Implications of Engineered Transmissible Hypovirulence for Biological Control of Chestnut Blight

Donald L. Nuss

Department of Molecular Oncology and Virology, Roche Institute of Molecular Biology, Roche Research Center, Nutley, NJ 07110 USA

ABSTRACT. The recent application of molecular analysis to the study of hypovirulence-associated viral RNAs has revealed considerable new information regarding the genetic organization, expression strategy and possible origin of these genetic elements and has resulted in the identification of viral genes that are capable of conferring specific hypovirulence-associated traits. These studies have culminated in the construction of an infectious cDNA clone of a hypovirulence-associated viral RNA. Combined, these developments provide exciting new opportunities for examining the molecular basis of transmissible hypovirulence and for engineering hypovirulent strains for improved biological control of chestnut blight.

Choi and Nuss recently reported the generation of an infectious cDNA clone of a hypovirulence-associated viral dsRNA of *Cryphonectria parasitica* (Murr.) Barr (9). This development represents the culmination of a three-part strategy that was adopted several years ago in an effort to understand the molecular basis of transmissible hypovirulence. The first part of the strategy involved the direct analysis of hypovirulence-associated dsRNAs in order to identify gross structural properties of these elements that would then serve as landmarks in subsequent analyses (18, 29). The second component involved cloning, sequence analysis and *in vitro* expression studies to determine the genetic organization and general expression scheme of a hypovirulence-associated viral dsRNA (10, 11, 24, 27, 28). The third part of the strategy was to introduce cDNA copies of portions of the viral dsRNAs into virulent *C. parasitica* strains by DNA-mediated transformation (8). The goal of this last component was to identify the genetic information responsible for hypovirulence and hypovirulence-associated traits, such as reduced sporulation, and thereby, to provide direct evidence that the viral dsRNA is responsible for alterations of fungal phenotype. The recent observations that transformation with a full-length cDNA copy of a hypovirulence-associated viral dsRNA conferred the complete hypovirulence phenotype and that a cytoplasmic viral dsRNA was resurrected from the chromosomally integrated cDNA copy (9) surpassed the expectations of this strategy. The availability of an infectious cDNA copy of a hypovirulence-associated viral dsRNA provides obvious new experimental approaches for determining the molecular basis of hypovirulence. For example, it is now possible to precisely alter the genetic composition of the viral dsRNA by mutagenesis of its cDNA copy. The fact that an autonomously replicating cytoplasmic dsRNA form of the viral RNA is resurrected from cDNA copies that are integrated into the fungal genome also has interesting implications for current efforts to restore the American chestnut. It is the latter topic that is the focus of this article. Sections of the article are adapted from a recent review to which the reader is referred for a more detailed development of specific molecular aspects of transmissible hypovirulence (22).

METHODS

Experimental methods used for the molecular characterization of viral dsRNAs, construction of a full-length L-dsRNA infectious cDNA clone and DNA-mediated transformations are described in detail in the following journal articles (8, 9, 12, 24, 27, 29).

RESULTS AND DISCUSSION

As indicated in the introduction, a basic understanding of the structural and organizational properties of a hypovirulence-associated viral RNA was a prerequisite to the construction of an infectious cDNA clone. A brief description of these properties is also useful to prepare the reader for discussions of the practical implications that an infectious cDNA clone provides for biological control of chestnut blight. Efforts by our laboratory to define the molecular characteristics of hypovirulence-associated viral genetic elements focused primarily on the large viral dsRNA, L-dsRNA, associated with hypovirulent *C. parasitica* strain EP713. It is important to preface a discussion of the structural and functional characteristics of this element by stressing that not all hypovirulence-associated viral dsRNAs conform to the genetic organization typified by this prototype. The dsRNAs associated with different hypovirulent *C. parasitica* strains exhibit considerable diversity in terms of concentration, number and size of components and sequence similarity (5, 13, 15, 20, 23). Nevertheless, each of the hypovirulence-associated dsRNAs that have so far been subjected to molecular analysis appear to be related to EP713 L-dsRNA in terms of organization (14, 17), and many hypovirulent *C. parasitica* strains contain a large dsRNA species of the same approximate size as L-dsRNA. Thus, it is also clear that the EP713 L-dsRNA is not atypical of the viral genetic elements associated with hypovirulent *C. parasitica* strains.

