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Durbahn, Christine Mary, Ph.D.

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MOLECULAR CHARACTERIZATION OF dsRNA-ASSOCIATED HYPOVIRULENCE IN MICHIGAN ISOLATES OF *CRYPHONECTRIA PARASITICA*

By

Christine M. Durbahn

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ABSTRACT

MOLECULAR CHARACTERIZATION OF dsRNA-ASSOCIATED HYPOVIRULENCE IN MICHIGAN ISOLATES OF *CRYPHONECTRIA PARASITICA*

By

Christine M. Durbahn

Cryphonectria parasitica, the causal organism of chestnut blight, was responsible for the demise of the American chestnut in eastern North America. Throughout Europe and in several locations in North America, including Michigan, chestnut trees are surviving even though they are infected with *C. parasitica.* This biological control of chestnut blight is thought to be due to the appearance of hypovirulent strains of the fungus which contain double-stranded RNA molecules that vary in size, concentration, and homology. The objective of this project was to gain an understanding of the biology and molecular structure of the dsRNA genomes involved in dsRNA-associated hypovirulence in Michigan strains of *C. parasitica.* This study focused on the biological function, genomic structure, and genomic organization of the dsRNA found in strain GH2. A strain containing two different dsRNA genomes was constructed, which was less virulent than the parental strains and which yielded asexual progeny that contained one, both, or neither of the parental genome types. Overlapping cDNA clones spanning nearly the entire length of the largest segment of dsRNA purified from the Michigan hypovirulent isolate GH2 were generated, and the nucleotide sequence of cloned inserts was determined.

The combined nucleotide sequence of these clones, totaling 9,608 nucleotides, includes 8,625 base pairs, which encode one open reading frame (ORF). The deduced amino acid sequence of this ORF (2,874 amino acids) includes putative RNA helicase, RNA-dependent RNA polymerase, and protease domains with a genomic organization similar to that observed for ORF B of hypovirulence-associated dsRNA molecules isolated from two other *C. parasitica* strains including one from Europe and one from New Jersey. Absent from the Michigan dsRNA molecule is a sequence similar to ORF A, which is apparently responsible for the down-regulation of the fungal enzyme laccase in European hypovirulent isolates. Correspondingly, isolate GH2 and other Michigan hypovirulent isolates accumulate the enzyme laccase at levels similar to those of virulent strains. Further study of the protein products encoded by the dsRNA may lead to a better understanding of the mechanism responsible for dsRNA-associated hypovirulence.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

ABTS	2,2'-azino-bis(3-ethylbenz-thiazoline-6-sulfonic acid)
BaYMV	barley yellow mosaic virus
bp	base pairs
cDNA	complementary DNA
DMOP	2,6-dimethoxy-phenol
dsRNA	double-stranded RNA
kb	kilobases
kd	kilodaltons
ORF	open reading frame
PAGE	polyacrylamide gel electrophoresis
PDA	potato dextrose agar
sci	single-conidial isolate
VLP	virus-like particle

Chapter I

INTRODUCTION

History of chestnut blight

The American chestnut tree (*Castanea dentata* [Marsh.] Borkh.) was once a major component of the hardwood forests in eastern North America. In the early part of this century, over 3 billion chestnut trees were killed by the disease called chestnut blight. In 1904, the fungal phytopathogen *Cryphonectria parasitica* (Murr.) Barr, the causal organism of chestnut blight, was isolated for the first time in North America from an American chestnut tree at the Bronx Zoo in New York (Anagnostakis, 1982). It is believed to have entered the United States on nursery stock of Oriental chestnuts (reviewed by MacDonald and Fulbright, 1991). Chestnut blight spread at epidemic rates throughout its natural range in eastern North America, killing over 3.5 billion chestnut trees by the mid-1950's (Hepting, 1974). Early researchers found that *C. parasitica* enters its host through wounds, such as branch scars, and penetrates the host periderm, phloem, and cambium, forming a canker that expands and girdles the tree (Bramble, 1936). Attempts to control this ascomycetous pathogen by applying fungicides were unsuccessful, as *C. parasitica* was either unaffected or could acquire resistance to all of the compounds tested (Jaynes and Van Alfen, 1978). Breeding programs were also started in an attempt to introduce into *C. dentata* resistance genes from the more resistant oriental species, such as *C. crenata* (Siebold and Zucc.) and *C. mollissima* (Blume) (MacDonald and Fulbright, 1991). Unfortunately, this approach was not quick or effective enough to have any impact on the epidemic.

In 1938, chestnut blight spread from North America to Europe, where *C. parasitica* attacked the European chestnut (*Castanea sativa* [Mill.]) (Anagnostakis, 1982). Blighted trees were first observed in northern Italy, and the disease spread at epidemic rates, similar to what had occurred in North America. By the 1950's, the disease had spread throughout Italy, however, unlike American chestnut trees, some European trees were surviving even though they were infected by the chestnut blight fungus. Italian plant pathologist Antonio Biraghi was the first to notice these surviving trees, and he characterized them as possibly being resistant to chestnut blight. He observed that cankers were not leading to lethal infections, and that the fungus remained in the outer layer of bark (Biraghi, 1953). It was later determined that the trees were not resistant. Instead, *C. parasitica* strains isolated from these trees by the French mycologist Jean Grente were less virulent than other strains and were termed hypovirulent (Grente, 1965). Other phenotypic characteristics originally associated with these European hypovirulent strains included a color change from orange to white and a reduction in sporulation (Grente, 1965).

Surprisingly, existing lethal cankers could be converted to non-lethal by inoculating them with mycelia from the hypovirulent strains (Grente and Berthelay-Sauret, 1978). It was determined that this was due to transmission of a cytoplasmic factor from the hypovirulent strain to the virulent strain. The hyphae of the two strains would anastomose, or fuse together, forming a bridge through which cytoplasm could pass from one strain to another. As a result of this process, the virulent strain was converted to hypovirulent (Grente and Berthelay-Sauret, 1978). A converted strain would also assume the other phenotypic traits associated with hypovirulence, including white pigmentation and reduced sporulation (Grente and Berthelay-Sauret, 1978).

Researchers in North America were excited by these findings, but at that time, no surviving or recovering trees had been observed on this continent. It was soon found that European hypovirulent strains could convert virulent, North American strains of *C. parasitica* to hypovirulent. When a canker on an American chestnut tree was inoculated with a European hypovirulent strain, the rate of canker expansion was reduced compared to virulent strains, and swelling and the formation of callus tissue was seen at the canker margins (Van Alfen *et al.*, 1975). Although hypovirulence was known to be transmissible and, therefore, thought to be cytoplasmic in nature, the factor responsible for the reduction in virulence was still unknown. In 1977, Day *et al.* reported that hypovirulent forms of *C. parasitica* contained double-stranded ribonucleic acid (dsRNA) molecules, while virulent strains did not. A causal relationship between the presence of dsRNA and hypovirulence was implied, and it was suggested that the dsRNA may be of viral origin, since most fungal viruses (mycoviruses) are composed of dsRNA. Hypovirulent strains were first found in North America in 1976 (Elliston *et al.*, 1977). Surviving American chestnut trees in Michigan were tested for hypovirulent isolates as researchers noted the similarity between the Michigan and European situations (Fulbright *et al.*, 1983).

Review of fungal viruses

Viruses, whose hosts are in the plant or animal kingdoms, have been studied since the late 19th century. In contrast, mycoviruses were not described until 1962. A disease of the cultivated edible mushroom *Agaricus bisporus* was first observed at a mushroom farm owned by La France Brothers in Pennsylvania and was therefore named La France disease (Buck, 1986). Mushrooms infected with this disease had long thin stipes and small caps. La France disease was proposed to be of viral origin in 1960 (Gandy) and this theory was confirmed through electron microscopy in 1962 (Hollings). The genome of this virus, like most mycoviruses discovered since then, was found to be composed of dsRNA (Buck, 1986). Mycoviruses, or unencapsidated dsRNA molecules, have been detected in all classes of fungi. The majority of virus infections of fungi, however, are symptomless (Hollings, 1978). Several of the exceptions are discussed below.

Ty elements in yeast

One class of mycoviruses that has been studied extensively are the retrovirus-like transposable elements found in many species of yeast, including *Saccharomyces cerevisiae.* There are 30-35 copies of a Ty element in the average yeast cell, and each has the ability to move or transpose within the genome of its host (Boeke and Garfinkel, 1988). In many cells, Ty elements are symptomless, however they can have a major effect on adjacent gene expression when inserted in the 5' region of a gene (Winston, 1988). Transposition occurs through a cytoplasmic RNA intermediate enclosed within virus-like particles (VLPs) that are composed of sugars and lipids, rather than virally encoded proteins. Also contained within the VLP is a reverse transcriptase, which catalyzes the synthesis of a complementary DNA (cDNA) copy of the Ty element (Boeke *et al.*, 1985). This cDNA copy is then exported from the VLP and is integrated into host chromosomal DNA (Liebman and Picologlou, 1988). New RNA intermediates are transcribed from these cDNA copies in the host chromosome and are encapsidated in VLPs (Boeke *et al.*, 1985).

Ty elements are related structurally and functionally to retroviruses, found in mammalian cells, and are members of a family of retrovirus-like transposons known as retrotransposons (Boeke and Garfinkel, 1988). The DNA form of both Ty elements and retroviruses is characterized by direct repeats at the 5'- and 3'- ends of the molecule, which are known as long terminal repeats (LTRs) and are ~334 bp in length (Elder *et al.*, 1983). The internal sequence of Ty elements contains two overlapping open reading frames (ORFs). The first, *tya*, encodes a protein that is homologous to DNA binding proteins and corresponds to the *gag* proteins of retroviruses. The second ORF, *tyb*, encodes a polypeptide with homology to the reverse transcriptase and endonuclease regions of the retroviral *pol* gene (Clare and Farabaugh, 1985). A third gene associated with retroviral genomes, *env*, encodes the coat protein that encapsidates the virus. This gene is not present in retrotransposons, and thus is not found in the Ty element genome. An ORF corresponding to the *env* gene was not expected, because Ty elements are encapsidated in VLPs rather than a virally encoded coat protein and do not have an extracellular stage in their life cycle, which would require a coat protein (Clare and Farabaugh, 1985). The study of retrotransposons is greatly facilitated by the vast amount of genetic information available for *S. cerevisiae*. A complete understanding of retrotransposition in yeast could lead to identification of pharmacological products that could affect the replication of mammalian retroviruses (Boeke and Garfinkel, 1988).

Killer viruses in yeast

Another interesting group of mycoviruses found in yeast are the killer viruses. Members of this group of viruses confer a selective advantage to their hosts by producing a toxin that is secreted from the host cell, killing cells of the same species that lack the particular virus or an immunity factor. This type of killer system has been identified in eight yeast genera, however, it was first described in *S. cerevisiae* (Bruenn, 1986).

S. cerevisiae killer virus particles are icosahedral, contain a dsRNA genome, and are approximately 30-40 nm in diameter (Bruenn, 1986). Each cell contains 100-3000 virus particles, all of which are located in the cytoplasm, but do not appear to be associated with the nucleus or mitochondria (Bruenn, 1980). A dsRNA molecule of ~4.8 kb has been associated with all dsRNA viruses of *S. cerevisiae* and is known as L dsRNA. This is the largest size class of dsRNA molecules in yeast, and it encodes the capsid polypeptide as well a replicase (Diamond *et al.*, 1989). Many of the viruses also contain a 1.9 kb dsRNA (M), which encodes the extracellular killer toxin as well as resistance to this toxin. M is encapsidated separately from L dsRNA (Leibowitz, 1988). There are several subgroups of M dsRNAs, each of which encodes a separate toxin. Two killer types, K1 and K2, have been extensively studied and have specific M dsRNAs known as M1 and M2, respectively. While killer cells are resistant to their own toxin, K1 cells are sensitive to K2 toxin and vice versa. Interestingly, M1 and M2 can not co-exist in the same cell. When a strain containing both M1 and M2 was constructed through mating, the M1 dsRNA had a competitive advantage over M2, which was not detected in progeny cells. This may be due to an advantage of M1 over M2 in obtaining a specific cellular component necessary for replication (Leibowitz, 1988). Similar constraints on co-existence have been postulated with strains of plant viruses and among similar plasmids in bacteria.

The K1 toxin has been extensively studied and consists of two protein subunits linked by disulfide bonds (Zhu *et al.*, 1987). Both subunits are encoded on one M dsRNA as a single prepropolypeptide, which is then further processed (Zhu *et al.*, 1987). It appears to be this precursor protein that is responsible for conferring immunity to the infected cell (Boone *et al.*, 1986). It is believed that the K1 toxin acts by binding to a cell wall β-glucan receptor, then forming a lethal cation channel in the plasma membrane of sensitive cells (Bussey, 1981; Kagan,1983; Boone *et al.*, 1990). The M2 dsRNA that encodes the K2 toxin, which has structural and functional properties similar to those of the K1 toxin, appears to be common in yeast strains used in wine making (Thomas *et al.*, 1991). During wine fermentation, desirable strains which secrete K2 toxin prevent the growth of cells sensitive to the toxin. This would ensure a non-contaminated, uniform yeast culture, which is optimal for the best flavor of the wine.

Viruses of plant pathogenic fungi

The killer system in *Ustilago maydis*

Ustilago maydis, the causal organism of corn smut, is a Basidiomycete, which causes the formation of galls on leaves, stems and in corncobs (Koltin, 1988). *U. maydis* is also a host for three dsRNA mycoviruses, known as P1, P4 and P6 (Koltin, 1986). These viruses are responsible for the production of a toxin effective against sensitive strains within the Ustilaginales (Nuss and Koltin, 1990). This phenomenon is very similar to that of the killer system found in yeast. A similar situation is found with *U. maydis* killer viruses as with *S. cerevisiae* killer viruses in that viruses P1 and P4 can coexist and function together in a single cell, while neither P1 or P4 can coexist with P6 (Koltin, 1988). The basic dsRNA banding patterns have been determined for each virus. P1 contains 6 dsRNA segments, P4 has 7 segments, and P6 has 5 segments. These dsRNA segments have been divided into three size classes designated heavy (H), medium (M) and light (L), referring to the molecular weight of each class (Koltin, 1988). Class H molecules range from 4.5 - 6.7 kb and encode the capsid protein in all three viruses, while class M molecules are 0.9 - 1.7 kb and encode the toxin in the P1 and P6 viruses (Koltin, 1988). The role of class L molecules, which are derived from one end of the M dsRNA, is much less clear (Field *et al.*, 1983). The L dsRNA from P1 has been sequenced and was determined to be 355 bp in length, but no open reading frames have been identified (Chang *et al.*, 1988). Previously, L dsRNA was thought to be involved in immunity to the toxin (Peery, 1982), however, molecular evidence seems to contradict this hypothesis (Chang *et al.*, 1988).

Each of the *U. maydis* viruses, P1, P4 and P6, differs in the specificity of its secreted toxin, KP1, KP4 and KP6, respectively. Killer secreting strains are resistant to the toxin they produce, but not to the other two toxins (Koltin, 1986). The majority of toxin research has been performed on KP6, which is composed of two independent protein subunits (Peery *et al.*, 1987). These subunits are synthesized as a single preprotoxin that is further processed into two secreted polypeptides (Tao *et al.*, 1990). The mode of action of this toxin is unknown, however cell wall receptors appear to be involved (Steinlauf *et al.*, 1988). There are also specific nuclear genes, P1r, P4r and P6r, responsible for resistance to each toxin, KP1, KP4 and KP6, respectively (Koltin, 1988).

Disease-factors in *Ophiostoma ulmi*

The ascomycete *Ophiostoma ulmi*, the causal organism of Dutch elm disease, is a devastating disease of elm species in Europe, North America and Southwest Asia. There are two major subgroups of this fungus, one is a highly pathogenic, aggressive strain, which is quickly replacing the other, a non-aggressive strain (Rogers *et al.*, 1988). Cytoplasmically transmissible disease factors (d-factors) have been identified in both of these subgroups of *O. ulmi*. Diseased isolates are characterized by sectors of weak or abnormal growth, reduced viability of conidia, and a reduction in the ability to sexually reproduce (Brasier, 1983).

While unencapsidated dsRNA segments have been identified in both diseased and healthy *O. ulmi* strains, transmission of the diseased state to a healthy strain was correlated with the transmission of 10 dsRNA segments (Rogers *et al.*, 1988). dsRNA in diseased strains has also been found to copurify with the mitochondria, which are deficient in cytochrome aa_3 (Rogers *et al.*, 1987). It is not known if the dsRNA is directly responsible for this deficiency, but suppression of respiration may be a mechanism by which fungal growth is reduced (Rogers *et al.*, 1987).

The possible use of dsRNA d-factors as a biological control is very exciting, however, there are at least two barriers to overcome. First, since the dsRNA is not transmitted through the sexual cycle of the fungus, a large number of uninfected ascospores capable of causing disease will be produced (Brasier, 1986). Also, the large number of vegetative compatibility groups within *O. ulmi* limits the spread of d-factors through hyphal anastomosis. Even with these limitations, the number of naturally occurring diseased strains is quite high (up to 40% in some locations in Europe), leading researchers to believe that biocontrol may be possible (Brasier, 1986).

dsRNA-associated virulence in *Rhizoctonia solani*

The soil-borne plant pathogen *Rhizoctonia solani* causes damping-off in over 100 plant species and is found throughout the world (Baker, 1970). Initial studies on the relationship between virulence and the presence of dsRNA molecules indicated that dsRNA may be associated with hypovirulence in *R. solani* (Castanho and Butler, 1978). Debilitated strains of *R. solani* contained dsRNA molecules while no dsRNA was recovered from healthy strains. The debilitation phenotype, as well as the dsRNA, was transmitted from hypovirulent to virulent strains upon hyphal anastomosis (Castanho and Butler, 1978).

Recently, the relationship between dsRNA and virulence was re-examined using *R. solani* strains isolated from various soil types and host plants in Israel (Ichielevich-Auster *et al.*, 1985). Of 109 strains characterized, approximately one third were nonpathogenic on 11 host plants tested and were termed hypovirulent. Nine strains (5 virulent and 4 hypovirulent) were chosen for further study of the relationship between dsRNA and virulence. No difference in growth rate in culture was seen among virulent and hypovirulent strains (Ichielevich-Auster *et al.*, 1985). dsRNA molecules were detected in both virulent and hypovirulent strains, however, virus particles were detected only in virulent strains (Finkler *et al.*, 1988). Extraction of dsRNA from the virus particles revealed the presence of two dsRNA segments within the particles. Other non-encapsidated dsRNA molecules, that did not cross-hybridize with the dsRNA from virus particles, were detected in both virulent and hypovirulent *R. solani* strains (Finkler *et al.*, 1988). Transmission experiments, using genetically-marked strains, revealed that a virulent strain can convert a hypovirulent strain to virulence by transmission of the viral dsRNA through hyphal anastomosis (Finkler *et al.*, 1985).

The correlation of the presence of a dsRNA virus to virulence in *R. solani* by Finkler *et al.* (1988) seems to contradict earlier results reported by Castanho and Butler (1978), in which the presence of dsRNA was correlated to hypovirulence. Upon re-examination of the virulent strain used in the earlier study, both unencapsidated dsRNA and viruses were identified. Since culture debilitation and hypovirulence are separate phenotypic traits, it has been proposed that the debilitation seen by Castanho and Butler (1978) is associated with an unencapsidated dsRNA, while virulence is correlated with virus particles (Nuss and Koltin, 1990).

The Reoviridae

Members of the largest family of dsRNA viruses, the Reoviridae, all have a dsRNA genome consisting of 10, 11 or 12 segments (Joklik, 1983). The type member of this family, respiratory enteric orphan virus (reovirus), was isolated from humans, and is not associated with any known disease. Other members of this family, however, can cause very serious diseases of plants, animals and insects (Joklik, 1983). Viruses within this family are divided into six genera which vary in host range, and in the number and size of dsRNA molecules within a viral genome.

Two genera of the Reoviridae family infect plants, the fijiviruses and the phytoreoviruses. The fijivirus genus is divided into three subgroups, including fiji disease virus (subgroup I), which is the type member of the genus, maize rough dwarf virus (subgroup II), and oat sterile dwarf virus (subgroup III) (Nuss and Dall, 1990). Each subgroup is distinguished on the basis of serology and electrophoretic genome profile. All fijiviruses have 10 dsRNA genome segments, which are contained within spiked virions (Pereira, 1991). Fijiviruses are transmitted by planthoppers and only infect graminaceous plants (Francki and Boccardo, 1983).

Phytoreoviruses are characterized by 12 dsRNA segments ranging in size from 851 bp - >3 kb (Nuss and Dall, 1990). The type member of this genus, wound tumor virus (WTV), was only recently isolated from a naturally infected plant (Hillman *et al.*, 1991). Previous to 1991, all WTV isolates studied originated from a single progenitor, which was isolated from a leafhopper

(Hillman *et al.*, 1991). The virus was then transferred to (*via* leafhoppers) and maintained in many species of dicotyledonous plants. Variation of the nucleotide sequence between individual segments of the type strain and the newly isolated strain ranged from only 1 - 3% (Hillman *et al.*, 1991).

Each of the 12 dsRNA genome segments of WTV contains a single ORF that encodes a unique protein. The 5'- and 3'-terminal ends of each segment contain a 6 and 4 bp consensus sequence, respectively. Just internal to each consensus sequence is a 6-14 bp inverted repeat, the length and nucleic acid sequence of which varies between segments (Nuss and Dall, 1990). Every WTV virion contains one copy of each segment, and it is thought that each of the 12 segments interacts with the coat protein to insure proper virion assembly (Anzola *et al.*, 1987). It was proposed that the 5'- and 3'-terminal consensus sequences and inverted repeats act as recognition signals during packaging (Anzola *et al.*, 1987).

Replication of WTV is similar to other members of the Reoviridae family. An RNA-dependent RNA polymerase is present within the virion. This RNA polymerase transcribes ssRNA copies from the viral dsRNA (Nuss and Peterson, 1980). Replication occurs in the cytoplasm within viroplasms, which are composed of viral proteins and dsRNA (Francki and Boccardo, 1983). A current hypothesis regarding replication suggests that viral RNA is synthesized in the viroplasm where mature particles are assembled. These particles then move from the viroplasm into the cytoplasm of the host cell (Francki and Boccardo, 1983). Viroplasms have also been identified in the cytoplasm of leafhopper cells (Chiu *et al.*, 1970).

As the name WTV implies, this virus has the ability to cause tumors in some host plants, including clover (Black, 1945). Another member of the phytoreoviridae, rice gall dwarf virus, also produces tumors in infected rice plants, however the mechanism of tumor induction is unknown (Nuss and Dall, 1990).

Genera of the Reoviridae that infect animals include orthoreovirus, orbivirus, and rotavirus, which differ primarily in the number of dsRNA segments per genome and in disease severity (Joklik, 1983). These viruses enter their hosts through the gastrointestinal tract, where primary replication is thought to occur. The virus then spreads to other organs, where it enters host cells, uncoats, and is transcribed and translated (Sharpe and Feilds, 1983). In many cases, viroplasms similar to those seen in WTV appear to be formed. Some of these viruses appear to be asymptomatic, while others can cause very severe diseases (Pereira, 1991).

Other dsRNA viruses found in plants

A final group of dsRNA molecules found within plants are collectively known as cryptoviruses. These viruses are considered to be cryptic, because they cause no visible symptoms. The cryptoviruses are transmissible only through pollen and seeds and are present in low concentrations in the infected host plant (Boccardo *et al.*, 1987). Studies have shown that the dsRNA of beet cryptic virus 1 (BCV1) can be translated *in vitro*, producing two polypeptides, one from each of the two dsRNA segments present in BCV1. One of the proteins is thought to be involved in viral replication, while the other functions as the coat protein (Accotto *et al.*, 1987). RNA-dependent RNA polymerase activity has been identified in all cryptovirus particles tested, and is thought to be responsible for replication (Boccardo and Accotto, 1988). There is also evidence that the dsRNA from *Phaseolus vulgaris* cv. Black Turtle specifically cross-hybridizes to chloroplast DNA (cpDNA) from *P. vulgaris* cv. Black Turtle and to mung bean cpDNA (Wakarchuk and Hamilton, 1990). However, these dsRNA molecules have not been associated with virus particles and, therefore, may not be cryptoviruses (Boccardo *et al.*, 1987). dsRNA from *P. vulgaris* cv. Black Turtle also cross-hybridized to DNA from plant species that do not contain dsRNA. Results of these studies may eventually point to the evolutionary origin of these dsRNA molecules.

dsRNA in *Cryphonectria parasitica*

dsRNA-containing hypovirulent isolates of *C. parasitica* have been collected from many locations throughout France and Italy, as well as in several locations in North America. The dsRNA molecules in *C. parasitica* have many viral properties, but should not be considered true viruses. For example, they do not have an extracellular state, are non-infectious, and are only transmitted through hyphal anastomosis (Nuss and Koltin, 1990). A coat protein that would encapsidate the dsRNA has not been identified, but virus-like particles (VLPs) have been reported to be associated with dsRNA in the cytoplasm of hypovirulent *C. parasitica* strains (Dodds, 1980; Hansen *et al.*, 1985). Similar vesicles have been found in virulent strains, however they do not contain dsRNA. Fungal polysaccharides have been detected in vesicles from virulent and hypovirulent strains, suggesting that these vesicles could function in fungal cell wall synthesis (Hansen *et al.*, 1985). An RNA-dependent RNA polymerase associated with dsRNA-containing-vesicles has also been identified and is thought to function in the replication of the dsRNA (Hansen *et al.*, 1985).

