229.

Baulcombe, D.C. (1996). Mechanisms of pathogen-derived resistance to viruses in transgenic plants. Plant Cell **8,** 1833–1844.

Chen, B., Choi, G.H., and Nuss, D.L. (1993). Mitotic stability and nuclear inheritance of integrated viral cDNA in engineered hypovirulent strains of the chestnut blight fungus. EMBO J. **12,** 2991–2998.

Chen, B., Choi, G.H., and Nuss, D.L. (1994a). Attenuation of fungal virulence by synthetic infectious hypovirus transcripts. Science **264,** 1762–1764.

Chen, B., Craven, M.G., Choi, G.H., and Nuss, D.L. (1994b). cDNA-derived hypovirus RNA in transformed chestnut blight fungus is spliced and trimmed of vector nucleotides. Virology **202,** 441–448.

Chen, B., Chen, C.-H., Bowman, B.H., and Nuss, D.L. (1996a). Phenotypic changes associated with wild-type and mutant hypovirus RNA transfection of plant pathogenic fungi phylogenetically related to *Cryphonetria parasitica.* Phytopathology **86,** 301–310.

Chen, B., Gao, S., Choi, G.H., and Nuss, D.L. (1996b). Extensive alteration of fungal gene transcript accumulation and alteration of G-protein regulated cAMP levels by virulence-attenuating hypovirus. Proc. Natl. Acad. Sci. USA **93,** 7996–8000.

Choi, G.H., and Nuss, D.L. (1992a). Hypovirulence of chestnut blight fungus conferred by an infectious viral cDNA. Science **257,** 800–803.

Choi, G.H., and Nuss, D.L. (1992b). A viral gene confers hypovirulence-associated traits to the chestnut blight fungus. EMBO J. **11,** 473–477.

Choi, G.H., Pawlyk, D.M., and Nuss, D.L. (1991a). The autocatalytic protease p29 encoded by a hypovirulence-associated virus of the chestnut blight fungus resembles the poty-virus-encoded protease HC-Pro. Virology **183,** 747–752.

Choi, G.H., Shapira, R., and Nuss, D.L. (1991b). Cotranslational autoproteolysis involved in gene expression from a double-stranded RNA genetic element associated with hypovirulence of the chestnut blight fungus. Proc. Natl. Acad. Sci. USA **88,** 1167–1171.

Choi, G.H., Larson, T.G., and Nuss, D.L. (1992). Molecular analysis of the laccase gene from the chestnut blight fungus and selective suppression of its expression in an isogenic hypovirulent strain. Mol. Plant-Microbe Interact. **5,** 119–128.

Choi, G.H., Pawlyk, D.M., Rae, B., Shapira, R., and Nuss, D.L. (1993). Molecular analysis and overexpression of the gene encoding endothiapepsin, an aspartic protease from *Cryphonectria parasitica.* Gene **125,** 135–141.

Choi, G.H., Chen, B., and Nuss, D.L. (1995). Virus-mediated or transgenic suppression of a G-protein α subunit and attenuation of fungal virulence. Proc. Natl. Acad. Sci. USA **92,** 305–309.

Chung, P.-H., Bedker, P.J., and Hillman, B.I. (1994). Diversity of *Cryphonectria parasitica* hypovirulence-associated double-stranded RNAs within a chestnut population in New Jersey. Phytopathology **84,** 984–990.

Craven, M.G., Pawlyk, D.M., Choi, G.H., and Nuss, D.L. (1993). Papain-like protease p29 as a symptom determinant encoded by a hypovirulence-associated virus of the chestnut blight fungus. J. Virol. **67,** 6513–6521.

Dodds, J.A. (1980). Association of type 1 viral-like dsRNA with club-shaped particles in hypovirulent strains of *Endothia parasitica.* Virology **107,** 1–12.

Elliston, J.E. (1978). Pathogenicity and sporulation in normal and diseased strains of *Endothia parasitica* in American chestnut. In Proceedings of the American Chestnut Symposium, W.L. MacDonald, F.C. Cech, J. Luchok, and C. Smith, eds (Morgantown, WV: West Virginia University Press), pp. 95–100.

Elliston, J.E. (1985). Characterization of dsRNA-free and dsRNA-containing strains of *Endothia parasitica* in relation to hypovirulence. Phytopathology **75,** 151–158.