The current view of the genetic organization and basic expression strategy of EP713 L-dsRNA (Figure 1) has been derived by a combination of cDNA cloning, nucleo-

tide sequence analysis, *in vitro* expression studies and mutagenesis (10, 11, 24, 27, 28). L-dsRNA was found to contain a stretch of approximately 40 adenosine residues (poly A) at the 3'-end of one strand that was base paired to a stretch of uridine residues (poly U) found at the 5'-terminus of the complimentary strand (18). Sequence analysis of cDNA clones representing the entire L-dsRNA revealed a length of 12,712 bp, excluding the poly A/poly U domain, and two large coding domains, ORF A (622 codons) and ORF B (3,165 codons), located within the poly A-containing strand. Additional dsRNA species present in strain EP713 were shown to be derived from L-dsRNA by internal deletion events (26). The generation of internally deleted defective RNA molecules may be a common feature of hypovirulence-associated viral dsRNAs.

Proteolytic processing has been found to play a fundamental role in the expression of the two coding domains present in L-dsRNA. ORF A encodes a 69 kDa polyprotein that is autocatalytically processed to produce two proteins, p29 from the amino-terminal portion and p40 from the carboxy-terminus. The catalytic domain was mapped to p29 and the cleavage dipeptide was identified as Gly 248/ Gly-249 (10, 11). A 48 kDa polypeptide, designated p48, also was shown to be released from the amino-terminal portion of the ORF B-encoded polyprotein during *in vitro* translation (28). Again the catalytic domain was mapped to p48 and the cleavage dipeptide was identified as Gly418/ Ala-419. Although additional details of ORF B processing remain to be determined, two conserved sequence motifs have been identified within the carboxy-terminal portion of

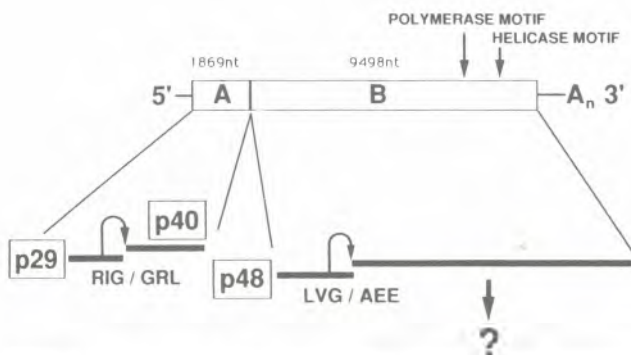

Figure 1. Current view of the genetic organization and basic expression strategy for EP713 L-dsRNA. The 5'-proximal coding domain, ORF A (622 codons), encodes two polypeptides, p29 and p40, that are released from a polyprotein, p69, by an autocatalytic event mediated by p29. Cleavage occurs between Gly-248 and Gly-249 during translation and is dependent upon the essential Cys-162 and His-215 (10, 11). Expression of ORF B (3,165 codons) also involves an autoproteolytic event in which a 48 kDa polypeptide, p48, is released from the N-terminal portion of the encoded polyprotein. In this case, cleavage occurs between Gly-418 and Ala-419 and is dependent upon essential residues Cys-341 and His-388 (28). Both p29 and p48 resemble papain-like proteinases and one proteolytic domain may have arisen as a result of a duplication event of the other (19, 28). Computer-assisted analysis revealed five distinct domains within the L-dsRNA coding regions that showed significant sequence similarity to previously described domains within plant potyvirus-encoded polyproteins (19). These include putative RNA-dependent RNA polymerase and RNA helicase mofits located in the approximate portions of ORF B indicated by the arrows. Adapted from Reference 27, Figure 9.