Hypovirulence-associated dsRNA molecules from Europe and North America have many similarities, including: cytoplasmic transmissibility, correlation with reduction in virulence, association with membrane-bound particles, and effects on fungal growth and morphology. However, major differences have been identified. The most striking differences were the lack of pigmentation and suppressed sporulation associated with European dsRNA, while North American hypovirulent strains were fully pigmented and sporulation was not as noticeably suppressed. dsRNA from several European strains cross-hybridized upon northern analysis, however, they did not cross-hybridize to dsRNA isolated from North American strains of *C. parasitica* (L'Hostis *et al.*, 1985). Furthermore, northern analysis performed on dsRNA from *C. parasitica* isolates from West Virginia, Michigan, France and Italy revealed several distinct homology groups (Paul and Fulbright, 1988). dsRNA isolated from West Virginia strains did not cross-hybridize to dsRNA from Michigan *C. parasitica* strains, and none of the North American strains tested cross-hybridized to dsRNA of French or Italian origin (Paul and Fulbright, 1988). Recently, a *C. parasitica* isolate collected in New Jersey was found to contain dsRNA that cross-hybridized to dsRNA of European origin (Hillman *et al.*, 1992). This was the first report of homology between dsRNA from North American and European isolates.

Within the last five years, the basic genomic organization of the dsRNA associated with the French hypovirulent strain EP713 has been elucidated. Upon gel electrophoresis, dsRNA from strain EP713 can be grouped into three size classes, large (L), medium (M) and small(S). The L-dsRNA is a single band ~12.7 kb in length. The M-dsRNA size class consists of one or more bands 8.0 - 10.0 kb in length while S-dsRNAs consist of two or more bands which range in size from 0.6 - 1.7 kb (Hiremath *et al.*, 1986). The M- and S-dsRNA size classes result from internal deletions of the L-dsRNA (Shapira *et al.*, 1991b). The 3'-terminus of all dsRNA size classes is characterized by a stretch of polyadenylic acid (poly[A]) (Hiremath *et al.*, 1986).

The L-dsRNA, which has been cloned and sequenced, is 12,712 bp in length. The strand of L-dsRNA terminating with 3' poly(A) is the coding strand and contains two large open reading frames designated ORF A and ORF B (Shapira *et al.*, 1991a). ORF A, which has been studied extensively, is 1,869 nucleotides (nt) in length and encodes two polypeptides, p29 and p40 (Choi *et al.*, 1991a). The protein p29 is a protease, which is autocatalytically released from the ORF A polyprotein during translation (Choi *et al.*, 1991a). The amino acid sequence of this protease is similar to that of the potyvirus helper component protease, known as HC-Pro (Choi *et al.*, 1991b). A function for p40 has not been determined. Virulent *C. parasitica* protoplasts were transformed with a cDNA copy of ORF A, which was found to confer phenotypic traits associated with European hypovirulent isolates, but did not reduce virulence (Choi and Nuss, 1992a). These traits included loss of pigmentation, suppression of sporulation, and a reduction in the accumulation of the fungal enzyme laccase. Although the biological function of laccase in *C. parasitica* is unknown, the enzyme is down-regulated in European dsRNA-containing hypovirulent isolates (Rigling *et al.*, 1989; Hillman *et al.*, 1990; Rigling and Van Alfen, 1991; Choi *et al.*, 1992).

ORF B has been partially characterized and is 9,498 nt in length. An autocatalytic protease similar to that seen in ORF A has been identified using *in vitro* translation of ORF B. This protease, a 48-kd polypeptide (p48), was cleaved from the amino-terminal portion of the ORF B polyprotein (Shapira *et al.*, 1991a). While no other polypeptides have been identified, several regions of nucleic acid similarity between ORF B and other viruses have been suggested. Putative helicase and RNA-dependent RNA polymerase domains have been proposed using amino acid comparisons of ORF B to several other viruses, including the potyvirus tobacco vein mottling virus (Koonin *et al.*, 1991).

Recently, Choi and Nuss (1992b) have transformed virulent *C. parasitica* protoplasts with a full-length cDNA copy of the dsRNA from strain EP713. Transformants were hypovirulent and possessed all of the phenotypic traits normally associated with dsRNA from EP713. This was the first time dsRNA was conclusively demonstrated to be responsible for hypovirulence.

Significantly, full-length EP713 L-dsRNA molecules were isolated from the cytoplasm of transformants. These dsRNA molecules were produced from the cDNA copy, which was integrated into the chromosome of the fungus. These cytoplasmic dsRNAs could be transferred, *via* hyphal anastomosis, to virulent strains, converting them to hypovirulent. As a biological control, these genetically engineered hypovirulent strains appear to have a major advantage over naturally occurring *C. parasitica* isolates in that the cDNA copy of the dsRNA is integrated into the fungal genome. Upon mating these strains with virulent strains, ascospore (sexual) progeny contained the cDNA copy of L-dsRNA in their genomes, produced L-dsRNA, and were hypovirulent (Choi and Nuss, 1992b). dsRNA is not transmitted to ascospore progeny in naturally occurring hypovirulent strains (Anagnostakis, 1982).

Hypovirulence in Michigan

Many blighted, but surviving, stands of American chestnut can be found within Michigan. Most of the state is not within the natural range of American chestnut trees, however, trees can be found throughout the state as pioneers moving west planted chestnut seedlings (Fulbright *et al.*, 1988). In fact, all recovering chestnut stands within Michigan are located outside of the natural range (MacDonald and Fulbright, 1991). It is thought that these stands are recovering because of the presence of hypovirulent *C. parasitica* isolates within the natural population (Fulbright *et al.*, 1985). Of the isolates collected to date, all but two hypovirulent isolates contained dsRNA. One of these isolates

(CL25) is of great interest since it was the first non-dsRNA containing hypovirulent isolate of *C. parasitica* to be discovered (Fulbright, 1985) and further investigation of these strains is presently underway (Mahanti, 1991).

When comparing dsRNA-containing Michigan hypovirulent isolates, obvious differences in culture morphology can be identified. Isolates have coloration ranging from dark to bright orange, have uneven to smooth margins, and have a slow to moderate growth rate (Fulbright *et al.*, 1983). At the molecular level, isolation of dsRNA followed by gel electrophoresis reveals three general banding patterns among hypovirulent isolates. The most predominant type was first identified in *C. parasitica* strain Grand Haven 2 (GH2), which contains three dsRNA segments of ~9.0, 3.5 and 0.8 kb in length. Many strains containing GH2-like dsRNA possess only the large, ~9.0 kb segment (Fulbright *et al.*, 1983). A second dsRNA genome consists of two smaller dsRNA segments ~2.8 and 1.6 kb in length. This genome has only been identified in *C. parasitica* isolates from Roscommon, Michigan and was first isolated from strain RC1 (Fulbright *et al.*, 1983). The third class of dsRNA genomes in Michigan has only been identified in one isolate of *C. parasitica*, GNC, and contains a single dsRNA segment with a size of ~10 kb.

Upon northern analysis, dsRNA isolated from strains GH2, RC1, and GNC did not cross-hybridize (Paul and Fulbright, 1988; P. McManus, Michigan State University, personal communication). There appear to be two distinct homology groups within the dsRNA genome of strain GH2. The two larger segments cross-hybridize to each other, but not to the ~0.8-kb segment

(Tartaglia *et al.*, 1986). By sequence analysis of cDNA clones, it was determined that the ~0.8-kb segment does not have the ability to produce any protein products (D. Nuss, Roche Institute of Molecular Biology, personal communication), and thus appears to be a satellite of the larger segments (D. Fulbright, Michigan State University, personal communication). Further analysis of the ~9.0- and 3.5-kb segments of the GH2 dsRNA genome revealed that the 3.5-kb segment probably results from an internal deletion within the 9.0-kb segment, as both the 5' and 3' ends of the two segments are homologous (Tartaglia *et al.*, 1986).

Objectives of this study

The overall objective of my dissertataion research was to further investigate the role of dsRNA molecules in the reduction of virulence in Michigan isolates of *C. parasitica.* Using isolates that were known to function in nature as biocontrols of chestnut blight, I was first able to multiply infect a virulent isolate with two dsRNA genomes, creating a super-debilitated strain. These experiments provided valuable information about the segregation and potential packaging of individual segments within a dsRNA genome.

Second, I began a detailed molecular analysis of the ~9.0-kb dsRNA segment found within isolate GH2. By generating and sequencing cDNA clones of this dsRNA, the basic genomic organization has been elucidated. Using this information, it was possible to compare, at the nucleic acid and amino acid levels, North American and European hypovirulence-associated dsRNA molecules. These comparisons are important in determining the similarity between these geographically distinct dsRNA genomes, which may reveal evolutionary relationships between them. By determining which regions are similar between vastly different dsRNA genomes, one may be able to identify regions that have functional significance and gain further insight into the mechanism of dsRNA-associated hypovirulence.

The final phase of my project concerned the expression of the fungal enzyme laccase, which is thought to be down-regulated by dsRNA in European hypovirulent strains of *C. parasitica* and has been implicated as a virulence factor (Rigling *et al.*, 1989). The specific region of dsRNA that must be present and expressed in the fungus for the down-regulation of the nuclearly-encoded enzyme laccase, has been identified in *C. parasitica* strain EP713 (Choi and Nuss, 1992a). Michigan dsRNA-containing isolates had never been assayed for the accumulation of laccase. Several biochemical analyses for the presence of the enzyme were employed to determine if dsRNA within Michigan isolates had the ability to down-regulate this fungal enzyme. The ability of dsRNA to down-regulate laccase is important in determining whether laccase is a virulence factor in Michigan isolates of *C. parasitica* and in identifying differences between dsRNA genomes isolated from European and Michigan strains of *C. parasitica.*

The ultimate goal of this project is to obtain a complete understanding of how dsRNA within *C. parasitica* causes a reduction in fungal virulence and, thereby, serves as a biological control of chestnut blight in Michigan. The work presented in this dissertation represents several of the steps necessary to attain this goal.

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Chapter II

Characterization of a *Cryphonectria parasitica* strain infected with multiple dsRNA genomes

Introduction

Cryphonectria parasitica (Murr.) Barr, the fungal pathogen responsible for chestnut blight, destroyed the mature American chestnut forest (*Castanea dentata* [Marsh] Borkh.) in the earlier part of this century. The European chestnut (*C. sativa* Miller) in Italy and France, and the American chestnut in a few locations in North America, including Michigan, are surviving despite repeated infection by *C. parasitica* (Anagnostakis, 1982; Fulbright *et al.*, 1983). Trees within these stands are frequently found to be infected with hypovirulent forms of *C. parasitica.* Besides reduced virulence, hypovirulent strains often demonstrate several phenotypic traits that distinguish them from virulent strains including changes in growth rate, culture morphology, sporulation and pigmentation (Van Alfen, 1982).

Most hypovirulent strains of *C. parasitica* isolated from surviving chestnut trees in Europe and North America harbor double-stranded RNA (dsRNA) molecules (Dodds, 1980). One of these dsRNA molecules is known to be responsible for the expression of the hypovirulence phenotype (Choi and Nuss, 1992), yet the molecular mechanism by which this occurs is unknown (Nuss and Koltin, 1990). The dsRNA molecules isolated from different hypovirulent strains frequently vary in size, number of size classes, concentration and homology (L'Hostis *et al.*, 1985; Paul and Fulbright, 1988). The dsRNA purified from Michigan isolates GH2 and RC1 do not cross-hybridize and include 3 and 2 segments respectively, representing different size classes of dsRNA (Paul and Fulbright, 1988). Hypovirulence-associated dsRNA genomes are thought to be packaged in membrane vesicles (Van Alfen, 1982; Newhouse *et al.*, 1990). It is not known if all of the size classes of dsRNA in a particular genome are always packaged together in a single vesicle, or how the molecules in different size classes are transmitted to asexual progeny.

Based on changes in dsRNA banding patterns after asexual segregation, it has been postulated that a hypovirulent strain of *C. parasitica* (EP-60) harbors more than one dsRNA genome (Elliston, 1985). This is the only observed case of infection of *C. parasitica* with multiple dsRNA genomes. It is not known if the various dsRNA molecules of a single genome found within the cytoplasm of *C. parasitica* interact with one another, or if they can act independently. There may be limitations in *C. parasitica* to maintaining and/or transmitting more than one dsRNA genome, since only one multiply-infected strain has been isolated (Elliston, 1985).

The goal of this study was to construct strains of *C. parasitica* infected with multiple dsRNA genomes, then to study the transmission of different size classes of molecules from this mixture of dsRNA genomes. A multiply-infected hypovirulent strain was constructed by transferring the two non-homologous dsRNA genomes (GH2 and RC1) to a virulent strain (CL1-16). In this paper we report on the segregation patterns of these dsRNA molecules, as well as their interaction and effect on fungal virulence.

Materials and methods

Cultures and growth conditions The *C. parasitica* cultures used in this study are listed in Table 2.1 and are shown in Figure 2.1. These strains are vegetatively compatible and all additional strains used for this work were derived from these cultures. The fungus was grown on potato-dextrose agar (PDA; Difco,Detroit, MI) at room temperature under cool white fluorescent lights with a 16-h photoperiod (Garrod *et al.*, 1985). Cultures were stored on PDA slants at 4°C. Cultures used for dsRNA isolation were grown on cellophane-covered PDA plates for 7 days or in stationary culture in Endothia complete broth without glucose (Puhalla and Anagnostakis, 1971) for 14-21 days.

Strain construction To reduce the potential for interference resulting from different nuclear backgrounds, the dsRNA molecules from the two hypovirulent isolates (GH2 and RC1) were transferred to the common nuclear background of CL1-16, a virulent single-conidial isolate of the bark isolate CL1 (Fulbright *et al.*, 1983). Transfer of dsRNA from each of the hypovirulent isolates to CL1-16

Table 2.1. Isolates of *C. parasitica* used in this study.

Isolate designation	dsRNA[a]	Virulence[b]	Reference
CL1-16	-	V	Paul and Fulbright, 1988
GH2	+	H	Fulbright *et al.*, 1983 Paul and Fulbright, 1988
RC1	+	H	Fulbright *et al.*, 1983 Paul and Fulbright, 1988

[a] +, dsRNA is present; -, dsRNA is not detectable.

[b] V, determined virulent in virulence assays; H, determined hypovirulent in virulence assays.

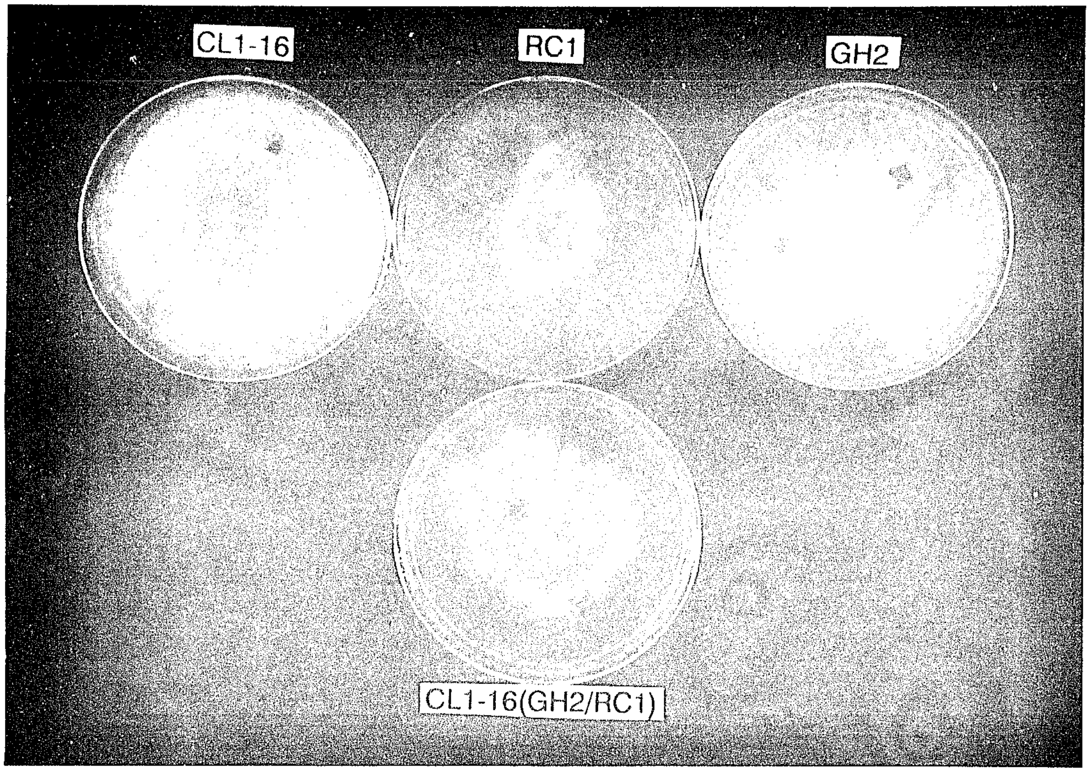

Figure 2.1. Culture morphology of *C. parasitica* strains used in this study.

was accomplished by pairing a hypovirulent isolate with CL1-16 on PDA as previously described (Anagnostakis and Day, 1979). The construction of a multiply-infected strain from two singly-infected strains was performed as described above, except the two hypovirulent strains, CL1-16(GH2) and CL1-16(RC1), were paired and conversion of either strain was deduced by noting a significant decrease in growth of one or both isolates at the margin of the two colonies. Subcultures of the newly constructed strains were taken from the margin of the colony and placed on PDA. In all cases, transfer of dsRNA was confirmed by polyacrylamide gel electrophoresis of the dsRNA molecules. Also, culture morphology changes were noted and virulence assays performed. Converted strains were given designations listing the source of the dsRNA in parentheses (Table 2.2).

Single-conidial isolation and virulence assays Single pycnidiospores (conidia) were isolated from the multiply-infected strain CL1-16(GH2/RC1) by removing 10 mm plugs from 10-day-old *C. parasitica* cultures and placing them in sterile distilled water. Plates containing PDA were inoculated with ten-fold serial dilutions of the spore suspensions. After three days, individual germinating spores were identified, cut from the agar surface and subcultured on PDA. Virulence was determined by inoculating Golden Delicious apple fruit with mycelial plugs taken from PDA plates and measuring the discolored area resulting after three weeks of growth at room temperature (Fulbright, 1984).

Double-stranded RNA isolation and cDNA cloning Double-stranded RNA was isolated as described (Morris and Dodds, 1979). Electrophoresis was performed using 5% polyacrylamide gels and stained with ethidium bromide. The large (9.0 kb) dsRNA band of strain GH2 (Tartaglia *et al.*, 1986) was cut from polyacrylamide gels and eluted with the Elutrap system as described by the manufacturer (Schleicher and Schuell, Keene, NH). A cDNA library was generated from the electroeluted 9.0 kb GH2 dsRNA segment as described by Rae *et al.* (1989). The RC1 cDNA library was made as described for GH2 except that cDNA clones were generated from total RC1 dsRNA (not a single segment).

Northern blot analysis Electrophoresis of dsRNA for northern blot analysis was performed using a 1.2% (w/v) agarose gel. The dsRNA was then denatured by treating the gel as described by Shapira *et al.* (1991) and transferred to nylon membrane (MSI, Westboro, MA) by capillary blotting using 20X SSC. Prehybridization was performed in 50% formamide (v/v), 5 X SSC, sonicated denatured salmon sperm DNA (250 μg/ml), 5 X Denhardt's at 42°C for a minimum of two hours (Ausubel *et al.*, 1987). One of two cDNA clones (pGH234 and pR49), generated from GH2 and RC1 dsRNA, respectively, was labeled with [α-^{32}P]dCTP by the random primer method (Feinberg and Vogelstein, 1983) and added to the prehybridization mix. Hybridizations were performed at 42°C for a minimum of 6 hours (Paul and Fulbright, 1988).

Results

Multiple infection Transfer of dsRNA into CL1-16 from hypovirulent isolates GH2 and RC1 was verified by a significant reduction in virulence (Table 2.2) and similar dsRNA electrophoretic banding patterns (Figure 2.2, Figure 2.3 step 1). Pairing the resulting hypovirulent strains CL1-16(GH2) and CL1-16(RC1) resulted in strain CL1-16(GH2/RC1), which contained dsRNA that co-migrated with the dsRNA from both sources (Figure 2.3 step 2) and was more debilitated in culture than either parent. Multiple infection was verified by examinations of dsRNA electrophoretic banding pattern profiles and northern analysis in which dsRNA of both parents was observed (Figures 2.2, 2.4 and 2.5). Although strain RC1 is characterized by 2 dsRNA bands upon gel electrophoresis (Figure 2.2), the larger 2.8-kb band is sometimes lost or degraded during the isolation procedure and therefore the 1.6-kb molecule would be the only band visualized by northern analysis (Figure 2.4). CL1-16(GH2/RC1) was less virulent than either parent in virulence assays performed on apple fruit (Table 2.2).

Identification of dsRNA molecules present in single-conidial isolates To determine if dsRNA segments from two different hypovirulent strains segregate independently, single-conidial isolates were obtained from CL1-16(GH2/RC1) (Figure 2.3, step 3). These isolates were grouped according to

Table 2.2. Characteristics of *C. parasitica* strain CL1-16 after infection with dsRNA genomes.

Strain[a]	Virulence tests[b] (mm^2)	dsRNA molecules detected GH2	dsRNA molecules detected RC1
CL1-16	3125 a	-	-
CL1-16(GH2)	1062 b	+	-
CL1-16(RC1)	831 b	-	+
CL1-16(GH2/RC1)	224 c	+	+

[a]Strains in parentheses served as the source dsRNA, which was transferred into the virulent strain CL1-16.

[b]Virulence data are derived from mean lesion area on inoculated Golden Delicious apple fruit of three replicates per strain. Means followed by the same letter do not differ significantly ($P=0.05$) according to Tukey's honestly significant test.

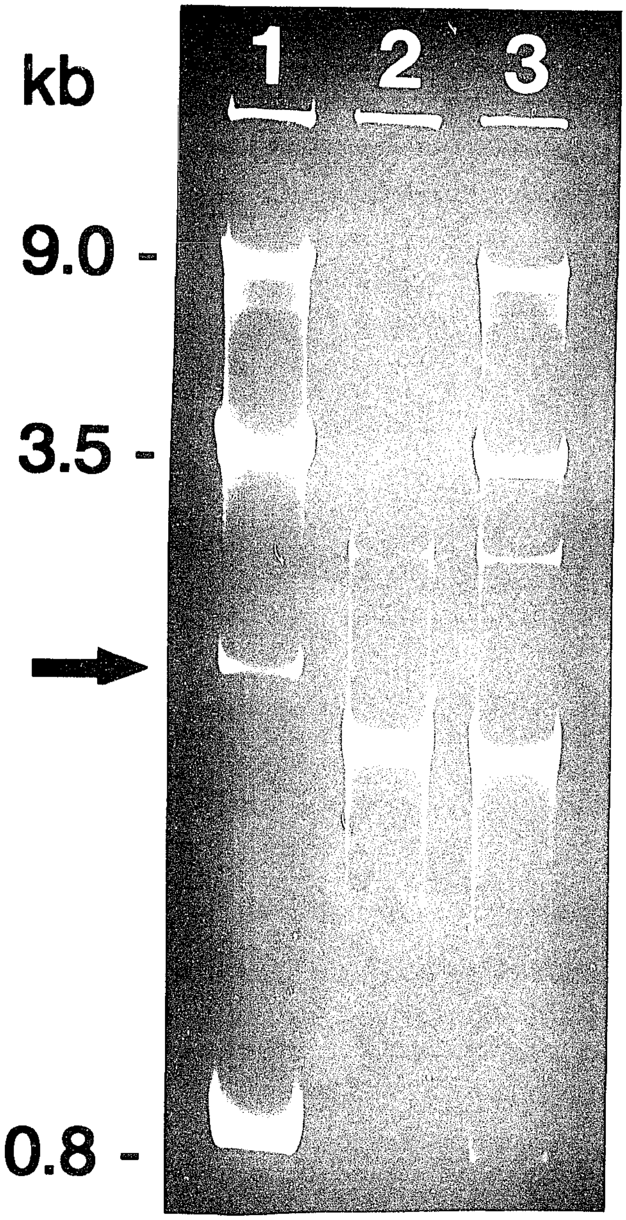

Figure 2.2. Banding patterns of dsRNA from converted strains of *C. parasitica.* dsRNA was electrophoresed in a 5% polyacrylamide gel and stained with ethidium bromide. Lanes: 1, CL1-16(GH2); 2, CL1-16(RC1); 3, CL1-16(GH2/RC1). Sizes of dsRNA molecules present in strain GH2 are indicated in kilobases (kb). Arrow indicates a dsRNA band which is sometimes present in strain GH2.