Enebak, S.A., MacDonald, W.L., and Hillman, B.I. (1994). Effect of dsRNA associated with isolates of *Cryphonectria parasitica* from the central Appalachians and their relatedness to other dsRNAs from North America and Europe. Phytopathology **84,** 528–534.

Fahima, T., Kazmierczak, P., Hansen, D.R., Pfeiffer, P., and Van Alfen, N.K. (1993). Membrane-associated replication of an unencapsidated double-stranded RNA of the fungus, *Cryphonectria parasitica.* Virology **195,** 81–89.

Fahima, T., Wu, Y., Zhang, L., and Van Alfen, N.K. (1994). Identification of the putative RNA polymerase of *Cryphonectria* hypovirus in a solubilized replication complex. J. Virol. **68,** 6116–6119.

Flavell, R.B. (1994). Inactivation of gene expression in plants as a consequence of specific sequence duplication. Proc. Natl. Acad. Sci. USA **91,** 3490–3496.

Gao, S., and Nuss, D.L. (1996). Distinct roles for two G-protein α subunits in fungal virulence, morphology, and reproduction revealed by targeted gene disruption. Proc. Natl. Acad. Sci. USA, in press.

Gao, S., and Shain, L. (1995). Activity of polygalacturonase produced by *Cryphonectria parasitica* in chestnut bark and its inhibition by extracts from American and Chinese chestnut. Physiol. Mol. Plant Pathol. **46,** 199–213.

Gao, S., Choi, G.H., Shain, L., and Nuss, D.L. (1996). Cloning and targeted disruption of *epng-1,* encoding the major *in vitro* extracellular endopolygalacturonase of the chestnut blight fungus, *Cryphonectria parasitica.* Appl. Environ. Microbiol. **62,** 1984–1990.

Gilman, A.G. (1987). G proteins: Transducers of receptor-generated signals. Annu. Rev. Biochem. **56,** 615–649.

Handelsman, J., and Stabb, E.V. (1996). Biocontrol of soilborne plant pathogens. Plant Cell **8,** 1855–1869.

Hansen, D.R., Van Alfen, N.K., Gillies, K., and Powell, N.A. (1985). Naked dsRNA associated with hypovirulence of *Endothia parasitica* is packaged in fungal vesicles. J. Gen. Virol. **66,** 2605–2614.

Hebard, F.V., Griffin, G.J., and Elkins, J.R. (1984). Developmental histopathology of cankers incited by hypovirulent isolates of *Endothia parasitica* on susceptible and resistant chestnut trees. Phytopathology **74,** 140–149.

Heiniger, U., and Rigling, D. (1994). Biological control of chestnut blight in Europe. Annu. Rev. Phytopathol. **32,** 581–599.

Hillman, B.I., Tian, Y., Bedker, P.J., and Brown, M.P. (1992). A North American hypovirulent isolate of the chestnut blight fungus with European isolate-related dsRNA. J. Gen. Virol. **73,** 681–686.

Hillman, B.I., Halpern, B.T., and Brown, M.P. (1994). A viral dsRNA element of the chestnut blight fungus with a distinct genetic organization. Virology **201,** 241–250.

Kazmierczak, P., Pfeiffer, P., Zhang, L., and Van Alfen, N.K. (1996). Transcriptional repression of specific host genes by the mycovirus *Cryphonectria* hypovirus 1. J. Virol. **70,** 1137–1142.

Koonin, E.V., Choi, G.H., Nuss, D.L., Shapira, R., and Carrington, J.C. (1991). Evidence for common ancestry of a chestnut blight hypovirulence-associated double-stranded RNA and a group of positive-strand RNA plant viruses. Proc. Natl. Acad. Sci. USA **88,** 10647–10651.

Larson, T.G., Choi, G.H., and Nuss, D.L. (1992). Regulatory pathways governing modulation of fungal gene expression by a virulence-attenuating mycovirus. EMBO J. **11,** 4539–4548.

L'Hostis, B., Hiremathm, S.T., Rhoads, R.E., and Ghabrial, S.A. (1985). Lack of sequence homology between double-stranded RNA from European and American hypovirulent strains of *Endothia parasitica.* J. Gen. Virol. **66,** 351–355.

Lindbo, J.A., and Dougherty, W.G. (1992). Untranslatable transcripts of the tobacco etch virus coat protein gene sequence can interfere with tobacco etch virus replication in transgenic plants and protoplasts. Virology **189,** 725–733.

