GENETIC ANALYSIS OF VEGETATIVE INCOMPATIBILITY POLYMORPHISMS AND HORIZONTAL TRANSMISSION IN THE CHESTNUT BLIGHT FUNGUS, *CRYPHONECTRIA PARASITICA*

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ABSTRACT

GENETIC ANALYSIS OF VEGETATIVE INCOMPATIBILITY POLYMORPHISMS AND HORIZONTAL TRANSMISSION IN THE CHESTNUT BLIGHT FUNGUS, *CRYPHONECTRIA PARASITICA*

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Vegetative incompatibility polymorphisms in *Cryphonectria parasitica* are known to have quite variable effects upon the horizontal (cytoplasmic) transmission of hypoviruses. This study examined the effects of individual vegetative incompatibility (*vic*) genes upon the horizontal transmission of nuclei, dsRNA hypoviruses, and a senescence-inducing agent in *C. parasitica*. A genetic analysis identified three new *vic* loci named *vic3*, *vic4* and *vic5*, each with two alleles, that produce incompatibility through allelic interactions. The effects of these three *vic* loci as well as *vic1* and *vic2* upon heterokaryon formation were tested using complementary color mutations (*cre* and *br*) under nonselective growth conditions. Heterokaryons were found to form between strains homoallelic at all *vic* loci, when grown on potato dextrose agar and chestnut tissue. Heteroallelism at any of the five *vic* loci produced barrages and prevented heterokaryon formation on chestnut tissue. Hyphal tips from heterokaryons contained both nuclear types at variable ratios. The effects of all five *vic* loci upon the horizontal transmission of hypoviruses were examined. Heteroallelism at only *vic1* produced a nonreciprocal (unilateral) transmission where the *vic1-2* recipient does not become infected with virus, but the *vic1-1* recipient always becomes infected. Heteroallelism at *vic2* prevented viral transmission but epistasis from other *vic* genes could decrease this barrier. Evidence indicates that allele *vic1-1* is epistatic over allele *vic2-1* when both occur in a recipient thereby reducing the transmission barrier caused by *vic2*. Alleles *vic4-2* and *vic5-2* may also cause a unilateral reduction in the *vic2* transmission barrier. Heteroallelism at *vic3* also causes unilateral transmission. Heteroallelism at *vic1* was found to be epistatic over heteroallelism at *vic3*. Heteroallelism at *vic4* and *vic5*, individually and together, did not hinder hypovirus transmission. An unknown hypovirulence-inducing agent was found to be phenotypically similar to the suppressive senescence syndrome found in other fungi. Transmission of this agent occurred between compatible strains, was uninhibited by heteroallelism at *vic4*, but was prevented by *vic2*. The senescence phenotype was found to be characterized by elevated levels of respiration through the alternative oxidase, and to produce conidia with variable degrees of senescence, implicating mitochondrial dysfunction in the senescence syndrome.

Dedicated with love to

Mom and Dad

for their love and for their encouragement through the years
of my fascination with the wonders of life

"Be exalted, O God, above the heavens; let your glory be over all the earth."
(Psalm 57:5)

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Introduction

The origin and consequences of genetic diversity represent two central issues in biology. How does genetic variability arise? How is the genesis of novel genetic information controlled? The primary source of genetic variation in eukaryotes lies with the complementary processes of mutation and mixis. In many filamentous fungi genetic variation can also occur in somatic cells through the natural formation of heterokaryons and heteroplasmons. These novel genetic states are quite unusual when considered in the context of most other eukaryotes (Andrews, 1991).

The transmission of genetic variation in most eukaryotes occurs primarily in the vertical dimension through sexual or clonal reproduction, that is, from parent to offspring; some evidence also suggests that horizontal gene transmission[1], that is, transmission from individual to individual, has also occurred although rarely (Kidwell, 1993). The situation with many filamentous fungi is quite different. In filamentous fungi the opportunity for horizontal transmission is associated with the body plan of the organism. Mycelia are frequently coenocytic in nature and form interconnected networks through the ramification and anastomosing of hyphae. The same processes governing vegetative cell fusions within

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[1] The term horizontal transmission has been used in different, though related, senses by various authors. Kidwell (1993) defines it as "nonsexual transfer of genetic information between genomes"; Smith et al (1992) used the term to refer to gene transfer between species. The term has also been used for the transmission of plasmids via conjugation between bacteria (Simonsen 1991), and for the transmission of chromosomal genes between bacteria of the same species (Guttman and Dykhuizen 1995). Griffiths (1995) uses the term in reference to the transmission of plasmids between fungal individuals within a species and between species. In all cases, genetic elements are transferred between individuals rather than from parent to offspring. It is in this sense that the transmission of viruses and nuclei between fungal individuals is referred to here as horizontal.

a single individual (genet) may also occur between genetically different individuals when they make contact. Horizontal gene transmission is therefore made possible through the direct cellular fusions of one mycelium with another, and the subsequent mixing of their protoplasms. This provides the opportunity for a remarkable cohabitation of nuclear and mitochondrial genomes within the shared cytoplasm of the somatic cells, and for coenocytic fungi, stable heterokaryons and heteroplasmons can sometimes form. The traditional concept of organismal individuality would therefore seem to be quite compromised in fungi. For many years, following the classic study by Buller (1931), the concept of the "compound mycelium" held that this creation of composite mycelia occurred frequently and improved the fitness of the organism. More recently, the compound mycelium concept has been challenged by the recognition that many filamentous fungi possess genetically regulated mechanisms that significantly limit the occurrence of intraspecific somatic cell fusions (Todd and Rayner, 1980).

Somatic or vegetative cell fusion and the genetic mechanisms that regulate the viability of the fusions have been found in the Ascomycetes (Glass and Kuldau, 1992) and Basidiomycetes (Casselton and Economou 1985) although the function of these systems is not the same for each of these groups. Interestingly, other organisms whose lifestyle and body plan provide unique opportunities for somatic cell interactions also exhibit similar systems: the plasmodial slimemolds (Myxomycetes) possess an elaborate somatic cell fusion system (Lane 1987), and evidence also indicates that the Zygomycetes (Griffin and Perrin 1960), and the filamentous bacteria, Actinomycetes, are capable of undergoing cell fusions (Waksman 1967). Buss (1987) points out that even colonial sessile marine animals such as the Ascidians and Bryozoans possess somatic cell compatibility systems. In the Ascomycetes, vegetative cell incompatibility systems have been found to limit the viability of intermycelial vegetative cell fusions to some, but not all, close relatives, and to mediate the degree of cytoplasmic continuity between fused hyphae (Glass and Kuldau, 1992).

Although the vegetative cell incompatibility systems in the Ascomycetes have the effect of maintaining the genetic (nuclear and mitochondrial) integrity of the individual (genet), it is not clear from present knowledge whether this constitutes their raison d'être. Several hypotheses have been proposed to explain the primary function of the genes controlling vegetative incompatibility, including such disparate ideas as reproductive organ development and ecological competition. Another hypothesis for the primary function of these genes is that they have evolved to limit the infectious transmission of cytoplasmic parasites (Caten 1972; Nauta and Hoekstra 1994). Limitations upon cytoplasmic continuity between mycelia not only constrain the movement of nuclei and mitochondrial genes, but also limit the horizontal transmission of intracellular parasitic genetic elements.

Viruses and plasmids are common in fungi, and several transposons are also known (Kistler and Miao, 1992; Nuss and Koltin, 1990; Griffiths, 1995). These mobile genetic elements are capable of being transmitted between individual mycelia through vegetative cell fusions. In fact, horizontal movement without direct cytoplasmic contact has not been detected. Although horizontal transmission, particularly of viruses, has been documented many times in fungi, the effects of the vegetative cell fusion systems upon the transmission process are still not well understood. The horizontal transmission of double-stranded (ds) RNA viruses in the chestnut blight pathogen, *Cryphonectria parasitica*, has been studied using many fungal strains from nature. These studies have shown that the effects of different vegetative incompatibility genotypes (polymorphisms) upon viral infection are complex and quite variable, suggesting that the polygenic nature of the polymorphisms is responsible.

The elucidation of the influence of vegetative cell incompatibility systems in the Ascomycetes upon horizontal transmission must be set in the context of the genetic basis of the incompatibility. One of the unique features of Ascomycete vegetative incompatibility is that it is regulated by several (many) genetic loci, each of which is capable of independently eliciting an incompatibility reaction. Preliminary studies in several Ascomycetes further indicate that each genetic locus may have a unique effect upon the fused cells, such as differences in the rapidity or extent of cell death (Garnjobst and Wilson 1956). Therefore the effects of vegetative cell incompatibility upon the cytoplasmic transmission of intracellular genetic elements must be considered on a gene by gene basis.

The purpose of this research was to investigate the effects of the vegetative incompatibility system of the Ascomycete *C. parasitica* upon horizontal (cytoplasmic) transmission. *C. parasitica* has been chosen for this study because its virulence can be reduced by cytoplasmic infection with viruses and transmissible cytoplasmic respiratory-deficiency mutations. Both of these debilitating genetic elements are strictly intracellular and their horizontal transmission has been reported to be limited by vegetative incompatibility (Anagnostakis and Day 1979; MacDonald and Fulbright, 1991). This work consists of two objectives. The first concerns the elucidation of the genetic basis of vegetative incompatibility by identifying new vegetative incompatibility (*vic*) genes. The second objective concerns the effects that individual *vic* genes have upon horizontal transmission of different genetic elements, including nuclei, dsRNA viruses, and a cytoplasmic senescence-inducing agent.

Vegetative incompatibility and heterokaryon formation

Gene transfer in filamentous fungi commonly occurs through nuclear transfer. The state where two or more different nuclei cohabit a common cytoplasm is termed a heterokaryon. The nature of the heterokaryotic condition in fungi is quite variable. For example, in Basidiomycetes the mating process requires the formation of a specialized heterokaryotic condition known as a dikaryon where strict developmental control, operating via clamp connections, maintains one of each nuclear type in each hyphal cell. Dikaryons are formed between strains of complementary mating types, and are the necessary prelude to sexual recombination. Ascomycetes do not require the formation of somatic dikaryons for sexual reproduction although ascogenous hyphae are dikaryotic, and many species can form different types of heterokaryons during somatic growth. The formation of heterokaryons in Ascomycetes results from hyphal fusions between conspecifics or even between different species. Vegetative incompatibility results when hyphal fusions create heterokaryons of incompatible nuclei but the heterokaryotic state is limited by the death of the fused cells. Recent reviews of vegetative incompatibility are Esser and Blaich (1994), Leslie (1993), and Glass and Kuldau (1992).

Two types of heterokaryons are known to form in Ascomycetes. In one type of heterokaryon nuclei from each individual migrate into the hyphal tips. Proliferation of the heterokaryon is therefore possible via hyphal tip extension and branching. Fungi in which this type of heterokaryon has been found are *Neurospora crassa* (Beadle and Coonradt, 1944), *Aspergillus nidulans* (Pontecorvo, 1953), *Penicillium cyclopium* (Rees and Jinks, 1952), and *Sclerotinia sclerotiorum* (Ford et al., 1995). In the second type of heterokaryon the different nuclear types are restricted to the fused hyphal cells and do not migrate throughout the mycelium or into the hyphal tips. Although these heterokaryotic cells do not grow or divide, they are capable of nutritionally sustaining mycelial growth well beyond the fusion cells (Adams et al., 1987). This second type of heterokaryon has been described in *Verticillium dahliae* (Puhalla and Mayfield, 1974), *Gibberella fujikuroi* (Puhalla and Spieth, 1985), *Gibberella zeae* (Adams et al., 1987), and *Magnaporthe grisea* (Crawford et al., 1986).

Heterokaryons have also been formed through protoplast fusion. Adams et al. (1987) found that heterokaryons formed by protoplast fusion in *G. zeae* differed from heterokaryons produced by hyphal fusions. Heterokaryosis following hyphal fusions of vegetatively compatible strains in *G. zeae* is confined to the fused cells. When heterokaryons were formed through protoplast fusion between vegetatively compatible complementing auxotrophic strains, the resulting colony initially produced prototrophic hyphal tips, but subsequently segregated into auxotrophic hyphal tips, suggesting nuclear segregation. In contrast, although heterokaryosis would not occur between vegetatively incompatible strains following mycelial contact, stable self-perpetuating heterokaryons of incompatible strains would form after protoplast fusion. The resulting colonies had an unusual, inhibited growth morphology, and produced prototrophic conidia. Adams et al. (1987) concluded that heteroploids had formed rather than heterokaryons between the vegetatively incompatible protoplasts. Tolmsoff (1983) has suggested that aneuploidy and heteroploidy may be natural mechanisms of variation in fungi.

The physiological basis of the cell death caused by vegetative incompatibility has not been examined in detail. Garnjobst and Wilson (1956) observed that the protoplasm of the fused cells of incompatible strains of *N. crassa* became granular and vacuolated. This degeneration involved one or a few cells in each hypha, and was sharply demarcated by septa with plugged pores. When cell degeneration occurred more slowly, two or three consecutive septae would become plugged, along with less degeneration in the more distal cells. Eventually the vacuoles disappeared and the protoplasm shrank leaving the old cell walls in place. The viable cells behind the plugged septa would then regrow, often within the old cell walls. Hyphal regrowth within old cell walls has also been observed by Jacobson (1993). Microinjection of protoplasm from one hypha into an incompatible hypha showed that the protoplasm was sufficient to induce cell death in the recipient cell (Garnjobst and Wilson, 1956). Transmission electron micrographs of compatible and incompatible cell fusions in *C. parasitica* showed that the incompatible fusions resulted in a granular appearance of the protoplasm, vacuolation, and subsequent collapse of the cell walls (Newhouse and MacDonald 1991). The association of vegetative cell death with organ development in *P. anserina*, and the complexity of the genetic regulation of cell death in this species, suggests that it may be appropriate to consider the cell death response of vegetative incompatibility as analagous to apoptosis (programmed cell death) which has been found to be important in the development of higher eukaryotes (Ellis et al., 1991).

Heterokaryon formation, whether restricted to fused hyphae or maintained in hyphal tips, is controlled by vegetative incompatibility genes. To understand the consequences of vegetative cell fusions in fungi, we must understand the gene interactions that limit the viability of the fusions.

The Genetic Basis of Vegetative Incompatibility

Genetic analyses of vegetative incompatibility in Ascomycetes have been conducted on only a few species. The best understood vegetative compatibility (vc) systems are those of *Podospora anserina* and *Neurospora crassa*. These two species along with *C. parasitica* will be reviewed here. In addition, the Basidiomycetes have a fascinating compatibility system controlled by mating type genes that regulates fusions between vegetative hyphae, but toward quite different developmental ends (Casselton and Economou, 1985). The plasmodial protists (Myxomycetes) present another interesting system where the viability of somatic cell fusions are controlled by multiple loci (Lane, 1987). Basidiomycete mating type compatibility and Myxomycete somatic cell compatibility will not be reviewed here.

Vegetative incompatibility polymorphisms in Ascomycetes are produced by polygenic systems. In the following reviews the term allelic vegetative incompatibility refers to an incompatibility reaction between strains caused by heteroallelism at any one or more vegetative incompatibility (*vic* or *het*) loci. Strains homoallelic at each *vic* locus are vegetatively compatible and do not exhibit the incompatibility reaction. The term nonallelic vegetative incompatibility refers to an incompatibility response produced by the presence of certain alleles at different loci in a common cytoplasm, resulting from either hyphal fusions or recombination.

Podospora anserina

The best understood vegetative (protoplasmic) incompatibility system is that of *P. anserina*. The genetic picture of vegetative incompatibility that has emerged for this species includes allelic gene interactions, nonallelic gene interactions and suppressor genes. The suppressor genes, in particular, have provided intriguing clues as to the biological function of vegetative incompatibility genes. The detection of vegetative incompatibility in *P. anserina* has been based upon the development of reaction zones called barrages between contacting mycelia. Rizet (1952) was the first to subject the barrage phenomenon in *P. anserina* to genetic analysis. Thirteen loci have been discovered so far which interact in an allelic or nonallelic fashion to cause vegetative incompatibility reactions. Nine loci are known to be involved in five nonallelic gene interactions. Five of the nine loci (*c,d,e,r,v*) were found in wild-type strains, and four of the loci (*f,g,k,l*) were produced by mutagenesis. Allelic incompatibility is controlled by five loci (*b,q,s,v,z*), one of which, *v*, also functions in the nonallelic system.

Nonallelic vegetative incompatibility involves lethal cellular reactions resulting from the interactions between the gene combinations, *C/D*, *C/E*, *G/F*, *L/K*, or *V/R*, where each capital letter represents a particular allele at a locus designated by that letter. These lethal cellular reactions will occur when hyphae from two strains fuse, each of which carries one of the alleles of these pairs, or when recombination reassorts these alleles into a single individual. Several lethal interactions are produced from *c/d* and *c/e* interactions because these three loci are multiallelic. Cell death resulting from any of the nonallelic gene interactions is suppressed by the cooccurrence of two mutations, a recessive mutation in the ***modA*** gene, and a dominant mutation in the ***modB*** gene (Boucherie and Bernet, 1974; 1980).