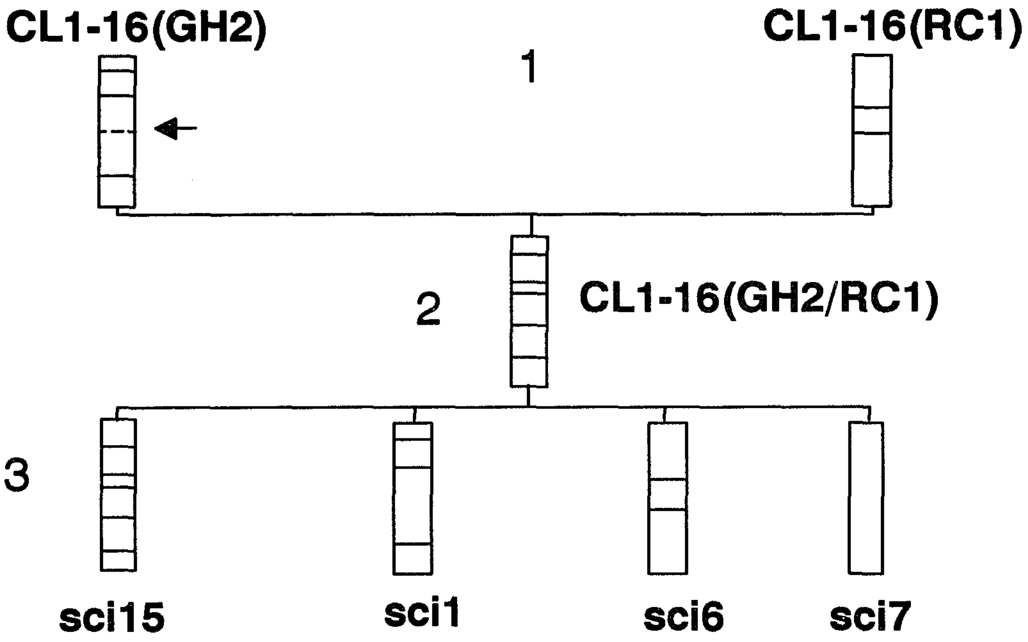

Figure 2.3. Diagrammatic representation of dsRNA banding patterns in ethidium bromide stained polyacrylamide gels. Thick horizontal lines represent dsRNA bands in a gel. Numbers designate steps referred to in text. Arrow indicates a dsRNA band which is sometimes present in strain GH2 and can be seen in lane 1 of Figure 2.2. This band is only included in step one of the diagram.

morphological phenotype and 2 - 5 representatives of each group were chosen for virulence and dsRNA assays. Single-conidial isolates from CL1-16(GH2/RC1) exhibited segregation of the dsRNA segments, resulting in strains without dsRNA or containing dsRNA electrophoretic banding patterns similar to RC1, GH2 and GH2/RC1 (Figures 2.2, 2.4 and 2.5). No exchange of dsRNA segments was observed to occur between genomes. Virulence of the various single-conidial isolates showing specific dsRNA electrophoretic banding patterns was comparable to the parental hypovirulent strains with similar banding patterns (Table 2.3).

Discussion

The primary goal of this study was to analyze the segregation and maintenance of two non-homologous dsRNA genomes introduced into a single nuclear background. The dsRNA genomes associated with isolates GH2 and RC1 segregated independently without any mixture of individual segments from the two genomes occurring in transmission to asexual progeny. When dsRNA was present in single-conidial isolates, the banding pattern profiles always included both or either complete genome, suggesting that both size classes of dsRNA molecules of RC1 are packaged together and separately from the GH2 dsRNA genome.

The strain CL1-16(GH2/RC1) was found to be less virulent than strains CL1-16(GH2) and CL1-16(RC1), suggesting that these two dsRNA genomes act additively. It is possible that the RC1 and GH2 dsRNA genomes use a

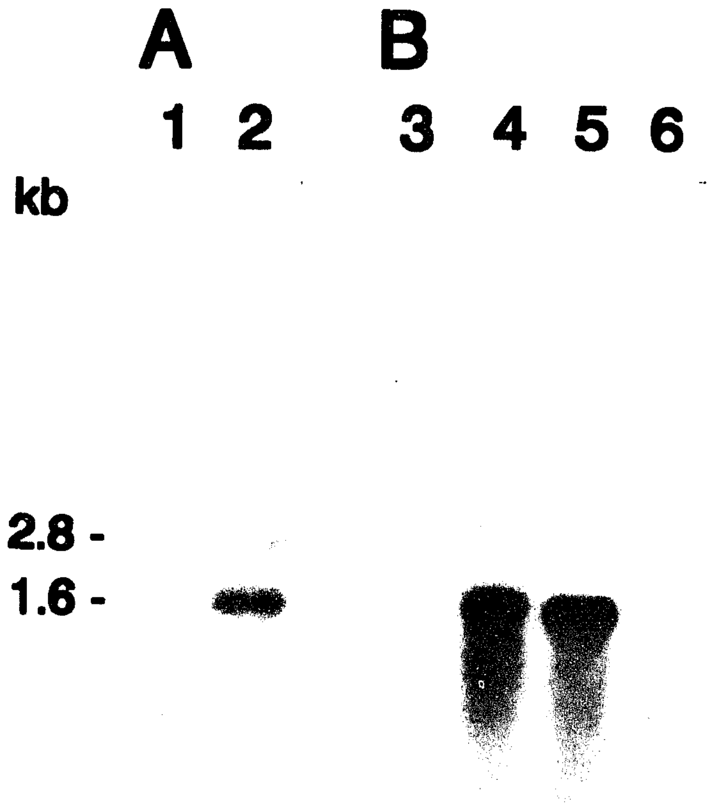

Figure 2.4. Northern blot analysis of dsRNA. dsRNA was isolated from the multiply-infected strain CL1-16(GH2/RC1) and several single-conidial isolates (sci) from this strain. The blot was probed with the ^{32}P-labeled cDNA clone pR49, which was generated from RC1 dsRNA. The 2.8-kb band is sometimes lost during isolation of dsRNA. Panel A lanes: 1, GH2; 2, RC1. Panel B lanes: 3, CL1-16; 4, CL1-16(GH2/RC1); 5, CL1-16(GH2/RC1)sci6; 6, CL1-16(GH2/RC1)sci1.

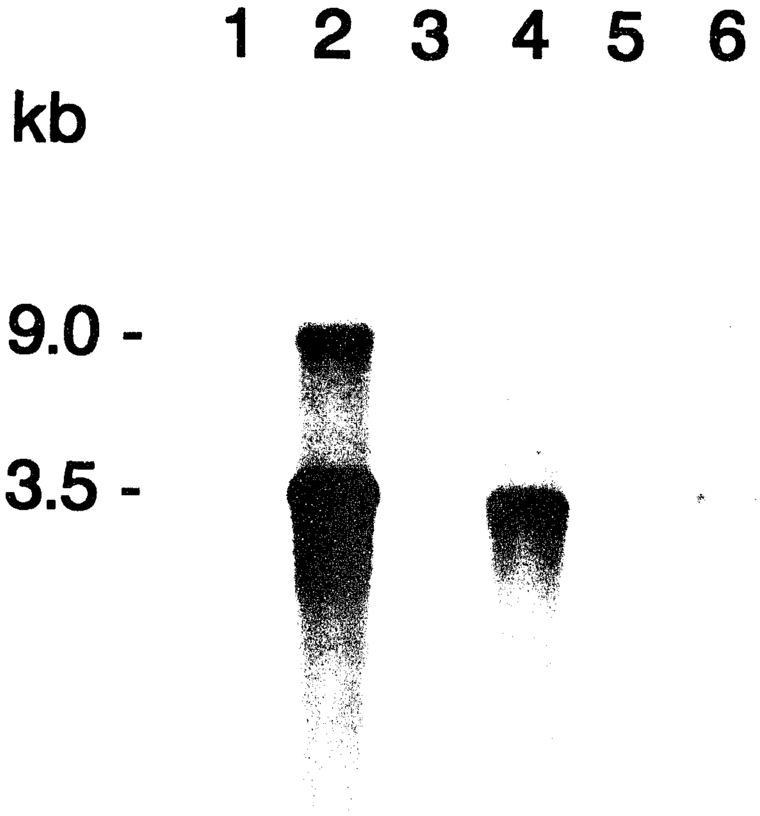

Figure 2.5. Northern blot analysis of dsRNA probed with the cDNA clone pGH234. This cDNA clone was generated from GH2 dsRNA. Lanes: 1, CL1-16; 2, GH2; 3, RC1; 4, CL1-16(GH2/RC1); 5, CL1-16(GH2/RC1)sci6; 6, CL1-16(GH2/RC1)sci1.

Table 2.3. dsRNA content of single-conidial isolates of strain CL1-16(GH2/RC1).

Strain	dsRNA molecules detected[a]	
	GH2	RC1
sci 1	+	-
sci 2	+	-
sci 3	+	+
sci 6	-	+
sci 7	-	-
sci 11	-	+
sci 12	-	+
sci 19	+	+
sci 20	-	-
sci 21	+	-
sci 26	-	-
sci 36	+	-
sci 38	+	+
sci 42	+	+
sci 48	+	-
sci 50	-	-
sci 100	+	+
sci 103	-	-
sci 115	-	+
sci 116	-	-
sci 122	-	-
sci 130	-	-
sci 133	-	+
sci 137	-	-

Table 2.3 cont.

sci 140	-	-
sci 144	+	-
sci 145	+	+
sci B1	-	-
sci B4	-	-
sci B5	-	-
sci B8	-	-
sci B9	-	+
sci B11	+	+
sci B12	+	-
sci B16	+	-
sci B22	+	+
sci B30	-	-
sci B31	+	-
sci B35	-	-
sci B40	+	+
sci B42	-	-
sci B50	-	-
sci B51	+	+
sci B52	+	-
sci B73	+	-
sci B77	-	-
sci B81	-	-
sci B82	+	-
sci B85	+	+
sci B96	-	-
sci B102	-	-

[a] +, dsRNA is present; -, dsRNA is not detectable.

different mechanism for reduction of fungal virulence and function independently of one another. This hypothesis is substantiated by morphological characteristics of the three cultures, as the multiply-infected strain possesses the phenotypic characteristics associated with each dsRNA genome (Figure 2.1). Another explanation, however, is that the two genomes do act by a similar mechanism, and the combination of the two amplifies their effects.

The results of our study concur with those of Elliston (1985), who observed a distinct culture morphology and level of virulence that could be identified with an individual dsRNA genome upon isolating single conidia from each multiply-infected strain. An additive effect conferred by the presence of two dsRNA genomes was not observed by Elliston (1985) in contrast to my studies. It is unknown if there was any sequence similarity between the two dsRNA genomes in strain Elliston's experiment. It is possible that the two dsRNA genomes used similar mechanisms for reduction of fungal virulence, and therefore did not additively affect virulence. The lack of hybridization analysis of single-conidial isolates from those studies makes interpretation of dsRNA segregation patterns more difficult. The use of northern analysis to detect the presence of dsRNA in single-conidial isolates of strain CL1-16(GH2/RC1) conclusively demonstrated that the GH2 and RC1 dsRNA genomes segregate independently from one another among asexual progeny.

Acknowledgements

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Chapter III

Molecular characterization of a dsRNA molecule from the Michigan *Cryphonectria parasitica* isolate GH2

To be submitted for publication as C. Durbahn Smart, D.L. Nuss and D.W. Fulbright

Introduction

Some isolates of the fungal pathogen *Cryphonectria parasitica* ([Murr.] Barr), the causal agent of chestnut blight, have been found to contain virus-like molecules of dsRNA. There is a correlative relationship between the presence of dsRNA in the cytoplasm of *C. parasitica* and a reduction in fungal virulence (hypovirulence) (Fulbright, 1984). Recently, a dsRNA molecule from a European isolate of *C. parasitica* has been shown to be directly responsible for hypovirulence (Choi and Nuss, 1992b). Hypovirulent strains have been studied for their involvement in the biological control of chestnut blight (MacDonald and Fulbright, 1991). These strains also offer an intriguing model system in which to study fungal-viral interactions, such as viral effects on fungal metabolism, virus replication and transfer among fungal strains, and origins of the dsRNA molecules.

Studies on the European hypovirulent strain of *C. parasitica*, EP713, have shown that the largest dsRNA segment within this strain (L-dsRNA) is 12,712 base pairs in length and contains two large open reading frames (ORFs), known as ORF A and ORF B (Shapira *et al.*, 1991a). The junction between ORF A and ORF B consists of a one base pair overlap of the two ORFs, suggesting that a -1 frameshift may occur following the termination of ORF A and prior to the initiation of ORF B (Shapira *et al.*, 1991a). ORF A produces a polyprotein from which an autocatalytic protease is cleaved producing two smaller polypeptides (Choi *et al.*, 1991a). This protease is similar at the amino acid level to those found in the helper component of several members of the potyvirus family (Choi *et al.*, 1991b). ORF B, which is ~ 9.5 kb in length, encodes a polyprotein that includes a protease domain (48 kd) which contains amino acid motifs similar to those found in ORF A (Shapira *et al.*, 1991a). Within the deduced amino acid sequence of ORF B, two other putative domains have been identified, which would encode an RNA-dependent RNA polymerase and an RNA helicase (Koonin *et al.*, 1991). It has been hypothesized that the dsRNA viruses of *C. parasitica* originated from ssRNA viruses (Shapira *et al.*, 1991a; Koonin *et al.*, 1991), as the putative domains were reported to be similar to those of ssRNA viruses, including barley yellow mosaic virus (BaYMV) which is a poty-like virus that is transmitted through a fungal vector (Koonin *et al.*, 1991). Recently, virulent *C. parasitica* protoplasts were transformed with full-length cDNA clones of the L-dsRNA (Choi and Nuss, 1992b). Transformants were hypovirulent, confirming that in *C. parasitica* strain EP713 the dsRNA is responsible for the reduction in fungal virulence (Choi and Nuss, 1992b).

Sequence analysis has also been performed on cDNA clones spanning the 5' and 3' termini of dsRNA isolated from the New Jersey hypovirulent *C. parasitica* strain NB58 (Hillman *et al.*, 1992). The dsRNA from strain NB58 cross-hybridized with that of EP713, and there was nucleotide sequence identity of 65-70% at the 3'-termini of the molecules. Strain NB58 contains a single segment of dsRNA ~ 12.5 kb in length, which is slightly smaller than the L-dsRNA of strain EP713 (Hillman *et al.*, 1992). This was the first report of cross-hybridization of dsRNA isolated from a North American strain of *C. parasitica* to dsRNA from a European strain. The complete nucleotide sequence of the dsRNA molecule isolated from strain NB58 is now known (B. Hillman, Rutgers University, personal communication).

Hypovirulent forms of *C. parasitica* have been isolated from recovering American chestnut stands throughout Michigan (Fulbright *et al.*, 1983). The culture morphology of the Michigan hypovirulent strains, which are orange in color and sporulate abundantly, differs from that of isolates from Europe which are white with suppressed sporulation (Fulbright *et al.*, 1983; Anagnostakis, 1982). One hypovirulent *C. parasitica* isolate, GH2, was collected in 1980 from an American chestnut (*Castanea dentata* [Marsh] Borkh.) grove located in Grand Haven, Michigan, where the trees have been surviving chestnut blight

(Fulbright *et al.*, 1983). This hypovirulent strain has been used successfully as a biological control of chestnut blight in Michigan (Fulbright *et al.*, 1983; Garrod *et al.*, 1985). GH2 contains three dsRNA segments approximately 9.0 kb, 3.5 kb, and 0.8 kb in size (Tartaglia *et al.*, 1986). Upon northern analysis, the 9.0- and 3.5-kb segments were found to share sequence homology, while the 0.8-kb segment did not cross-hybridize with either of the larger segments (Tartaglia *et al.*, 1986; Paul and Fulbright, 1988). It is speculated that the 3.5-kb segment occurs as the result of an internal deletion of the largest dsRNA segment in isolate GH2, as the dsRNA molecules within each size class are 3'-polyadenylated (Tartaglia *et al.*, 1986; Shapira *et al.*, 1991b). Northern analysis also revealed that dsRNA from isolate GH2 did not cross-hybridize with dsRNA genomes of European origin (Paul and Fulbright, 1988).

In this study, we report on sequence analysis of cDNA clones generated from the ~ 9.0-kb dsRNA segment of isolate GH2 and on the comparison of the GH2 dsRNA sequence to that of the other dsRNA molecules isolated from *C. parasitica.*

Materials and methods

Cultures and growth conditions *C. parasitica* isolate GH2 was collected from a non-lethal canker on an American chestnut tree in Grand Haven, Michigan (Fulbright *et al.*, 1983). *C. parasitica* cultures were grown on potato dextrose agar (PDA; Difco, Detroit, MI) at room temperature under cool-white, fluorescent lights with a 16 h photoperiod (Garrod *et al.*, 1985). Cultures were stored on PDA slants at 4°C. Cultures used for dsRNA isolation were grown in stationary culture in Endothia complete broth without glucose (Puhalla and Anagnostakis, 1971) for 14-21 days or on cellophane-covered PDA plates for 5-10 days.

Double-stranded RNA isolation and cDNA cloning Standard molecular genetic techniques were performed as described by Sambrook *et al.* (1989). Double-stranded RNA was isolated using CF-11 cellulose (Whatman) column chromatography as described by Morris and Dodds (1979). Electrophoresis was performed using 5% polyacrylamide gels, which were stained with ethidium bromide following electrophoresis. The largest dsRNA segment of strain GH2 was cut from polyacrylamide gels and eluted from the gel using the Elutrap system as described by the manufacturer (Schleicher and Schuell, Keene, NH). Using oligo $(dT)_{12\text{-}18}$ as a primer for first strand synthesis, a cDNA library was generated from the electroeluted dsRNA segment, as described by Hillman *et al.* (1992). Double-stranded cDNA was ligated into the cloning vector pUC9, which had 5' poly(dG) extensions (Pharmacia, Piscataway, NJ), and recombinant plasmids were used to transform *Escherichia coli* strain HB-101 (BRL, Gaithersburg, MD). This cDNA library did not contain dsRNA sequences from the 5' region of the GH2 dsRNA molecule. Therefore, a second cDNA library was constructed using a specific oligonucleotide primer for first strand synthesis. This primer was generated using a sequence known to be near the 5' end of the GH2 dsRNA molecule. The cloning vector was again pUC9, however *E. coli* strain DH5-α (BRL) was transformed with the recombinant plasmids.

Dot blot and Southern analysis of cDNA clones was performed using ^{32}P-labeled GH2 dsRNA as a probe, to ensure that each cDNA clone originated from the GH2 dsRNA. The location of each cDNA clone, with respect to the 3' poly(A) tail of the dsRNA, was determined using Southern hybridization, and a map of the cDNA clones was generated.

Sequence analysis of cDNA clones DNA sequence was obtained from double-stranded DNA plasmid templates containing cDNA inserts. The plasmids were first denatured using the method of Zhang *et al.* (1988), followed by dideoxy terminator sequencing reactions using Sequenase (United States Biochemical, Cleveland, OH). The ends of each insert were sequenced using the -20 universal primer (United States Biochemical) and the M13 reverse primer (Promega, Madison, WI). Additional sequence was obtained using synthetic oligonucleotide primers specific for each cDNA clone. Nucleotide sequence analysis was performed using Editbase software (Neils Neilsen, Purdue University). Deduced amino acid sequence comparisons were made using the programs of the University of Wisconsin Genetics Computer Group.

Results

Cloning and sequence analysis of dsRNA from isolate GH2 dsRNA was isolated from *C. parasitica* strain GH2, and three segments were identified. The largest segment, > 9.0 kb, was purified from polyacrylamide gels, and a partial cDNA library was constructed in the plasmid vector pUC9 (Figure 3.1). cDNA clones spanned the entire length of the dsRNA molecule, with the exception of the extreme 5' end. At least two independent clones were identified for much of the length of the dsRNA molecule. Cloned inserts were sequenced and were found to extend 9,608 nucleotides from the 3' end of the dsRNA molecule (Figure 3.2). The 3' end was polyadenylated, confirming the results of Tartaglia *et al.* (1986). The strand of dsRNA that terminates with this 3' poly(A) appears to be the coding strand, as sequence analysis revealed a single, long ORF, of 8,625 nucleotides, ending 857 base pairs (bp) from the 3' terminus of the dsRNA molecule (Figures 3.1 and 3.2). No other large ORFs were identified in any of the remaining five reading frames (Figure 3.3).

Computer alignments of deduced amino acid sequences A comparison of the deduced amino acid sequence of cDNA clones from GH2 dsRNA to amino acid sequences deduced from DNA sequences in the GenBank/EMBL database revealed similarity with only one sequence. This was the sequence of the dsRNA from *C. parasitica* strain EP713, however, the sequence of the dsRNA from strain NB58 has not yet been entered into the database (B. Hillman,

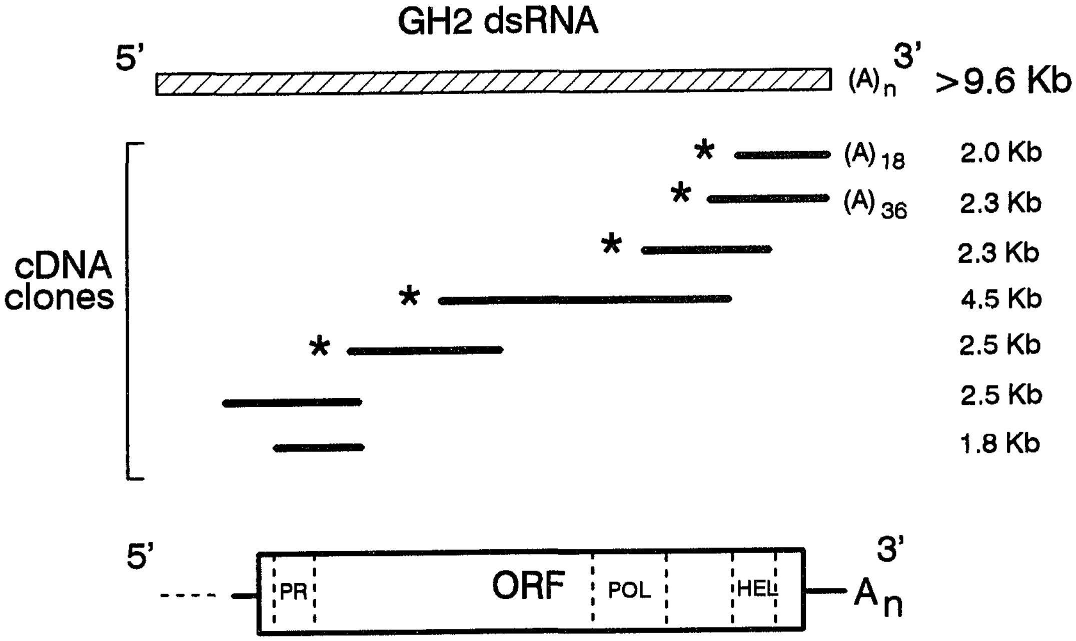

Figure 3.1. Map of cDNA clones of dsRNA from *C. parasitica* strain GH2. The dsRNA is represented as a hatched box. Individual cDNA clones are shown as solid bars. The open reading frame (ORF) is shown as a rectangle below the cDNA clones. Putative functional domains are delineated with dashed lines: PR, protease; POL, RNA-dependent RNA polymerase; HEL, RNA Helicase. cDNA clones marked with an asterisk were generated by D. Nuss and D. Fulbright.