ORF B. These include the motifs for a RNA-dependent RNA polymerase (19) and a RNA helicase (27). Interestingly, computer-assisted analysis of the L-dsRNA coding domains revealed a total of five distinct domains, including the two proteases, the RNA helicase and the RNA polymerase, that showed significant sequence similarity to previously described domains within potyvirus polyproteins (19). These similarities suggest a possible ancestry for L-dsRNA and the single-stranded RNA genomes of the plant infecting potyviruses.

Although much remains to be determined about the structural and genetic properties of hypovirulence-associated viral dsRNAs, the progress obtained to date for EP713 L-dsRNA has fulfilled the major requirements of the first two components of the original experimental strategy outlined in the introduction. The feasibility of the third part of the strategy was recently demonstrated in studies by Choi and Nuss (8) in which ORF A was shown to confer hypovirulence-associated traits such as reduced pigmentation, reduced lactase synthesis and suppressed conidiation when introduced into a virulent *C. parasitica* strain by DNA-mediated transformation. Unfortunately, the ORF A transformed strains were not hypovirulent. Nevertheless, these results were important because they provided direct evidence for a cause and effect relationship between the viral dsRNA present in a hypovirulent *C. parasitica* strain and specific traits associated with that strain. In addition, they demonstrated that these phenotypic traits are not the result of some general reaction of the fungus to replicating viral RNA, but are caused by the action of specific viral-encoded gene products. Finally, these studies showed that hypovirulence-associated traits such as suppressed sporulation could be uncoupled from reduced virulence.

Although transformation studies aimed at determining the contribution that individual viral-encoded polypeptides make to the hypovirulent phenotype are intrinsically interesting, until recently, practical application of the information gained from those studies was limited for several reasons. The fact that hypovirulence-associated viral genetic information is in the form of RNA rather than DNA precludes easy genetic manipulations. Furthermore, these viral genetic elements are not infectious in the classical sense and have never been re-introduced into fungal strains in a form that was subsequently able to replicate. Therefore, even if one could genetically manipulate the viral dsRNAs, it was unclear how that altered element could be re-introduced into the fungus in a functional form. In this context, the recent development of an infectious full-length cDNA clone of EP713 L-dsRNA represents a major advancement in overcoming these limitations (9).

The transformation vector (pXH9) used to deliver the full-length cDNA copy of L-dsRNA into virulent *C. parasitica* strains is illustrated in Figure 2. The L-dsRNA cDNA was placed under the control of the *C. parasitica* glyceraldehyde-3-phosphate dehydrogenase gene (gpd-1) promoter and terminator sequences in a plasmid that also contained the *Escherichia coli* hygromycin B phosphotransferase gene as a selectable marker. As was observed