Bernet (1992a) has shown that the nonallelic vegetative incompatibility genes *c,d,e,r* and *v* (hereafter, *c-v*) have pleiotropic effects. Gene interactions between the pairs of loci *c/d*, *c/e*, and *r/v* were found to be necessary for the development of aerial organs, including protoperithecia, perithecia, and aerial hyphae. Strains with the double mutation ***modA modB*** also demonstrated a connection between nonallelic incompatibility and aerial organ development because of their pleiotropic effects: ***modA modB*** abolished nonallelic incompatibility as well as the development of protoperithecia and aerial hyphae (Boucherie and Bernet, 1974; 1980). Bernet (1992a) has shown that each of the gene interactions *c/d*, *c/e*, and *r/v* are involved in cell death. Evidence suggests that for each of these three gene interactions one of the gene products (*d, e, r*) is synthesized in the vegetative cells while the other gene product (*c, v*) is synthesized in the perithecia. Bernet (1992a) concludes that the development of reproductive organs are aided by the degradative functions of the ***mod*** and *c-v* genes which transform fertile stationary-phase cells into a source of nutrients for the developing organs. The suppression of both nonallelic vegetative incompatibility and developmentally associated cell death by ***modA modB*** mutations suggests that these two processes are related.

Other mutations have been found in *P. anserina* which involve the regulation of cell death. Mutations resulting in large perithecia (*lpr*) have been described which also have pleiotropic effects (Bernet, 1991). The *lpr* mutations (*lprA, B, C, D, E, F*) lead to the increased development of protoperithecia and aerial hyphae and also to the early death of stationary-phase cells. The mutations ***modA modB***, which suppressed nonallelic vegetative incompatibility, also suppressed the *lpr* mutations. In addition, *lprB* maps at the *d* incompatibility locus.

Bernet (1992a) has proposed an interesting regulatory system for the development of perithecia based upon the phenotypic evidence from the nonallelic incompatibility genes and the suppressor genes. He notes that the products of the nonallelic incompatibility genes *c* and *v* are diffusible through hyphae whereas the gene products of ***d, e, r, modA***, and ***modB*** are not. Furthermore, Asselineau et al. (1981) showed that the gene products of *d, e, r*, and *modB* may be associated with the plasma membrane. Bernet (1992a) therefore proposes that the products of genes *c* and *v* may be analagous to peptide hormones and the products of ***d, e***, and ***r*** to their receptors. The polypeptide products of genes *c* and *v* may then diffuse away from perithecia through the surrounding hyphae, bind to their receptors, and signal the inception of a cell death process which will provide the developing perithecia with nutrients. This suggested mechanism would also account for the asymmetry in the cell death response observed between strains incompatible at these loci (Labarere et al., 1974). What then is the relationship between nonallelic vegetative incompatibility which results from anastomoses between different strains and the cell death associated with aerial organ formation? Bernet (1992a) suggests that nonallelic vegetative incompatibility results from mutations in genes whose normal function lies in the application of cell death to development.

Recent work is beginning to elucidate the biochemical basis of nonallelic vegetative incompatibility (Begueret et al., 1994; Saupe et al., 1994; Saupe et al., 1995). Several alleles at loci *c* (*het-c*) and *e* (*het-e*) have been cloned. The *e* locus polypeptide has two structural features homologous to known proteins. The carboxy terminal region contains a repeat of 42 amino acids that shows similarity to B subunits of trimeric G proteins. The number of repeats varies among *e* alleles from 3 to 12. The amino terminal region has sequence similarity to GTP-binding consensus sequences of GTPases. Single amino acid differences in the GTP-binding domain prevents the incompatibility reaction. The structure of these proteins suggests that they are involved in signal transduction. Four wildtype *c* alleles have been cloned that have distinct specificities for *e* alleles (Saupe et al., 1995). The polypeptides differ from each other by 1 to 15 amino acids, and show similarity to a protein from pig brain that catalyzes the exchange of glycolipids between cellular membranes (Saupe et al., 1994). Gene disruptions of *c* caused defective ascospore production, indicating that *c* has an essential role in development.

Allelic vegetative incompatibility in *P. anserina* is controlled by five known loci, *b, q, s, v,* and *z*. The most detailed molecular understanding of allelic vegetative incompatibility comes from the cloning of the three *s* locus alleles: *s, S* and the neutral allele s^x (Turcq et al., 1990; Deleu et al., 1993). Alleles *s* and *S* encode 30 kDa polypeptides which differ at 14 amino acids. The function of the polypeptides could not be inferred from present databases. Sequencing of *s* and *S* alleles from four different strains each showed that the amino acid sequences of the two alleles is completely conserved. The neutral allele, s^x, differed from *s* by containing a direct duplication of 46 bp, and by failing to produce any detectable protein. Remarkably, Deleu et al. (1993) found that a single amino acid difference between the *s* and *S* alleles is sufficient to induce vegetative incompatibility. Four sequences with *s* specificity were also cloned from the species *Podospora comata*. In contrast to *P. anserina*, these four protein sequences differed by up to 12 amino acids even though they conferred the same incompatibility specificity. Turcq et al. (1991) inactivated the *s* and *S* alleles by gene disruption, and were thereby able to inhibit incompatibility. They also claimed that the *s* genes were not essential for cell viability. Unfortunately, no data (and no methods) were incorporated into their paper to support this claim.

Allelic incompatibility is suppressed in *P. anserina* by mutations in the gene *modD* while nonallelic incompatibility is not so suppressed. The initial work on the *modD* mutations indicated that they affected the transition from the stationary phase into a developmental phase where aerial and reproductive organs are formed (Labarere and Bernet, 1979, Durrens et al., 1979, Durrens and Bernet, 1982). Specifically, Labarere and Bernet (1979) showed that exit from stationary phase depends upon proteolytic activity which is dependent upon the *modD* gene. This proteolytic activity has also been found to be suppressed by the *modB* gene, which as mentioned above, also suppresses the proteolytic activities caused by the nonallelic incompatibility genes. Because of this confluence in the effects of the *mod* genes, Bernet (1992b) has suggested that the only difference between allelic and nonallelic vegetative incompatibility is the trigger mechanism. Bernet (1992b) further speculates that the effects of the various allelic incompatibility genes upon the *modD* gene might represent redundancy in the developmental control system.

Neurospora crassa

Neurospora crassa was one of the first Ascomycetes where heterokaryosis was documented (Beadle and Coonradt, 1944), and subsequently became the first fungus where heterokaryon incompatibility was genetically characterized. Ten vegetative (heterokaryon) incompatibility (*het*) loci have been identified in *N. crassa* (Perkins, 1988); two new *het* loci have recently been reported (Min et al. 1994; Ohrnberger et al. 1994). These loci produce incompatibility through allelic gene interactions although there is now evidence that nonallelic incompatibility is also present in this species (Jacobson, 1993). Garnjobst (1953, 1955) identified and named the vegetative incompatibility genes *het-c* and *het-d*, the first such genes to be identified for any fungus. The identification of *het-i* by Pittenger (1964) and *het-e* by Wilson and Garnjobst (1966) followed. Holloway also (1955) studied the actions of four loci that either prevented the formation of heterokaryons or inhibited the growth of heterokaryotic mycelium. These genes were referred to as incompatibility genes, but they have not been incorporated into other analyses of vegetative incompatibility so their relationship to identified *het* genes is unknown. The mating type locus of *N. crassa* also has a vegetative incompatibility function (Perkins 1988). The number of *het* genes was extended to 10 (*het5-het10*) by Mylyk (1975) using duplication-producing chromosomal rearrangements which allowed the detection of segmental aneuploids heterozygous at different incompatibility loci.

The nature of the incompatibility reaction in *N. crassa* has been addressed by several studies. Garnjobst and Wilson (1956) showed that the incompatibility response followed hyphal fusion between strains differing at *het-c* and *het-d*. The physiological basis of incompatibility was addressed by Wilson et al. (1961) who did reciprocal injection studies using the cytoplasm from strains which represented all combinations of the alleles of *het-c* and *het-d*. Lethal cytoplasmic reactions occurred when the cytoplasm from a hypha of one strain was injected into incompatible recipient strains but no lethal reactions appeared when the cytoplasms from compatible strains were mixed. Several types of degradative treatments were also done to the extracted cytoplasms to identify the nature of the active compounds. Proteases were found to eliminate the lethal effects which indicated that the unknown factor(s) was probably a protein.

Suppressor mutations of vegetative incompatibility have recently been reported in *N. crassa* (Arganoza et al., 1994b). The specificity of these mutations was found to vary greatly: some suppressor mutants suppressed only certain alleles of a *het* locus, others suppressed both alleles at the locus, and others suppressed the incompatibility due to heteroallelism at three *het* loci. Remarkably, spontaneous suppressor mutations were found to arise fairly frequently. Heterokaryon incompatibility due to the mating type genes *A* and *a* (recently termed idiomorphs) has been found to be suppressed by the *tol* gene (Newmeyer, 1970). Jacobson (1991) has shown that other *Neurospora* species have a mutant *tol* gene. Introgression of the *tol*^{T} gene from the pseudohomothallic species *N. tetrasperma* into *N. crassa* resulted in suppression of mating type vegetative incompatibility while introgression of the *N. crassa* tol^C gene into *N. tetrasperma* allowed mating type vegetative incompatibility to appear, and thereby eliminated pseudohomothallism.

Cryphonectria parasitica

The genetic basis of the vegetative incompatibility system of *C. parasitica* has been studied by Anagnostakis (1977, 1980, 1982, 1988). The genes directly responsible for initiating the vegetative incompatibility reactions have been termed *vic* genes in *C. parasitica* rather than *het* genes as in *Neurospora* although the two designations may refer to homologous genes. Prior to the present study two *vic* loci had been identified using a classical genetic approach. These two loci are unlinked and have been designated *vic1* and *vic2* (earlier classification labelled them *B* and *C*, respectively). Each locus has two known alleles that produce an incompatible reaction through allelic interactions. Nonallelic vegetative incompatibility reactions have not been found in *C. parasitica*. Reference to a third incompatibility gene, *vic3*, has recently appeared in publication (Rizwana and Powell 1992). However, the use of the name in this paper is incorrect as demonstrated by the genetic analyses of Anagnostakis (1982) and this dissertation (chapter 3). The mating-type locus in *C. parasitica* does not have a vegetative incompatibility function.

Individual vegetative incompatibility genotypes (polymorphisms) have been referred to as vegetative compatibility (vc) types by most authors and designated by a number or a letter. The number of vegetative incompatibility loci in *C. parasitica* has been estimated to be between five and seven. This estimate is based upon the recovery of 106 vc types from more than 1200 progeny from sexual crosses between vc type 5 and vc type 10 strains (Anagnostakis 1982). If incompatibility were exclusively produced by allelic interactions, seven different *vic* loci would be needed to account for this many vc types ($2^7 = 128$ total vc types from seven loci). Anagnostakis also estimated the total number of *vic* genes segregating in this cross by the proportion of progeny compatible with the male parent. In this estimate only five *vic* loci are required to account for these ratios. Explanations offered to account for these discordant estimates were the presence of nonallelic or epistatic interactions, or different levels of vegetative incompatibility comparable to that seen in Myxomycetes. It should be noted that none of these proposed explanations was directly supported by these data. Two simpler explanations are possible. First, the proportion of progeny compatible with the male parental vc type may underrepresent the total number of *vic* genes segregating in the cross if some of the genes are closely linked. Secondly, the methods used to detect vegetative incompatibility are not always sufficiently sensitive (see Chapter 2). The vegetative compatibility genotypes of vc types 5 and 10 are of interest because they represent strains, relative to each other, with the largest number of heteroallelic *vic* loci yet known, and both of these vc types have been collected several times from natural cankers in Connecticut (Anagnostakis, 1982).

Heterokaryon formation was found to occur in *C. parasitica* using four different auxotrophic mutants derived from a single strain and placed under selective conditions (Puhalla and Anagnostakis 1971). However, attempts at forming heterokaryons using morphological and auxotrophic mutants under nonselective conditions were not very successful, and led the authors to conclude that heterokaryosis is not common in this species in nature. Anagnostakis (1981) later found a strain that was a stable heterokaryon with regard to mating type. Parasexual recombination has recently been reported in this species after protoplast fusion (Rizwana and Powell 1995).

The specificity of vegetative incompatibility in *C. parasitica* has been reported to be unstable after UV mutagenesis (Rizwana and Powell, 1992). Mutagenesis of *vic1* and *vic2* genes was inferred from the gain and loss of incompatiblity between strains which differed at these loci. In both cases the change was transient because the incompatibility of the mutant would revert back to its original vc type upon subculturing. Changes in vc type were also reported in this study from sectors of the strains. Two points should be kept in mind concerning this study. First, the cell death phenomenon which constitutes the vegetative incompatibility reaction is undoubtedly a complex physiological process that includes many genes. Random mutagenesis could have affected other genes involved in the regulation or expression of the incompatibility reaction. The suppressor mutations which affect vegetative incompatibility in *P. anserina* and *N. crassa* demonstrate this complexity. Secondly, genetic support for the inference that the mutagenesis specifically affected *vic1* and *vic2* is lacking.

The occurrence and horizontal transmission of viruses and genetic elements in filamentous fungi

Vegetative incompatibility genes not only limit heterokaryon formation but also limit the intermycelial transmission of mobile cytoplasmic genetic elements. Ascomycetes and Basidiomycetes are parasitized by viruses and other types of genetic elements that reside in the cytoplasm. Reviews of these parasitic elements and their effects upon their hosts can be found in Ghabrial (1980), Buck (1986), Nuss and Koltin (1990), Kistler and Miao (1992) and Griffiths (1995).

Fungal viruses may or may not be encapsidated, and most are composed of dsRNA (Buck, 1986). Several intracellular locations have been found for fungal viruses including mitochondria (Polashock and Hillman, 1994), free in the cytoplasm (Yamashita et al. 1973), and in vesicles of host origin (Hansen et al 1985). The distribution of viruses within hyphae is known to be variable, and the titer of a virus can vary at different locations in the mycelium (Ghabrial, 1980). Intracellular distributions of viruses are also subject to the developmental state of the mycelium. Ultrastructural studies found viruses to be free in the cytoplasm in younger hyphae, but also present within membrane-bound vesicles and vacuoles in older hyphae (see references in Buck, 1986).

What is the general occurrence of viruses and plasmids of fungi in nature? Survey work to meaningfully address this question is now becoming available. The most information about the diversity and abundance of parasitic genetic elements in nature has been accumulated on dsRNA viruses. Buck (1986) states that more than 100 different fungi are known to harbor viruses. The within-species diversity of viruses and dsRNA elements is best known for *C. parasitica* where many different dsRNAs have been found (Nuss, 1992; Paul and Fulbright, 1988; Fulbright, 1990; Enebak et al., 1994a; Enebak et al.1994b). The geographic distribution of the dsRNAs in populations of *C. parasitica* appears to include much of the range of the fungus in eastern North America, and *C. parasitica* populations in Europe, China, and Japan (Fulbright et al., 1983; Anagnostakis, 1987; Heiniger and Rigling, 1994). An interesting recent study of sequence divergence among the dsRNAs from *C. parasitica* isolates collected in a limited geographic area showed genetic drift although most of the nucleotide changes did not alter the deduced translation products (Chung et al., 1994). A survey of wildtype isolates of *Neurospora* showed that seven of 36 carried dsRNA (Myers et al. 1988). All of the dsRNA-containing strains were from geographically different regions, and three of the seven dsRNAs showed cross homology. A survey of *Ustilago maydis* isolates from nature showed that all of them carried dsRNA (Seroussi et al., 1989). DsRNAs have been found in all of the major taxonomic groups of the rusts and in most of the species surveyed (Zhang et al., 1994).

Plasmids also appear to be quite common in filamentous fungi (Griffiths, 1995). Fungal plasmids occur as either circular or linear types, and are almost exclusively located in the mitochondria (Griffiths, 1995). Several plant pathogenic fungi have been found to harbor plasmids, including *Gaeumannomyces graminis*, *Fusarium oxysporum*, *Nectria haematococca*, and *Rhizoctonia solani* (reviewed in Samac and Leong, 1989). A recent study of 61 field isolates of *Fusarium oxysporum* from Japan found that each of six formae speciales carried a different linear plasmid (Hirota et al., 1992). A survey of 114 field isolates of *Rhizoctonia solani* showed that nearly half harbored plasmids and that homologous plasmids were frequently found in the same vegetative compatibility group (Miyasaki et al., 1990).