Figure 3.2. Nucleotide and deduced amino acid sequences of GH2 dsRNA cDNA clones. Nucleotide sequence is numbered on the left, and deduced amino acid sequence is numbered on the right.

```
   1 CTTTGGAGGAACTTTAAATTGTATTAATTTTGCATTTGCAAGAGTTAGCCCATCTCTAAAATGAGGCTATCGCTGTGAACAGCCTTAAAGACCCTCTGGG
                                                                  M  Q  E  E  L  Q  N  N  Q  P  G  S  G    13
 101 CAAACTGATTATGAATTGAGAGTTAGACTTCTCAGAAAAAGTCTGGTCTTGTCCTAGATTATGCAGGAGGAACTCCAAAACAACCAGCCGGGATCTGGCT
     S  S  R  S  G  R  D  T  S  V  N  K  V  A  P  T  R  S  G  S  V  P  D  D  T  R  V  I  T  Q  S  R  P  S  47
 201 CTTCCCGCAGTGGCCGCGACACATCTGTGAATAAAGTCGCTCCGACACGGTCTGGGTCTGTGCCGGATGACACCAGGGTGATTACCCAATCCAGGCCTTC
       T  S  S  G  D  K  K  K  S  V  G  D  D  G  F  I  P  T  L  S  P  R  E  F  L  D  C  T  S  Y  G  G  A   80
 301 TACTTCTTCAGGCGACAAGAAGAAGTCCGTTGGTGATGACGGTTTCATCCCAACCCTGTCTCCTCGGGAGTTTCTTGACTGCACATCCTATGGAGGTGCA
      R  V  G  G  E  P  P  L  A  K  F  T  E  N  P  C  Y  P  V  P  L  G  E  Q  L  K  L  S  P  D  F  T  V    113
 401 CGGGTGGGTGGTGAACCCCCGTTAGCCAAATTCACTGAGAACCCCTGTTACCCTGTACCACTTGGGGAACAGTTAAAGCTAAGTCCAGACTTCACAGTCT
     C  R  C  Q  V  H  I  R  P  A  S  Y  C  L  T  D  L  K  G  C  A  L  K  Q  V  G  N  H  P  V  I  Q  A  A  147
 501 GCAGATGTCAGGTGCATATCCGACCTGCATCTTACTGCCTAACGGATCTGAAAGGGTGTGCTCTGAAGCAGGTTGGGAACCATCCGGTTATCCAAGCTGC
       A  K  K  Q  N  T  F  C  H  A  S  E  D  V  R  R  L  D  R  T  F  V  E  L  V  C  N  S  L  P  N  Q  P   180
 601 TGCAAAGAAACAGAACACATTTTGTCATGCTTCTGAAGATGTTAGGAGATTAGATAGGACTTTCGTGGAACTTGTTTGCAATTCCTTACCTAATCAGCCT
      A  F  S  V  F  E  G  N  P  L  A  N  R  L  A  S  S  H  S  A  D  V  K  D  F  G  M  G  Y  C  A  L  S    213
 701 GCTTTTTCAGTGTTTGAAGGAAATCCATTGGCTAATAGGTTGGCATCATCACATTCTGCCGACGTCAAGGATTTCGGCATGGGTTATTGTGCATTGTCTG
     V  L  R  P  K  L  R  W  R  S  A  R  V  L  G  P  D  C  L  L  G  D  F  D  K  V  F  N  W  A  G  L  K  Q  247
 801 TCCTGCGTCCTAAACTCAGGTGGCGGTCTGCCAGAGTTCTTGGACCCGATTGTCTCCTTGGCGACTTTGATAAGGTCTTTAATTGGGCAGGGCTTAAACA
       F  K  A  M  T  F  V  E  V  Y  R  G  F  Y  H  L  V  T  V  P  G  A  K  G  V  D  L  E  A  G  E  N  L   280
 901 ATTTAAAGCGATGACGTTTGTCGAAGTCTATCGTGGATTCTATCATCTGGTGACTGTGCCTGGCGCGAAGGGGGTGGACCTTGAGGCCGGTGAGAACTTG
      K  N  E  I  S  K  I  L  E  K  N  P  D  A  R  V  G  T  G  S  G  V  D  N  G  T  Y  L  T  P  D  Y  F    313
1001 AAGAATGAAATCTCAAAAATCCTTGAGAAGAACCCTGATGCAAGGGTAGGTACTGGGTCGGGTGTTGACAATGGCACCTACTTGACTCCCGACTATTTTG
     E  E  L  G  A  E  A  E  Y  L  E  E  E  D  L  P  L  D  L  C  W  K  E  M  F  P  P  S  Y  G  Y  S  A  L  347
1101 AAGAGTTAGGTGCTGAGGCTGAGTACTTGGAGGAGGAAGATCTTCCGCTCGACCTCTGCTGGAAGGAGATGTTCCCACCATCTTATGGGTACTCTGCACT
       F  G  M  H  V  G  D  F  G  D  E  M  D  V  F  K  F  L  A  N  I  D  L  M  V  Y  E  Y  V  N  F  G  I   380
1201 GTTTGGTATGCACGTAGGTGACTTTGGTGACGAGATGGACGTCTTCAAGTTCCTAGCAAATATAGATCTGATGGTGTATGAGTACGTTAATTTTGGCATT
      G  D  G  F  C  H  V  A  E  G  S  Q  T  K  D  S  E  L  H  L  D  K  A  T  L  K  A  G  L  H  R  M  I    413
1301 GGGGATGGTTTTTGTCATGTCGCCGAGGGTTCCCAGACCAAGGACAGTGAGCTTCATCTAGATAAAGCTACGCTGAAGGCTGGCTTGCACAGGATGATTG
     E  A  D  W  N  F  G  L  L  P  V  E  L  T  T  A  V  V  M  G  T  I  G  D  V  E  P  T  L  D  V  V  A  T  447
1401 AGGCTGACTGGAATTTCGGTTTGCTTCCTGTTGAGCTAACCACGGCTGTTGTTATGGGCACAATTGGCGATGTGGAGCCAACGTTGGATGTCGTTGCAAC
       N  Y  F  A  A  Q  M  E  E  Q  P  E  P  I  P  V  E  E  Q  Y  K  F  T  V  G  E  N  G  W  M  S  S  D   480
1501 GAATTACTTCGCTGCGCAGATGGAAGAACAACCTGAGCCTATCCCCGTGGAGGAACAATATAAATTCACGGTGGGTGAAAATGGTTGGATGTCTTCTGAC
      F  F  G  I  C  D  G  F  A  K  S  L  F  D  S  F  G  S  G  K  G  Q  L  E  V  S  P  A  L  W  N  E  I    513
1601 TTCTTCGGCATCTGCGACGGTTTCGCTAAATCTTTGTTCGATTCTTTTGGTTCTGGTAAAGGGCAATTGGAGGTATCTCCAGCCCTATGGAATGAAATAC
     R  A  A  W  D  G  E  P  I  I  G  D  P  D  E  T  N  Y  P  V  P  G  S  F  L  V  E  L  S  D  D  S  E  W  547
1701 GCGCTGCCTGGGACGGTGAACCAATCATCGGCGACCCAGATGAGACAAACTACCCTGTACCTGGGTCATTTCTTGTGGAACTTTCAGATGACTCAGAATG
       L  K  V  P  D  R  P  V  G  T  H  K  R  V  M  P  P  D  F  V  L  V  T  H  P  D  H  V  A  R  F  A  H   580
1801 GCTCAAAGTTCCTGATAGGCCAGTGGGCACACATAAGAGGGTTATGCCCCCTGATTTTGTATTGGTTACGCATCCGGACCATGTTGCAAGATTTGCACAT
      E  G  W  E  T  V  S  L  P  M  D  S  K  A  F  I  S  L  G  Q  A  I  L  E  K  G  L  Y  A  A  L  D  M    613
1901 GAAGGATGGGAGACTGTCTCCCTCCCAATGGACAGCAAAGCCTTTATATCTTTAGGGCAGGCGATTCTTGAGAAAGGCTTGTATGCAGCTCTAGACATGA
     K  A  M  H  E  T  L  F  E  H  I  K  A  A  M  P  I  C  E  Q  S  D  L  I  Y  L  V  S  G  T  T  F  H  Y  647
2001 AGGCTATGCATGAGACACTCTTCGAGCATATCAAGGCAGCAATGCCTATATGCGAACAGAGTGATCTCATCTACCTTGTGTCCGGGACGACGTTCCATTA
       F  L  A  S  V  F  P  E  K  Q  V  F  E  V  C  P  V  P  R  E  D  N  G  V  C  P  E  F  Y  L  G  H  Y   680
2101 CTTCCTTGCCTCTGTGTTCCCTGAAAAACAAGTCTTTGAGGTGTGTCCAGTTCCACGCGAGGACAATGGTGTTTGTCCTGAGTTCTATCTTGGACACTAC
      F  K  D  V  T  V  D  P  G  F  G  F  G  V  G  R  L  Y  N  K  W  L  T  A  P  K  Y  L  N  E  L  E  W    713
2201 TTCAAAGATGTCACTGTCGATCCTGGTTTTGGTTTTGGAGTTGGAAGACTATATAACAAATGGTTAACCGCTCCGAAATATCTGAATGAGTTAGAGTGGG
     E  G  E  F  R  Y  I  E  P  P  K  F  H  S  I  A  P  W  A  A  D  R  W  Q  I  S  S  P  S  I  G  F  K  P  747
2301 AAGGTGAGTTCAGGTACATTGAACCACCTAAGTTTCACTCTATAGCACCGTGGGCGGCTGATAGATGGCAGATTTCCTCTCCATCAATTGGCTTTAAACC
       Y  D  D  F  V  K  K  T  S  F  Q  E  N  E  T  I  K  G  Y  F  S  L  G  S  C  E  S  I  T  K  E  T  R   780
2401 CTACGATGACTTTGTCAAGAAAACTTCATTTCAGGAAAATGAAACCATCAAAGGTTATTTCTCTCTTGGTTCTTGTGAGTCAATAACGAAAGAAACTCGT
      S  A  L  A  W  L  R  S  L  P  V  K  W  E  V  D  K  R  W  T  Y  L  F  E  G  T  D  Y  V  E  S  P  F    813
2501 TCGGCTCTTGCTTGGTTACGCTCTTGCCTGTGAAATGGGAGGTTGACAAAAGGTGGACGTACCTTTTTGAGGGTACTGACTACGTGGAGTCTCCTTTCA
     T  N  H  A  T  Y  L  H  K  F  D  W  V  V  H  H  G  G  S  G  V  T  N  T  C  C  A  V  R  V  P  Q  T  I  847
2601 CGAACCATGCTACCTATCTCCACAAATTTGATTGGGTAGTCCATCACGGAGGATCGGGTGTGACCAATACGTGTTGTGCGGTTCGTGTCCCTCAAACCAT
       L  P  Q  V  G  D  Q  F  V  W  E  D  A  L  K  E  H  T  I  P  L  H  V  P  E  D  E  L  R  A  L  L  F   880
2701 CTTGCCTCAGGTTGGCGACCAATTTGTCTGGGAAGATGCACTGAAGGAACACACAATTCCACTACATGTGCCAGAGGATGAGTTGCGTGCTCTCCTCTTC
      H  D  R  S  K  P  I  T  K  P  D  W  A  K  V  V  K  D  G  P  Q  W  W  V  D  A  L  V  D  N  D  A  S    913
2801 CATGATAGGAGCAAACCTATAACTAAACCCGACTGGGCCAAAGTCGTTAAAGATGGTCCTCAATGGTGGGTAGATGCTCTTGTGGACAATGATGCTTCTC
     P  V  Q  P  F  R  I  L  N  H  C  C  H  D  W  D  A  Y  G  W  K  P  T  D  L  V  V  G  Q  N  H  D  W  W  947
2901 CCGTTCAACCGTTTAGGATTCTGAACCACTGCTGCCATGATTGGGATGCATATGGTTGGAAGCCGACGGATCTAGTAGTTGGGCAGAATCATGATTGGTG
       F  T  L  F  N  S  A  W  E  R  G  E  F  E  L  T  L  K  F  V  R  K  V  C  K  P  T  G  S  P  L  V  G   980
3001 GTTCACTCTGTTCAACTCTGCCTGGGAACGTGGAGAGTTTGAGTTGACGTTAAAATTTGTCCGTAAGGTGTGCAAGCCAACTGGCTCACCACTCGTTGGG
      W  S  G  F  S  L  T  Q  T  Y  P  E  H  A  H  G  W  L  T  S  E  R  L  E  F  E  P  F  A  K  K  A  F    1013
3101 TGGTCTGGGTTTAGCCTTACCCAGACATACCCTGAACACGCTCATGGCTGGCTGACCTCCGAAAGGTTAGAGTTCGAGCCAtTCGCCAAAAAGGCTTTTT
     S  A  R  Y  I  A  V  N  E  E  T  K  Y  R  E  L  G  S  R  A  L  T  S  R  S  V  H  N  P  Y  V  H  M  N  1047
3201 CTGCACGGTATATTGCCGTGAATGAAGAAACGAAGTACAGAGAGCTAGGTTCTAGAGCTCTGACTTCGCGGTCTGTGCACAACCCTTATGTGCACATGAA
       P  R  N  I  D  C  P  C  G  G  R  G  Y  D  F  G  G  K  C  E  R  C  F  L  Q  G  L  D  N  G  K  L  D   1080
3301 TCCACGCAATATCGACTGCCCTTGCGGGGGTAGAGGCTACGATTTCGGTGGAAAATGTGAACGTTGTTTTCTTCAGGGTTTAGATAATGGCAAGCTTGAT
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Figure 3.2

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       A  I  D  L  E  A  I  T  T  T  V  Y  R  G  F  E  R  K  N  K  P  C  R  K  D  V  T  F  R  K  S  T  A     1113
3401  GCCATTGACCTGGAGGCCATTACAACTACGGTGTACAGGGGTTTCGAACGTAAAAACAAGCCCTGTCGAAAAGATGTCACATTCAGGAAGAGCACGGCGA

      S  F  W  V  R  S  R  K  R  Y  Y  Q  L  D  S  R  V  V  K  N  T  A  S  P  T  L  A  R  A  L  L  D  Q  I   1147
3501  GTTTCTGGGTGAGATCACGAAAGAGGTACTATCAGTTGGATTCACGTGTTGTCAAAAACACTGCATCACCAACACTTGCTAGAGCCCTCTTGGATCAAAT

        Q  D  M  S  D  V  A  N  V  E  A  F  W  E  Y  T  N  S  K  P  P  H  Y  T  Y  K  T  S  R  H  M  L  N    1180
3601  CCAGGACATGTCTGATGTGGCAAACGTCGAAGCTTTCTGGGAGTACACAAACTCAAAACCTCCCCACTACACCTATAAGACGAGTAGGCATATGCTGAAC

       H  L  I  S  E  Q  A  R  L  H  V  D  V  G  M  S  N  I  A  T  D  V  V  K  A  L  G  G  V  L  S  V  G     1213
3701  CACTTGATTAGTGAGCAGGCTCGTCTTCATGTTGATGTGGGTATGTCTAATATCGCAACGGATGTTGTGAAGGCACTTGGTGGGGTTCTTTCTGTTGGTT

      S  F  G  A  L  Y  S  R  L  A  N  A  N  C  F  S  S  S  G  R  D  F  M  K  K  R  W  W  V  L  V  D  A  L   1247
3801  CTTTCGGGGCACTATACTCACGTCTCGCAAATGCCAATTGTTTCTCGTCGTCCGGTCGGGATTTCATGAAGAAGCGTTGGTGGGTTCTGGTTGATGCTTT

        R  H  F  D  D  L  T  R  S  V  G  I  K  A  M  P  E  L  V  R  G  I  F  P  E  L  P  Q  A  S  V  T  S    1280
3901  GAGGCACTTCGATGATCTGACTAGAAGTGTTGGTATAAAAGCTATGCCAGAATTGGTGCGTGGTATATTTCCTGAGCTCCCTCAAGCTTCAGTCACCTCT

       V  I  P  A  S  F  A  L  S  R  P  L  R  T  R  V  A  G  K  L  W  M  D  Q  M  R  A  Q  P  A  S  V  R     1313
4001  GTTATCCCAGCTTCCTTCGCTTTGTCAAGACCATTGAGGACAAGGGTGGCAGGTAAACTCTGGATGGATCAGATGCGAGCACAACCAGCGTCGGTTCGGG

      V  H  L  F  N  I  R  L  P  V  L  G  K  D  F  G  V  F  H  A  F  V  E  F  D  G  W  F  W  E  L  Q  Q  I   1347
4101  TGCACTTGTTCAACATACGGTTACCGGTGCTTGGTAAAGATTTTGGTGTCTTCCATGCATTTGTCGAATTTGATGGCTGGTTCTGGGAATTGCAACAAAT

        S  Q  E  K  C  R  I  N  R  S  K  F  P  P  E  A  S  S  D  R  P  L  A  K  T  I  L  V  K  S  P  I  V    1380
4201  TTCTCAAGAAAAATGCCGTATCAACCGTAGCAAGTTCCCTCCTGAAGCTAGCTCTGATCGTCCTTTGGCAAAAACTATCCTGGTTAAATCGCCCATTGTT

       G  S  L  D  L  R  R  I  S  R  E  F  D  G  I  G  Y  K  I  L  G  D  N  C  L  V  F  A  N  M  L  V  Y     1413
4301  GGTTCTCTGGACCTAAGGAGAATCTCACGCGAATTTGACGGGATAGGATACAAGATCTTGGGTGACAATTGTCTCGTTTTTGCGAATATGTTGGTGTATC

      L  L  T  G  T  V  I  P  W  R  H  F  G  I  F  G  K  D  L  S  L  N  I  Q  K  E  L  M  Q  W  A  S  S  H   1447
4401  TTCTGACTGGGACAGTGATACCTTGGAGACACTTTGGGATCTTCGGTAAAGATTTGTCACTTAACATTCAGAAGGAACTAATGCAGTGGGCCTCATCACA

        F  F  L  H  E  D  E  Q  R  L  Q  T  R  D  N  N  A  A  G  L  T  H  T  G  Q  T  V  S  S  I  K  S  W    1480
4501  TTTCTTCCTTCATGAGGATGAGCAACGATTGCAGACCCGTGACAATAATGCTGCTGGTCTGACGCACACTGGTCAAACGGTTTCCTCAATAAAATCTTGG

       T  G  P  K  R  F  I  R  D  Y  G  L  T  C  V  Q  K  L  E  A  S  L  E  A  Y  S  D  D  P  D  I  D  C     1513
4601  ACAGGGCCAAAACGGTTCATAAGAGATTACGGTCTTACCTGTGTTCAGAAACTTGAAGCCTCTTTGGAGGCCTACAGTGATGATCCAGATATAGACTGTC

      P  Q  E  R  D  H  M  L  D  F  M  Q  F  A  I  T  K  F  G  L  S  G  A  I  V  S  R  A  I  L  S  R  R  V   1547
4701  CCCAGGAACGTGATCACATGTTGGACTTCATGCAATTCGCAATTACAAAGTTTGGTTTGTCTGGTGCCATAGTGTCAAGAGCGATCTTGTCTCGTCGTGT

        R  R  M  A  T  S  G  R  K  W  K  F  M  H  H  L  L  V  L  F  K  Q  V  V  Q  T  R  L  G  E  D  A  M    1580
4801  CCGTAGGATGGCGACCTCGGGTCGGAAATGGAAATTTATGCACCATCTGCTTGTTCTTTTCAAGCAGGTGGTACAAACACGACTGGGAGAGGATGCTATG

       G  V  L  T  A  T  S  N  L  R  G  G  L  R  G  G  K  K  V  G  W  T  P  I  L  S  I  S  V  P  R  H  W     1613
4901  GGTGTTTTAACTGCAACCTCGAACCTACGCGGTGGCCTCCGCGGAGGTAAGAAGGTTGGTTGGACTCCCATACTTAGCATAAGTGTCCCTAGGCATTGGT

      F  R  S  G  N  R  L  V  T  V  D  H  L  P  E  N  L  N  M  R  T  K  K  R  V  Q  L  D  L  P  Q  I  A  K   1647
5001  TTCGCTCTGGTAATCGGTTGGTCACAGTTGACCATCTCCCTGAGAATTTGAACATGAGGACAAAGAAACGTGTTCAACTGGATTTGCCCCAAATAGCGAA

        R  Y  Q  H  Y  F  G  V  D  P  P  P  L  G  F  K  W  I  R  P  G  E  Y  E  I  G  V  K  V  P  V  R  T    1680
5101  GAGGTATCAACACTATTTTGGGGTGGATCCTCCGCCACTAGGTTTCAAGTGGATTCGACCTGGAGAATACGAGATTGGCGTGAAGGTTCCTGTGAGAACT

       N  L  P  K  M  D  S  L  T  Q  E  L  C  H  E  L  Q  E  L  H  P  F  E  L  G  V  F  S  L  R  F  G  T     1713
5201  AACTTGCCAAAAATGGATTCTCTTACGCAAGAGCTCTGTCATGAGCTCCAAGAATTACACCCTTTTGAGCTTGGGGTGTTTTCCTTGCGCTTCGGAACAG

      A  Q  M  A  E  E  V  T  N  R  Y  F  A  G  G  F  K  E  G  T  L  I  P  E  Q  D  Q  E  E  L  A  Q  A  I   1747
5301  CTCAGATGGCCGAGGAAGTCACAAATCGGTACTTCGCTGGTGGATTCAAAGAGGGGACACTCATACCTGAGCAAGATCAGGAGGAACTTGCTCAGGCGAT

        F  E  N  E  S  H  L  F  S  D  T  Q  L  I  S  P  E  E  V  W  K  K  W  H  R  N  Y  S  A  G  F  P  F    1780
5401  ATTTGAAAATGAGTCTCACCTTTTCTCTGACACCCAACTCATATCTCCGGAAGAGGTATGGAAGAAGTGGCATAGAAACTATTCTGCTGGTTTCCCGTTC

       R  F  T  D  R  G  N  S  S  R  Q  K  L  I  D  A  V  G  G  K  E  R  F  L  Q  C  V  R  D  Y  I  E  S     1813
5501  CGTTTCACTGACCGTGGAAATTCAAGTCGTCAAAAGTTGATTGATGCTGTTGGCGGGAAGGAAAGGTTTCTTCAGTGTGTTAGAGATTATATAGAGTCTC

      P  E  A  F  P  T  V  S  H  A  F  I  K  D  E  V  L  P  K  S  Y  V  E  R  E  K  I  R  T  I  I  A  Q  D   1847
5601  CTGAAGCATTTCCGACCGTCAGCCATGCGTTTATTAAGGATGAGGTTTTACCAAAATCTTATGTTGAACGTGAGAAAATTCGCACAATCATTGCACAAGA

        P  L  N  Y  Y  L  S  M  A  V  Q  G  D  A  A  K  R  L  D  P  S  S  F  S  A  V  G  V  S  R  S  H  G    1880
5701  TCCTTTGAACTACTATCTATCAATGGCAGTTCAGGGAGACGCTGCAAAAAGACTTGACCCATCTTCATTCTCGGCTGTTGGTGTATCTCGATCTCATGGA

       E  M  S  A  L  A  E  K  H  L  A  Y  K  H  H  T  A  M  D  V  T  A  M  D  S  T  A  S  I  D  A  V  G     1913
5801  GAGATGTCCGCTTTGGCTGAAAAACATCTTGCTTACAAGCATCATACCGCAATGGATGTGACAGCCATGGATTCCACTGCATCCATTGACGCAGTGGGGG

      V  I  K  K  L  R  K  K  G  F  Q  K  H  S  Q  R  D  A  I  E  S  A  I  D  A  T  Y  D  N  L  V  A  S  W   1947
5901  TAATCAAGAAGCTACGGAAAAAAGGTTTCCAAAAACACTCTCAACGTGATGCTATCGAGAGTGCTATTGATGCAACCTATGACAATCTCGTTGCTTCTTG

        I  I  D  I  H  S  G  R  A  R  F  K  R  Q  G  L  S  T  G  H  A  T  T  T  P  S  N  T  E  Y  M  R  V    1980
6001  GATTATTGACATACACTCTGGGAGGGCGCGTTTCAAACGCCAAGGTCTTTCTACCGGTCATGCTACAACGACACCATCGAACACTGAGTATATGCGTGTG

       L  M  L  Y  S  W  K  Q  I  T  G  R  P  Y  S  E  F  Y  D  C  V  K  F  S  S  F  S  D  D  N  F  W  S     2013
6101  TTGATGTTGTATTCGTGGAAGCAGATAACCGGAAGACCCTACTCTGAATTCTATGATTGTGTAAAATTCAGTTCCTTCTCAGATGACAATTTTTGGTCTA

      T  N  L  D  E  N  V  F  S  G  R  L  V  S  D  F  W  L  S  R  G  V  Q  V  R  V  E  G  V  S  D  S  L  S   2047
6201  CTAATCTCGATGAAAATGTGTTTTCAGGTAGATTGGTATCTGACTTCTGGTTGAGTCGTGGTGTGCAAGTTCGTGTCGAAGGTGTTTCAGACAGTCTTTC

        D  L  S  F  L  A  K  K  F  S  F  E  Q  K  H  L  D  E  V  A  S  L  T  G  A  H  P  K  V  A  I  V  H    2080
6301  TGACTTATCTTTTTTGGCGAAGAAGTTTTCTTTTGAACAAAAGCACTTAGATGAGGTTGCCTCTTTGACAGGTGCACACCCTAAGGTTGCTATTGTCCAT

       D  I  N  R  L  L  T  K  F  S  D  Y  K  K  K  N  T  L  R  Y  R  W  E  K  F  T  A  L  Q  L  N  C  A     2113
6401  GACATCAATCGATTACTCACAAAATTCTCTGACTATAAGAAGAAGAATACACTTCGGTATAGGTGGGAGAAGTTCACTGCTCTTCAGCTGAACTGTGCCC

      H  Y  P  E  V  H  E  K  V  G  E  Y  L  D  V  M  E  K  L  L  L  K  R  K  S  G  R  L  F  M  K  Q  H  P   2147
6501  ACTACCCAGAAGTGCATGAAAAAGTGGGTGAGTATCTTGATGTTATGGAAAAACTTCTGTTGAAGAGGAAATCGGGAAGGTTGTTTATGAAACAACACCC

        R  T  S  Y  D  D  V  L  R  L  M  Y  L  P  T  D  K  T  R  Q  S  L  L  V  S  T  H  E  P  D  L  M  E    2180
6601  TAGGACAAGTTATGATGACGTCTTGAGGTTGATGTACCTGCCAACTGATAAGACTCGCCAGAGTCTATTAGTTTCAACACATGAACCTGATCTCATGGAG

       K  V  H  D  W  W  N  T  L  Q  V  D  I  M  T  F  D  S  T  A  N  T  Y  G  R  I  L  S  Q  F  A  G  L     2213
6701  AAGGTTCATGATTGGTGGAACACTCTTCAGGTCGACATTATGACATTCGACTCAACGGCGAACACATATGGTCGTATTCTTTCACAATTCGCAGGCCTGT
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Figure 3.2 (cont.)

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        L  E  I  G  G  L  N  V  E  D  P  G  L  F  L  K  G  P  G  E  Y  P  H  D  P  E  F  T  L  E  H  H  I  Y  2247
6801  TGGAGATAGGTGGTCTAAATGTTGAGGACCCAGGGTTGTTCTTGAAAGGCCCTGGGGAATATCCTCATGACCCAGAGTTCACTCTAGAGCATCATATCTA

         L  L  N  G  C  P  E  T  Y  E  K  M  E  I  L  A  Q  K  T  P  F  S  T  F  M  D  I  P  G  F  W  A  R   2280
6901  CCTGTTGAATGGATGTCCAGAGACCTATGAAAAAATGGAGATTCTTGCTCAGAAGACTCCATTTTCAACATTCATGGACATTCCTGGTTTCTGGGCCAGG

        R  E  Y  Y  D  I  S  E  S  M  A  N  A  L  R  V  K  V  S  L  L  L  G  I  Y  T  L  V  A  W  L  E  Q    2313
7001  CGGGAATACTACGATATCAGTGAGAGCATGGCTAATGCCCTCCGTGTCAAAGTCAGTTTGCTTCTTGGTATATACACTTTGGTTGCTTGGTTGGAACAGG

        G  L  M  T  V  P  I  I  G  P  A  Y  R  L  L  A  T  A  K  Y  V  S  E  K  A  Y  S  R  L  N  S  L  Y  Y  2347
7101  GCTTAATGACTGTTCCAATAATTGGACCCGCTTACAGGTTGCTGGCGACAGCAAAGTATGTTAGCGAGAAAGCTTATTCGAGGCTCAATTCACTATACTA

         A  V  F  G  D  S  S  A  I  I  S  A  M  M  P  K  D  R  Y  L  M  L  K  V  I  A  Y  R  V  W  M  V  T   2380
7201  TGCAGTTTTTGGTGACTCATCTGCGATCATATCAGCGATGATGCCAAAGGATCGTTACCTTATGCTTAAGGTGATTGCTTATCGTGTGTGGATGGTGACA

        T  P  I  D  C  F  A  F  D  G  G  I  E  R  A  Q  E  W  V  D  A  C  L  K  F  G  Q  D  I  H  Q  I  A    2413
7301  ACTCCGATCGACTGCTTTGCTTTCGATGGTGGTATCGAGAGAGCTCAAGAATGGGTGGACGCGTGTCTTAAGTTTGGGCAAGACATACATCAAATTGCTC

        L  D  F  D  I  S  S  I  L  P  T  P  G  T  G  E  R  E  K  Q  G  T  P  T  G  W  T  G  I  D  H  A  D  S  2447
7401  TAGACTTTGACATAAGTTCTATATTGCCTACACCTGGCACAGGCGAGCGTGAAAAACAGGGTACACCAACGGGATGGACTGGAATTGATCATGCAGATTC

         V  C  S  V  Q  E  A  L  L  E  E  K  T  P  M  V  T  G  V  P  G  A  G  K  S  T  D  F  V  I  S  L  K   2480
7501  TGTGTGTAGTGTTCAAGAGGCTCTGCTTGAGGAAAAGACACCCATGGTCACAGGTGTCCCTGGTGCTGGGAAATCTACGGATTTTGTCATCAGCCTGAAG

        Q  K  Y  E  T  V  I  V  A  C  P  R  Q  I  L  V  K  N  N  P  V  A  Q  T  K  L  Y  S  G  C  E  D  N    2513
7601  CAGAAATATGAAACGGTGATCGTTGCCTGCCCCCGTCAAATTCTAGTTAAAAACAACCCTGTAGCGCAGACAAAACTATATTCGGGTTGTGAGGACAATT

        L  I  R  G  Y  I  N  F  G  T  A  G  Y  L  R  R  T  L  A  D  L  P  E  S  T  I  L  C  L  D  E  F  H  E  2547
7701  TGATAAGGGGCTACATCAACTTTGGTACTGCTGGCTACTTGCGTAGAACACTTGCAGATTTACCTGAAAGCACAATACTATGTCTGGATGAATTCCATGA

         M  D  E  D  S  L  W  L  L  D  R  Y  R  G  Q  C  V  V  I  T  A  T  P  D  F  Y  G  S  Q  R  F  S  E   2580
7801  GATGGATGAGGACAGTTTATGGCTGTTAGACAGATATAGAGGACAATGTGTTGTTATCACAGCAACACCTGACTTTTATGGTAGCCAAAGGTTCTCTGAG

        V  R  L  S  K  G  R  N  S  A  W  T  I  Q  D  D  F  R  D  T  P  G  K  L  E  D  G  W  N  C  L  M  E    2613
7901  GTACGTCTTTCAAAAGGTAGGAATTCTGCCTGGACAATACAAGACGACTTCAGAGACACTCCCGGAAAGCTAGAAGATGGATGGAATTGCCTAATGGAAA

        S  A  K  T  N  D  R  V  L  M  I  V  P  S  I  Q  D  V  E  T  C  K  R  H  A  A  Q  L  V  T  N  K  R  V  2647
8001  GTGCAAAGACGAACGATCGTGTACTGATGATCGTCCCAAGTATTCAAGATGTGGAGACATGCAAGCGACATGCAGCACAACTTGTTACGAACAAACGAGT

         C  G  L  Y  R  G  Q  N  T  V  T  E  A  D  W  Y  F  A  T  S  I  V  D  A  G  L  T  I  P  G  L  T  K   2680
8101  CTGTGGATTGTACAGAGGGCAAAACACTGTTACAGAAGCTGATTGGTATTTCGCAACATCAATTGTTGACGCAGGTTTGACTATACCAGGGTTGACGAAA

        I  I  D  L  G  W  S  L  G  Y  K  H  G  K  F  I  K  R  P  S  S  R  N  I  S  A  Q  R  R  G  R  T  G    2713
8201  ATCATAGATCTTGGTTGGTCGTTGGGTTACAAGCATGCCAAGTTCATCAAGCGTCCTTCTTCTCGCAACATATCTGCTCAGAGAAGGGGAAGAACGGGAC

        R  T  C  A  G  Q  Y  I  R  L  I  K  D  Y  D  D  S  N  W  D  F  S  T  Q  F  Q  C  F  S  W  S  T  A  K  2747
8301  GGACTTGTGCAGGCCAGTACATACGACTAATCAAAGATTACGATGATTCCAACTGGGACTTTTCGACACAGTTCCAATGCTTCAGTTGGTCGACTGCTAA

         K  W  D  P  N  F  R  R  G  K  C  R  T  P  G  M  I  E  A  L  P  G  G  Y  E  S  V  F  G  E  G  D  W   2780
8401  AAAGTGGGATCCAAACTTCAGACGTGGTAAGTGTCGCACACCAGGTATGATAGAAGCTCTACCTGGGGGTTACGAATCTGTCTTCGGTGAAGGCGATTGG

        S  M  V  L  Y  A  V  F  M  Y  D  A  R  L  D  V  N  R  A  R  A  S  Y  Q  A  M  R  K  F  P  E  R  K    2813
8501  TCAATGGTGCTTTATGCAGTTTTTATGTATGATGCACGCTTAGATGTGAACCGTGCGCGAGCCAGTTACCAGGCTATGCGTAAATTCCCTGAACGTAAAG

        E  F  S  H  L  T  G  R  I  E  N  F  H  F  D  D  L  F  M  V  E  D  K  L  K  R  F  Q  L  P  G  Q  N  G  2847
8601  AATTCAGTCATCTTACTGGGAGGATCGAAAACTTCCACTTTGATGACCTGTTCATGGTTGAAGACAAACTTAAACGGTTCCAACTTCCTGGGCAGAACGG

         N  F  W  S  W  D  L  H  S  C  V  Q  V  D  F  E  Q  K  C  P  S  H  L  L  D  V  D  *                     2874
8701  GAACTTCTGGTCATGGGATTTGCATTCCTGTGTCCAGGTGGACTTCGAACAAAAATGCCCATCTCATTTACTAGACGTTGACTAGTACAATAACAACGGT

8801  ACATTTCTTTTGTCATTTCTTTTGTCAGTGTATCCACAACCTGTGCGAGCCCGCGAAGGCCTAAAACTAGCAATTGGGGAGAACTTTGCCGTAATCAATG

8901  GCCTCTGAGGCTGAAACTGGGGTAAACATTTTGGGACAACCTCCTATGGCATTTAGAGGTTTTTGCCCAATTTCCCAGAAGTAGGAAGGCATGATGGTGC

9001  CCTCACACCTTGTGGGTTACGCCAAAGTTGGACAAGGACTGCGGGTCGCTTGAAGTCGAATCTCCCATCACCAGTATCTGGAGTAGACCAGACTGGGACC

9101  TTTTTAGGTTGCTAAAGGTCCTACGGGGCTTAGTAAACCGTCGTTCTCACAATCTAGGAGTGGAAAAGCAGACCTCAGTCTCGCGCTGAGATGGCGCTTA

9201  GTACGCCTAGTGTATTGGGTGGTTCCCAAGCACACGACGAAAATAAACCAAACAGGTTGTTTTTTTCCTTTATATTTAAAGCGTTCCTTAGTTCTCTGG

9301  AAAATGAGACCAGAGCTGGACGATATTTAAAATTCAAAAATATCTTTCTTCCCGTTAAAAGAAGATAAAGATCAACTTGGATTAACCTTTGGCCAAAGGG

9401  TAATTGTTCAATGGAGATTGAGACCATTGCTACCCAACTGGAAAAGGTGCTAGGAAGCTTTGTTCCGTGTGACGCACTGCAGGTACGTTCATGTTCAAAT

9501  TAACCAGATAGTAATAACGGGCTGGTAACAAAGTGGAGCAATCCTTTTGCGTTTTAGCGGCGGCACCGTATATACGGGCGTCGCTTTCGCAAAAAAAAAA

9601  AAAAAAAA
```