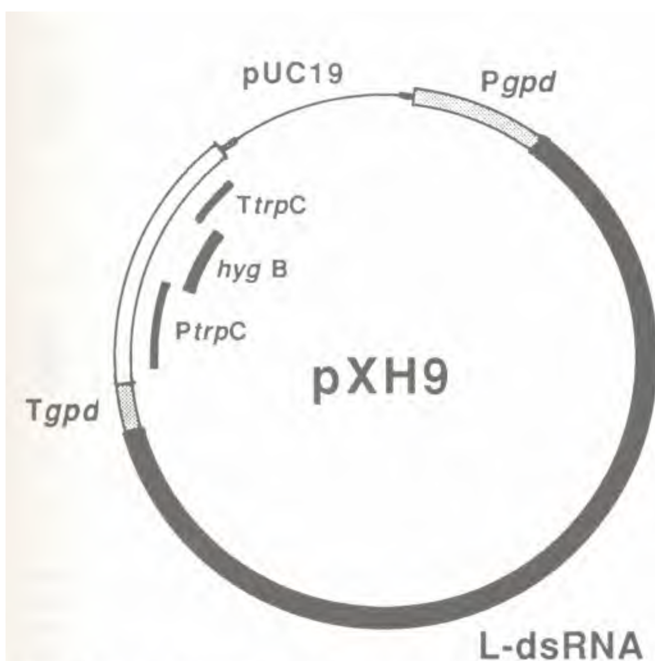

Figure 2. Plasmid pXH9 was used to introduce a full-length cDNA copy of EP713 L-dsRNA into virulent *C. parasitica* strain EP155 by DNA-mediated transformation (9). The plasmid contains the entire L-dsRNA cDNA sequence fused upstream to the *C. parasitica* glyceraldehyde-3-phosphate dehydrogenase (*gpd*-1) promoter and fused downstream to the gpd-terminator. pXH9 also contains the *Escherichia coli* hygromycin B phosphotransferase gene as a selectable marker flanked by the *Aspergillus nidulans trpC*. promoter and terminator domains, all in a pUC19 background.

for the ORF A transformants (8), most of the hygromycin-resistant fungal colonies resulting from transformation of virulent strain EP155 with pXH9 exhibited traits such as reduced pigmentation and suppressed sporulation. However, unlike the ORF A transformants, the pXH9 transformants were also found to harbor a cytoplasmically replicating form of L-dsRNA that was shown with the aid of a molecular tag to be resurrected from the integrated cDNA copy. The resurrected viral RNA was shown to convert compatible virulent strains to hypovirulence following anastomosis, and both the original transformants and the strains converted by the resurrected L-dsRNA were found to be hypovirulent. Thus strains transformed with the full-length L-dsRNA cDNA copy exhibited the entire hypovirulence phenotype. These results constitute direct formal evidence that a viral-like dsRNA is the genetic determinant responsible for transmissible hypovirulence in the chestnut blight fungus and have interesting implications for current efforts to restore the American chestnut.

As illustrated in Figure 3A, there are three potential modes of transmission of hypovirulence-associated viral dsRNA. Efficient transmission of viral dsRNA to a virulent strain occurs only following anastomosis (5). However, the formation of viable anastomoses is limited to strains of the same or very closely related vegetative compatibility groups (1). Consequently, viral dsRNAs are not efficiently transmitted to incompatible strains. A second potential mode of transmission is through asexual spores or conidia. Although the overall level of conidiation is reduced in many hypovirulent strains (15), viral dsRNAs are transmitted through the conidia that are produced, however, at a variable frequency (25). A third potential mode of transmission is through sexual spores (ascospores) resulting from mating. Mating compatibility in *C. parasitica* is controlled by a single mating type locus involving two alleles designated (A) and (a) (3). Thus strains representing different vegetative compatibility groups can mate provided that they are of opposite mating types and the progeny can be of different vegetative compatibility groups than the parental strains due to allelic rearrangement. However, available evidence indicates that hypovirulence-associated viral dsRNAs are not transmitted through ascospores at readily detectable levels (3, 4, 16). As a consequence of this transmission pattern, the introduction of a natural hypovirulent *C. parasitica* strain into a forest ecosystem results in the efficient conversion of only those virulent strains that are members of closely related vegetative compatibility groups. Provided that the compatible strains represent a significant portion of the population, this could result in effective disease control. If, however, the vegetative compatibility structure of the population is complex, as appears to be the case in the eastern deciduous forest of the United States (7), the proportion of virulent strains that would be susceptible to conversion would be small, thus limiting vegetative spread and effectiveness of disease control (2, 6, 7, 21).