The most extensive search for plasmids in fungi has been carried out in *Neurospora* species (reviewed in Griffiths, 1995). In *Neurospora,* seven different homology groups have been found that represent circular plasmids, and four homology groups have been found among the linear plasmids (Griffiths, 1995). Yang and Griffiths (1993) conducted a worldwide survey with 171 strains of *N. intermedia* and *N. crassa* and found that most of them carried either linear or circular plasmids, and some strains carried both. Using Southern hybridizations they found that some plasmids are globally widespread. Another extensive survey of plasmids in natural populations of *Neurospora* species showed that the distribution of seven different homology groups is world wide and includes several species (Arganoza et al., 1994). Distinct frequency distributions have been found for two plasmids with different phenotypic effects upon a single host species. In a Hawaiian population of *N. intermedia*, Debets et al. (1995) found that a cryptic circular plasmid was present in 74% of the isolates while a senescence-inducing linear plasmid was present in only 38% of the isolates, and both frequencies were maintained over time.

The infectious transmission of fungal viruses and plasmids is only known to occur through hyphal fusions which permit cytoplasmic contact (plasmogamy) between individual mycelia and has been documented in a number of fungi (Table 1). Extracellular routes for infection are not known even though a few examples of lytic fungal viruses are known (Ghabrial, 1980). The cytoplasmic spread of a virus through a single mycelium has been observed in *C. parasitica* to occur more rapidly than hyphal tip growth (Martin and Van Alfen, 1991; personal observations). Viral transmission can also occur vertically (intergenerationally) via asexual and sexual spores although sexual spores are virus-free in some species (Buck 1986).

What limits the horizontal transmission of viruses and genetic elements in fungi? Because infection requires intracellular passage any processes which prevent cytoplasmic

Table 1. Examples of horizontal (cytoplasmic) transmission of parasitic genetic elements in filamentous fungi. All transmissions were observed between individuals of a species unless otherwise indicated.

fungal species	**genetic element**[a]	**reference**
Schizophyllum commune	VLPs and plaques	Koltin et al. (1973)
Rhizoctonia solani	degenerative disease	Castanho and Butler (1978)
Ustilago maydis	VLPs	Wood and Bozarth (1973)
Melampsora lini	dsRNA	Lawrence et al. (1988)
Agaricus bisporus	virus	Gandy (1960)
Colletotrichum lindemuthianum	virus	Delhotal et al. (1976)
Helminthosporium victoriae	disease	Lindberg (1959)
Gaeumannomyces graminis	VLPs	Rawlinson et al. (1973)
Ophiostoma ulmi	d-factors, unencapsidated dsRNA	Rogers et al. (1986)
Ophiostoma novo-ulmi	degenerative disease	Charter et al. (1993)
Cryphonectria parasitica	dsRNA hypoviruses; respiratory defects; senescence agent	Anagnostakis and Day (1979); Mahanti et al (1993); Huber et al. (1994); Monteiro-Vitorello et al. (1995)
Aspergillus amstelodami	vegetative death (*vgd*) cytoplasmic mutation	Caten (1972)
Aspergillus niger	virus	Lhoas (1970)
Penicillium stoloniferum	dsRNA virus	Lhoas (1971)
Penicillium chrysogenum	lytic plaques	Lemke et al. (1973)
Podospora anserina	senescence	Marcou (1961)
Neurospora crassa	*kalilo* DNA plasmid, senescence; *Tad* transposon	Debets et al. (1994); Kinsey (1990); Griffiths et al. (1990)
Neurospora intermedia	*kalilo* DNA plasmid; mt chromosome, mt plasmids	Griffiths et al. (1990); Debets et al. (1994); Collins and Saville (1990)
N. intermedia → *N. crassa*	*kalilo* DNA plasmid	Griffiths et al. (1990)

[a] Designations for the transmissible genetic elements or agents are those used by the authors.

contact between mycelia would effectively prevent infection. Therefore, vegetative incompatibility is generally regarded as a primary limitation. However, the effects of vegetative incompatibility upon horizontal transmission of viruses and genetic elements have not been thoroughly studied in any fungal species. Certainly one of the hindrances has been the polygenic nature of the vegetative incompatibility systems which produce a high number of incompatibility phenotypes. The effects of vegetative incompatibility upon horizontal transmission have been considered primarily in *Aspergillus amstelodami*, *Ophiostoma ulmi*, *N. crassa*, and *C. parasitica*.

The cytoplasmic transmission of the spontaneous mutation known as vegetative death (*vgd*) in *A. amstelodami* was examined in relationship to different vegetative incompatibility genotypes (Caten, 1972). Vegetatively compatible strains freely permitted transmission of the *vgd* mutation while differences at two or more vegetative incompatibility loci prevented transmission. Infectivity was also found to be differentially limited by specific *het* genes. The *hetB* locus prevented cytoplasmic transmission, while *hetA* reduced transmission by 80% (Caten, 1972; Handley and Caten, 1973). Caten (1972) suggested an additive effect upon the inhibition of transmission due to increasing numbers of heteroallelic *het* loci.

Cytoplasmic transmission of the d-factor in *O. ulmi* has also been found to be limited by vegetative incompatibility. Brasier (1984) has identified four different classes of vegetative incompatibility reactions in *O. ulmi* that can be distinguished phenotypically: wide (w) incompatibility reaction zones, a narrow (n) reaction zones, and two types referred to as line and line-gap (l/lg) reaction zones. The w reactions were quite restrictive of d-factor transmission while the n reactions allowed transmission about half of the time. Some of the weakest incompatibility reactions (l/lg) permitted d-factor transmission in 100% of the transmission tests. Genetic analysis indicated that the w reactions are caused by a single *w* locus, the n reactions may be caused by more than one other locus, and the lg reactions are caused by a single weak locus.

The horizontal transmission of plasmids and the effects of vegetative incompatibility upon plasmid transmission in fungi have been documented in several studies. Recently, Debets et al. (1994) tested the transmission barrier to plasmids imposed by *het-c*, *het-d*, *het-e*, and the mating type locus in *N. crassa*. The barrier created by *het-c* reduced transmission more than the other three loci. Mitochondrial plasmids in *Neurospora* have also been shown to transfer from one mitochondrial genotype to another at a high frequency during vegetatively incompatible mycelial interactions (Collins and Saville, 1990). This study also found a nonparental combination of nuclei and mitochondria in one conidium following an incompatible mycelial contact. Collins and Saville (1990) conclude that vegetative fusions may be an important source of mitochondrial genetic variation in natural populations. Circumstantial evidence for plasmid transmission in *Neurospora* in nature is also indicated by the association of plasmids with different mitochondrial DNA types, and by the occurrence of homologous plasmids in different species (Arganoza et al., 1994a; Yang and Griffiths, 1993). The horizontal transmission of the senescence plasmid *kalilo* has been observed in the laboratory between the species *N. intermedia* and *N. crassa* (Griffiths et al., 1990).

Horizontal transmission of viruses and genetic elements in *C. parasitica*

The horizontal (cytoplasmic) transmission of viruses and genetic elements has received more attention in *C. parasitica* than in any other fungus due to the biological control known as transmissible hypovirulence. French workers first discovered that the hypovirulence phenotype could be transmitted to virulent strains through hyphal fusions in the laboratory, and that virulent strains *in planta* could be converted to the hypovirulent phenotype upon contact with hypovirulent strains (Grent, 1965; Grent and Berthelay-Sauret, 1978). The spread of hypovirulence through the *C. parasitica* population in Italy is thought to have dramatically reduced the severity of the disease (see references in Heiniger and Rigling, 1994). Interest in the infectious spread of hypovirulence has not only been motivated by the widespread appearance of hypovirulence in Italy, but also by the localized presence of hypovirulence in North America (Fulbright et al. 1983; Griffin 1986; MacDonald and Fulbright 1991). This has provided the incentive for studies of the effects of vegetative incompatibility upon the horizontal transmission of dsRNA.

How important is vegetative incompatibility in restricting the horizontal transmission of viruses in *C. parasitica*? Two general observations can be made. First, dsRNA viruses are able to freely pass between vegetatively compatible strains by way of hyphal fusions. Secondly, some vegetative incompatibility reactions prevent horizontal transmission, but others do not. Results obtained from dsRNA virus transmission studies have commonly relied on pairing several strains of different compatibility types harboring dsRNA with a number of strains representing many compatibility types which do not harbor dsRNA. The results repeatedly have demonstrated that horizontal transmission is variable given a particular dsRNA-containing donor and various potential recipients of different vc types: some strains (vc types) will always become infected with dsRNA, other vc types will become infected at a reduced frequency, and still others will never become infected. This result has been observed many times (e.g. Anagnostakis and Day 1979; Anagnostakis 1983, 1984a; Kuhlman and Bhattacharyya 1984; Kuhlman et al. 1984).

Differences in infectivity were related by Anagnostakis (1983) to "strong" versus "weak" barrage reactions which were distinguished by whether or not pycnidia formed along the border of the reaction zone. Most weak barrage reactions permitted rapid transmission while strong barrages were associated with little or no infection. Recently, Liu and Milgroom (1996) have presented interesting evidence that the successive addition of heteroallelic *vic* loci presents an increasingly effective barrier to transmission. The weakness of this study is that the vc genotypes of the strains are not known so that differences in transmission cannot be definitively attributed to the cumulative effects of *vic* loci rather than the particular effects of individual *vic* loci. Horizontal transmission has also been found to occur in nontransitive steps (Anagnostakis, 1983; Kuhlman et al. 1984; Fulbright et al.,1988). Nontransitive transmission ocurs when strain A can infect strain B, and strain B can infect strain C, but strain A cannot (or infrequently) infect strain C. Anagnostakis (1983) and Fulbright et al. (1988) referred to this phenomenon as a transmission network and suggested that such networks could facilitate the movement of viruses through populations of incompatible strains.

Two other studies further characterized the relationship between vegetative incompatibility polymorphisms and susceptibility to dsRNA infection by advancing two concepts concerning horizontal transmission. First, Kuhlman and Bhattacharyya (1984) and Kuhlman et al. (1984) presented the concept of "broad conversion capacity" which refers to the ability of a particular dsRNA-containing strain to infect other strains in several vegetative compatibility groups. Secondly, these studies described "conversion groups" which highlighted the potential complex nontransitive nature of transmission in populations with several incompatibility genotypes. Conversion groups are composed of more than one vegetative compatibility genotype where dsRNA transmission readily occurs among the vc types of the conversion group but generally not (or infrequently) between vc types in different conversion groups. Nine different conversion groups were described using cluster analysis based upon frequency and rate of conversion (Kuhlman et al., 1984). Each of the nine conversion groups overlapped with at least one other conversion group; that is, certain strains were present in more than one group, thereby linking all nine groups together in a network (Kuhlman et al., 1984). Presumably, a dsRNA virus could enter any strain in this network of conversion groups and eventually become transmitted to all of the vegetative compatibility groups within the nine conversion groups. Whether this can occur in nature has not been tested.

The characterization of conversion groups also raised important conceptual and methodological issues. Kuhlman et al. (1984) suggested that vegetative compatibility groups are not discrete units but rather form a continuum. Apparently, this concept arises from the described conversion groups and from "multiple-merge" strains that they have identified. The multiple-merge strains were described as appearing to be vegetatively compatible (=merging) with strains from different compatibility groups. However, they describe their compatibility assays as being unreliable and inconsistent. In chapter 2, I have examined strains from some of their multiple-merge vc types and provide a different interpretation. Although the concept of conversion groups is sound (and provided with a genetic basis by this dissertation, Chapter 4), the Kuhlman et al. (1984) concept of a vegetative compatibility continuum based upon multiple-merge strains still lacks support.

The pathogen: *Cryphonectria parasitica*

Cryphonectria parasitica (Murr.) Barr is a filamentous ascomycete in the Diaporthales. The pathogen was originally described by Murrill (1906) and named *Diaportha parasitica* Murrill. Anderson and Anderson (1912) transferred the species to the genus *Endothia*. Barr (1978) produced a monograph on the Diaporthales of North America and transferred *Endothia parasitica* and four other species of *Endothia* to the genus *Cryphonectria*. Subsequent studies by Micales and Stipes (1987) concur with Barr, but Griffin et al. (1986) prefer the genus *Endothia*. In Barr's (1978) classification the genus *Endothia* is in the family Gnomoniaceae (subfamily Mamianioideae, tribe Endothieae) and *Cryphonectria* is placed in a separate family, Valsaceae (subfamily Valsoideae, tribe Diaportheae).

C. parasitica was introduced into the United States near the turn of the century, probably from China or Japan on imported chestnut trees (Anagnostakis, 1987). The first published report of the disease and the original description of the fungus as a new species were based on diseased American chestnut trees (*Castanea dentata*) within the New York Zoological Gardens (Merkel, 1906). The disease spread rapidly throughout the natural range of the American chestnut in eastern North America. Both natural agents and human activities are attributed to be responsible for the rapid dissemination which by 1945 included the entire natural range of the American chestnut (Griffin, 1986). This pathogen was also introduced into Europe and first noticed on European chestnut trees (*Castanea sativa*) in Italy in 1938 (Griffin, 1986). The extent and severity of the disease in *C. dentata* and *C. sativa* populations rank chestnut blight as one of the worst plant disease pandemics witnessed and caused by humans.

C. parasitica reproduces asexually through conidia (pycnidiospores) produced in pycnidia which develop in stroma. Sexual reproduction occurs through ascospores that are produced within perithecia embedded in the stroma. Dissemination of the conidia, which are extruded as sticky masses on tendrils, has been documented on insects, birds and mammals (Anagnostakis, 1987). Wind dispersal of the conidia is not thought to be significant. Ascospores are ejected from perithecia and may be wind dispersed.

Infection of the tree frequently occurs at the base of branches or at wound sites. The cankers that develop from virulent infections appear as depressed regions of bark where necrosis of the underlying tissues has resulted from mycelial ingress and expansion. The vascular cambium is killed by virulent strains of the fungus so that several cankers on a large tree or single cankers on small trees will girdle and kill the distal portions of the tree. *C. dentata* remains as a frequent component of the eastern deciduous forest in North America because epicormic sprouts develop when the above-ground portion of the tree dies. The sprouts are, in turn, infected by the fungus after several years, then die, and are replaced by new epicormic sprouts, repeating the cycle.

The mating system of *C. parasitica* includes both outcrossing and self-fertilization, and the unusual biological phenomenon of multiple paternity has also been demonstrated in the laboratory (Anagnostakis, 1982b). A study of the outcrossing rate by Milgroom et al. (1993) found that natural populations did exhibit a mixed mating system (both outcrossing and self-fertilization). A comparison of the genetic diversity of *C. parasitica* in China with the North American population using restriction fragment length polymorphisms showed greater genetic diversity in China as would be expected due to founder effects in the North American population (Milgroom et al., 1992). The genetic diversity of populations has also been examined using vegetative incompatibility groups. Vegetative compatibility diversity has been found to be high in some North American populations (MacDonald and Double, 1978; Anagnostakis and Kranz, 1987; Milgroom et al., 1991). However, Milgroom et al. (1993) found that the outcrossing rate suggested by molecular markers was much higher than that indicated by vegetative compatibility diversity. Other work has also shown that the vegetative compatibility diversity of a population may significantly under represent the resident genetic diversity (Liu and Milgroom, 1992). The frequency of vegetative compatibility groups has also been found to be skewed in two population surveys. Anagnostakis and Kranz (1987) and MacDonald and Double (1978) report observations of populations over several years where a single vc type was much more common than others. The diversity of vc groups has been found to be higher in Connecticut than in Europe (France, Corsica, and Italy) (Anagnostakis et al., 1986).

The chestnut blight disease is of particular interest to the study of host/parasite relationships because of the occurrence of horizontally transmissible hyperparasites that reduce fungal virulence. *C. parasitica* was first officially recorded in Europe (Italy) on

Castanea sativa by Biraghi in 1938 (Heiniger and Rigling, 1994). In 1951, Biraghi found superficial, healing cankers on *C. sativa*. Grente (1965) later isolated unusual strains of *C. parasitica* from healing cankers that were reduced in virulence, and when coinoculated with normal strains in trees, resulted in the development of nonlethal cankers. The term hypovirulence was coined by Grente (1965) to describe these strains. Day et al. (1977) found that the transmissible hypovirulence phenotype was associated with dsRNA. Proof that dsRNA caused hypovirulence was recently provided by Choi and Nuss (1992) who transformed *C. parasitica* with a full-length cDNA of hypovirus CHV1-713 which produced the associated hypovirulence traits and caused the reappearance of cytoplasmic dsRNA.

However, not all cases of hypovirulence can be attributed to dsRNA. Transmissible hypovirulence has also been found to be caused by unknown cytoplasmic factors (Fulbright, 1985; Mahanti et al. 1993; Huber et al., 1994). In the work by Fulbright and colleagues, hypovirulent strains collected in the field were able to transmit a hypovirulence phenotype to virulent strains but no dsRNA was detected. Some of these strains from nature have a senescence phenotype reminiscent of *Neurospora* senescence that is probably caused by the hypovirulence agent (Chapter 5). To test the possibility that the senescence agent causes mitochondrial dysfunction, Monteiro-Vitorello et al. (1995) induced mitochondrial mutations that debilitated the fungus, causing abnormal respiration and reducing virulence, and were horizontally transmissible. Curiously, strains infected with certain dsRNAs produce conidia bearing a nuclear mutation called *flat* that reduces virulence and segregates as a single locus (Anagnostakis, 1984b; personal observations).