Figure 3.2 (cont.)

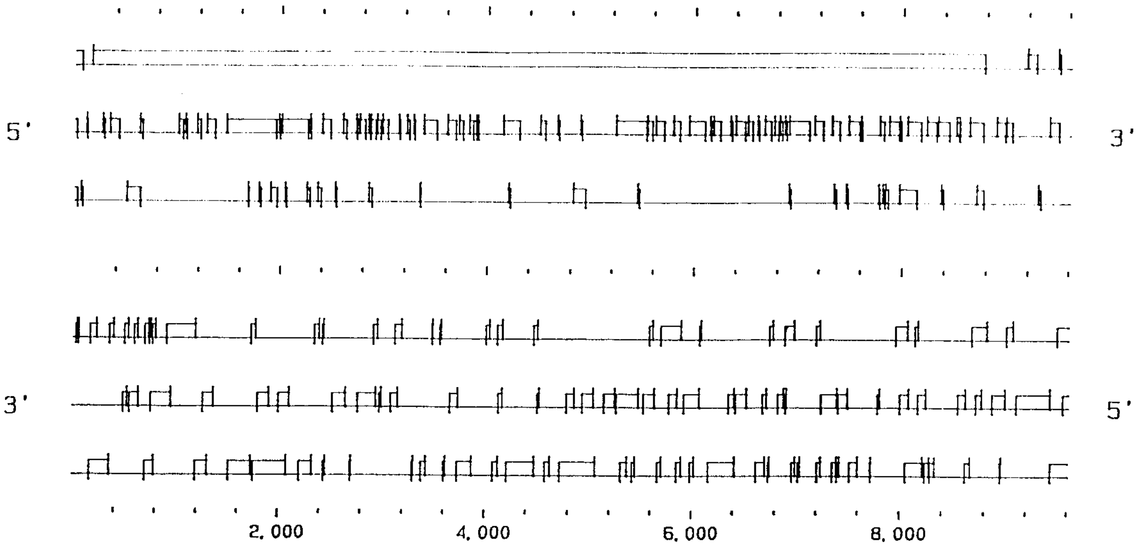
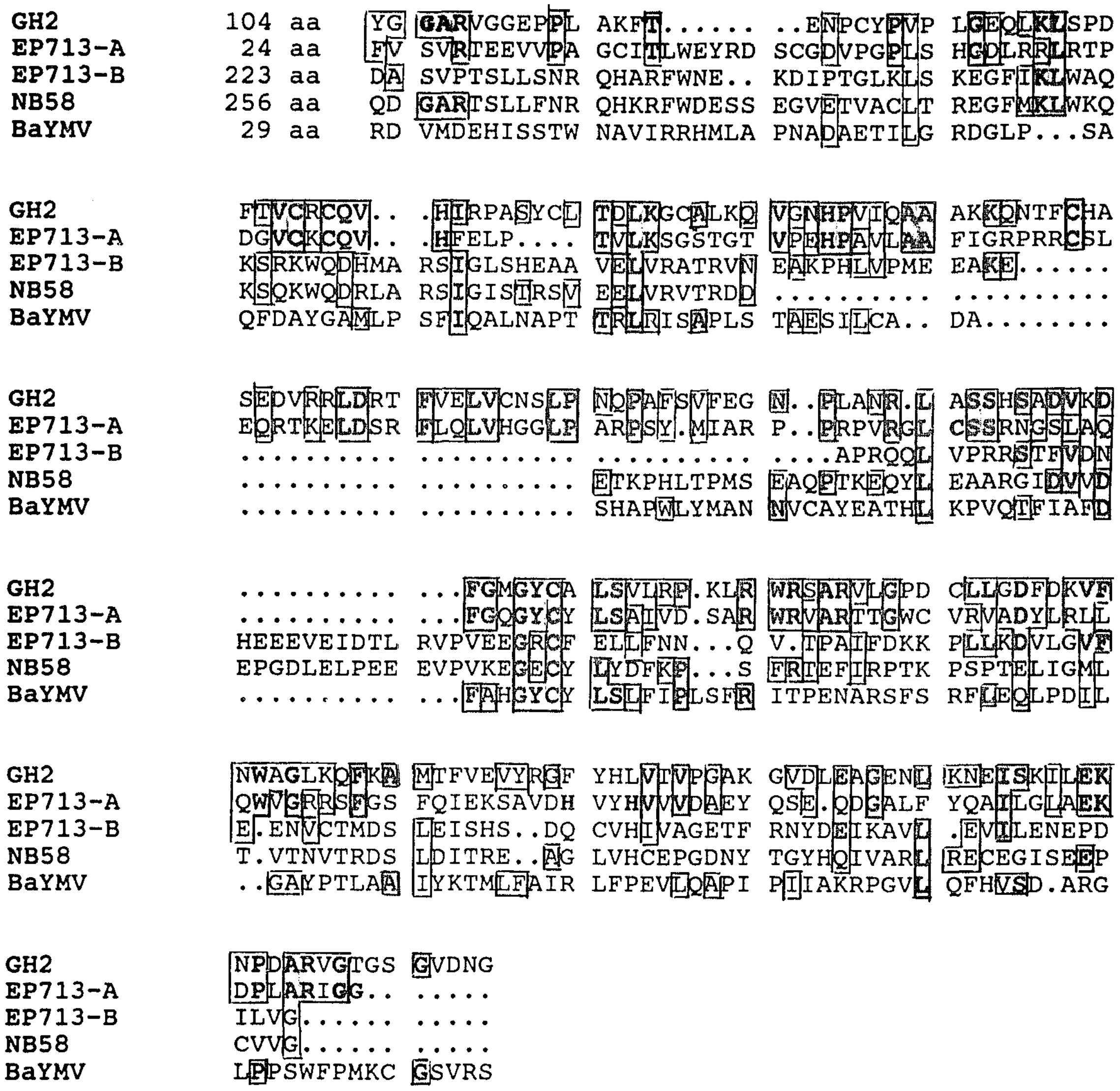


Figure 3.3. Open reading frames in the six reading frames of the cDNA sequence. Rectangles represent ORF's. Hatch marks above the rectangle represent the start of an ORF, hatch marks below the rectangle represent the end of an ORF. The DNA sequence is numbered in base pairs.

Rutgers University, personal communication). The GH2 nucleotide sequence is 38% identical to both those from EP713 and NB58; the deduced amino acid sequence of the GH2 ORF is 19% identical to that of EP713 ORF B and 18% identical to that from NB58 ORF B. Putative protease, RNA-dependent RNA polymerase, and RNA helicase domains have been identified in dsRNA molecules isolated from *C. parasitica* strain EP713 (Koonin *et al.*, 1991). These domains appear to be conserved in GH2 dsRNA, as well. The deduced amino acid sequence of dsRNA from strains EP713, NB58 and GH2 were aligned, and some similarity was identified within these putative domains (Figures 3.4-3.6). Within the protease domain, alignments were made between the GH2 ORF and putative proteases identified in ORF A and ORF B of EP713, ORF B of NB58, and RNA 1 of BaYMV, a ssRNA plant virus. A putative protease domain was identified in the GH2 ORF and appeared to be most similar to the domain identified in ORF A of strain EP713 (Figure 3.4).

A putative domain encoding an RNA-dependent RNA polymerase, which would be involved in viral replication, has also been identified in the GH2 ORF. This domain is interesting, because the overall structure is more similar to BaYMV than to either dsRNA from *C. parasitica.* There is a 153 amino acid insertion in the center of the domain in both EP713 and NB58, however in BaYMV and GH2, this region does not exist (Figure 3.5).

The final putative domain, an RNA helicase, which would also be involved in viral replication, can be identified in all four deduced amino acid sequences (Figure 3.6). This domain is located in what would be the carboxy-



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GH2       104 aa  YG GARVGGEPPL AKFT...... ..ENPCYPVP LGEQLKLSPD
EP713-A    24 aa  FV SVRTEEVVPA GCITLWEYRD SCGDVPGPLS HGDLRRLRTP
EP713-B   223 aa  DA SVPTSLLSNR QHARFWNE.. KDIPTGLKLS KEGFIKLWAQ
NB58      256 aa  QD GARTSLLFNR QHKRFWDESS EGVETVACLT REGFMKLWKQ
BaYMV      29 aa  RD VMDEHISSTW NAVIRRHMLA PNADAETILG RDGLP...SA

GH2               FTVCRCQV.. .HIRPASYCL TDLKGCALKQ VGNHPVIQAA AKKQNTFCHA
EP713-A           DGVCKCQV.. .HFELP.... TVLKSGSTGT VPEHPAVLAA FIGRPRRCSL
EP713-B           KSRKWQDHMA RSIGLSHEAA VELVRATRVN EAKPHLVPME EAKE......
NB58              KSQKWQDRLA RSIGISTRSV EELVRVTRDD .......... ..........
BaYMV             QFDAYGAMLP SFIQALNAPT TRLRISAPLS TAESILCA.. DA........

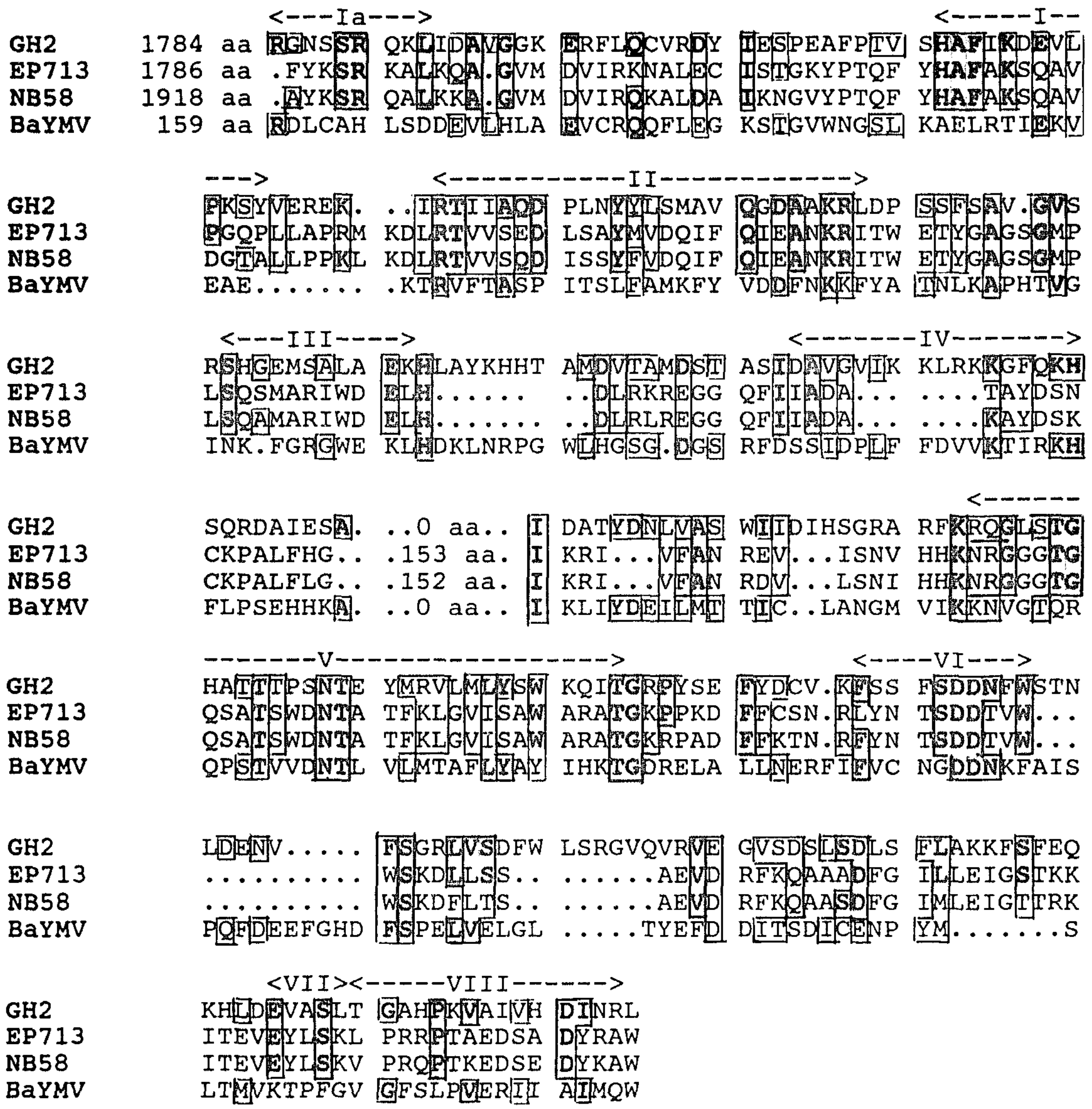
GH2               SEDVRRLDRT FVELVCNSLP NQPAFSVFEG N..PLANR.L ASSHSADVKD
EP713-A           EQRTKELDSR FLQLVHGGLP ARPSY.MIAR P..PRPVRGL CSSRNGSLAQ
EP713-B           .......... .......... .......... ....APRQQL VPRRSTFVDN
NB58              .......... .......... ETKPHLTPMS EAQPTKEQYL EAARGIDVVD
BaYMV             .......... .......... SHAPWLYMAN NVCAYEATHL KPVQTFIAFD

GH2               .......... ...FGMGYCA LSVLRP.KLR WRSARVLGPD CLLGDFDKVF
EP713-A           .......... ...FGQGYCY LSAIVD.SAR WRVARTTGWC VRVADYLRLL
EP713-B           HEEEVEIDTL RVPVEEGRCF ELLFNN...Q V.TPAIFDKK PLLKDVLGVF
NB58              EPGDLELPEE EVPVKEGECY LYDFKP...S FRTEFIRPTK PSPTELIGML
BaYMV             .......... ...FAHGYCY LSLFIPLSFR ITPENARSFS RFLEQLPDIL

GH2               NWAGLKQFKA MTFVEVYRGF YHLVIVPGAK GVDLEAGENL KNEISKILEK
EP713-A           QWVGRRSFGS FQIEKSAVDH VYHVVVDAEY QSE.QDGALF YQAILGLAEK
EP713-B           E.ENVCTMDS LEISHS..DQ CVHIVAGETF RNYDEIKAVL .EVILENEPD
NB58              T.VTNVTRDS LDITRE..AG LVHCEPGDNY TGYHQIVARL RECEGISEEP
BaYMV             ..GAYPTLAA IYKTMLFAIR LFPEVLQAPI PIIAKRPGVL QFHVSD.ARG

GH2               NPDARVGTGS GVDNG
EP713-A           DPLARIGG.. .....
EP713-B           ILVG...... .....
NB58              CVVG...... .....
BaYMV             LPPSWFPMKC GSVRS
```

Figure 3.4. Alignment of putative protease domains. The distance between the N terminus of each polyprotein and the beginning of this domain is indicated. Amino acid residues identical to those in GH2 ORF are boxed and shaded, while similar residues are boxed. Residues in bold type were determined to be necessary for autocatalytic processing in EP713-A (Choi *et al.*, 1991b). Similar amino acid residues were determined as follows: G and A; S and T; D, E, N, and Q; K and R; L, I, V, and M; F, Y, and W.