The predicted transmission pattern for engineered hypovirulent strains containing integrated cDNA copies of L-dsRNA differs from that observed for natural hypovirulent strains in two fundamental ways (Figure 3B). Firstly, nearly all, rather than a fraction, of the asexual spores would be expected to carry L-dsRNA genetic information either in the form of integrated cDNA from which L-dsRNA can be resurrected, or in the combined forms of integrated cDNA and resurrected dsRNA. Secondly, and more significantly, the integrated cDNA form has the potential for transmission into the progeny of sexual crosses between the engineered hypovirulent strain and virulent vegetatively incompatible strains of the opposite mating type. Nuclear inheritance of the integrated cDNA would be followed by resurrection of the cytoplasmic L-dsRNA form in the progeny, a portion of which would represent vegetative compatibility groups different from the parental strains. This in turn should result in expanded vegetative dissemination. The predicted ability of the cDNA form of L-dsRNA to be introduced into new vegetative compatibility groups as a result of mating, coupled with a higher transmission frequency through asexual spores, should result in significantly increased dissemination and sustainability of the hypovirulence phenotype after field introduction of a genetically engineered *C. parasitica* strain.

Assessment of the practical implications related to this newly acquired ability to genetically engineer hypovirulent *C. parasitica* strains will require a multiphased approach. This will include studies designed to examine meiotic and mitotic stability of the integrated L-dsRNA cDNA, testing