Nuclei with the *flat* mutation are horizontally transmissible under laboratory conditions (personal observations).

The hyperparasites

All of the viruses and viruslike genetic elements described from *C. parasitica* thus far are composed of dsRNA. An excellent review of these dsRNA viruses was published by Nuss (1992). The recent characterization of three dsRNAs from *C. parasitica* that are associated with, or known to cause, hypovirulence has lead to the erection of a new virus family, the Hypoviridae (Hillman et al., 1995). This new family of viruses is interesting in several respects. The dsRNAs are unencapsidated but associated with pleomorphic membranous vesicles of host origin. The lack of a capsid precludes the dsRNAs from being infectious through extracellular routes as is typical for other viruses. Therefore hypoviruses must infect new hosts through an intracellular route, that is, through cytoplasmic contact between hosts. Fusions between hyphae of different fungal individuals provide the means for horizontal (infectious) transmission.

The dsRNA genome of hypoviruses is not segmented but internal deletions will produce defective replicating molecules of dsRNA that vary in size, number, and concentration (Shapira et al., 1991). The structural organization of hypoviruses includes a 3' poly(A) tract base paired to a 5' poly(U) tract at one end, and a consensus 28 nucleotide 3' terminal sequence (Nuss, 1992). The most thoroughly characterized of the hypoviruses is CHV1-713 which is of European origin. The L dsRNA of CHV1-713 is 12712 base pairs long, excluding the polyA-polyU domain (Choi and Nuss, 1992). The

L dsRNA contains two open reading frames, each of which encodes a polyprotein. Both polyproteins undergo proteolytic (autocatalytic) processing to produce the active protein species. Sequence similarities between several coding domains within the L-dsRNA and conserved motifs within potyvirus proteins include a putative RNA helicase domain and RNA-dependent RNA polymerase domain (Nuss, 1992). Hypovirus CHV3-GH2 from Michigan has been partially sequenced and found to have a large open reading frame with significant similarities to CHV1-713 (Durbahn, 1992; Smart and Fulbright, 1996). Although the hypoviruses are unlike conventional viruses in terms of lacking both a capsid and an extracellular infection capability, justification for considering them to be viruses is based upon their similarity to viral genomes in genetic organization and mode of expression (Nuss, 1992).

Viruses and dsRNA elements infecting *C. parasitica* are widespread in the native and naturalized range of the fungus although their frequency in fungal populations can vary widely. For example, Double et al. (1985) surveyed over 1000 isolates of *C. parasitica* from West Virginia and only found nine that contained detectable levels of dsRNA. But two other surveys which totaled 360 isolates from virulent cankers in West Virginia, Virginia, and Maryland found that 25% of the isolates contained the SR-2 dsRNA species (Likins, 1990; Sillick and MacDonald, 1988).

Hypoviruses constitute the best characterized and probably most frequent group of viruses that infect *C. parasitica*. Studies of the relationships among dsRNA viruses and elements in several regions of North America have revealed considerable diversity. A survey of dsRNAs from six localities in Michigan showed that hypovirus CHV3-GH2 hybridized to dsRNAs from five of the localities (Paul and Fulbright, 1988). However, dsRNA from Michigan isolate RC1 did not hybridize to other Michigan dsRNAs or to dsRNAs from West Virginia and Tennessee (Paul and Fulbright, 1988). Further North American geographical diversity is demonstrated by the lack of cross-hybridization between dsRNAs from fungal isolates from Maryland (SR-2), Pennsylvania (D2), and West Virginia (C-18) (Enebak et al., 1994b). European and North American dsRNAs were shown to be nonhomologous in hybridization studies by L'Hostis et al. (1985), although hypovirus CHV2-NB58 from New Jersey has been found to hybridize with two dsRNAs from Europe (Hillman et al., 1992). CHV2-NB58 may have originated from earlier releases of European hypovirulent strains in the same region (Hillman et al. 1992).

Recently new types of dsRNA viruses and elements have been found in *C. parasitica*. A hypovirulent West Virginia isolate (C-18) was found which contained a dsRNA similar to reoviruses (Enebak et al. 1994a). This dsRNA has 11 segments, at least seven of them are genetically unique, and it is associated with icosahedral particles in the mycelium. Another dsRNA element has been found in the mitochondria of a hypovirulent isolate from New Jersey (Polashock and Hillman, 1994). This dsRNA is small (2728-bp), lacks polyadenylated termini, is apparently unencapsidated, and is ancestrally related to the yeast cytoplasmic T and W dsRNAs rather than the Potyviridae.

What effects do dsRNA viruses and elements have upon their fungal host? Are the effects the result of a general debilitation, or are specific cellular functions afflicted? Although the causal connection between dsRNA and hypovirulence in *C. parasitica* has been established (Choi and Nuss, 1992), the mechanism by which virulence is reduced is not yet known. Infection with certain dsRNAs has been found to reduce fungal growth rate on synthetic media and live chestnut tissue, and to alter gross morphology on synthetic medium (Anagnostakis and Waggoner, 1981; Elliston, 1985; Hillman et al., 1990; Nuss, 1992). Each of these abnormalities may be related to reduced virulence.

Other phenotypic and molecular alterations due to dsRNA infection that may be ecologically important have also been found. In particular, the production of both conidia and ascospores can be reduced by some species of hypovirus (Elliston, 1985; Nuss, 1992). The first evidence for changes of gene expression in the fungal host was provided by Powell and Van Alfen (1987a, 1987b). Exciting recent work by Choi et al. (1995) has shown that the accumulation of a GTP-binding protein (G protein) is reduced in hypovirus-infected strains. This is significant because G proteins are important components of signal transduction pathways in eukaryotes, and their suppression could potentially broadly affect gene expression, hence pathogenesis, in the fungus. The reduction of several types of host molecules has also been demonstrated in hypovirus-infected, hypovirulent strains including pigment (Hillman et al., 1990), oxalate (Havir and Anagnostakis 1983; Vannini et al., 1993), extra and intracellular laccase (Choi et al. 1992; Rigling and Van Alfen 1993), a cell surface protein (Carpenter et al. 1992), cutinase (Varley et al. 1992), and a putative mating type pheromone (Zhang et al. 1993). The specific reduction of laccase is of particular interest because it has been implicated in lignin degradation and pathogenesis in other fungi (Nuss, 1992). Choi et al. (1992) have demonstrated the repression of laccase mRNA levels by hypovirus CHV1-713. Larson et al. (1992) have shown that dsRNA interferes with the cellular signalling processes that are necessary for laccase induction.

The effects of hypoviruses and dsRNA elements upon *C. parasitica* are not uniform however. Some viruses such as CHV3-GH2 reduce virulence but do not reduce sporulation, pigmentation, or laccase production (Durbahn 1992). Elliston (1978, 1985) has demonstrated that dsRNAs can also have quite varied effects upon fungal virulence, ranging from severely debilitating to benign. A recently described dsRNA element from the central Appalachians had no apparent effect upon its fungal host (Enebak et al, 1994b).

Vegetative incompatibility in fungi: function and consequences

The biological purpose and consequences of vegetative incompatibility systems in filamentous fungi have received attention in only a few species although vc groups have been frequently used as phenotypic markers in population studies. Several hypotheses have been proposed to explain the function and evolution of vegetative incompatibility in fungi. These will be briefly discussed.

I will refer to the first hypothesis as the parasite defense hypothesis. This idea was proposed by Caten (1972) who suggested that vegetative incompatibility responses evolved as a cellular defense reaction against cytoplasmic infection. Since fungal viruses and parasitic genetic elements require cytoplasmic contact between mycelia for transmission, any process that prevents or limits such contact will limit infection. Numerous studies with *C. parasitica* (reviewed above) and some work with *O. ulmi* have shown that vegetative incompatibility can limit the horizontal transmission of dsRNAs and the d-factor, respectively. Recent work with *Neurospora* has shown that vegetative incompatibility can limit the transmission of plasmids as well (Debets et al., 1994).

The second hypothesis is related to the first but sufficiently distinct to require separate treatment. I will call this hypothesis the somatic cell parasite hypothesis after Buss (1987). Cellular defense mechanisms might also have evolved to protect the genetic integrity of individuals against less fit, and thereby parasitic, somatic cells (or nuclei) rather than viruses or other genetic elements that cause reduced fitness. Buss (1987) has developed the idea that the evolution of cellular differentiation in multicellular organisms required a means for protecting the individual from harmful somatic cell variants that might arise. The coenocytic nature of many fungi further provides the opportunity for nuclei and mitochondria of less fit individuals to gain reproductive access. The situation with fungi is, by analogy at least, similar to that of the sedentary, colonial, marine metazoa. Buss (1987) considers the restriction of somatic cell parasitism by compatibility systems in organisms capable of fusion to be a control upon the units of selection. He writes:

> The coupling of historecognition with intraspecific competition strongly implies that the fusion/rejection loci of clonal invertebrates are genes which act to control the units of selection. Fusion results in a competition between cell lineages, and rejection results in competition between individuals. The decision to fuse or to reject is a decision to compete at the level of the cell or at the level of the individual. (p.150)

Two studies have attempted to model the effects of vegetative incompatibility upon limiting parasitic nuclear invasion. Hartl et al. (1975) modeled the situation where a parasitic nuclear gene is competitively superior in a heterokaryon, but less fit as a homokaryon. They showed that a parasitic nuclear gene could theoretically explain the evolution of two vc groups. A recent study by Nauta and Hoekstra (1994) considered whether protection from a parasitic nuclear gene could account for the evolution of numerous vc groups, as are found in nature. Nauta and Hoekstra found that the conditions necessary for the evolution of numerous vc groups are quite restrictive, suggesting that protection from somatic cell parasites may not provide a sufficient selective pressure. Nauta and Hoekstra (1994) also modeled the effects of parasitic cytoplasmic elements upon the evolution of vc group diversity and found that selection caused by cytoplasmic parasites may be more likely to favor the evolution of high numbers of vc groups although this model was still unsatisfactory.

Both of these models do not consider important aspects of the biology of vegetative incompatibility. In considering the selective pressure caused by less fit nuclear genes (nuclei), neither model addressed the situation where heterokaryons in some Ascomycetes such as *Gibberella fujikuroi* are restricted to the fused cells and do not proliferate, thereby eliminating or significantly limiting the possibility of somatic cell parasitism (Puhalla and Spieth, 1985). Concerning the model based upon cytoplasmic parasites, Nauta and Hoekstra note that they did not consider that some incompatibility barriers do not prevent viral infection, as is well documented in *C. parasitica*.

The third hypothesis suggests that intraspecific competition provides the selection pressure for the evolution of vc polymorphisms (Rayner 1991; Rayner et al., 1984). Individuals of sessile, colonial organisms with limited substrate, such as fungi (and also plants and some marine metazoans), are incapable of relocation to new substrate in the same manner as mobile organisms. In this case, vegetative incompatibility in fungi delimits the boundaries of the foraging space for a particular individual.

Pleiotropy could also explain the existence of vegetative incompatibility where the incompatibility genes have other primary cellular functions. For example, the numerous loci capable of initiating vegetative incompatibility reactions could represent mutations in genes which normally function as homo- or heteromultimeric proteins with other purposes (Deleu et al., 1993). The vegetative incompatibility function of the mating type genes in *Neurospora* is a good example of pleiotropy (Perkins, 1988). The interesting work by Bernet and colleagues (see references in Bernet 1992a, 1992b) has also provided evidence that some vegetative incompatibility genes have cellular functions other than intraspecific fusion and rejection responses, particularly in regard to the development of reproductive organs (reviewed above). As already mentioned, the work by Turq et al. (1991) addresses the question of pleiotropic effects (i.e. cell viability, reproductive organ development), but it cannot be evaluated because of the absence of data and methods. Surprisingly, none of the works with which I am familiar have considered the possibility that the genes involved in allelic and nonallelic vegetative incompatibility may have different primary functions (delimitation of conspecifics versus development, respectively) that converge upon a common programmed cell death response.

Esser (1971) and Esser and Blaich (1973, 1994) postulated that nonallelic vegetative incompatibility in *P. anserina* may exist to limit outcrossing by producing sterility barriers between strains. This has been criticized because *P. anserina* is pseudohomothallic (Bernet, 1992a). However, Jacobson (1995) has recently demonstrated that vegetative incompatibility specifically limited outcrossing in the pseudohomothallic species *N. tetrasperma*. Vegetative incompatibility has not been found to limit fertilization in ascomycetes such as *N. crassa* and *C. parasitica*.

Lastly, another possible function for vegetative incompatibility may be the maintenance of physiological or developmental independence (Rayner, 1991; Rayner et al., 1984). Two different mycelia growing on a common substrate could experience different microenvironmental conditions that require different responses. Developmental or physiological signals translocated through one mycelium in response to one environment may be inappropriate for the neighboring mycelium.

Dissertation Content

The questions addressed in this dissertation consider the genetic regulation of vegetative cell fusions and the concomitant effects of vegetative cell incompatibility upon the horizontal transmission of cytoplasmic genetic elements. Chapter 2 presents a new method for detecting vegetative incompatibility between strains of *C. parasitica* and applies this method to the evaluation of strains purportedly exhibiting compatibility with more than one vc genotype. Chapter 3 consists of a genetic analysis which identified three new *vic* loci: *vic3*, *vic4*, and *vic5*. This study also examined the relationship between vegetative incompatibility and heterokaryon formation using complementary pigmentation biosynthesis mutations. Chapter 4 examines the effects of individual *vic* genes upon the horizontal (cytoplasmic) transmission of two different dsRNA hypoviruses and an uncharacterized dsRNA element. Chapter 5 examines the effects of several *vic* genes upon the horizontal transmission of an unidentified senescence-inducing agent, and provides a preliminary physiological characterization of the senescence syndrome.

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Chapter 2

Detection of Vegetative Incompatibility in *Cryphonectria parasitica*

Abstract

Vegetative incompatibility in *Cryphonectria parasitica* is usually identified by barrage development, that is, a zone of visibly inhibited mycelial growth where conspecific mycelia make direct contact. The development of observable barrages between certain incompatible genotypes can be difficult to identify on the traditional assay medium, potato dextrose agar. This study has found that barrage testing conducted on autoclaved chestnut cortex/phloem tissue is more sensitive and reproducible than tests performed on potato dextrose agar. The new wood/agar vegetative compatibility (vc) assay permitted the identification of previously unrecognized nonparental vc types among the progeny of a sexual cross. Representative strains from two vc types reported to be compatible with several different vc types were found, instead, to be incompatible with these other vc types with the new assay. In addition, barrage development was detectable in strains infected with debilitating hypoviruses using the wood/agar vc assay.

Vegetative (heterokaryon) incompatibility in Ascomycetes is the condition whereby stable vegetative cell fusions cannot form between conspecifics due to the presence of particular alleles at any of several incompatibility loci (Glass and Kuldau, 1992; Leslie, 1993). The incompatibility reaction which limits heterokaryon formation occurs after hyphal fusion and generally results in either cell death or heterokaryons with abnormal growth. In allelic incompatibility systems, such as in *Neurospora crassa* and *Cryphonectria parasitica*, compatible vegetative cell fusions require both interacting individuals to be homoallelic at all vegetative incompatibility loci; heteroallelism at any of the incompatibility loci results in a rejection response. Nonallelic vegetative incompatibility systems, such as in *Podospora anserina*, render vegetative cell fusions incompatible when particular alleles are present at different loci. The detection of vegetative incompatibility has generally been accomplished with heterokaryon tests or barrage tests. Heterokaryon tests rely upon the complementation of mutations in compatible strains that results in a positive selection phenotype, and the absence of selected growth when complementation is prevented by incompatible cell fusions. In contrast, the barrage test detects incompatible vegetative cell fusions by the development of a zone of inhibited mycelial growth and dead cells (called a barrage) between two anastomosing mycelia, and can be done with wild type strains. The use of barrage tests to detect vegetative incompatibility depends upon the visible development of the incompatibility reaction.