MacDonald, W.L., and Fulbright, D.W. (1991). Biological control of chestnut blight: Use and limitations of transmissible hypovirulence. Plant Dis. **75,** 656–661.

Napoli, C., Lemieux, C., and Jorgensen, R. (1990). Introduction of a chimeric chalcone synthase gene into petunia results in reversible cosuppression of homologous genes *in trans*. Plant Cell **2,** 279–289.

Newhouse, J.R., Hoch, H.C., and MacDonald, W.L. (1983). The ultrastructure of *Endothia parasitica*. Comparison of a virulent with a hypovirulent isolate. Can. J. Bot. **61,** 389–399.

Nuss, D.L. (1992). Biological control of chestnut blight: An example of virus mediated attenuation of fungal pathogenesis. Microbiol. Rev. **56,** 561–576.

Paul, C.P., and Fulbright, D.W. (1988). Double-stranded RNA molecules from Michigan hypovirulent isolates of *Endothia parasitica* vary in size and sequence homology. Phytopathology **78,** 751–755.

Polashoch, J.J., and Hillman, B.I. (1994). A small mitochondrial double-stranded (ds) RNA element associated with a hypovirulent strain of the chestnut blight fungus and ancestrally related to yeast cytoplasmic T and W dsRNAs. Proc. Natl. Acad. Sci. USA **91,** 8680–8684.

Powell, W.A., and Van Alfen, N.K. (1987a). Differential accumulation of poly(A)$^+$ RNA between virulent and double-stranded RNA-induced hypovirulent strains of *Cryphonectria (Endothia) parasitica*. Mol. Cell. Biol. **7,** 3688–3693.

Powell, W.A., and Van Alfen, N.K. (1987b). Two nonhomologous viruses of *Cryphonectria (Endothia) parasitica* reduce accumulation of specific virulence-associated polypeptides. J. Bacteriol. **169,** 5324–5326.

Rigling, D., and Van Alfen, N.K. (1991). Regulation of laccase biosynthesis in the plant pathogenic fungus *Cryphonectria parasitica* by double-stranded RNA. J. Bacteriol. **173,** 8000–8003.

Shapira, R., and Nuss, D.L. (1991). Gene expression by a hypovirulence-associated virus of the chestnut blight fungus involves two papain-like activities. J. Biol. Chem. **266,** 19419–19425.

Shapira, R., Choi, G.H., Hillman, B.I., and Nuss, D.L. (1991a). The contribution of defective RNAs to the complexity of viral-encoded double-stranded RNA populations present in hypovirulent strains of the chestnut blight fungus, *Cryphonectria parasitica*. EMBO J. **10,** 741–746.

Shapira, R., Choi, G.H., and Nuss, D.L. (1991b). Virus-like genetic organization and expression strategy for a double-stranded RNA genetic element associated with biological control of chestnut blight. EMBO J. **10,** 731–739.

Turner, G.E., and Borkovich, K.A. (1993). Identification of a G protein α subunit from *Neurospora crassa* that is a member of the G_i family. J. Biol. Chem. **268,** 14805–14811.

Varley, D.A., Podila, G.K., and Hiremath, S.T. (1992). Cutinase in *Cryphonectria parasitica*, the chestnut blight fungus: Suppression of cutinase gene expression in isogenic hypovirulent strains containing double-stranded RNA. Mol. Cell. Biol. **12,** 4539–4544.

Wang, P., and Nuss, D.L. (1995). Induction of a *Cryphonectria parasitica* cellobiohydrolase I gene is suppressed by hypovirus infection and regulation by a G-protein–linked signaling pathway involved in fungal pathogenesis. Proc. Natl. Acad. Sci. USA **92,** 11529–11533.

Zhang, L., Churchill, A.C.L., Kazmierczak, P., Kim, D., and Van Alfen, N.K. (1993). Hypovirulence-associated traits induced by a mycovirus of *Cryphonectria parasitica* are mimicked by targeted inactivation of a host gene. Mol. Cell. Biol. **13,** 7782–7792.

Zhang, L., Villalon, Y., Sun, P., Kazmierczak, P., and Van Alfen, N.K. (1994). Virus-associated down-regulation of the gene encoding cryparin, an abundant cell-surface protein from the chestnut blight fungus, *Cryphonectria parasitica*. Gene **139,** 59–64.