```
                <---Ia--->                                      <-----I--
GH2     1784 aa RGNSSR QKLIDAVGGK ERFLQCVRDY IESPEAFPTV SHAFIKDEVL
EP713   1786 aa .FYKSR KALKQA.GVM DVIRKNALEC ISTGKYPTQF YHAFAKSQAV
NB58    1918 aa .AYKSR QALKKA.GVM DVIRQKALDA IKNGVYPTQF YHAFAKSQAV
BaYMV    159 aa RDLCAH LSDDEVLHLA EVCRQQFLEG KSTGVWNGSL KAELRTIEKV

              --->        <-----------II------------>
GH2           PKSYVEREK. ..IRTIIAQD PLNYYLSMAV QGDAAKRLDP SSFSAV.GVS
EP713         PGQPLLAPRM KDLRTVVSED LSAYMVDQIF QIEANKRITW ETYGAGSGMP
NB58          DGTALLPPKL KDLRTVVSQD ISSYFVDQIF QIEANKRITW ETYGAGSGMP
BaYMV         EAE....... .KTRVFTASP ITSLFAMKFY VDDFNKKFYA TNLKAPHTVG

               <---III---->                        <-------IV------->
GH2           RSHGEMSALA EKHLAYKHHT AMDVTAMDST ASIDAVGVIK KLRKKGFQKH
EP713         LSQSMARIWD ELH....... ..DLRKREGG QFIIADA... ....TAYDSN
NB58          LSQAMARIWD ELH....... ..DLRLREGG QFIIADA... ....KAYDSK
BaYMV         INK.FGRGWE KLHDKLNRPG WLHGSG.DGS RFDSSIDPLF FDVVKTIRKH

                                                           <------
GH2           SQRDAIESA. ..0 aa.. I DATYDNLVAS WIIDIHSGRA RFKRQGLSTG
EP713         CKPALFHG.. .153 aa. I KRI...VFAN REV...ISNV HHKNRGGGTG
NB58          CKPALFLG.. .152 aa. I KRI...VFAN RDV...LSNI HHKNRGGGTG
BaYMV         FLPSEHHKA. ..0 aa.. I KLIYDEILMT TIC..LANGM VIKKNVGTQR

              -------V----------------->            <----VI--->
GH2           HATTTPSNTE YMRVLMLYSW KQITGRPYSE FYDCV.KFSS FSDDNFWSTN
EP713         QSATSWDNTA TFKLGVISAW ARATGKPPKD FFCSN.RLYN TSDDTVW...
NB58          QSATSWDNTA TFKLGVISAW ARATGKRPAD FFKTN.RFYN TSDDTVW...
BaYMV         QPSTVVDNTL VLMTAFLYAY IHKTGDRELA LLNERFIFVC NGDDNKFAIS

GH2           LDENV..... FSGRLVSDFW LSRGVQVRVE GVSDSLSDLS FLAKKFSFEQ
EP713         .......... WSKDLLSS.. ......AEVD RFKQAAADFG ILLEIGSTKK
NB58          .......... WSKDFLTS.. ......AEVD RFKQAASDFG IMLEIGTTRK
BaYMV         PQFDEEFGHD FSPELVELGL .....TYEFD DITSDICENP YM.......S