In *Cryphonectria parasitica*, the chestnut blight pathogen, barrage tests have been relied upon to test for vegetative incompatibility and to identify vegetative compatibility (vc) groups. The customary method for barrage testing with *C. parasitica* utilizes potato dextrose agar (PDA) as the growth substrate and subjects paired cultures to four days of growth in the dark, followed by a 16 hour daily photoperiod for several more days (Anagnostakis 1977, 1984). Under these conditions, vegetatively incompatible strains develop a visible barrage zone that may be bordered by pycnidia where they make contact while compatible strains produce a confluent mycelium. While many workers have used this method (e.g. Anagnostakis,1977; Kuhlman et al., 1984), my experience has been that mycelial interactions are often ambiguous under these conditions, making interpretation of the interactions difficult. Atypical incompatibility reactions have also been noted in other studies. For example, compatibility groups have been described where certain member strains sometimes produce barrages among themselves (Kuhlman and Bhattacharyya, 1984). In another study, the term "multiple-merge" vc group was used for *C. parasitica* strains which were apparently vegetatively compatible with more than one vc group although the authors indicated that the incompatibility reactions were difficult to evaluate (Kuhlman et al., 1984). Barrage reactions have also been classified as "strong" versus "weak" to discriminate between obvious visible differences in the development of the reaction zone (Anagnostakis, 1983). I have found that the identification of the so-called weak incompatibility reactions can be especially problematic.

This work was undertaken in conjunction with a genetic analysis of vegetative incompatibility polymorphisms in *C. parasitica* in order to find a method for producing more distinctive and reproducible barrage reactions to facilitate identification of vegetative incompatibility genes. The enhancement of the detection of weak incompatibility reactions was of particular concern. The new technique was then applied to several instances of anomolous vegetative incompatibility reactions reported in the literature to see if these strains had unusual incompatibility phenotypes or if the anomolous incompatibility reactions had a methodological basis.

MATERIALS AND METHODS

Strains. *Cryphonectria parasitica* strains used in this study are presented in Table 1. Cryphonectria hypovirus CHV3-GH2 was originally obtained from a nonlethal canker on a chestnut tree in Grand Haven, Michigan (Fulbright et al, 1983). Cryphonectria hypovirus CHV1-713 was obtained from strain Ep713. Hypovirus CHV1-713 was transferred to strain 389.7 through three steps of transmission which were accomplished by pairing a donor (hypovirus infected) strain with a virus-free recipient on potato dextrose agar (PDA). First, Ep713 was used to infect strain Ep78; the resulting strain, Ep78(713) was then used to infect P1.9. Strain P1.9(713) was used to infect 389.7. Strain P1.9 is an ascosporic progeny from a cross between strains Ep155 and 80-2c. Hypovirus CHV3-GH2 was transmitted into strain 389.7 by first infecting Ep289 with CHV3-GH2 by pairing strain GH2 with Ep289. The resulting strain, Ep289(GH2), was then used to infect 389.7. Transfer of the viruses was confirmed by dsRNA extraction of the infected strains following the procedures of Morris and Dodds (1979) as modified by Fulbright et al. (1983).

Sexual crosses. The protoperithecial strain was grown on autoclaved chestnut stems about 6 cm long which were placed in disposable 15 mm x 100 mm Petri dishes with about 20 ml of molten 1.5% water agar (Sigma) to hold the stems in place. Prior to autoclaving, strips of bark were cut off the stems which exposed strips of cortex and phloem tissues alternating between strips of intact bark. Crosses were performed by spreading conidia as spermatia on the protoperithecial strain that had been growing on the stems for two to three weeks. The inoculated plates were kept in an incubator with an eight hour photoperiod at 22° C.

Ascospores were collected by carefully extracting intact perithecia which were embedded in mycelium beneath the bark using dissecting needles. Perithecia crushed or found to be oozing ascospores during removal were not used. The intact perithecia were then placed into a drop of sterile water on a microscope slide to wash off conidia which

Table 1. Strains of *Cryphonectria parasitica* used in this study.

Strains	Source[a]	Vegetative compatibility types [b]	Hypovirus present
389.7	I	vc 5	-
Ep243	II	vc 56	-
Ep289	II	vc 71	-
Ep78	ATCC 38752	vc 17	-
Ep155	ATCC 38755	vc 40	-
Ep713	ATCC 52571	vc 40	CHV1-713
80-2c	III	unnamed	
P1.9	Ep155 X 80-2c	recombinant	-
D1.29	389.7 X Ep243	recombinant	-
D3.31	389.7 X Ep243	recombinant	-
GH2	IV	unnamed	CHV3-GH2
Ep78(713)	this study	vc 17	CHV1-713
P1.9(713)	this study	recombinant	CHV1-713
Ep289(GH2)	this study	vc 71	CHV3-GH2
389.7(GH2)	this study	vc 5	CHV3-GH2
389.7(713)	this study	vc 5	CHV1-713
16-7-3	V	vc theta	-
9-11-2	V	vc Q	-
114	II	vc 7	-
157	II	vc 42	-
158	II	vc 43	-
159	II	vc 44	-

Strains	Source[a]	Vegetative compatibility types [b]	Hypovirus present
37	II	vc 19	-
241	II	vc 54	-

[a] Strains are from the following sources: I, UV mutagenized conidium from Ep389 described in chapter 3; II, Sandra Anagnostakis, Connecticut Agricultural Experiment Station; III, single conidium from strain 80-2 described in chapter 3; IV, Dennis Fulbright, Michigan State University; V, William MacDonald, West Virginia University.

[b] All of the vc types designated by a number represent the Connecticut system of vegetative compatibility grouping. Vc types theta and Q represent the West Virginia system of vegetative compatibility grouping. The recombinant (nonparental) vc types are progeny from either cross 389.7 X Ep243, or progeny from cross Ep155 X 80-2c. Strain 80-2c has not been given a vc type designation although the vc genotype has been described in chapter 3.

may have been sticking to the surface of the perithecia. This procedure was done under a dissecting microscope (40X). If many conidia were found to wash off of a perithecium, the perithecium was transferred to one or more additional drops of sterile water to wash off the majority of loose conidia. The washed perithecia were then transferred to a clean drop of water on a microscope slide and crushed with sterile dissecting needles under a dissecting scope (40X). The ascospores and asci that spilled from the ruptured perithecium were removed with a pipetman and spread with water on Petri plates of 1.5% water agar (Sigma) supplemented with nicotinamide at 0.002 mg/ml. Single ascospores were collected from the water agar soon after germination (24-36 hours after plating) using a dissecting scope (40X) and a finely beveled cutting tool. Ascospores germinate more quickly than conidia on water agar thereby insuring that ascospores could be distinguished from conidial contaminants. Germinated ascospores were subsequently transferred to PDA, generally eight per plate.

Barrage tests. Barrage tests were conducted on American chestnut stems harvested throughout the year. Cut stems were placed at 4° C with their bottom ends in water if they were not immediately prepared for barrage testing. The stems were cut transversely into sections about 1.5-2 cm long. The diameter of the stems varied from about 10-15 mm; the primary size limitation for the stems concerned the depth of the Petri dish. When stems were too thick to place in standard disposable 15 mm x 100 mm Petri dishes without coming into contact with the lid, they were bissected longitudinally. To prepare the stems for barrage testing, the outer layer of periderm and bark was carefully cut off with a sharp knife, leaving an inner layer of cortex and phloem still attached to the stem. The cuts were imprecise as to which tissue layers remained, although both cortex and phloem tissues remained attached to the stem to varying degrees. *C. parasitica* was found to grow well on the cortex and phloem tissues that occur between the secondary xylem and the outer bark, but grew poorly on the secondary xylem and the intact outer bark themselves. These small pieces of stem were autoclaved for twenty minutes at about 120° C. Eight pieces of autoclaved stem were then arranged in 1.5% molten water agar (Sigma) in disposable 15 mm x 100 mm Petri dishes with the exposed cortex/phloem surface sticking above the agar (Figure 1).

The development of barrages occurred on the exposed cortex/phloem surface of the stems. Pairs of fungal strains were placed on the agar at either end of the stem pieces so that the mycelia would grow and contact each other on the stem surface. It was useful to dip the transversely cut ends of the stems in the molten agar when embedding them because the fungus grows across the agar more quickly than across the secondary xylem surface. With this method, eight unknown strains could be assayed against a single tester strain in one petri dish. In order to insure that the opposing strains in a barrage test made contact on the surface of the wood, it was preferable to use strains that were at approximately the same age or stage of growth (i.e. active growth or stationary phase) so that neither strain overgrew the wood faster than the other. Every barrage test was performed at least twice. Barrage tests were conducted in plates subjected to three different light regimens. Plates were either placed under a daily photoperiod of 12 hours light, or four to six days of darkness followed by a 12 hour photoperiod, or total darkness. Cultures were kept at room temperature (about 25° C). Cultures subjected to light were placed under cool white flourescent lights (34 watts) at a distance of 25-30 cm. This method for detecting vegetative incompatibility will be called the wood/agar vc assay.

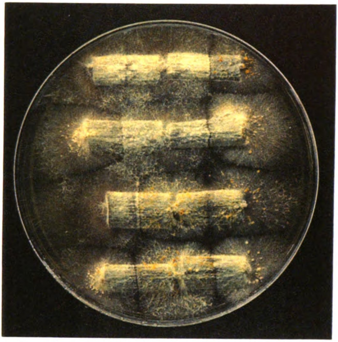
Barrage tests were also conducted on potato dextrose agar (PDA; Difco) as described by Anagnostakis (1977, 1984). Small cubes of PDA (2 mm^3) with mycelium were placed about 5-7 mm apart on PDA for the barrage tests. Inoculated plates were exposed to the same light regimens as described above and were kept at room temperature (about 25° C). A variation of this test was also used where red dye was added to the PDA (Rizwana and Powell, 1992; Kohn et al., 1990). Red dye (FD&C #40) and red food color (McCormick) were both used in separate experiments (35 drops per liter PDA) to test their enhancement of the visibility of barrage reactions.

RESULTS

Visible vegetative incompatibility reactions were found to be more discernible when strains made vegetative contact in the wood/agar vc assay rather than in the traditional PDA vc assay. The appearance of the barrages produced by the progeny from cross 389.7 X Ep243 were of two general types. A prominent type of barrage appeared in the wood/agar vc assay after five to seven days that consisted of a conspicuous gap between the mycelia at their zone of contact (Figure 1). When strains producing this prominent barrage were subjected to the PDA vc assay, a distinct region of thin mycelium without obvious pycnidia developed as a reaction zone where the mycelia made contact after about seven days. Following 1-2 weeks exposure to light, the barrage on PDA was often bordered by rows of pycnidia.

A second type of barrage was also present between some progeny when they made contact in the wood/agar vc assay. This barrage was morphologically like the first, but always appeared as a narrower gap (about 0.5 mm wide) between the confronting mycelia (Figure 1). In the PDA vc assay, this weak incompatibility reaction sometimes appeared as a subtle narrow gap in the development of pycnidia across the expanse of both mycelia, but did not appear as a break in the confluence of the two mycelia. Repeated pairings of weakly incompatible strains showed that these strains would sometimes appear to be compatible, that is, lack a barrage, when tested with the PDA vc assay. Following prolonged exposure to light (two months or more), pycnidia did not develop as a border along the weak type of incompatibility reaction on PDA as they did with the strong type of incompatibility reaction. The visibility of the weak incompatibility reactions was not enhanced by the addition of either of the red dyes to PDA.

The development of barrages in both the wood/agar vc assay and PDA vc assay was subject to modification by environmental conditions. Mycelial growth in the wood/agar vc assay showed some variability that may be attributable to differences in the chestnut tissue, but this is uncertain. In some cases, mycelial growth was thin and not vigorous. When this occurred, barrages were difficult to discern and the barrage test was rejected and repeated. Barrages developed in the wood/agar vc assay under all light conditions tested. However, the weakest barrages, particularly between strain 9-11-2 (vc type Q) and strain 241 (vc type 54) were most clearly produced in complete darkness. Barrages produced in the wood/agar vc assay were most discrete when the mycelium grew robustly, and were not enhanced by the development of rows of bordering pycnidia as occurs on



389.7	389.7	EP243
D3.31	389.7	D1.29
389.7	EP243	EP243
D3.31	EP243	D1.29

Figure 1. Barrage tests using the wood/agar vc assay between parental strains 389.7 and Ep243 and two progeny from the cross of these strains. The order of the strains in the plate is presented below the photo. Weak and prominent incompatibility reactions are present. The weak incompatibility reactions occur between strain pairs 389.7/D3.31 and Ep243/D1.29. The other incompatibility reactions are of the prominent type.

PDA.

The barrage tests using the PDA vc assay showed variable results. Cultures kept continuously in the dark were unusable for discerning incompatibility as previously reported (Anagnostakis, 1977). Cultures subjected to either a 12 hour photoperiod, or four to six days of darkness followed by several days of a 12 hour photoperiod, were relatively comparable to each other in the development and variability of the strong incompatibility reactions. The strong type of barrage became more evident on PDA when the mycelia produced relatively more pycnidia and less aerial mycelium. Barrages on PDA were also easier to observe with increasing time: one week or more of exposure to light produced the clearest barrage development. As stated above, the weak incompatibility reactions were sometimes (but not always) visible on PDA as a subtle decrease in pycnidia at the border of the confronting mycelia. Detection of this gap in the distribution of pycnidia along the contact zone was dependent upon the presence of environmental conditions that favored pycnidial development. Therefore, when environmental conditions were such that dense aerial mycelium developed rather than pycnidia, this weak barrage was not visible in the PDA vc assay.

The wood/agar vc assay demonstrated the presence of two additional vegetative compatibility types among the progeny of a cross between strains 389.7 (vc 5) and Ep243 (vc 56) that were previously missed in sexual crosses between these two compatibility types (Anagnostakis, 1988; Huber and Fulbright, 1994). The progeny from this cross consisted of the two parental vc types which formed conspicuous barrages with the opposite parental type, and two nonparental vc types, each of which was strongly incompatible with one parental type and weakly incompatible with the other (Table 2, Figure 1). Several replicates of barrage tests of the progeny using the PDA vc assay showed that the weak incompatibility reactions were visible in some tests but not in others. The previously published reports of crosses between these two vc types indicated that they differed at a single vegetative incompatibility (*vic*) locus. However, this new assay indicated that strains 389.7 and Ep243 were heteroallelic at two *vic* loci, one which produces a relatively prominent barrage and one which produces a very weak barrage.

Both the PDA vc assay and the new wood/agar vc assay were also applied to strains that represented two "multiple-merge" vc types (Q and theta) and the vc types with which they were reported to be compatible (Kuhlman et al., 1984). Because both vc types Q and theta were reported to be mutually compatible with vc type 54, all of the vc types apparently compatible with either Q or theta were tested in all combinations to look for other unusual compatibility reactions.

The wood/agar vc assay was found to resolve ten different pairings of strains from the two multiple-merge groups that the PDA vc assay typed as either compatible or equivocal (Tables 3 and 4). Vc type theta was previously reported by Kuhlman et al. (1984) as being compatible with vc types 7 and 54. I found that the PDA vc assay did show incompatibility between vc types theta and 54, but did not produce a clear barrage between theta and 7 (Table 3). However, the wood/agar vc assay produced clear barrages between these strains (Table 4). The barrage produced between vc theta and vc 7 was very narrow and similar in appearance to the weak barrages observed among some of the progeny from cross 389.7 x Ep243. Vc type Q was reported by Kuhlman et al. (1984) as being

Table 2. Vegetative compatibility types of the progeny from cross 389.7 X Ep243 based upon the wood/agar vc assay.

	parental vc types	
	389	Ep243
compatible progeny	14	11
weakly incompatible progeny*	15	16

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* A weak vegetative incompatibility reaction is characterized by a narrower barrage in the wood/agar vc assay. Each progeny that produced a weak incompatibility reaction with one parental vc type always produced a strong incompatibility reaction (broader barrage) with the other parental vc type. The progeny in each of these two nonparental vc types are compatible among themselves and strongly incompatible with the other nonparental vc type.

compatible with vc types 19, 42, 43, 44, and 54. I found that the PDA vc assay showed two of these combinations to be compatible and two to be equivocal (Table 3). In contrast, barrage tests using the wood/agar vc assay showed that vc type Q was incompatible with vc types 19, 42, 44, and 54 (Table 4). Vc type 54 was found to be very weakly incompatible with vc type Q such that visible barrage formation was dependent on environmental conditions: particularly weak (narrow) barrages were evident between these vc types when they were kept in total darkness, but barrages were not always visible when assays were conducted in light. Vc type Q and vc type 43 were found to be vegetatively compatible indicating that they represent a single vc type (Table 4).

Barrage tests were also performed on strains which contained hypoviruses CHV1-713 and CHV3-GH2 (Figure 2). Both of these viruses are known to cause altered growth of the fungal host. Incompatibility tests conducted with the wood/agar vc assay showed that barrages were still produced by the hypovirus-infected strains. The barrages produced by the virus-infected strains were sometimes broader in appearance and less discrete than those produced by virus-free strains (Figure 2), but still discernible. Occasionally particular subcultures would grow too thinly in the wood/agar vc assay for clear barrage development, but replicate tests were always found with more vigorous growth.

Table 3. Barrage tests of strains representing vegetative compatibility types Q, theta, and the vc types with which they had been reported to be compatible (i.e. "multiple-merge" response) by Kuhlman et al. (1984). Barrage tests were based upon the PDA vc assay.