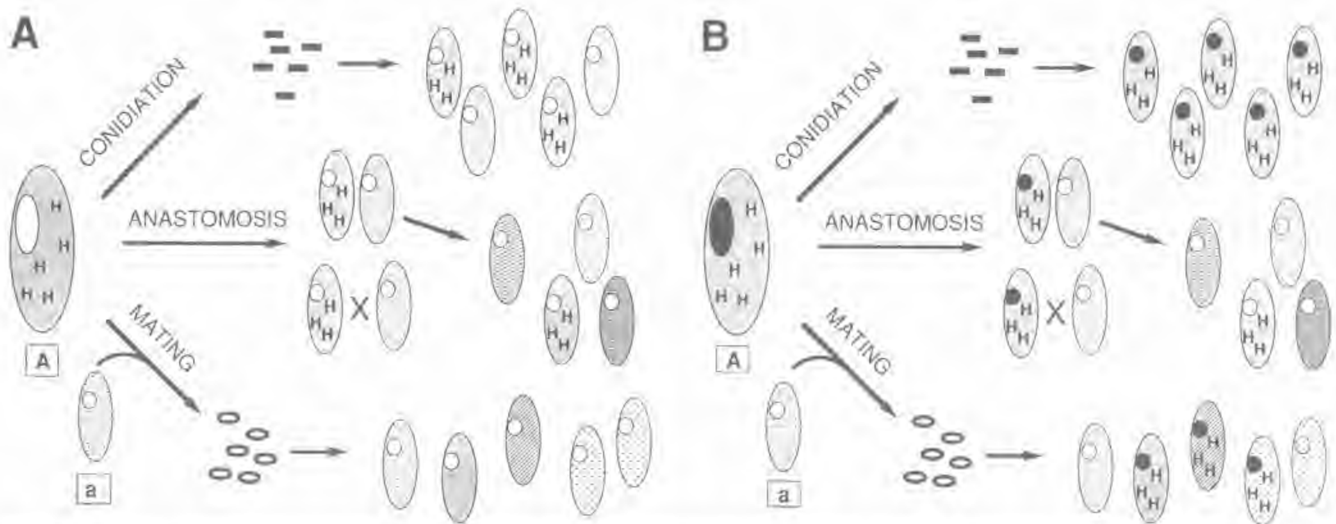

Figure 3. Predicted transmission of natural and engineered hypovirulence-associated viral dsNRAs. Panel A shows three potential modes of transmission of natural hypovirulent-associated viral dsRNAs. Fungal cells containing a nucleus (circle) and, in the case of hypovirulent strains, cytoplasmically replicating viral dsRNAs indicated by (H) are represented as ellipses reminiscent of the shape of a canker on infected chestnut trees. Different shading patterns indicate different vegetative compatibility groups. One potential mode of transmission is through asexual spores. Although the overall level of conidiation is reduced in many hypovirulent strains (15), viral dsRNA is transmitted through the conidia that are produced, however, at a variable rate (25). Efficient transmission of viral dsRNA to a virulent strain occurs only following anastomosis (5). However, viable anastomosis is limited to strains of the same or very closely related vegetative compatibility groups (1). Consequently, viral dsRNAs are not transmitted to incompatible strains, indicated by the X. A third potential mode of transmission is through ascospores resulting from mating. Mating compatibility in *C. parasitica* is controlled by a single mating type locus involving two allels designated (A) and (a) (3). Thus strains representing different vegetative compatibility groups can mate provided they are of opposite mating types. However, available evidence indicates that hypovirulence-associated viral dsRNAs are not transmitted through ascospores at readily detectable levels. In summary, natural hypovirulence-associated viral dsRNAs are transmitted efficiently via anastomosis, at a variable frequency through conidia and infrequently or not at all through ascospores. As indicated in Panel B, the predicted transmission pattern for a genetically engineered hypovirulent strain differs from a natural hypovirulent strain in two fundamental ways. First, nearly all, rather than a fraction, of the conidia would be expected to carry L-dsRNA genetic information either in the form of integrated cDNA (indicated by the solid filled nucleus) from which L-dsRNA (H) can be resurrected, or in the combined forms of integrated cDNA and resurrected dsRNA. Secondly, and more significantly for purposes of dissemination, the integrated cDNA form of L-dsRNA has the potential for transmission through ascospores. Due to allelic rearrangement, the progeny of a sexual cross can be of different vegetative compatibility groups than the parental strains. The introduction of the integrated L-dsNRA cDNA copy into progeny of different vegetative compatibility groups as a result of mating followed by resurrection of the L-dsRNA should, in turn, promote expanded vegetative dissemination. Adapted in part from reference 22, Figures 11 and 12.

of parameters such as host range and fitness under simulated field conditions and controlled field studies to determine biocontrol efficacy. The broad scope of these studies will necessitate a multidisciplinary approach involving input from plant pathologists, ecologists, foresters, mycologists and molecular biologists.

ACKNOWLEDGMENTS

I thank the following past and present members of our laboratory for productive and stimulating collaborations. Baoshan Chen, Gil Choi, Mark Craven, Brad Hillman, Thomas Larson, Diane Pawlyk, Brendan Rae, Roni Shapira, James Tartaglia and Ping Wang. Generous support by the Roche Institute of Molecular Biology and Hoffman-La Roche Inc. is gratefully acknowledged.

LITERATURE CITED

1. Anagnostakis, S.L. 1977. Vegetative incompatibility in *Endothia parasitica.* Exp. Mycol. 1:306-316.
2. Anagnostakis, S.L. 1982. Biological control of chestnut blight. Science 215:466-471.
3. Anagnostakis, S.L. 1984. The mycelia) biology of *Endothia parasitica II.* Vegetative incompatibility. Pages 499-507 in: D.H. Jennings and A.D.M. Rayner (ed.), The Ecology and Physiology of the Fungal Mycelium. Cambridge University Press, Cambridge, England.
4. Anagnostakis, S.L. 1984. Nuclear gene mutations in *Endothia (Cryphonearia) parasitica* that affect morphology and virulence. Phytopathology 74:561-565.
5. Anagnostakis, S.L. and Day, P.R. 1979. Hypovirulence conversion in *Endothia parasitica.* Phytopathology 69:1226-1229.
6. Anagnostakis, S.L., Hau, B. and Kranz, J. 1986. Diversity of vegetative compatibility groups of *Cryphonectria parasitica* in Connecticut and Europe. Plant Disease 70:536-538.
7. Anagnostakis, S.L. and Kranz, J. 1987. Population dynamics of *Cryphonectria parasitica* in a mixed-hardwood forest in Connecticut. Phytopathology 77:751-754.
8. Choi, G.H. and Nuss, D.L. 1991. A viral gene confers
9. Choi, G.H. and Nuss, D.L . 1992. Hypovirulence of chestnut blight fungus conferred by an infectious viral cDNA . Science. 257:800-803.
10. Choi, G.H., Pawlyk, D.M. and Nuss, D.L. 1991. The autocatalytic protease p29 encoded by a hypovirulence-associated virus of the chestnut blight fungus resembles the potyvirus-encoded protease HC-Pro. Virology 183:747-752.
11. Choi, G.H., Shapira, R and Nuss, D.L. 1991. Co-translational 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.
12. Churchill, A.C.L., Ciufetti, L.M., Hansen, D.R, Van Etten, H.D. and Van Alfen, N.K. 1990. Transformation of the fungal pathogen *Cryphonectria parasitica* with a variety of heterologous plasmids. Curr. Genet. 17:25-31.
13. Dodds, J.A. 1980. Revised estimates of the molecular weights of dsRNA segments in hypovirulent strains of *Endothia parasitica.* Phytopathology 70:1217-1220.