                <VII><-----VIII------>
GH2           KHLDEVASLT GAHPKVAIVH DINRL
EP713         ITEVEYLSKL PRRPTAEDSA DYRAW
NB58          ITEVEYLSKV PRQPTKEDSE DYKAW
BaYMV         LTMVKTPFGV GFSLPVERII AIMQW
```

Figure 3.5. Alignment of putative RNA-dependent RNA polymerase domains. The distance between the N terminus of each polyprotein and the beginning of this domain is indicated. Amino acid residues identical to those in GH2 ORF are boxed and shaded, while similar residues are boxed. Similar amino acid residues are as described in Figure 3.4. Conserved motifs found in all positive-strand RNA viral RNA-dependent RNA polymerases (Koonin *et al.*, 1991) are indicated by arrows above the sequence.

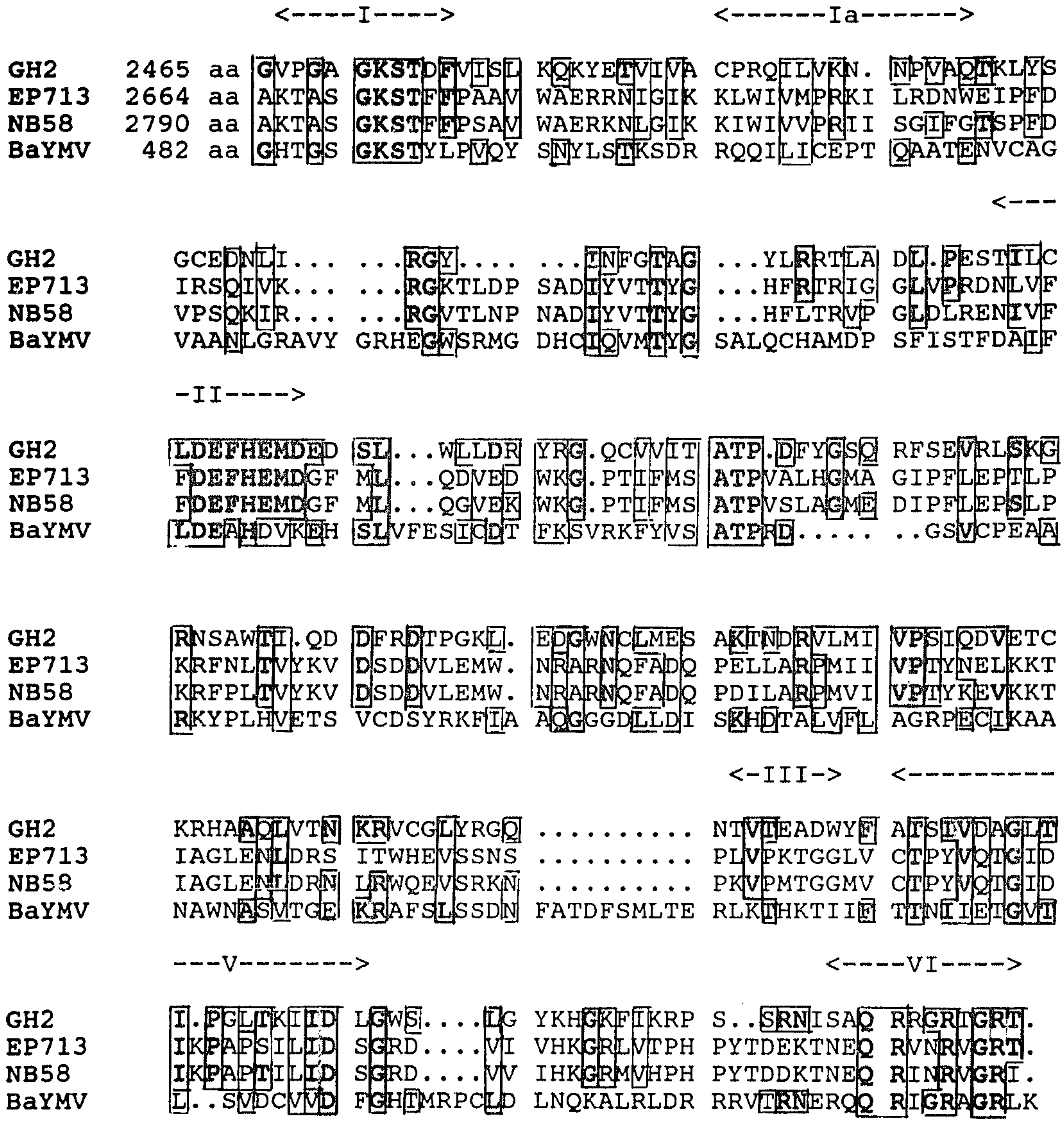


Figure 3.6. Alignment of putative RNA helicase domains. The distance between the N terminus of each polyprotein and the beginning of this domain is indicated. Amino acid residues identical to those in GH2 ORF are boxed and shaded, while similar residues are boxed. Similar amino acid residues are as described in Figure 3.4. Conserved motifs found in the helicase domain of many positive-strand RNA viruses (Hodgman, 1988) are indicated by arrows above the sequence.

terminal portion of the GH2 ORF and in ORF B of EP713 and of NB58 (Figure 3.7).

Discussion

The dsRNA from the Michigan hypovirulent *C. parasitica* isolate GH2 is intriguing, because it is presumably responsible for the reduction of fungal virulence and alteration of colony morphology. However, it does not alter fungal pigmentation or sporulation as is seen in many European hypovirulent isolates (Fulbright *et al.*, 1988). Also, dsRNA from isolate GH2 does not cross-hybridize with dsRNA from any other hypovirulent strain of *C. parasitica* outside of Michigan (Paul and Fulbright, 1988) yet it is homologous with 80% of the dsRNA molecules found in Michigan hypovirulent strains (Fulbright, 1990). I began a molecular analysis of the dsRNA associated with hypovirulence in isolate GH2, in order to identify the similarities, as well as the differences, between the dsRNA genomes of GH2 and other dsRNAs from *C. parasitica.*

The overall genomic organization of the largest segment of dsRNA from isolate GH2 is shown in Figures 3.1 and 3.3. I have identified a single, large ORF, 2,875 codons in length which may encode a polyprotein similar to that encoded by ORF B of EP713 dsRNA (Shapira *et al.*, 1991a). In the dsRNA characterized from EP713 and NB58, two ORFs were identified which are contiguous but distinguished by a reading frame shift. The characterization of

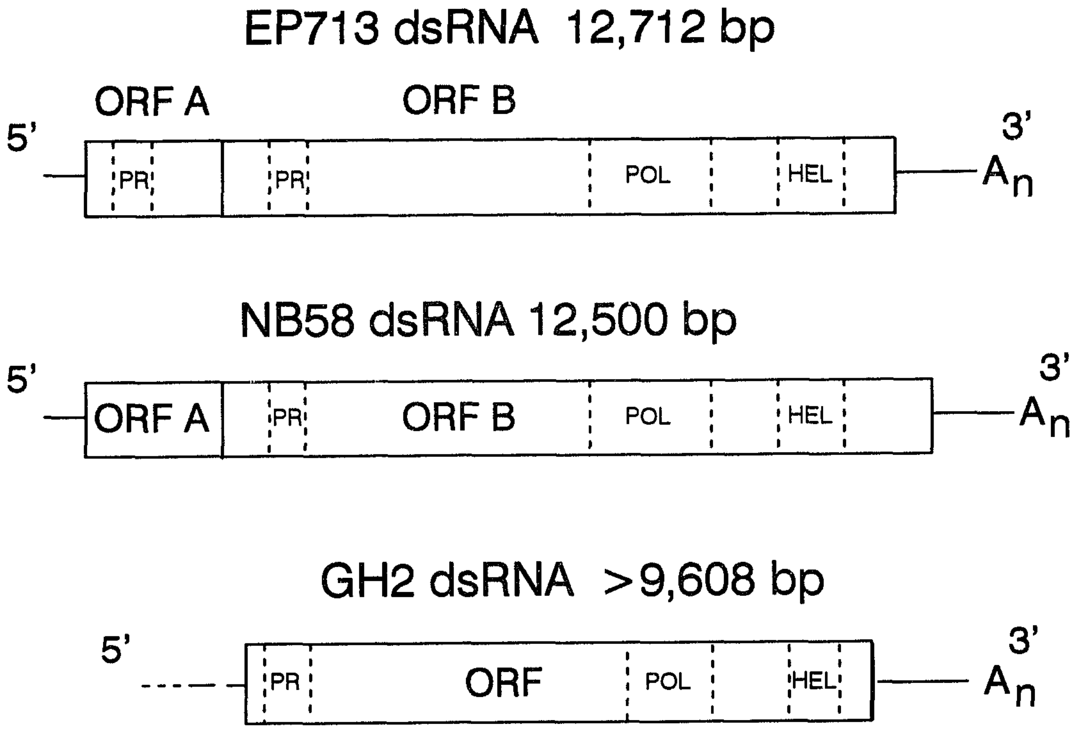


Figure 3.7. Genomic organization of protein coding regions of dsRNA from EP713, NB58 and GH2. Rectangles represent ORFs. Functional domains are delineated by dashed lines: PR, protease; POL, RNA-dependent RNA polymerase; HEL, RNA helicase.

cDNA clones of dsRNA from GH2 indicates that this dsRNA only encodes a single ORF and lacks the second ORF (ORF A) found in strain EP713. ORF A in EP713 dsRNA has been shown to be responsible for the phenotypic traits associated with European hypovirulent strains, such as reduction of pigmentation and sporulation, as well as the down-regulation of the fungal enzyme laccase (Choi and Nuss, 1992a). The fact that ORF A or an "ORF A-like" region is lacking on the GH2 dsRNA may be reflective of the differences in pigmentation, sporulation, and accumulation of laccase observed between Michigan and European hypovirulent strains (see Chapter IV). The deduced amino acid sequences of ORFs identified in dsRNA from *C. parasitica* strains GH2, EP713 and NB58 were aligned and compared. The amino acid sequence of the potyvirus-like BaYMV was included in the sequence comparisons since previous studies have shown a relationship between this ssRNA virus and the dsRNA in strain EP713 (Koonin *et al.*, 1991).

Two protease domains have been identified in the dsRNA of strain EP713. One domain was identified in ORF A by Choi *et al.* (1991a), while the second resides in the amino-terminal portion of ORF B (Shapira *et al.*, 1991a). The deduced amino acid sequence of the GH2 ORF appears to be most closely related to the domain identified in ORF A of strain EP713. However, the putative domain from GH2 differs from EP713 ORF A since it does not contain cysteine and histidine residues or the glycine dipeptide cleavage site, all of which are essential for autocatalytic processing (Choi *et al.*, 1991b). The presence of the amino acid sequence GYCALS within the GH2 ORF, which aligns with the GYCYLS motif of EP713 ORF A and BaYMV (Figure 3.4), suggests that this region of the polyprotein acts as a protease.

The overall level of similarity between the GH2 ORF and any other amino acid sequence of the putative RNA-dependent RNA polymerase domain was very low. The presence of the amino acid sequence SDD, which is also found in the deduced amino acid sequences of dsRNA from EP713 and NB58, is similar to the GDD sequence found in plant viral RNA-dependent RNA polymerases, suggesting that this region of the polyprotein acts as a polymerase. Interestingly, a large insertion (153 aa), which is present in the middle of the EP713 polymerase domain (Koonin *et al.*, 1991), is not present in the GH2 domain (Figure 3.5). This insertion is not present in the RNA-dependent RNA polymerase domains of BaYMV or several other ssRNA viruses within the potyvirus family (Koonin *et al.*, 1991).

The RNA helicase domain of the GH2 ORF deduced amino acid sequence represents the region that is most similar to deduced amino acid sequences of ORF B from either EP713 or NB58 (Figure 3.6). The sequence of seven exactly conserved residues within motif II are especially intriguing as they may represent an essential portion of the RNA helicase peptide.

The overall similarity of the genomic organization of the three dsRNA molecules that have been characterized at a molecular level, suggests that there is an evolutionary relationship between them (Figure 3.7). cDNA clones of the extreme 5' end of the GH2 dsRNA have not been obtained. This region may include sequences important for regulation of expression and/or replication of this virus-like molecule. The comparison between *C. parasitica* dsRNA sequences and the plant ssRNA virus BaYMV, which has a fungal vector, suggests that these dsRNA molecules may have originated from ssRNA viruses. The similarities which have been discovered between dsRNA from GH2, EP713 and NB58, provide insight into the mechanism of dsRNA replication and protein processing, but do not provide, at this time, further elucidation of the mechanism involved in the reduction of fungal virulence. Use of these cDNA clones in *in vitro* translation experiments and in the transformation of virulent *C. parasitica* strains may lead to a further understanding of the molecular mechanisms of dsRNA-associated hypovirulence.

Acknowledgements

The complete nucleotide sequence cDNA clones generated from the dsRNA of *C. parasitica* strain NB58 was the kind gift of Dr. Bradley Hillman, Rutgers University. cDNA clones representing the 3' end of GH2 dsRNA were generated by Donald Nuss, Roche Institute of Molecular biology, and Dennis Fulbright, Michigan State University. I gratefully acknowledge the technical assistance of Alvin Ravenscroft, Michigan State University, and Diane Pawlyk, Roche Institute of Molecular Biology.

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Chapter IV

Presence of laccase in hypovirulent strains of *Cryphonectria parasitica* recovered from Michigan

Introduction

Cryphonectria parasitica (Murr.) Barr, the causal organism of chestnut blight, was responsible for killing over three billion American chestnut (*Castanea dentata* [Marsh] Borkh.) trees in the Eastern hardwood forests of North America (Fulbright *et al.*, 1988). Chestnut trees are surviving in several localized areas in North America because of the presence of hypovirulent or less virulent forms of the fungus (MacDonald and Fulbright, 1991). Many strains of the fungus with the hypovirulent phenotype contain double-stranded RNA (dsRNA) molecules, which vary in size, homology and concentration among the different strains isolated (Paul and Fulbright, 1988). The molecular mechanism by which dsRNA causes the reduced virulence phenotype is unknown. However, the presence of dsRNA in *C. parasitica* isolates recovered from European chestnut trees (*C. sativa* Mill.) has been correlated with reduced accumulation of the enzyme laccase in these strains (Rigling *et al.*, 1989; Hillman *et al.*, 1990).

Laccase (benzenediol:oxygen oxidoreductase, EC 1.10.3.2) is a multicopper oxidase, which catalyzes the oxidation of organic substrates, such as mono-, di- and polyphenols, aminophenols, and diamines (Germann *et al.*, 1988). This enzyme is common throughout the plant and fungal kingdoms and has been purified from several sources, however, the exact biological function of laccase is still unknown. In *Aspergillus nidulans*, laccase is involved in pigmentation (Clutterbuck, 1972), while in *Botrytis cinerea* it has been shown to be involved in pathogenicity (Bar Nun *et al.*, 1988). It is also known that in many wood decaying fungi, including *Heterobasidium annoses*, laccase is involved in lignin degradation (Mayer, 1987). The genes encoding laccase have been cloned and sequenced from nuclear DNA of the fungi *A. nidulans* (Aramayo and Timberlake, 1990), *Coriolus hirsutus* (Kojima *et al.*, 1990), *Neurospora crassa* (Germann *et al.*, 1988) and, recently, *C. parasitica* (Choi *et al.*, 1992; Rigling and Van Alfen, 1991).

The dsRNA from the hypovirulent *C. parasitica* strain EP713, which is of European origin, contains two contiguous open reading frames (ORFs) (Shapira *et al.*, 1991). The first ORF, designated ORF A, encodes a polyprotein 622 amino acids in length, which is processed into two polypeptides. One of the polypeptides is an autocatalytic protease, but the function of the other is unknown (Choi *et al.*, 1991). The second ORF (ORF B), is 3165 amino acids in length and also encodes a polyprotein, which is processed into at least two polypeptides, one of which is an autocatalytic protease (Shapira *et al.*, 1991). Although several domains have been identified within ORF B, only the protease has been positively identified. The dsRNA from the European strain EP713 does not cross-hybridize to dsRNA from Michigan strains, so it is interesting to compare phenotypes caused by the presence of these dsRNA molecules.

The presence of hypovirulence-associated dsRNA molecules in the cytoplasm of *C. parasitica* causes not only a reduction in fungal virulence, but also alters other phenotypic traits. By comparing the associated phenotypes induced by different dsRNA genomes, we hope to gain a better understanding of dsRNA function. European hypovirulent strains, such as EP713, are white, sporulation is suppressed, and laccase activity is reduced (Anagnostakis, 1982), while Michigan hypovirulent strains exhibit normal orange pigmentation and continue to sporulate. ORF A, encoded by dsRNA isolated from strain EP713, was found to be responsible for the morphological phenotypic characteristics and the reduction in laccase accumulation (Choi and Nuss, 1991). When virulent *C. parasitica* protoplasts were transformed with a cDNA clone encoding ORF A, pigmentation, sporulation and laccase activity were reduced to levels similar to those of EP713 (Choi and Nuss, 1991). It has recently been determined that the EP713 dsRNA suppresses the accumulation of laccase by down-regulating laccase mRNA accumulation (Choi *et al.*, 1992; Rigling and Van Alfen, 1991).

Although a great deal is known about the regulation of laccase in European hypovirulent strains of *C. parasitica*, this enzyme has not been examined in Michigan hypovirulent strains containing dsRNA. In this study, both virulent and hypovirulent strains of *C. parasitica* isolated in Michigan were analyzed to determine if a relationship exists between the presence of dsRNA and a reduction in the fungal enzyme laccase. The correlation between hypovirulence and laccase activity was also examined in a hypovirulent *C. parasitica* strain (CL25), which does not contain dsRNA (Fulbright, 1985).

Materials and methods

Cultures and growth conditions Cultures used in this study are listed in Table 4.1. *C. parasitica* cultures were grown on potato dextrose agar (PDA, Difco, Detroit, MI) in Petri dishes at 25 °C with a 16 h photoperiod under cool white fluorescent lights (Garrod *et al.*, 1985). Cultures were stored on PDA slants at 4 °C.

Bavendamm assay for phenol oxidase activity To test for phenol oxidase activity, *C. parasitica* strains were grown on cellophane-covered Bavendamm medium containing 2% agar (Difco), 1.5% malt extract (Difco) and 0.5% tannic acid (Fluka, Switzerland) (Bavendamm, 1928). Petri dishes containing Bavendamm medium were inoculated with a portion of fungal mycelia 4 mm in diameter. Cultures were stored at 25 °C in the dark for 5 days. Presence of brown pigment in the medium indicated a positive reaction for laccase activity.

Table 4.1. Strains used in this study.

Strain	Virulence[a]	dsRNA[b]	Reference
CL1-16	V	-	Paul and Fulbright, 1988
GH2	H	+	Fulbright *et al.*, 1983 Paul and Fulbright, 1988
CL1-16 (GH2/RC1)	H	+	Durbahn Smart and Fulbright, unpublished (see Chap. II)
CL25	H	-	Fulbright, 1985
EP155	V	-	Anagnostakis, 1981
EP713	H	+	Anagnostakis, 1981

[a] V, virulent; H, hypovirulent

[b] +, contains dsRNA; -, no detectable dsRNA

Assay for laccase activity Fungal cultures were grown in 10 ml of Endothia minimal media (EMM) (Puhalla and Anagnostakis, 1979) in 25 ml Erlenmeyer flasks, three flasks per strain. Culture flasks were inoculated with a mycelial plug (5 mm in diameter) and shaken (225 RPM) at 25°C in the dark. The cultures were maintained in the dark to induce laccase expression (Hillman *et al.*, 1990). After 7 days of growth, mycelia were pelleted by centrifugation at 10,000 RPM in a Sorvall SS-34 rotor for 5 min. The supernatant fluid was used for protein determination and for laccase enzyme assays (Rigling *et al.*, 1989).

Protein assays were performed using the Bio Rad Bradford solution according to the manufacturer (Bio Rad). Laccase activity was measured with 2,6-dimethoxy-phenol (DMOP, Fluka, Switzerland) as a substrate (Bollag, 1979). Culture supernatant (0.2 ml) was added to 0.8 ml 2.5mM DMOP in 80 mM sodium tartrate buffer pH 3.0. Absorbance at 468 nm (OD_{468}) was measured in a disposable, 10 mm pathlength cuvette at 0 and 5 min using a Gilford Response spectrophotometer.

Native polyacrylamide gel electrophoresis Culture supernatant proteins (0.5 μg) were separated by electrophoresis using anionic polyacrylamide gels run at 50 V for ~15 h as described (Keleti and Lederer 1974). After electrophoresis, gels were incubated for at least 30 min in 80mM sodium tartrate buffer (pH 3.0) with 2.5mM 2,2'-azino-bis(3-ethylbenz-thiazoline-6-sulfonic acid) (ABTS), a substrate of laccase, until a green precipitate was formed as a product of laccase activity.

Isolation of dsRNA *C. parasitica* strains were grown for 7 days on cellophane-covered PDA medium. The fungus was removed from the cellophane, and 3 g (fresh weight) samples were used for dsRNA purification. dsRNA was isolated by CF-11 cellulose (Whatman) column chromatography as described by Morris and Dodds (1979). dsRNA was resolved using 5% PAGE and was visualized by ethidium bromide staining.

Results and discussion

To characterize the expression of the extracellular laccase in Michigan *C. parasitica* strains, laccase activity was assayed by growth on Bavendamm medium, by a spectrophotometric assay, and by native gel activity staining. When grown on Bavendamm medium, all strains tested except EP713 caused the media to turn brown due to the oxidation of tannic acid, indicating the presence of a phenol oxidase (Figure 4.1). These results indicate that Michigan hypovirulent strains do accumulate laccase enzyme, while EP713 has reduced laccase activity, as reported (Hillman *et al.*, 1990; Rigling *et al.*, 1989). To quantify the relative amount of laccase in each sample, the spectrophotometric assay was used. Each strain was grown in liquid Endothia minimal medium in the dark for seven days. Minimal medium, which contained no protein, was

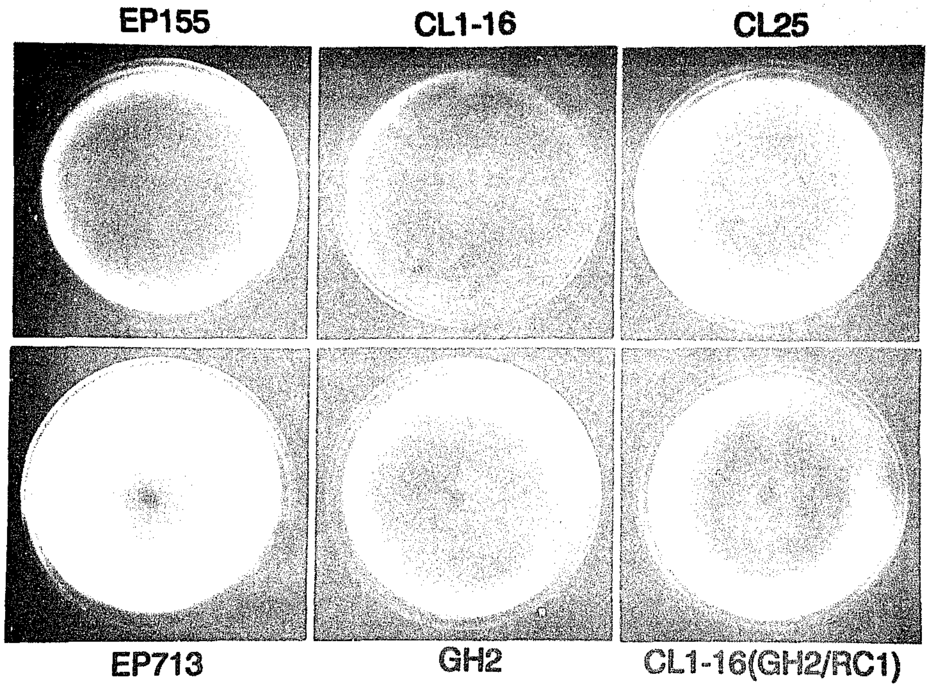

Figure 4.1. Virulent and hypovirulent isolates of *C. parasitica* grown on Bavendamm medium. Brown pigment in the medium indicates laccase activity.

used so that protein concentrations of the culture supernatant could be accurately determined. The absorbance change at 468 nm due to laccase activity using DMOP as a substrate was measured after 5 min with a spectrophotometer. The increase in OD_{468} appeared to be similar for culture supernatants from all isolates assayed except for strain EP713. It has been reported that laccase activity in EP713 is down-regulated (Hillman *et al.*, 1990; Rigling *et al.*, 1989), but in our assays specific activity in the culture supernatant from EP713 was the highest of the strains tested (Table 4.2). Repeated assays using a single culture supernatant were consistent, but activity varied considerably between different flasks of the same strains and between replicate experiments. Other researchers have also noted inconsistency in laccase expression in culture by northern analysis (Choi *et al.*, 1992). This variability was partially corrected by the addition of low levels of the protein synthesis inhibitor cycloheximide (Choi *et al.*, 1992). A recent report describing the regulation of laccase biosynthesis in *C. parasitica*, Rigling and Van Alfen (1991) presented spectrophotometric data on laccase activity from only one of three replicate flasks assayed rather than taking the average of all replicates in each experiment, as is presented here. Results of native polyacrylamide gel electrophoresis followed by staining with the substrate ABTS were similar to those of spectrophotometric assays. Culture supernatants containing 0.5 μg protein from each strain produced a diffuse band, staining with approximately equal intensity (Figure 4.2). In each gel stained, one or two samples had a band which was lighter in intensity than the others (Figure 4.2, lane 2), however

Table 4.2. Laccase activity assays. Activity was measured using three replicates per strain.

Strain	Activity[a]
EP713	0.427±0.079
CL1-16	0.342±0.233
CL1-16 (GH2/RC1)	0.148±0.047
GH2	0.134±0.074
CL25	0.240±0.029
EP155	0.250[b]

[a] ΔOD_{468} after 5 min / mg protein

[b] Only one measurement performed

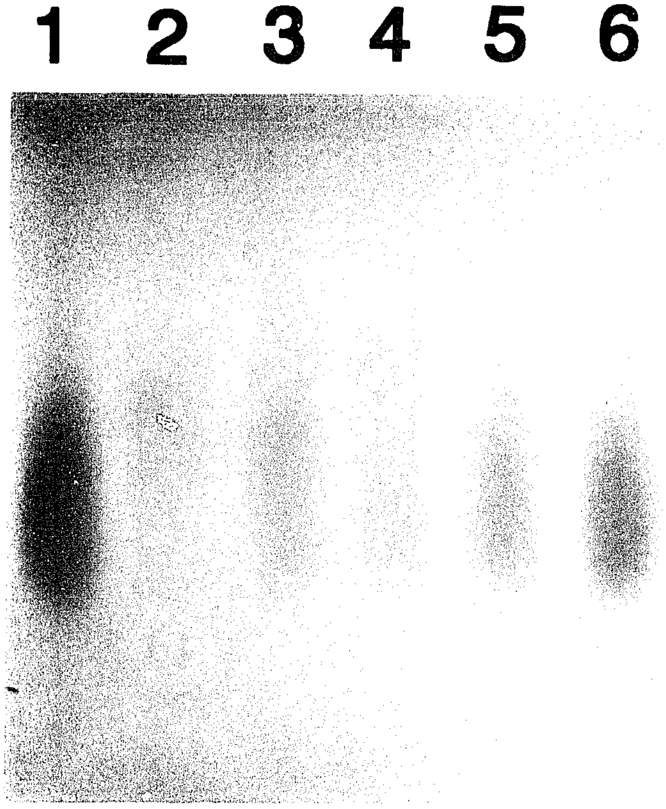

Figure 4.2. Laccase activity in a native protein polyacrylamide gel. Culture supernatant proteins (0.5 μg) were run in a 7.5% anionic gel system. Following electrophoresis, the gel was incubated in the presence of ABTS, a substrate of laccase. Coloration in the gel indicates laccase activity. Lanes: 1, EP713; 2, EP155; 3, CL25; 4, CL1-16; 5, CL1-16(GH2/RC1); 6, GH2.

the strain which produced a lighter band varied from gel to gel. As with the spectrophotometric assays, culture supernatant from strain EP713 appeared to have as much laccase activity as those from the other strains used.

All *C. parasitica* strains tested to date have some extracellular laccase activity (Rigling *et al.*, 1989; Choi *et al.*, 1992). Even strains in which laccase activity is known to be reduced, such as EP713, contained measurable activity (Rigling and Van Alfen, 1991). Perhaps the spectrophotometric assays and native gel staining used in this study were too sensitive for the amounts of laccase present in culture supernatant from each strain. Another possibility is that since neither of the substrates used was specific for laccase, the assays detected the activity of other phenoloxidases. For this reason, the Bavendamm assay is the best test for the presence of laccase in *C. parasitica*, as the results are consistent and indicate a gross, but measurable change in the ability to oxidize tannic acid.

The most significant observation of this study was that both virulent and hypovirulent Michigan *C. parasitica* strains produced laccase when grown on Bavendamm medium. The Michigan dsRNA-containing hypovirulent isolate GH2 had laccase activity as did strain CL1-16(GH2/RC1), in which the dsRNA from two hypovirulent strains was transferred into the nuclear background of the virulent CL1-16 (see Chapter II). Therefore, it appears that these dsRNA genomes do not have the ability, either individually or in combination, to down-regulate laccase. It is also interesting to note that CL25, a hypovirulent non-dsRNA containing Michigan isolate (Fulbright, 1985), also shows laccase activity. Thus, there is no association between hypovirulence and a reduction in laccase in Michigan *C. parasitica* isolates.

dsRNA molecules were detected in four of the seven *C. parasitica* strains used (Figure 4.3). There was no correlation between the presence of dsRNA molecules in a strain and a positive reaction on Bavendamm's agar. This is in contrast to the European strains tested in which the virulent, non-dsRNA-containing strain had high laccase activity, while activity in the hypovirulent, dsRNA-containing strain was reduced (Figure 4.2). This finding is significant, since this is the first report of a hypovirulent dsRNA-containing *C. parasitica* strain which does not down regulate the fungal enzyme laccase. Because the overall genomic organization of dsRNA isolated from *C. parasitica* strains EP713 (European origin) and GH2 (Michigan origin) is similar (see Chapter III), we find it most interesting that GH2 does not down regulate laccase. The ORF A region of the dsRNA from strain EP713 appears to be responsible for the reduction in laccase activity (Choi and Nuss, 1992). Correspondingly, sequences similar to ORF A that are necessary for the down-regulation of laccase in EP713 appear to be absent in strain GH2 (see Chapter III).

The ability of laccase to degrade lignin (Lewis and Yamamoto, 1990), which reportedly can act as a defense mechanism of the chestnut tree (Hebard *et al.*, 1984), and the close correlation between a reduction in laccase and hypovirulence in European *C. parasitica* strains, has lead to the suggestion that reduction of laccase accumulation may be fully or partially responsible for

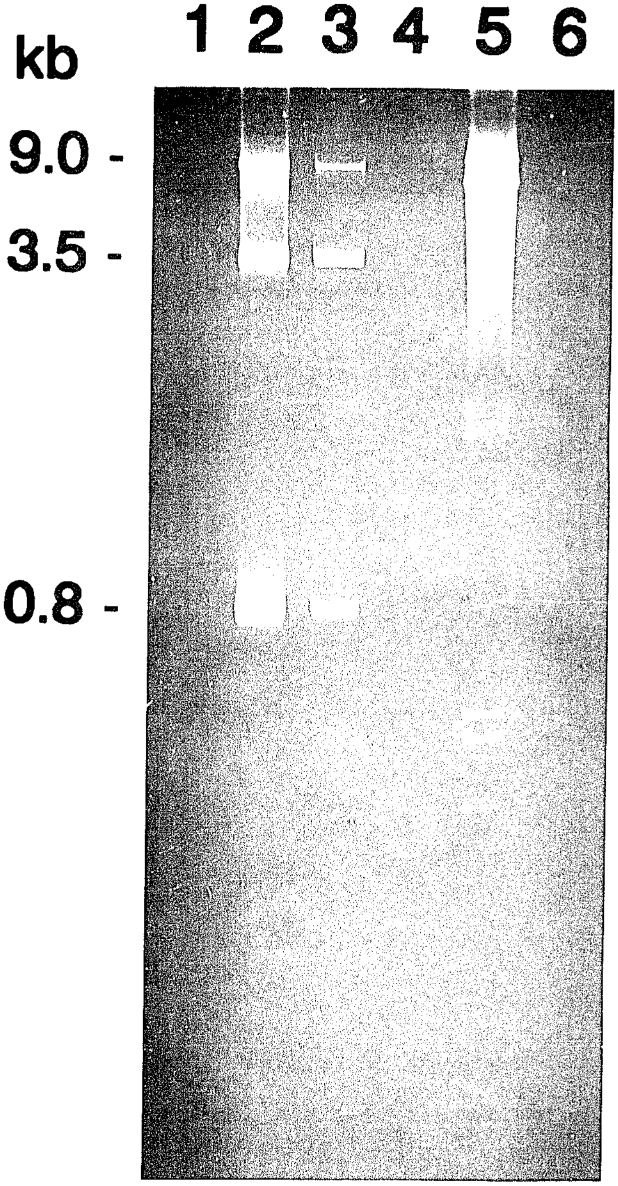

Figure 4.3. Banding patterns of dsRNA from Michigan and European *C. parasitica* isolates. The dsRNA was electrophoresed in a 5% polyacrylamide gel and stained with ethidium bromide. Strain CL25 is a Michigan hypovirulent strain, which does not contain dsRNA. Strains CL1-16(GH2/RC1) and GH2 are Michigan hypovirulent strains, which contain dsRNA. CL1-16 and EP155 are virulent, non-dsRNA-containing strains from Michigan and Connecticut, respectively. EP713 is a hypovirulent strain containing European dsRNA. Lanes: 1, CL25; 2, CL1-16(GH2/RC1); 3, GH2; 4, CL1-16; 5, EP713; 6, EP155. Approximate sizes of dsRNA molecules present in strain GH2 are indicated in kilobases (kb).

hypovirulence (Rigling *et al.*, 1989; Choi *et al.*, 1992). Results of this study clearly indicate that reduction in laccase accumulation is not responsible for hypovirulence in Michigan strains of *C. parasitica.*

Conclusions

Based on the Bavendamm assay for laccase activity, I have found no correlation between laccase activity and the hypovirulence phenotype in Michigan isolates of *C. parasitica.* Results from other laboratories, when basing their work on European-derived dsRNA have found that a correlation does exist. This is indicative of the diversity found among *C. parasitica* dsRNA genomes (Paul and Fulbright, 1988; L'Hostis *et al.*, 1985; Fulbright *et al.*, 1988). Finally, use of previously described assays for laccase activity with the substrates DMOP or ABTS yielded inconsistent data within a single experiment, as well as between experiments. Therefore, I conclude that the Bavendamm assay is the most reliable test for laccase activity at the present time.

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Chapter V

Discussion

A great deal of knowledge has been gained about mycoviruses, since their discovery thirty years ago. The majority of mycoviruses have genomes composed of double-stranded RNA (Buck, 1986). While most mycovirus infections are symptomless (Hollings, 1978), others have a dramatic effect on their fungal host (see Chapter I). The dsRNA virus-like molecules in *C. parasitica* are unique among mycoviruses in that they cause a reduction in virulence, but do not kill their host. The disease-factors in *Ophiostoma ulmi* have also been observed to reduce virulence in infected strains, but this phenomenon has been correlated with the simultaneous presence of ten dsRNA segments (Rogers *et al.*, 1987). In contrast, only one dsRNA segment is necessary for hypovirulence in *C. parasitica* (see Appendix A).

Most mycoviruses are encapsidated by protein coats that are virally encoded, while a few are contained within vesicles of fungal origin (Buck, 1986). The dsRNA genomes found within *C. parasitica* appear to be sequestered within vesicles which are located in the cytoplasm of the fungus (Dodds, 1980; Hansen *et al.*, 1985). When the dsRNA genomes of GH2 and

RC1 were combined into a common nuclear background, and single conidial isolates were collected, no mixing of dsRNA segments between genomes was observed. This suggested that all three dsRNA segments found in the Michigan hypovirulent isolate GH2 are packaged together, as are the two segments of the Michigan hypovirulent isolate RC1 (see Chapter II). In contrast, the individual dsRNA segments of multi-segmented genomes of some mycoviruses, such as the killer viruses in yeast, are packaged individually (Leibowitz *et al.*, 1988). The dsRNA genomes found in *C. parasitica* strains GH2 and RC1 also differ from those of the killer systems of yeast and *Ustilago maydis*, in that when two genomes are combined into a single nuclear background, both of the individual genomes can be maintained (see Chapter II). In the killer systems, one dsRNA genome appears to out compete the second dsRNA genome (Bruenn, 1986).