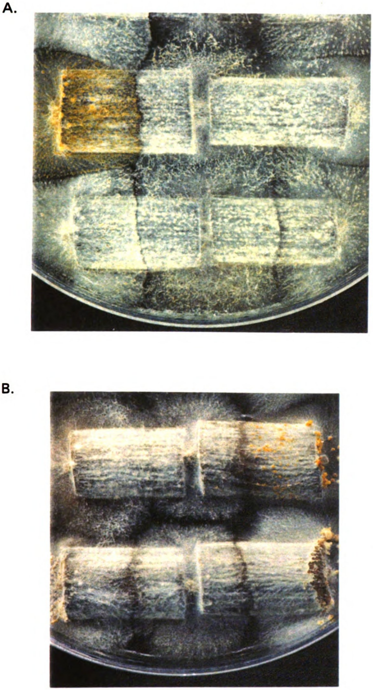
	241	159	158	157	114	37	16-7-3	9-11-2
9-11-2	+	±	+	±	-	±	-	+
16-7-3	-	-	-	-	±	-	+	
37	-	+	-	±	-	+		
114	-	-	-	-	+			
157	-	+	-	+				
158	±	-	+					
159	±	+						
241	+							

Note: + = compatible; - = incompatible; ± = results were equivocal because some replicate tests appeared compatible and others weakly incompatible.

Table 4. Barrage tests of strains representing vegetative compatibility types Q, theta, and the vc types with which they had been reported to be compatible (i.e. "multiple-merge" response) by Kuhlman et al. (1984). Barrage tests were based upon the wood/agar vc assay.

	241	159	158	157	114	37	16-7-3	9-11-2
9-11-2	-	-	+	-	-	-	-	+
16-7-3	-	-	-	-	-	-	+	
37	-	-	-	-	-	+		
114	-	-	-	-	+			
157	-	-	-	+				
158	-	-	+					
159	-	+						
241	+							

Note: + = compatible; - = incompatible



EP243 389.7(713) 389.7
D3.31 389.7(713) D1.29

389.7 389.7(GH2) EP243
D3.31 389.7(GH2) D1.29

Figure 2. Barrage tests of weak and strong incompatibility reactions using the wood/agar vc assay demonstrating the efficacy of the assay with virus-infected strains. The order of the strains in each plate is presented to the right of the photo. A. Barrage tests using strain 389.7(713). B. Barrage tests using strain 389.7(GH2).

DISCUSSION

This study has found that vegetative incompatibility polymorphisms in *C. parasitica* are more easily and reliably detected using the wood/agar vc assay rather than the traditional PDA vc assay. The new assay even detected a new vegetative incompatibility (*vic*) locus that was undetected in two previous studies. In addition, the new assay showed that strains representing vc types previously considered to be compatible with multiple vegetative compatibility types were found to be incompatible with these other types.

A genetic analysis of vegetative incompatibility found errors in two previous studies which analyzed the compatibility types of progeny from crosses between vc types 5 and 56 (Anagnostakis, 1988; Huber and Fulbright, 1994). These studies, which relied upon PDA as the growth substrate for the barrage tests, erroneously classified the progeny as exclusively parental vc types. When the progeny of this cross were subjected to the wood/agar vc assay, two nonparental vc types appeared. Each of these new recombinant vc types produced a weak type of incompatibility reaction with one of the parental types, rendering it difficult to detect and giving the appearance of only two classes of progeny using the PDA vc assay. Weak vegetative incompatibility reactions in *C. parasitica* have been previously identified by the absence of pycnidia bordering the barrage (Anagnostakis, 1983, 1988). The class of weak incompatibility reactions identified in this study using the wood/agar vc assay not only lacks pycnidia production, but also typically does not produce a distinct zone of inhibited mycelial growth on PDA as is characteristic of barrages.

Differences in the visible manifestations of vegetative incompatibility reactions have also been observed in other Ascomycete species. For example, four morphological types of barrage-like reactions have been described in *Ophiostoma ulmi* that differ in the extent to which the mycelium is affected (Brasier, 1984). These morphological differences in the incompatibility reactions in *O. ulmi* have been attributed to the effects of different vegetative incompatibility genes (Brasier, 1984).

Strains of *C. parasitica* that were apparently vegetatively compatible with several different vc types have been reported by Kuhlman et al. (1984). These strains were referred to as having a "multiple-merge" capability which refers to their failure to develop barrages with mutually incompatible strains. A reevaluation of the incompatibility relationships among strains representing these vc types using the PDA vc assay demonstrated several instances of either compatible or equivocal interactions. However, repeating these tests with the new wood/agar vc assay demonstrated that the equivocal incompatibility reactions, and three cases of compatible reactions, were actually weakly incompatible. The weakest incompatibility reaction (i.e. least prominent barrage) occurred between strains in vc types 54 and Q. The barrage produced between these strains was consistently observed when they were grown on chestnut tissue without light; but when grown with light, the visible barrage was sometimes not present. These analyses show that the weakest incompatibility reactions are the most easily obscured by environmental conditions. The observations by Kuhlman and Bhattacharyya (1984) that replications of incompatibility tests between certain strains did not consistently show incompatibility are similar to my observations of weak interactions. Proffer and Hart (1988) have also observed the mutiple-merge phenomenon among strains of *Leucocytospora kunzei*, but they did not report attempts to vary the conditions under which incompatibility was observed.

Are the vegetative incompatibility groups in *C. parasitica* discrete or do nontransitive effects mitigate incompatibility? Kuhlman et al. (1984) concluded that the existence of multiple-merge vc types demonstrates that the vegetative incompatibility system in *C. parasitica* does not produce discrete incompatibility groups, but instead produces a continuum of overlapping vc groups. My evaluation of strains from the putative multiple-merge vc types Q and theta does not support this concept. However, the suppression of specific vegetative incompatibility genes is conceivable as a consequence of suppressor mutations such as those recently found in *Neurospora crassa* that prevent vegetative incompatibility between certain incompatibility genotypes (Arganoza et al., 1994). The presence of similar mutations in natural populations of *C. parasitica* is unknown but a formal possibility. Some of the strains used in the studies by Kuhlman and Bhattacharrya (1984) and Kuhlman et al. (1984) could carry such mutations. It should also be noted that the strains used in this study representing vc types Q and theta are not the same strains used by Kuhlman et al. (1984). Therefore, it is possible that differences exist between individual strains within vc types Q and theta. In conclusion, the published reports of the multiple-merge phenomenon and the failure to consistently detect barrages between strains may be the result of two simultaneously confounding factors: differences between strains at weak incompatibility loci, and environmentally-induced inconsistencies in growth. A reevaluation of the other strains which fit the multiple-merge phenomenon is needed before the concept of nondiscrete vegetative compatibility types is accepted. The possibility of suppressor mutations should also be considered.

The infection of fungal strains with dsRNA viruses can also contribute to the apparent nondiscrete quality of some vegetative compatibility types in two ways. First, viral infection can significantly affect the growth and morphology of the mycelium, thereby obscuring barrage development on PDA (Anagnostakis, 1977). However, this problem was found to be diminished with the new wood/agar vc assay. Secondly, the horizontal transmission of viruses between different vegetative compatibility types may approximate a continuum due to nontransitive transmission effects. Here, the horizontal transmission of viruses needs to be kept conceptually distinct from vegetative incompatibility. While virus transmission is inhibited by some *vic* loci and modifiable by epistatic interactions between *vic* genes, it has not been found to be inhibited between all vegetative incompatibility polymorphisms (Anagnostakis, 1983; Fulbright et al., 1988; this dissertation).

The development of visible vegetative incompatibility reactions as a function of the growth environment is not unique to *Cryphonectria parasitica*. Vegetative incompatibility tests for *Ophiostoma ulmi* are conducted on elm sapwood agar medium because some types of incompatibility reactions cannot be observed on other media, such as potato dextrose agar, carrot agar, and a malt extract medium (Brasier, 1984). Croft and Dales (1984) have reported that heterokaryon incompatibility sometimes occurs in *Aspergillus* without any apparent mycelial reaction. Brasier (1984) has suggested that this situation with *Aspergillus* may also be due to the type of medium used. Visible vegetative incompatibility reactions are also not present between incompatible strains of *Cochliobolus heterostrophus* when grown on an agar medium (Leach and Yoder, 1983). Previous work on *C. parasitica* has demonstrated that the light regimen can influence barrage development (Anagnostakis, 1977).

The influence of the environment upon the development of visible barrages does not imply that the vegetative incompatibility reactions themselves are different at the cellular level. Barrages are a visible phenotype, produced in response to the cell death which occurs between fused incompatible cells, that would depend upon the degree of hyphal contact between confronting mycelia. Less contact between mycelia would provide less opportunity for hyphal fusions and, presumably, correspondingly fewer cells involved in the incompatibility response. The frequency of hyphal fusions has been shown to be affected by growth substrate in the Basidiomycete *Corticium vellereum* (Bourchier, 1957). It has not been tested whether the frequency of hyphal fusions is affected by growth substrate in *C. parasitica*. Barrage development on the chestnut cortex/phloem tissue may be more pronounced because of denser mycelial growth, alterations in the frequency of hyphal fusions, or unknown factors. An environmental influence upon the physiology of the incompatibility reaction itself has not been investigated to my knowledge.

Since the detection of weak vegetative incompatibility reactions in *C. parasitica* using the PDA vc assay has been found to be unreliable, the use of the wood/agar vc assay is recommended for vegetative incompatibility testing.

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Chapter 3

Vegetative Incompatibility Polymorphisms in *Cryphonectria parasitica*: new *vic* loci and heterokaryon formation.

Abstract

The genetic basis of vegetative incompatibility polymorphisms and their effects upon heterokaryon formation were examined in the chestnut blight pathogen, *Cryphonectria parasitica*. Three new vegetative incompatibility (*vic*) loci, designated *vic3*, *vic4*, and *vic5*, were identified; two alleles were found at each of the three new *vic* loci. These three loci, like *vic1* and *vic2*, function in an allelic manner: compatibility requires that each *vic* locus be homoallelic, whereas heteroallelism at any *vic* locus produces an incompatible reaction. Vegetatively compatible strains were found to form heterokaryons when grown under nonselective conditions on PDA and in the wood/agar vc assay. Two pigmentation mutations, *cre* (cream) and *br* (brown), were found to be tightly linked and to complement and form the wild type orange color when they occurred as heterokaryons or as meiotic recombinants. Hyphal tips subcultured from heterokaryons carried both nuclear types, and conidia consisted of either single nuclear type. Nuclear ratios in the hyphal tips from the heterokaryons varied, and corresponded to variations in mycelial color ranging from orange to orange/brown to brown. Heterokaryons were prevented from forming between strains heteroallelic at *vic1*, *vic2*, *vic3*, or *vic4*. Heteroallelism at *vic4* is occasionally associated with abnormal, flat sectors between anastomosing mycelia. Heteroallelism at *vic5* prevented heterokaryon formation in the wood/agar vc assay, but morphologically abnormal orange mycelium frequently formed between strains grown on PDA. The effects of *vic1*, *vic2*, and *vic3* upon heterokaryon formation were epistatic over the less restrictive effects of *vic4* and *vic5*. The effect of heteroallelism at both *vic4* and *vic5* was considered additive since no abnormal sectors appeared between contacting mycelia.

Filamentous Ascomycetes exhibit the ability to discriminate between conspecifics when they differ from each other at specific genetic loci (Glass and Kuldau, 1992; Leslie, 1993). Vegetative cell fusions between genetically compatible conspecifics may permit the formation of heterokaryons and heteroplasmons whereas genetic incompatibility will limit the formation of these chimeric genetic states. The vegetative or heterokaryon incompatibility systems in Ascomycetes which mediate these cellular acceptance/rejection responses have been found to be controlled by numerous genes. Both allelic and nonallelic gene interactions have been found that initiate incompatibility reactions. Allelic recognition systems, such as in *Neurospora crassa* and *Cryphonectria parasitica*, require homoallelism at each of the compatibility loci to produce compatible vegetative cell fusions; incompatible cellular fusions follow from heteroallelism at any of these loci. Nonallelic vegetative incompatibility, such as in *Podospora anserina*, occurs when particular alleles at different loci interact.

Genetic analyses of vegetative incompatibility in Ascomycetes have shown that numerous genetic loci typically control the elicitation of the incompatibility reaction. In *N. crassa* ten *het* (heterokaryon incompatibility) loci have been identified (Perkins, 1988); in *Aspergillus nidulans* eight *het* loci have been found (Anwar et al., 1993). In natural populations of *Podospora anserina*, five incompatibility loci that cause allelic incompatibility, and five that cause nonallelic incompatibility, have been identified; one of these loci is involved in both incompatibility systems (Bernet, 1992). Previous studies of *C. parasitica* have only identified and named two *vic* loci, although estimates that at least seven *vic* loci exist have been made based upon the number of nonparental vc types that have segregated from sexual crosses (Anagnostakis, 1982).

What is the relationship between vegetative cell incompatibility and the maintenance of individual genetic integrity in filamentous Ascomycetes? Fusions between vegetative cells allow the opportunity for protoplasmic contents to move from one individual (genotype) to another. Organelles and genetic elements that have been observed to move through vegetative hyphal fusions include nuclei (Beadle and Coonradt, 1944), mitochondrial chromosomes (Collins and Saville, 1990; Mahanti and Fulbright, 1995), plasmids (Griffiths et al., 1990), and viruses (Day et al. 1977; Buck, 1986; Nuss and Koltin, 1990). The effects of vegetative incompatibility upon the horizontal transmission of nuclei and cytoplasmic genetic elements are generally restrictive although many interesting exceptions have been found that indicate that incompatibility has quite variable effects upon transmission. For example, under laboratory conditions evidence indicates that individual incompatibility genes can have different effects upon the horizontal transmission of nuclei (Pittenger and Brawner, 1961), plasmids (Debets et al., 1994), viruses (Anagnostakis, 1983; Brasier, 1984) and other genetic elements (Handley and Caten, 1973). The effects of vegetative incompatibility upon cytoplasmic transmission are of particular interest in *Cryphonectria parasitica* (Murr.) Barr., the chestnut blight pathogen, where several different types of transmissible genetic elements capable of reducing fungal virulence have been found (Nuss, 1992; Monteiro-Vitorello et al, 1995; this study).

Cryphonectria parasitica (Ascomycota, Diaporthales) was introduced into North America near the turn of the century and subsequently infectiously spread throughout the *Castanea dentata* (American chestnut) population in about four decades (Anagnostakis, 1987; Griffin, 1986). The blight has also widely infected *Castanea sativa* in Europe (Heiniger and Rigling, 1994). Subsequently, a natural form of biological control which reduces fungal virulence has appeared on both continents. The primary agents known to be responsible for reducing fungal aggressiveness are cytoplasmically transmissible dsRNA viruses (hypoviruses). These viruses have spread through much of the *C. parasitica* population in Europe but are only locally present in the North American population (MacDonald and Fulbright, 1991; Heiniger and Rigling, 1994). The lack of infectious spread of hypoviruses in the North American population of *C. parasitica* has been attributed to the high number of resident vegetative incompatibility polymorphisms which have been thought to significantly restrict cytoplasmic transmission (Anagnostakis et al., 1986). However, confounding the attribution of reduced virus infectivity to vegetative incompatibility is the variability in transmission efficiency found among the many vegetative incompatibility polymorphisms in the North American population. This suggests that an understanding of the effects of vegetative incompatibility upon horizontal transmission depends upon understanding the effects of individual incompatibility genes.

Before the effects of vegetative incompatibility upon genotype integrity could be fully examined, it was imperative to identify all heteroallelic *vic* loci in each strain. Therefore, I sought to identify additional *vic* loci in *C. parasitica* for the purpose of studying their individual contributions to the maintenance of genotype integrity. In pursuit of this objective, I have been able to identify and name three new *vic* loci, and to examine the relationship between vegetative incompatibility and heterokaryon formation.

MATERIALS AND METHODS

***C. parasitica* strains and culture conditions.** The strains of *C. parasitica* used in this study, including their genetic markers and sources are listed in Table 1. Strain 80-2c is a single conidial isolate derived from strain 80-2. Strain 80-2 was collected from the field in West Virginia by W. L. MacDonald (West Virginia University) and has an irregular phenotype that varies from a flat morphology to abundant aerial mycelium. This strain has a variable color ranging from brown to white, often showing a blotchy appearance. It also harbors an uncharacterized dsRNA that probably originated from a dsRNA of Italian origin (Euro 7) used in field studies in West Virginia (W. L. MacDonald, personal communication). Strain 80-2c lacks dsRNA, has a normal growth phenotype, and has a dark brown pigmentation mutation (*br*). All *C. parasitica* strains were grown and stored on potato dextrose agar (PDA; Difco, Detroit, MI) at 4° C. Actively growing cultures were kept under cool white flourescent lights (34 watt) at a distance of 25-30 cm at room temperature (25° C). Long term storage was accomplished using the silica gel method used for Neurospora (Perkins, 1977).