14. Durbahn, C.M., Nuss, D.L. and Fulbright, D.W. 1992. Molecular analysis of the dsRNA associated with hypovirulence in a Michigan strain of the chestnut blight fungus *Cryphonectria parasitica.* (Abst.) Phytopathology 82:1077.
15. Elliston, J.E. 1985. Characterization of dsRNA-free and dsRNA-containing strains *of Endothia parasitica* in relation to hypovirulence. Phytopathology 75:151-158.
16. Fulbright, D.W., Paul, C.P. and Garrod, S.W. 1988. Hypovirulence: a natural control of chestnut blight. In K.G. Mukerji and K.L. Garg (ed.), Biocontrol of Plant Diseases. CRC Press, Boca Raton, Ha.
17. Hillman, B.I., Tian, Y., Bedker, PJ. and Brown, M.P. 1992. A North American hypovirulent isolate of the chestnut blight fungus with European isolate-related dsRNA. J. Gen. Viral. 73:681-686.
18. Hiremath, S., L'Hostis, B., Ghabrial, S.A. and Rhoads, R.E. 1986. Terminal structure of hypovirulence- associated dsRNA in the chestnut blight fungus *Endothia parasitica.* Nucleic Acids Res. 14:9877-9896.
19. 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.
20. L'Hostis, B., Hiremath, S.T., Rhoads, RE. and Ghabrial, S.A. 1985. Lack of sequence homology between double-stranded RNA from European and American hypovirulent strains of *Endothia parasitica.* J. Gen. Viral. 66:351-355.
21. MacDonald, W.L. and Fulbright, D.W. 1991. Biological control of chestnut blight: use and limitation of transmissible hypovirulence. Plant Disease 75:656-661.
22. Nuss, D.L. 1992. Biological control of chestnut blight: an example of virulence-mediated attenuation of fungal pathogenesis. Microbiological Reviews 56:561-576.
23. 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.
24. Rae, B.P., Hillman, BJ., Tartaglia, J. and Nuss, D.L. 1989. Characterization of double-stranded RNA genetic elements associated with biological control of chestnut blight: organization of terminal domains and identification of gene products. EMBO J. 8:657-663.
25. Russin, J.S. and Shain, L 1985. Disseminative fitness of *Endothia parasitica* containing different agents for cytoplasmic hypovirulence. Can. J. Bot. 65:54-57.
26. Shapira, R, Choi, G.H., Hillman, B.I. and Nuss, D.L. 1991. The contribution of defective RNAs to complexity of viral-encoded double-stranded RNA populations present in hypovirulent strains of the chestnut blight fungus, *Cryphonectria parasitica.* EMBO J. 10: 741-746.
27. Shapira, R, Choi, G.H. and Nuss, D.L. 1991. 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.
28. Shapira, R. and Nuss, D.L. 1991. Gene expression by a hypovirulence-associated virus of the chestnut blight fungus involves two papain-like protease activities. J. Biol. Chem. 266:19419-19425.
29. Tartaglia, J., Paul, C.P., Fulbright, D.W. and Nuss, D.L. 1986. Structural properties of double-stranded RNAs associated with biological control of chestnut blight fungus. Proc. Natl. Acad. Sci. USA 83: 9109-9113.