In *C. parasitica*, the effect on the fungal host when harboring both dsRNA genomes of GH2 and RC1 appeared to be additive, as the multiply-infected strain was less virulent than strains that contained either dsRNA genome individually (see Chapter II). This additive effect has not been reported for any other mycoviruses, and I hypothesize that the dsRNA genomes of isolates GH2 and RC1 have different modes of action in the reduction of fungal virulence. Future experiments could include sequencing the cDNA clones of RC1 dsRNA to determine if this dsRNA encodes a polypeptide. A comparison of the cDNA sequences obtained from GH2 and RC1 dsRNAs could provide information on the different factors responsible for the varied phenotypes associated with each genome, as well as the mechanism for reduction of virulence. The RC1 dsRNA genome is much smaller than that of GH2 and may represent the minimum complement of dsRNA necessary for reduction in virulence.

A comparison of the deduced amino acid sequences of the two open reading frames (ORFs) from dsRNA from *C. parasitica* strain EP713 with those of other mycoviruses, as well as plant viruses, revealed that EP713 was most closely related to positive-strand ssRNA viruses (Koonin, 1991). The ORF identified in the largest dsRNA segment from isolate GH2 has a similar genomic organization as those of dsRNA genomes from Europe and New Jersey (see Chapter III). GH2 is unique, however, in that only a single open reading frame was identified, rather than the two overlapping ORFs that were identified in the European and New Jersey *C. parasitica* isolates (see Chapter III). Now that the complete sequence of the GH2 ORF is known, *in vitro* translation studies could be performed to determine if proteolytic cleavage occurs, producing smaller peptides from this putative polyprotein. These proteins could be isolated, and antibodies raised to them, which could be used to determine when and where these proteins are expressed *in vivo*. Western blot analysis could also be performed to determine if antibodies raised to proteins produced by GH2 dsRNA were antigenically related to proteins produced by other *C. parasitica* hypovirulence-associated dsRNA molecules, as well as to those produced by other mycoviruses and plant viruses.

The enzyme laccase is produced by many plants and fungi, however, the exact biological function of this enzyme is, in many cases, unknown (Mayer, 1987). The down-regulation of laccase by the dsRNA associated with European strains of *C. parasitica* is the only report of a virus that is responsible for a reduction in laccase. No other plant or fungal virus has been identified that down-regulates laccase. Michigan hypovirulent strains, including GH2, once again appear to be unique among hypovirulent strains of *C. parasitica* by not down-regulating laccase.

A biological and molecular characterization of the dsRNA genome found in the Michigan hypovirulent *C. parasitica* strain GH2 has revealed that this viral-like genome appears to be quite different from other mycoviruses that have been described. While GH2 has a genomic organization that is similar to those of other dsRNA molecules purified from geographically distinct *C. parasitica* strains, it has several biological properties that are quite different. Those traits involve the effect of the dsRNA on pigmentation, sporulation, growth in culture, and accumulation of laccase.

An argument can be made that GH2 and EP713 dsRNAs are evolutionarily distinct. There is only 18% deduced amino acid sequence homology, no cross-hybridization upon northern analysis, and only one ORF exists in GH2 dsRNA, while two are present in EP713 dsRNA. dsRNA from strain EP713 results in a down-regulation of laccase, pigmentation, and sporulation, and while GH2 has a slower growth rate than uninfected strains, it has normal pigmentation, sporulation, and laccase production. On the other hand, it seems easier to support the argument that dsRNA from strains EP713 and GH2 are similar and perhaps evolutionarily related. Both reduce virulence, contain large dsRNA viruses that lack protein capsules and are contained in vesicles. The dsRNA molecules in each strain are 3' polyadenylated and contain a large open reading frame which is presumed to produce a polyprotein that contains putative RNA helicase, RNA-dependent RNA polymerase and protease domains. While a direct lineage between the dsRNA molecules isolated from strains EP713 and GH2 is doubtful, the similarities between these molecules suggest that an evolutionary relationship does exist.

The working hypothesis concerning the mechanism of dsRNA-associated hypovirulence is that a polypeptide, encoded for by dsRNA, down-regulates a *C. parasitica* gene or genes necessary for fungal virulence. The specific *C. parasitica* genes that may be down-regulated, reducing fungal virulence, are unknown. Another possibility is that the dsRNA itself, rather than a dsRNA encoded polypeptide, disrupts fungal processes, and thereby reduces virulence. Until a specific viral protein responsible for reduction in virulence is identified, both of these hypotheses are valid. However, since all of the dsRNA molecules sequenced to date contain open reading frames, the assumption is that the putative protein products would interact with the fungal host.

A survey of the research performed on the host-parasite (fungal-viral) interactions involved in hypovirulence reveals that the viral side of the interaction has been studied much more thoroughly than the fungal biology. It is possible that fungal factors are involved in viral maintenance within the cytoplasm of *C. parasitica*. This phenomenon has been observed in the yeast killer system (Bruenn, 1986). If this is the case in *C. parasitica*, strain GH2G3 may be the ideal strain in which to study these interactions, since all single-conidial isolates (asexual progeny) from strain GH2G3 contain dsRNA (see Appendix B). A simple experiment, transferring the dsRNA into a new nuclear background, collecting asexual progeny, and assaying for the presence of dsRNA, would reveal if the nucleus was involved.

The outlook for the survival of the American chestnut in North America is positive on several fronts. First, within Michigan, small stands of chestnut trees, which were declining only 5 years ago, now appear to be recovering and contain naturally occurring hypovirulent strains of *C. parasitica.* Second, artificial inoculation of hypovirulent strains around the margins of a potentially lethal canker can prevent it from killing the tree. This method is rather labor intensive, however, since each canker on a tree must be treated individually. The most exciting prospect for biological control of chestnut blight was elucidated through recent transformation experiments performed by Choi and Nuss (1992). In these experiments, the full-length cDNA clone of dsRNA from the European *C. parasitica* strain EP713 conferred hypovirulence to transformants containing the cDNA, as well as to both ascospores and conidia produced by the transformants. If these transformants are found to be stable over long periods of time and can induce hypovirulence in field situations, they have the potential to be effective biological controls, because they would not be inhibited by vegetative compatibility, as are natural isolates. Because sporulation is suppressed by the dsRNA in strain EP713, dsRNA molecules from other strains, such as GH2, may be more effective biocontrols and should continue to be studied. Also, the smaller dsRNA genome size found in *C. parasitica* strain RC1 may be more stable than larger genomes when integrated into the fungal genome, and may represent another effective biocontrol.

While hypovirulence will prevent the American chestnut from going extinct, I do not believe that the eastern forests of North America will ever again be predominantly composed of chestnut trees. Various oak species, which are now well established, have filled the void left by the death of the chestnut trees. Chestnut orchards, however, are gaining popularity among nut growers, and hypovirulent strains of *C. parasitica* could no doubt be effective as a biological control in an orchard situation. I am confident that future research will not only elucidate the mechanism responsible for hypovirulence, but will also enable us to have American chestnuts roasted over an open fire.

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APPENDICES

APPENDIX A

Virulence of *Cryphonectria parasitica* strains with various combinations of dsRNA molecules

Introduction

Chestnut blight, caused by *Cryphonectria parasitica*, is naturally controlled in several locations in Michigan by hypovirulent, or less virulent forms of the fungus (Fulbright *et al.* 1983). American chestnut trees (*Castanea dentata* [Marsh.] Borkh.) in one such location (Grand Haven) have been infected with chestnut blight since the 1940's, but are surviving due to protection by hypovirulent, double-stranded RNA containing strains of *C. parasitica.* A study of this site was performed by Fulbright *et al.*, (1983), and a brief summary of their results is presented below. Two hypovirulent isolates containing different dsRNA banding patterns upon polyacrylamide gel electrophoresis (PAGE) were collected at the Grand Haven site and were designated GH2 and GHU4. Isolate GHU4 had an extremely slow growth rate and did not produce pycnidia or conidia on chestnut stems. Isolate GH2 was more interesting in that, while reduced in virulence, it was more aggressive than GHU4 and produced swollen cankers that sporulated, demonstrating that hypovirulence may have disseminated through asexual spores (Fulbright *et al.*, 1983). It was later determined that dsRNA from *C. parasitica* isolates GH2 and GHU4 cross-hybridized upon northern analysis, although their dsRNA banding pattern and the molecular weights of individual dsRNA segments were different (Paul and Fulbright, 1988). We have chosen isolate GH2 as the focus of the present study.

C. parasitica strain GH2 is characterized by a dsRNA genome that contains three size classes of dsRNA molecules (Dodds, 1980; Tartaglia *et al.*, 1986). The largest is approximately 9.0 kb, while the smaller two are approximately 3.5 kb and 0.8 kb. The two largest molecules cross-hybridize upon northern analysis, while the smallest does not hybridize to either of the larger molecules (Tartaglia *et al.*, 1986). We have identified *C. parasitica* isolates which contain one, two or all three of the dsRNA molecules characteristic of the GH2 dsRNA genome. Interestingly, a *C. parasitica* strain containing only the smaller two dsRNA molecules has never been reported. My goal was to determine if the variability observed in the dsRNA banding pattern could be associated with changes in virulence and, therefore, if the number of segments or any one specific segment accounted for changes in aggressiveness of the isolates.

Materials and methods

Cultures and growth conditions The *C. parasitica* isolates CL1-16 and GH2 were recovered from Crystal Lake and Grand Haven, Michigan, respectively, in 1980 (Fulbright *et al.*, 1983). Isolates GNC, TG, M3, and F5 were recovered from an experimental plot at Crystal Lake in 1985 (Garrod *et al.*, 1985) in which GH2 was released in 1983. Cultures were maintained on PDA (Difco, Detroit, MI) at 25 °C under cool white, fluorescent lights with a 16 h photoperiod (Garrod *et al.*, 1985).

dsRNA analysis and culture conversions dsRNA isolation by column chromatography over CF-11 cellulose (Whatman) was performed as described by Morris and Dodds (1979). dsRNA from each strain was transferred into the nuclear background of the virulent, non-dsRNA-containing isolate CL1-16 via hyphal anastomosis as described by Anagnostakis and Day (1979). Proper transfer was confirmed by dsRNA isolation, which was separated on 5% polyacrylamide gels and stained with ethidium bromide. Strains were designated with the name of the recipient isolate (CL1-16) followed by the name of the dsRNA donor strain in parentheses (eg. CL1-16(GH2)). The conversion of the virulent isolate CL1-16 to hypovirulent, due to the transfer of dsRNA molecules from a donor hypovirulent strain, was confirmed by virulence assays in which Golden Delicious apple fruit were inoculated with the converted CL1-16 strain. Inoculation with the virulent isolate CL1-16 served as a control.

The virulence of each converted strain was determined based on the size of the lesion produced on the apple fruit when compared to the size of the lesion produced by the virulent strain, CL1-16.

Stem inoculations The 5 dsRNA-containing strains were inoculated on each of 5 American chestnut tree stems in a research plot in Frankfort, MI. Duplicate inoculations were performed in a separate plot at Russ Forest in southern Michigan. Stem inoculations were made using a sterile 5 mm cork borer and were approximately 3 mm deep, penetrating the bark and cambial layers of the stem. A fungal mycelial plug, 5 mm in diameter, was placed in the wound on the stem and was covered with tape to prevent desiccation. The virulent *C. parasitica* isolate, CL1-16, was not tested, because such inoculations would rapidly kill the small trees. Instead, canker areas were compared with those of CL1-16(GH2). The area of the canker caused by each strain was measured on each stem at 1, 2 and 3 months after inoculation. The mean and standard deviation of the canker areas were calculated. After 3 months, *C. parasitica* was re-isolated from each canker as described by Garrod *et al.* (1985). Bark samples were placed on PDA, and the resultant *C. parasitica* isolates subcultured. dsRNA isolation was performed on the subcultures to verify that the dsRNA content of each strain had not changed.

Results and discussion

Characterization of culture morphology and dsRNA content The unique culture morphology for each strain used in this study is shown in Figure A1. After dsRNA isolation and analysis on PAGE (Figure A2), it was observed that various culture morphologies were reflective of differing dsRNA genomes. Each hypovirulent strain contained dsRNA molecules with mobility in PAGE similar to 1, 2, or all 3 dsRNA size classes generally associated with strain GH2 (Figure A2). dsRNA was transferred from the hypovirulent strains GH2, M3, F5, TG, and GNC into the common nuclear background of the virulent strain CL1-16. The dsRNA banding pattern of the resulting strains were verified by PAGE to be consistent with those of the donor strains. Culture morphologies of the converted strains were also similar to those of the donor, hypovirulent strains. Strains CL1-16(M3) and CL1-16(GNC) each had only one dsRNA band observable after gel electrophoresis. The single dsRNA band in CL1-16(M3) appeared to be identical in size to that in strain CL1-16(GH2) (approximately 9 kb), however the band in strain CL1-16(GNC) was larger (approximately 10 kb). Strain CL1-16(F5) contained two bands of dsRNA identical in mobility to the two largest dsRNA segments found in CL1-16(GH2) (approximately 9.0 kb and 3.5 kb). Strain CL1-16(TG) contained three dsRNA bands similar in mobility to those in CL1-16(GH2), except that the middle band in CL1-16(TG) appeared to be smaller than the middle band in CL1-16(GH2)

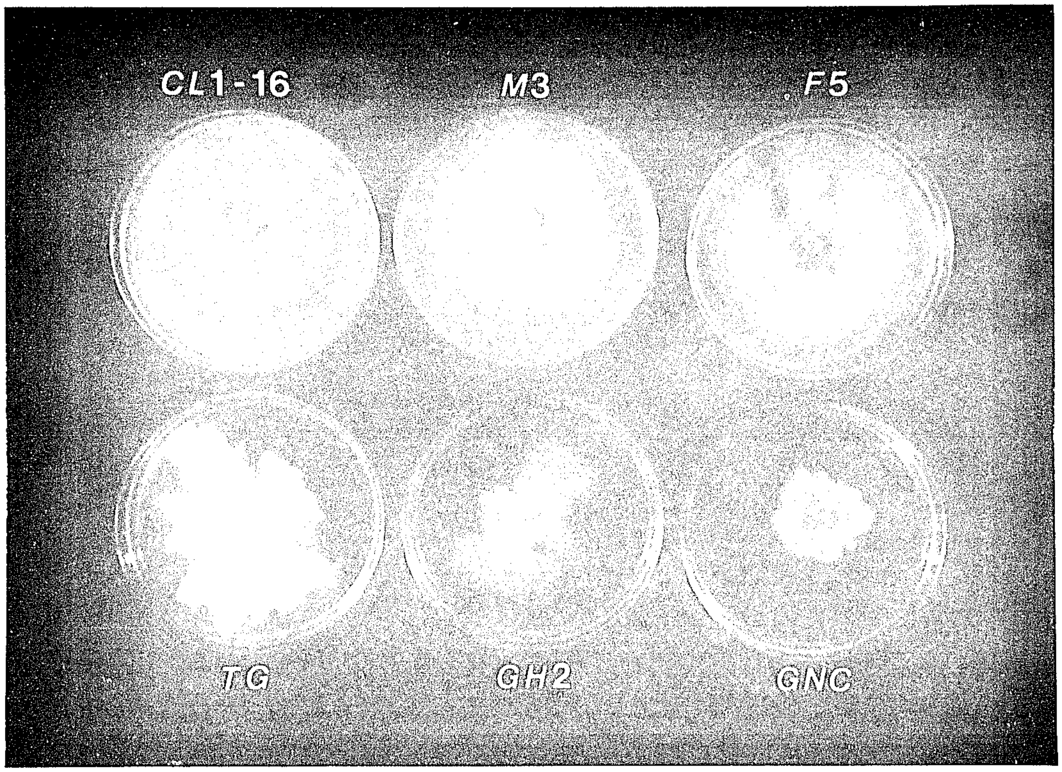

Figure A1. Culture morphology of isolates used in this study.

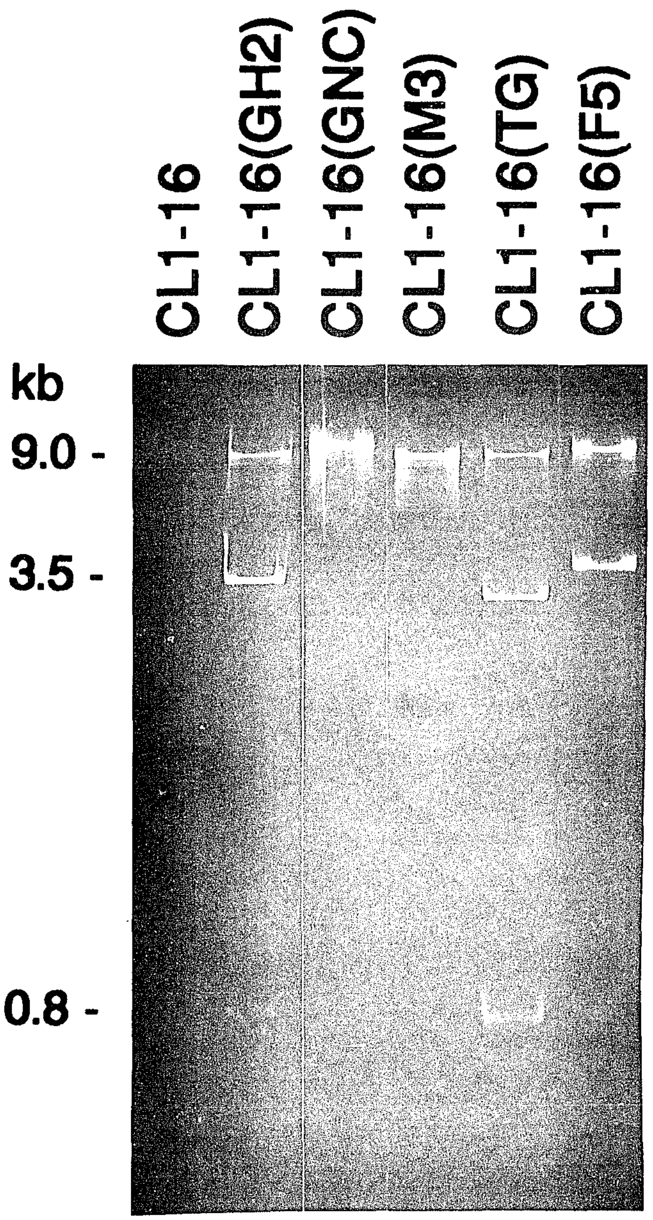

Figure A2. Ethidium bromide-stained dsRNA isolated from each strain inoculated into chestnut stems. dsRNA was separated by 5% PAGE. Molecular size is indicated in kilobases (kb).

(Figure A2).

Effect of dsRNA genomes on virulence To determine which dsRNA molecules in *C. parasitica* isolate GH2 might be required for the observed reduction in virulence, we obtained strains with various GH2 dsRNA banding patterns. The mean canker areas for each strain at each plot is shown in Figures A3 and A4. Strain CL1-16(M3) was clearly the most virulent of the isolates used in this study, while strain CL1-16(GNC) was the least virulent. The other three isolates were approximately equal in virulence, although strain CL1-16(TG) was slightly more virulent than strains CL1-16(F5) and CL1-16(GH2) (Figure A3).

My results indicate that a strain which contained only the largest molecule of the GH2 dsRNA genome was more virulent than strains which contained either two or all three molecules (Figures A2, A3, and A4). Strain CL1-16(M3), which has one dsRNA band, caused significantly larger cankers than strains CL1-16(F5), CL1-16(TG) and CL1-16(GH2), all of which had two or three bands. No clear differences were seen between strains CL1-16(F5), with two dsRNA bands and CL1-16(TG) and CL1-16(GH2), with three bands each. Perhaps if the study could have been continued for two years, small differences in virulence, which may exist between these strains would be observed. Unfortunately, a majority of the test trees died before further analysis could be made. Some of the trees died because of cankers of strain CL1-16(M3), and others died of natural cankers, which

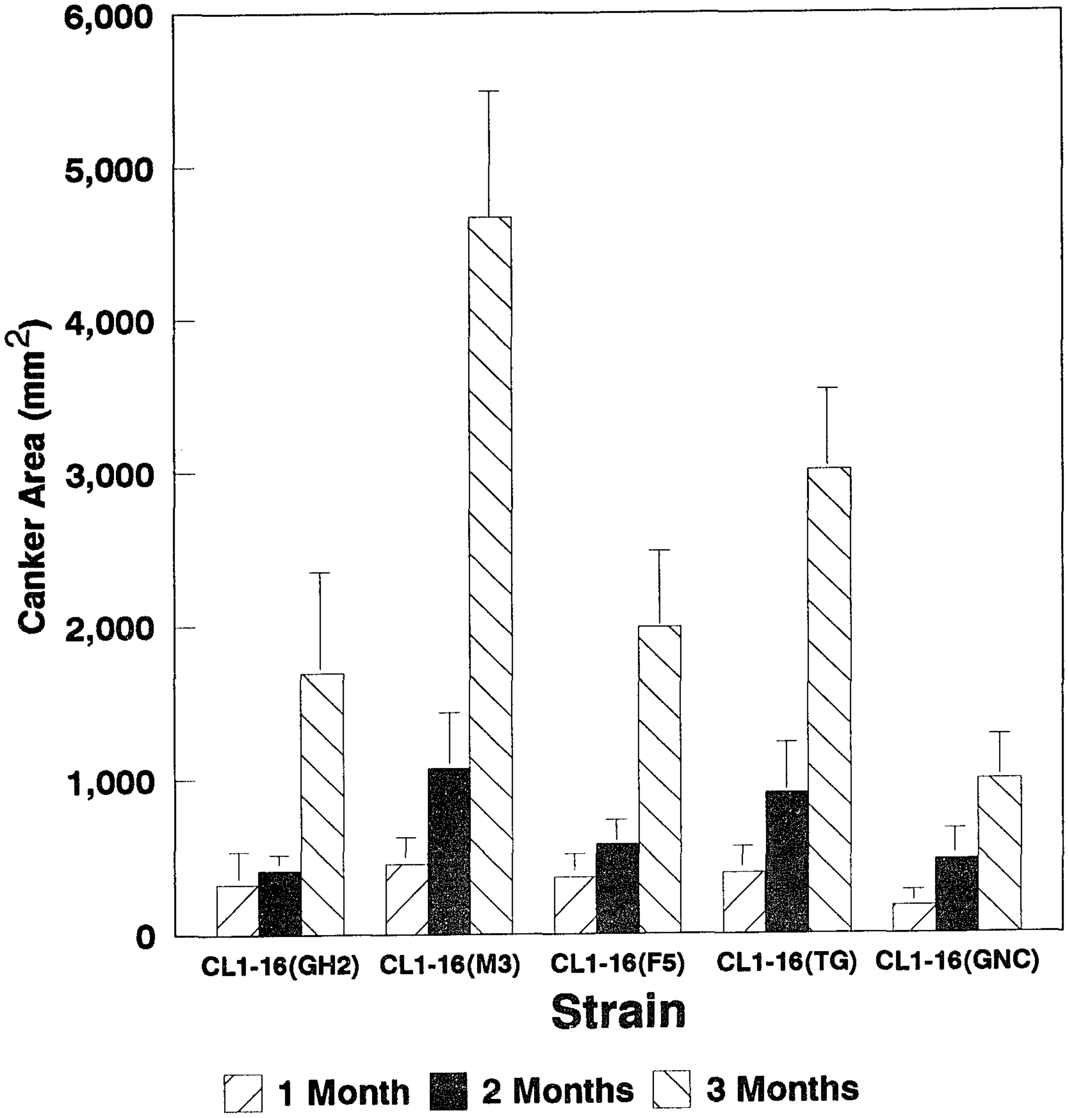

Figure A3. Mean canker area produced by each strain at the Frankfort plot. Bars represent an average of 5 inoculations. Standard deviation is indicated as a line above each bar.

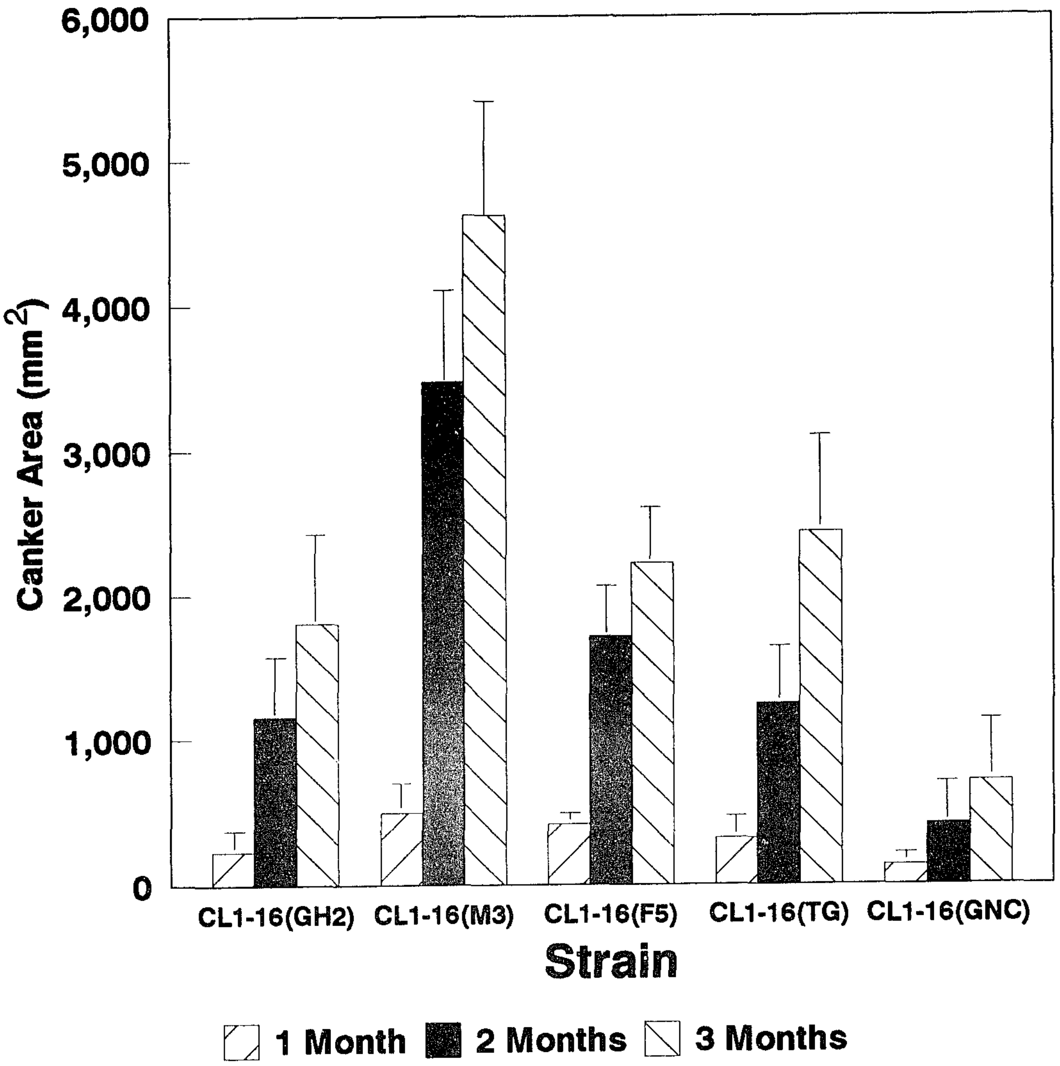

Figure A4. Mean canker area produced by each strain at the Russ Forest plot. Bars represent an average of 5 inoculations. Standard deviation is indicated as a line above each bar.

appeared during the course of the experiment.

Strain CL1-16(GNC) was least virulent of all the strains tested, and it contains only a single size dsRNA molecule. However, subsequent dot blot analysis revealed this molecule does not cross-hybridize to GH2 dsRNA (P. McManus, Michigan State University, personal communication) and thus is not relevant in analysis of GH2-associated hypovirulence.

Although the two small bands in the GH2 dsRNA genome are not necessary for the maintenance of dsRNA, as the 9.0-kb band can exist without the other two, there did appear to be an effect on virulence. Since we were unable to distinguish a difference in virulence between strains CL1-16(F5), containing the two top dsRNA bands, and CL1-16(GH2), containing all three bands, perhaps the smallest, 0.8-kb dsRNA molecule has little effect on virulence. This 0.8-kb molecule, which has been cloned and partially sequenced, does not encode a protein product (D. Nuss, Roche Institute of Molecular Biology, personal communication). The 9.0-kb band of the GH2 dsRNA genome, however, does contain at least one open reading frame capable of producing a polyprotein (see Chapter III). Since the 9.0- and 3.5-kb bands are homologous, it is conceivable that protein products involved in hypovirulence could be encoded by both molecules. Therefore, when the 3.5-kb band is present, in addition to the 9.0-kb band, a further reduction in virulence is seen.

There was remarkable consistency in canker area at 3 months between the two plots. The only major difference was that strain CL1-16(TG) was slightly more virulent at the Frankfort plot than at the Russ Forest plot. It is interesting to compare the growth rate of the cankers over the course of the 3 months. In each plot, mean canker area after one month was quite small and increased dramatically in the Russ Forest plot after 2 months, while in the Frankfort plot, a large increase in mean canker area was seen between 2 and 3 months. One possible explanation for this difference in canker growth rate is the amount of rainfall received by each plot during each month of the experiment. This work was started during a drought, and neither plot received any rain during the first month of the experiment. During the second month, southern Michigan, where Russ Forest is located, received rainfall, and the trees looked healthy at the second measuring. When the measurements for the second month were made at the Frankfort plot in northern Michigan, the trees still looked very wilted, and the Frankfort plot did not receive rainfall until the third month of the experiment. Perhaps the growth rate of the canker was a response to these environmental conditions. It is also possible that other factors including tree size or other environmental conditions specific for each plot played a role in the rate of canker growth.

Finally, the initial characterizations of *C. parasitica* isolate GNC, which is less virulent than isolate GH2, indicate that it may prove useful for future work. Clearly, further analysis is necessary to determine if the dsRNA genome within isolate GNC is homologous to any known *C. parasitica* dsRNA genomes, or if it represents a new homology group.

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APPENDIX B

Possible nuclear influence on dsRNA-associated hypovirulence

Cryphonectria parasitica (Murr.) Barr isolate GH2 is a naturally occurring, hypovirulent isolate associated with the biological control of chestnut blight. GH2 was recovered from an American chestnut (*Castanea dentata* [Marsh.] Borkh.) grove in Michigan (See Appendix A; Fulbright *et al.* 1983). To further characterize this isolate, single-conidial isolates (sci's) of GH2 were collected and grouped according to culture morphology and growth rate. Three groups were identified among the sci's of isolate GH2, and were designated fast, moderate and slow, referring to their growth rate on PDA in culture. There were approximately equal numbers of fast and moderate sci's, but slow growing sci's were quite rare. When assayed for the presence of dsRNA, using the standard isolation technique of Morris and Dodds (1979) followed by gel electrophoresis, sci's in the fast group did not contain dsRNA, while those in the moderate and slow groups had dsRNA banding patterns identical to *C. parasitica* isolate GH2.

Analysis of single-conidial isolates

To distinguish between those GH2 sci's in the moderate and slow groups, single conidia were isolated from a representative strain of each group. A summary of all single-conidial isolations performed is shown in Figure B1. From the moderate group, 60 sci's were collected from strain GH2L6 and were categorized into two groups; fast, containing no dsRNA, and moderate, having a GH2-type dsRNA banding pattern upon gel electrophoresis. The slow growing sci GH2G3 was unique in that it only produced sci's having moderate or slow growth rate. Of the 60 single conidia isolated from strain GH2G3, 15 were randomly assayed for the presence of dsRNA, and all were found to have the GH2-type banding pattern. Many of these sci's, however, only contained the top (~9.0 kb) dsRNA segment upon gel electrophoresis rather than the characteristic 3 band pattern. This is the only Michigan hypovirulent *C. parasitica* strain identified in which dsRNA can notbe lost upon single-conidial isolation. Two sci's from strain GH2G3, designated GH2G3-ss3 and GH2G3-ss16, were further characterized by collecting 25 sci's from each strain. All 50 sci's in this group had a moderate or slow growth rate, and all 10 which were assayed for the presence of dsRNA contained a single segment which had a molecular size of ~9.0 kb, identical to that of the top band of GH2 (see Figure B1 for a summary).

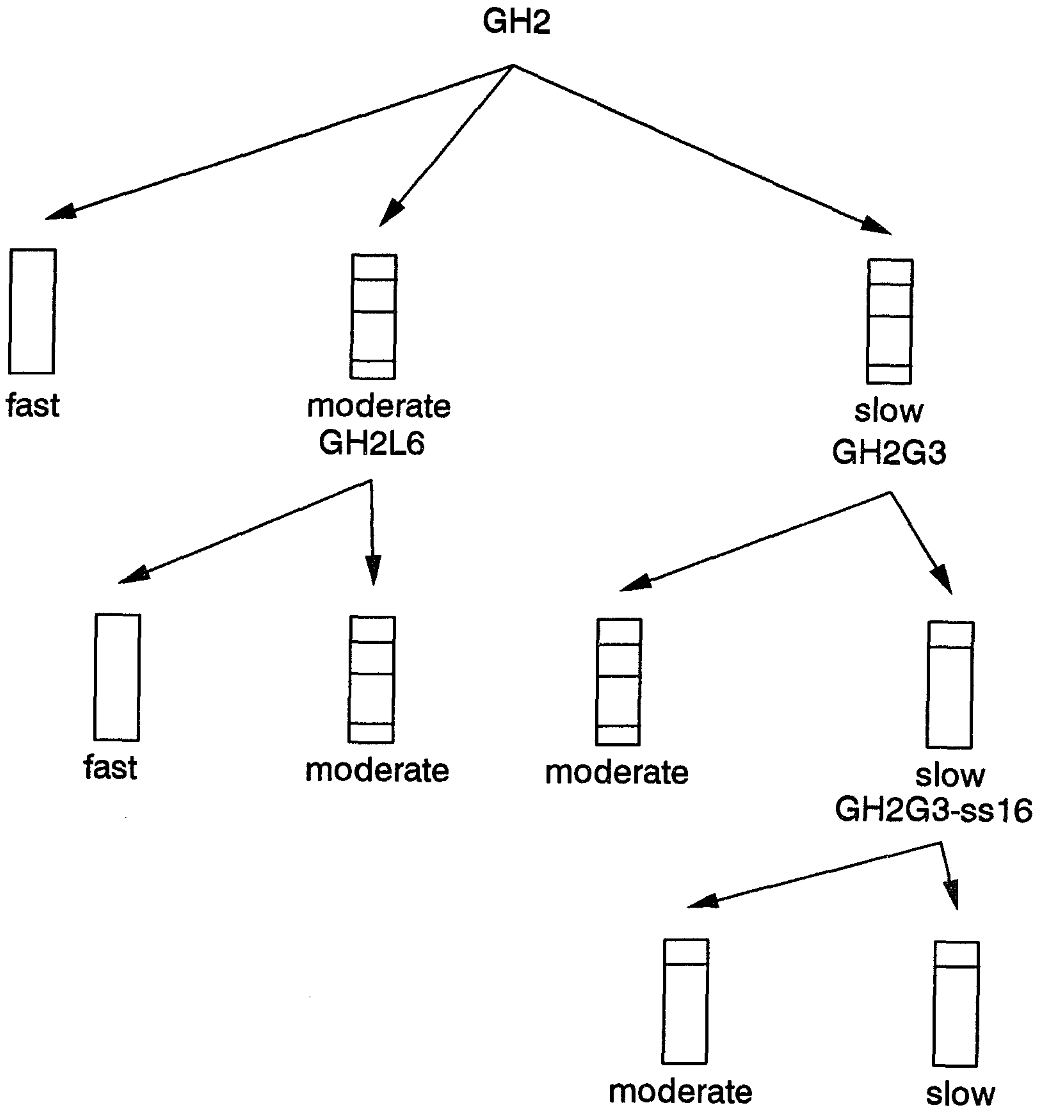

Figure B1. Summary of banding patterns of single-conidial isolates. Rectangular boxes represent the dsRNA banding patten observed in polyacrylamide gels. Arrows point to asexual progeny of each parental strain. Fast, moderate and slow refer to the growth rate in culture of each group.

Analysis of ascospore progeny

When dsRNA-containing *C. parasitica* strains are sexually crossed, resulting ascospore progeny have not been found to contain dsRNA (Day *et al.*, 1977). Because of our inability to collect dsRNA-free sci's from strains GH2G3, GH2G3-ss3 and GH2G3-ss16, sexual crosses were performed as described by Garrod *et al.*, (1983). A nuclear marker, such as color, was necessary for each strain in the cross, therefore a white *C. parasitica* strain,EP329, and GH2G3-ss16, which was orange were chosen. If there was a nuclear effect, which was enabling the dsRNA to be maintained at a high level, ascospore progeny might also be hypovirulent and maintain dsRNA. When strain EP329 was used as the maternal parent, 124 ascospores were collected of which 86 were white and 38 were orange. Virulence assays were performed on 62 of the ascospores, and two were initially found to have a low level of virulence. Upon subculturing, these two ascospores increased in virulence and neither contained dsRNA. Similar results were seen in the reciprocal cross, however no hypovirulent or dsRNA-containing ascospores were identified.

Conclusions

The mechanism by which strains such as GH2G3 maintain dsRNA in all conidia, while most other strains can not, is unknown. Perhaps it is a function of the dsRNA rather than a function of the host nucleus, since the dsRNA was not maintained through sexual crosses. It is also possible that another cytoplasmic element, such as a plasmid, enhances the stability of the dsRNA, but is also lost through meiosis. dsRNA stability and maintenance are two important characteristics in hypovirulent *C. parasitica* strains which are to be used as biological controls. Further study of GH2G3 may enhance our knowledge of how these functions are performed.

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