Sexual crosses. The protoperithecial strain was grown on autoclaved chestnut stems about 6 cm long which were placed in disposable 15 mm x 100 mm Petri dishes with about 20 ml of molten 1.5% water agar (Sigma) to hold the stems in place. Prior to autoclaving, strips of bark were cut off the stems to expose strips of cortex and phloem tissues alternating between strips of intact bark. Crosses were performed by spreading conidia as spermatia on the protoperithecial strain that had been growing for two to three weeks. The inoculated plates were kept in an incubator with an eight hour photoperiod at 22° C.

Perithecia developed most abundantly along the margin of intact bark and exposed cortex/phloem tissues. The time between application of the conidia (spermatia) and the appearance of perithecia was quite variable and ranged from three weeks to more than eight weeks.

Ascospores were collected by carefully extracting intact perithecia which were embedded in mycelium beneath the bark using dissecting needles. Perithecia crushed or found to be oozing ascospores during removal were not used. The intact perithecia were then placed into a drop of sterile water on a microscope slide to wash off conidia which may have been sticking to the surface of the perithecia. This procedure was done under a dissecting microscope (40X). If many conidia were found to wash off of a perithecium, the perithecium was transferred to one or more additional drops of sterile water to wash off the majority of loose conidia. The washed perithecia were then transferred to a clean drop of water on a microscope slide and crushed with sterile dissecting needles under a dissecting scope (40X). The ascospores and asci that spilled from the ruptured perithecium were removed with a pipetman and spread with water on Petri plates of 1.5% water agar (Sigma) or 1.5% noble agar (Difco; Detroit, MI). The water agar or noble agar were appropriately supplemented with growth nutrients when the progeny carried any of the auxotrophic mutations. Methionine was added to the noble agar at 0.1 mg/ml, and nicotinamide was added at 0.002 mg/ml. Single ascospores were collected from the agar soon after germination (24-36 hours after plating) using a dissecting scope (40X) and a finely beveled cutting tool. Ascospores germinate more quickly than conidia on water agar thereby insuring that ascospores could be distinguished from conidial contaminants.

Germinated ascospores were subsequently transferred to PDA, generally eight per plate.

Mutagenesis. Conidia from strain EP389 were subjected to UV mutagenesis to obtain an auxotrophic mutation for use as an additional genetic marker. Conidia were collected from EP389 and $2x10^8$ conidia were resuspended in 20 ml of sterile, distilled water in a 15 X 100 mm plastic disposable Petri dish. The conidia were exposed to 254 nm UV-light which was held 12 cm above the petri dish. During mutagenesis, conidia were kept suspended in the water with a stir bar. A red light was used for viewing the procedures in the dark. Conidia were exposed to UV light for 160 seconds which produced a 90% kill. The mutagenized conidia were then diluted and spread on plates of PDA (about 100 per plate) amended with sodium desoxycholate (50 mg/ml) to reduce colony size. Individual colonies which appeared after one week were transferred to a separate Petri plate containing water agar. Colonies which failed to grow on water agar were subsequently tested for specific nutritional requirements on water agar supplemented with various amino acids and vitamins following the method of Holliday (1956). A new strain designated 389.7 was thereby created that carried a nicotinamide requirement (*nic*) that segregated as a single locus (Tables 1 and 9).

Heterokaryon determination. When vegetatively compatible brown colored (*br*) and cream colored (*cre*) progeny derived from cross 389.7 *cre* X 80-2c *br* grew in contact with each other, orange color developed at the confluence of the two mycelia. To determine if this orange mycelium was a heterokaryon composed of the two different mutant nuclei, hyphal tips were collected from subcultures of the orange mycelium that had been placed upon water agar supplemented with methionine (0.1 mg/ml) or nicotinamide (0.002

mg/ml) as needed and allowed to grow for one to two weeks. Hyphal tips about 1 mm long were cut off along with small blocks of agar and placed on PDA. The cultures were kept under lights as described above which enhanced conidiation. Conidia from the hyphal tip cultures were collected with a sterile loop, resuspended in sterile water, and spread on PDA. Individual conidia were collected soon after germination (24-30 hours after being spread on PDA) and subcultured onto PDA (eight per plate). The conidial cultures were kept under cool white fluorescent lights at room temperature (25° C) until their color developed.

Vegetative incompatibility determination. Barrage tests were performed using the wood/agar vc assay described in chapter 2 rather than the traditional PDA vc assay described by Anagnostakis (1977, 1988). Heterokaryon determination was conducted under nonselective conditions on PDA which relied upon the complementation of *cre* and *br* color mutations. Vegetatively compatible *cre* and *br* strains developed orange colored mycelium at the confluence of the mycelia. Subcultures of cream and brown colored strains were placed about 5mm apart near one edge of a PDA plate and placed under cool white flourescent lights as described above. These strains grew across the PDA in continuous contact with each other. After 7 to 10 days, the color of the strains had developed sufficiently to clearly detect the orange color if present. Vegetatively incompatible *cre* and *br* strains did not develop orange mycelium when they made contact. When scoring the *vic* genotype of ascosporic progeny, up to eight pairings of unknown strains with tester strains were performed in one Petri plate. This was accomplished by pairing a subculture of an unknown strain about 5 mm from a subculture of a tester strain.

Table 1. Strains used in this study.

Strain	Relevant Genotype[a]	Source[b]
EP389	*vic1-1, 2-1, 3-1, 4-1, 5-1, cre, MAT1-1*	ATCC 38980
389.7	*vic1-1, 2-1, 3-1, 4-1, 5-1, cre, nic, MAT1-1*	UV mutagenesis of EP389
EP388	*vic1-2, 2-1, 3-1, 4-1, 5-1, met, MAT1-2*	ATCC 38979
EP289	*vic1-1, 2-2, 3-1, 4-1, 5-1, met, MAT1-2*	Conn. Agri. Exp. Sta.[c]
22508	*vic1-2, 2-2, 3-1, 4-1, 5-1, met, MAT1-2*	ATCC 22508
EP243	*vic1-1, 2-1, 3-1, 4-2, 5-2, MAT1-2*	Conn. Agri. Exp. Sta.[c]
EP29	*vic1-2, 2-2, 3-2, 4-1, 5-2, MAT1-1, MAT1-2*[d]	ATCC 38754
EP155	*MAT1-2*	ATCC 38755
80-2c	*vic1-2, 2-1, 3-1, 4-2, 5-2, br, MAT1-2*	conidium of 80-2[c]
A1.8	*vic1-2, 2-2, 3-1, 4-1, 5-1, cre, met*	389.7 X 22508
A1.10	*vic1-2, 2-1, 3-1, 4-1, 5-1, cre, nic*	389.7 X 22508
A1.13	*vic1-1, 2-2, 3-1, 4-1, 5-1, cre, met*	389.7 X 22508
F2.4	*vic1-1, 2-1, 3-1, 4-1, 5-2, cre, nic*	389.7 X 80-2c
F2.13	*vic1-2, 2-1, 3-1, 4-1, 5-2, cre, nic*	389.7 X 80-2c
F2.14	*vic1-2, 2-1, 3-1, 4-2, 5-2, cre, nic*	389.7 X 80-2c
F2.36	*vic1-1, 2-1, 3-1, 4-1, 5-1, br*	389.7 X 80-2c
F3.2	*vic1-1, 2-1, 3-1, 4-2, 5-1, cre*	389.7 X 80-2c
F3.4	*vic1-2, 2-1, 3-1, 4-1, 5-1, br, nic*	389.7 X 80-2c
F3.10	*vic1-1, 2-1, 3-1, 4-1, 5-2, br, nic*	389.7 X 80-2c
F3.12	*vic1-1, 2-1, 3-1, 4-2, 5-1, cre, nic*	389.7 X 80-2c
F3.13	*vic1-2, 2-1, 3-1, 4-2, 5-1, cre*	389.7 X 80-2c
F3.15	*vic1-2, 2-1, 3-1, 4-1, 5-2, br, nic*	389.7 X 80-2c
F3.16	*vic1-2, 2-1, 3-1, 4-2, 5-1, br, nic*	389.7 X 80-2c
F3.21	*vic1-1, 2-1, 3-1, 4-2, 5-2, cre, nic*	389.7 X 80-2c
F3.26	*vic1-1, 2-1, 3-1, 4-2, 5-1, br, nic*	389.7 X 80-2c
F3.39	*vic1-1, 2-1, 3-1, 4-2, 5-2, br, nic*	389.7 X 80-2c
F4.9	*vic1-1, 2-1, 3-1, 4-1, 5-2, br, MAT1-2*	389.7 X 80-2c
F4.13	*vic1-1, 2-1, 3-1, 4-1, 5-1, br, MAT1-2*	389.7 X 80-2c
J2.6	*vic1-1, 2-2, 3-1, 4-2, 5-1, cre, nic*	F3.16 X A1.13

Table 1 (cont'd).

J2.20	*vic1-2, 2-2, 3-1, 4-2, 5-1, cre, nic*	F3.16 X A1.13
J2.23	*vic1-2, 2-2, 3-1, 4-2, 5-1, br, nic*	F3.16 X A1.13
J2.34	*vic1-1, 2-2, 3-1, 4-2, 5-1, br, met*	F3.16 X A1.13
J2.43	*vic1-1, 2-2, 3-1, 4-1, 5-1, br*	F3.16 X A1.13
J2.57	*vic1-2, 2-2, 3-1, 4-1, 5-1, br, met, nic*	F3.16 X A1.13
J2.69	*vic1-1, 2-2, 3-1, 4-1, 5-1, br, nic*	F3.16 X A1.13
K1.9	*nic*[e]	EP29 X A1.10
K1.32	*cre, nic*[f]	EP29 X A1.10
K1.43	*vic1-2, 2-1, 3-2, 4-1, 5-1, cre*	EP29 X A1.10
K2.30	*vic1-2, 2-2, 3-2, 4-1, 5-1, cre*	EP29 X A1.10
K2.39	*cre*[f]	EP29 X A1.10
L1.6	*vic1-2, 2-2, 3-1, 4-2, 5-1, cre*	J2.23 X K2.30
L1.8	*vic1-2, 2-2, 3-2, 4-1, 5-1, br, nic*	J2.23 X K2.30
L1.17	*vic1-2, 2-2, 3-2, 4-2, 5-1, cre*	J2.23 X K2.30
L1.39	*vic1-2, 2-2, 3-2, 4-2, 5-1, br, nic*	J2.23 X K2.30
M1.5	*vic1-1, 2-2, 3-2, 4-1, 5-1, br, nic*	K2.30 X J2.69
M1.6	*vic1-1, 2-2, 3-2, 4-1, 5-1, cre*	K2.30 X J2.69
M1.21	*vic1-2, 2-2, 3-2, 4-1, 5-1, br*	K2.30 X J2.69
N1.9	*vic1-1, 2-1, 3-2, 4-1, 5-1, cre*	F4.13 X K1.43
N1.19	*vic1-1, 2-1, 3-2, 4-1, 5-1, br*	F4.13 X K1.43
N1.39	*vic1-2, 2-1, 3-2, 4-1, 5-1, br*	F4.13 X K1.43

[a] For brevity vegetative incompatibility genotypes have been written as *vic1-1,2-1* rather than as *vic1-1*, *vic2-1*.

[b] The sexual cross is indicated from which each strain is derived. Other sources of strains are also indicated.

[c] Strains EP289 and EP243 provided by S. L. Anagnostakis, Connecticut Agricultural Experiment Station; strain 80-2 provided by William MacDonald, West Virginia University.

[d] The vc genotype for EP29 is incomplete. Strain EP29 is heteroallelic at additional weak *vic* loci relative to the other strains in this study. See the results section for an explanation. EP29 has been found to be heterokaryotic with respect to the mating type locus (Anagnostakis, 1981).

[e] The vc genotype of K1.9 is unknown, but preliminary analysis suggests that it carries alleles *vic1-2*, *vic2-2*, *vic3-1*, and *vic4-1*.

[f] The vc genotypes of K1.32 and K2.39 are not known, but preliminary analysis suggests that they carry alleles *vic1-2*, *vic2-1*, *vic3-2*, and *vic4-1*.

A maximum of eight pairings were evenly placed around the perimeter of the Petri plate which permitted the paired *cre* and *br* strains to grow in contact with each other toward the center of the plate and provided ample growth to observe the formation of orange heterokaryons.

Results

Heterokaryon formation. Strains 389.7 *cre nic* and 80-2c *br* were crossed and ascosporic progeny were collected. To determine vc reactions among these sexual progeny, the ascosporic cultures were paired together using the PDA vc assay. Pairings between some cream and brown progeny showed the development of wildtype orange color where the mycelia made contact, indicating the possibility that complementation of genetic markers *cre* and *br* had occurred. To determine whether the orange mycelium was a heterokaryon, ten hyphal tip subcultures were collected from orange mycelium formed at the confluence of the 389.7 *cre nic* parent and F2.36 *br* progeny (Figure 1). Colonies grown from the hyphal tips varied in color from orange to orange-brown to brown. Conidia were collected from colonies representing the three color types (Table 2). Hyphal-tip colonies that produced orange and orange-brown mycelium yielded conidia that produced both cream and brown mycelia, demonstrating that the orange sector was composed of hyphae containing nuclei of each parental isolate, and thereby proving the heterokaryotic nature of the orange sector. This also demonstrated that the cream color mutation (*cre*) and the brown color mutation (*br*) are able to complement each other in a heterokaryon, permitting the development of wild type orange color.

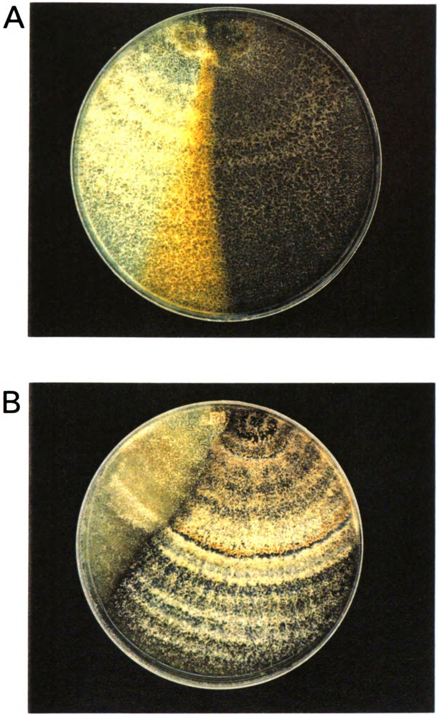

Figure 1. Heterokaryon formation between strains 389.7 *cre* and F2.36 *br* homoallelic at all *vic* loci (A), and the prevention of heterokaryon formation between 389.7 *cre* and F3.4 *br* heteroallelic at only *vic1* (B). The orange sector produced between the strains in (A) is heterokaryotic and exhibits wild type color due to complementation between the *cre* and *br* mutations.

Table 2. Ratios of *cre* (cream) to *br* (brown) nuclear types from heterokaryons composed of vegetatively compatible strains and one putative heterokaryon from strains heteroallelic at *vic5*.

Heterokaryon components[a]	**Subculture**	**Mycelium color**	**Number of conidia**		**ratio**
			cream	brown	
389.7 + F2.36	heterokaryon #1 from PDA	orange	12	97	1 : 8
	heterokaryon #2 from PDA	orange	61	145	1 : 2
	hyphal tip 1 from het #1	orange	57	47	1 : 1
	hyphal tip 2 from het #1	orange/brown	1	102	1 : 100
	hyphal tip 3 from het #1	brown	0	102	
	6th subculture from het #1	dark orange	0	110	
389.7 + F3.10	orange sector from PDA	orange	0	99	
	hyphal tip	orange	106	0	
	6th subculture	orange	0	110	

[a] The vegetative compatibility genotypes of the homokaryotic strains are: 389.7 *vic1-1, 2-1, 3-1, 4-1, 5-1*; F2.36 *vic1-1, 2-1, 3-1, 4-1, 5-1*; F3.10 *vic1-1, 2-1, 3-1, 4-1, 5-2*.

The differences in mycelial coloration of the hyphal tip colonies appeared to be due to a gene dosage effect brought about by varying numbers of nuclei representing each parent. Conidia were collected from two different orange heterokaryotic sectors formed between two different pairings of strains 389.7 and F2.36 (Table 2). Heterokaryotic sector number 1 produced *cre* to *br* conidia at a ratio of 1:2. Heterokaryotic sector number 2 produced *cre* to *br* conidia at a ratio of 1:8. The hyphal-tip subculture from heterokaryon number 2 that produced orange mycelium yielded *cre* and *br* conidia at a ratio of 1:1 (Table 2). The orange-brown hyphal-tip subculture yielded conidia resulting in *cre* and *br* colonies at a ratio of 1:100 (Table 2). The brown colored hyphal-tip subculture only yielded *br* conidia. Since *C. parasitica* conidia are uninucleate (Puhalla and Anagnostakis, 1971), the ratio of *cre* to *br* conidia represents the nuclear ratio of *cre* to *br* nuclei within the heterokaryotic mycelium assuming the nuclei are randomly distributed during conidiogenesis.

Six serial mass subcultures of heterokaryotic sector 2 were made to test the stability of the heterokaryons. All of the subcultures retained the orange color although the sixth was a darker orange. Only *br* conidia were collected from the sixth subculture (Table 2).

Restriction of heterokaryon formation by vegetative incompatibility. To determine whether vegetative incompatibility restricted heterokaryon formation, pairs of cream and brown ascosporic progeny, representing both those that formed heterokaryons and those that did not form heterokaryons, were chosen from cross 389.7 *cre nic* X 80-2c *br* and paired in the wood/agar vc assay. Those strains which were vegetatively compatible in the wood/agar vc assay were found to be those that formed heterokaryons on PDA, and those strains where a barrage formed in the wood/agar vc assay were those that failed to form heterokaryons on PDA (Figure 1). Vegetatively compatible *cre* and *br* strains were also found to form an orange colored zone of mycelium at their point of contact in the wood/agar vc assay.

Genetic analysis confirming the identity of *vic1* and *vic2*. Previous work by Anagnostakis (1980, 1982, 1988) identified and named two *vic* loci, *vic1* and *vic2*, using *C. parasitica* strains designated vc types 5, 8, 39, and 71 based on PDA vc testing. Confirmation of these vc genotypes was obtained through three sexual crosses evaluated with the wood/agar vc assay (Table 3). Strain 389.7 *cre nic* (vc type 5) was crossed with strain EP388 *met* (vc type 39). The resulting progeny segregated in a 1:1 ratio for each of the parental vegetative compatibility types indicating that the parents differed at a single *vic* locus as reported by Anagnostakis (1982). This locus has been previously named *vic1*, and EP388 *met* is considered to carry the allele *vic1-2*. Strain 389.7 *cre nic* was also crossed with EP289 *met* (vc type 71). These progeny also segregated in a 1:1 ratio for each of the parental vegetative compatibility types indicating that EP289 also differed from 389.7 at a single *vic* locus.

To confirm the vc genotypes of EP388 and EP289 as well as test the genotype of strain 22508 (vc type 8), strain 389.7 *cre nic* was crossed with strain 22508 *met* (Table 3). Strains 389.7 and 22508 were expected to differ from each other at both *vic1* and *vic2*. Therefore, if the vc genotype of 22508 is *vic1-2*, *vic2-2*, then EP388 and EP289 will be compatible with the two nonparental vc types. The progeny from this cross segregated in a 1:1:1:1 ratio consisting of the two parental types, and two nonparental types. The two nonparental classes of progeny were compatible with either EP388 or EP289 confirming the postulated genotypes of Anagnostakis (1988) and demonstrating that strain EP388 and strain EP289 differ from each other at two *vic* loci, and that 22508 is of genotype *vic1-2*, *vic2-2*. This analysis of *vic1* and *vic2* provided the tester strains that were subsequently used to identify new *vic* loci.

Genetic analysis identifying two new *vic* loci, *vic4* and *vic5*. Strain 389.7 *cre nic* was crossed with strain 80-2c *br* (Table 4). Initially, progeny from this cross were placed into four groups based upon the ability of *cre* and *br* progeny to form orange mycelium in heterokaryon tests on PDA. However each of these four color groups contained some pairs of *cre* and *br* strains that produced abnormal thin orange mycelium with conspicuously reduced conidia production (described in detail below). Therefore, the progeny in each of these four groups were subjected to barrage tests using the wood/agar vc assay. The wood/agar vc assay demonstrated that each of the four initial groups of progeny was composed of two vegetative compatibility groups: those strains that formed morphologically abnormal orange mycelium on PDA were found to form a barrage in the wood/agar vc assay. The progeny from cross 389.7 *cre nic* X 80-2c *br* therefore segregated into eight vegetative compatibility types: two parental vc types and six nonparental types. One of the nonparental groups was found to be compatible with the tester genotype *vic1-2*, *vic2-1* indicating that 80-2c carried the *vic1-2* allele. The other nonparental compatibility genotypes were not identifiable by any other known tester strains. Therefore, it was postulated that 80-2c was heteroallelic with respect to 389.7 at three *vic* loci: *vic1* and two new *vic* loci, *vic4* and a weak incompatibility locus designated

Table 3. Number of progeny in vegetative compatibility groups from three sexual crosses.

	Vegetative compatibility genotypes				
	vic1-1,2-1[a]	*vic1-2,2-1*	*vic1-1,2-2*	*vic1-2,2-2*	
cross	vc 5[b]	vc 39	vc 71	vc 8	total progeny
389.7 *cre nic* X EP388 *met*	19	21			40
389.7 *cre nic* X EP289 *met*	20		20		40
389.7 *cre nic* X 22508 *met*	17	11	16	18	62

[a] For brevity vegetative incompatibility genotypes have been written as *vic1-2,2-1* rather than as *vic1-1*, *vic2-1*.

[b] vc type 5 tester strain was 389.7; vc type 39 tester strain was EP388; vc type 71 tester strain was EP289; vc type 8 tester strain was 22508. Vegetative incompatibility tests were done using the wood/agar vc assay.

Table 4. Number of progeny in eight different vegetative compatibility genotypes that segregated from cross 389.7 *cre nic* X 80-2c *br*.

	Vegetative compatibility genotypes				
	vic1-1,2-1,3-1,4-1,5-1[a]	*vic1-1,2-1,3-1,4-1,5-2*	*vic1-2,2-1,3-1,4-1,5-1*	*vic1-2,2-1,3-1,4-1,5-2*	
	389.7/F2.36[b]	F2.4/F4.9	A1.10/F3.4	F2.13/F3.15	
number of progeny	19	20	22	28	
	vic1-1,2-1,3-1,4-2,5-1	*vic1-1,2-1,3-1,4-2,5-2*	*vic1-2,2-1,3-1,4-2,5-1*	*vic1-2,2-1,3-1,4-2,5-2*	
	F3.2/F3.26	F3.21/F3.39	F3.13/F3.16	F2.14/80-2c	total progeny
number of progeny	30	17	21	12	169

[a] For brevity vegetative incompatibility genotypes have been written as *vic1-1,2-1* rather than as *vic1-1*, *vic2-1*.

[b] Pairs of *cre* and *br* strains used in the compatibility testing for the genotype indicated above. Progeny from this cross were first separated into four groups using the heterokaryon vc assay with four pairs of vegetatively compatible strains: 389.7/F2.36, A1.10/F3.4, F3.2/F3.26, and F2.14/80-2c. Each of the four groups were composed of progeny which formed either morphologically normal orange sectors or morphologically abnormal orange sectors with the complementary colored tester. All progeny in each of these four groups failed to form orange heterokaryotic sectors with the tester strains from the other four groups. All strains in each of the four groups were then subjected to the wood/agar vc assay using the tester strains from their group plus a strain chosen from among the morphologically abnormal types. In this manner it was determined that the progeny consisted of eight compatibility groups; each of the original four color complementation groups consisted of strains which were homoallelic at *vic1*, *vic2*, *vic3*, and *vic4*, but heteroallelic at *vic5*. The barrage tests using the wood/agar vc assay revealed weak barrages due to *vic5*.

vic5 (Table 4). The name *vic3* was not used here because it had been used elsewhere (Rizwana and Powell, 1992; 1995), though erroneously. The status of *vic3* was not known at this juncture in the study, therefore the new loci were named *vic4* and *vic5*. Subsequent genetic analyses described below provide a proper genetic basis for *vic3*.

The presence of a new weak incompatibility locus, designated *vic5*, was validated by crossing 389.7 *cre nic* with strain F4.9 *br*. Strain F4.9, an ascosporic progeny representing one of the six nonparental vc genotypes from cross 389.7 *cre nic* X 80-2c *br*, formed a narrow barrage with 389.7 in the wood/agar vc test and formed an orange colored mycelium with greatly reduced conidiation when paired with 389.7 on PDA. Fifty progeny were collected and tested with the wood/agar vc assay: 29 were compatible with parent 389.7, and 21 were compatible with parent F4.9 indicating that the parents differed at a single *vic* locus. Since this locus segregated independently of *vic1* and *vic4*, it was tentatively named *vic5* pending additional analysis to test whether this gene was allelic with *vic2* or *vic3*. This cross demonstrated that the weak barrage in the wood/agar vc assay and the abnormal orange sectors on PDA could be attributed to a common cause: a single weak *vic* locus.

Cross 389.7 *cre nic* X 80-2c *br* included ascosporic progeny that were vegetatively compatible with parent 389.7. To confirm that these progeny had the same vc genotype as 389.7, one of the brown compatible progeny, F4.13 *br*, was crossed with 389.7 *cre nic*. All of the progeny from this cross were found to be compatible with the parental vc type verifying that the two parental strains were homoallelic at all *vic* loci.

Confirmation of the other new *vic* allele, designated *vic4-2*, segregating in cross 389.7

cre nic X 80-2c *br* proceeded as follows. Strain F3.16 *br nic* was found to form a weak barrage with parent 80-2c, suggesting that these two vc types were heteroallelic at *vic5*, but shared the new allele *vic4-2*. Therefore the *vic* genotype of F3.16 was postulated to be *vic1-2*, *vic2-1*, *vic4-2*, *vic5-1*. To test this prediction, F3.16 *br nic* was crossed with the tester strain A1.13 *cre met* which had the genotype *vic1-1*, *vic2-2*, *vic4-1*, *vic5-1*. The progeny from this cross segregated into eight vc types as expected using the PDA heterokaryon assay (Table 5). None of the orange heterokaryons from any of the eight groups developed the abnormal type of orange morphology indicating that *vic5* did not segregate in this cross. Of the eight vc types of progeny, four were compatible with tester strains representing the four combinations of alleles at *vic1* and *vic2*, as expected. The presence of four additional vc types (three nonparental types and one compatible with parent F3.16) verified that the parental strains were heteroallelic at *vic1*, *vic2*, and a third locus, *vic4*. The identification of the three remaining nonparental vc types from cross F3.16 *br nic* X A1.13 *cre met* will be described below.

The two remaining nonparental vc genotypes from cross 389.7 *cre nic* X 80-2c *br* were identified using the analysis of cross 389.7 *cre nic* X EP243. Prior analysis of strain EP243 showed that it differed from 389.7 at two *vic* loci, one of which caused a weak incompatibility reaction (Chapter 2). Parent EP243 and the two nonparental vc genotypes from this cross were found to be compatible with three of the nonparental vc genotypes from cross 389.7 *cre nic* X 80-2c *br*. One of the nonparental vc types from 389.7 *cre nic* X EP243 was found to be compatible with strain F4.9 thereby proving that EP243 carries *vic5-2*. Consequently, the vc genotype of EP243, and the corresponding compatible

Table 5. Number of progeny in eight different vegetative compatibility genotypes that segregated from cross A1.13 *cre met* X F3.16 *br nic*.

	Vegetative compatibility genotypes				
	vic1-1,2-1,3-1,4-1,5-1[a] 389.7/F2.36[b]	*vic1-2,2-1,3-1,4-1,5-1* A1.10/F3.4	*vic1-1,2-2,3-1,4-1,5-1* A1.13/J2.43	*vic1-2,2-2,3-1,4-1,5-1* A1.8/J2.57	
number of progeny	12	7	7	12	
	vic1-1,2-1,3-1,4-2,5-1 F3.12/F3.26	*vic1-2,2-1,3-1,4-2,5-1* F3.13/F3.16	*vic1-1,2-2,3-1,4-2,5-1* J2.6/J2.34	*vic1-2,2-2,3-1,4-2,5-1* J2.20/J2.23	total progeny
number of progeny	10	9	20	17	94

[a] For brevity vegetative incompatibility genotypes have been written as *vic1-1,2-1* rather than as *vic1-1, vic2-1*.

[b] Pairs of *cre* and *br* strains used in the vegetative compatibility testing for the genotype indicated above. Vegetative incompatibility was determined using the heterokaryon assay.

nonparental vc type from 389.7 *cre nic* X 80-2c *br*, is *vic1-1*, *vic2-1*, *vic4-2*, *vic5-2*. The other nonparental type from cross 389.7 *cre nic* X EP243, and the corresponding compatible nonparental vc type from cross 389.7 *cre nic* X 80-2c *br*, is therefore *vic1-1*, *vic2-1*, *vic4-2*, *vic5-1*.

The final nonparental vc genotype from cross 389.7 *cre nic* X 80-2c *br*, represented by F3.15 *br nic*, was identified because of the abnormal orange sectors on PDA and weak incompatibility reaction in the wood/agar vc assay that occurred when F3.15 grew in contact with tester A1.13 *cre met*, indicating that these vc genotypes differed at only *vic5*. This fit expectations based upon the other nonparental genotypes.

The preceeding analysis of the vc genotype of strain 80-2c has shown that it is heteroallelic with respect to 389.7 at *vic1* and two new loci, *vic4* and *vic5*. Strain EP243 was also found to be heteroallelic relative to 389.7 at *vic4* and *vic5*, and to carry the same alleles at loci *vic4* and *vic5* that 80-2c carries. The demonstration that gene *vic5-2* is not allelic with locus *vic2* will be presented below in the analysis of cross EP29 x A1.10 *cre nic*.

Unusual mycelial interactions associated with heteroallelism at *vic4* and *vic5*. Occasionally, *cre* and *br* strains heteroallelic at only *vic4* produced irregularly shaped sectors with dark brown, slightly orange-tinged, appressed mycelium where the two strains made contact on PDA. These sectors of appressed mycelium never appeared when the strains made contact in the wood/agar vc assay. The incompatibility reaction (barrage) produced by *vic5* in the wood/agar vc assay was less broad and more easily obscured by weak mycelial growth than the reactions produced by *vic1*, *vic2*, *vic3*, and *vic4*. When *cre* and *br* strains heteroallelic at only *vic5* grew in contact with each other on PDA, no barrage was visible and a sector of thin orange mycelium with greatly reduced conidiation developed at their confluence (Figure 2). However, orange mycelium never formed when *cre* and *br* strains heteroallelic at only *vic5* made contact in the wood/agar vc assay. The abnormal sectors produced in association with *vic4* and *vic5* were maintained upon subculturing.

To determine if the orange mycelium was heterokaryotic that appeared when strains heteroallelic at only *vic5* made contact on PDA, ten hyphal tip subcultures were collected from a subculture of orange mycelium produced by the contact of strains 389.7 *cre nic* and F3.10 *br nic* (*vic1-1*, *vic2-1*, *vic3-1*, *vic4-1*, *vic5-2*), an ascosporic progeny from cross 389.7 *cre nic* X 80-2c *br*. The resulting colonies that grew from the hyphal tips were light orange in color, and none showed the variation from orange to brown found among the hyphal tip cultures of the vegetatively compatible *cre* and *br* strains. Conidia were collected from the original orange sector and from the fifth of a series of subcultures that retained the orange color. In both cases *br* conidia were collected (Table 2). In contrast, conidia collected from a representative light orange hyphal tip colony produced only *cre* conidia. These results suggest that the abnormal orange sectors produced between strains 389.7 and F3.10 are heterokaryotic because both nuclear types were derived from the mycelium; however, both nuclear types were not recovered from a single hyphal tip.

Identification of the remaining nonparental vc genotypes from cross F3.16 *br nic* X A1.13 *cre met*. Three nonparental vc types from cross F3.16 *br nic* X A1.13 *cre met* remained to be identified. One of these nonparental vc types could be identified using

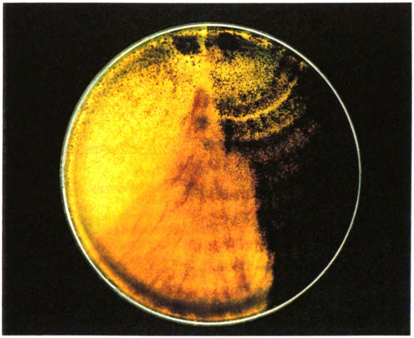

Figure 2. An orange sector of abnormal mycelium has formed between strains 389.7 *cre* and F3.10 *br* heteroallelic at only *vic5*. The orange sector has thinner mycelium and less conidia than the normal mycelium of the *cre* and *br* strains. The plate has back lighting.

strain F3.26 *br nic*. The two remaining nonparental vc genotypes were distinguished by crossing J2.23 *br nic* with K2.30 *cre*. Four classes of ascosporic progeny resulted from this cross (Table 7). Therefore the vc genotype of J2.23 was determined to be *vic1-2*, *vic2-2*, *vic3-1*, *vic4-2*, *vic5-1*. Cross J2.23 *br nic* X K2.30 *cre* will be discussed in more detail below in relation to the identification and characterization of *vic3*.

Genetic analysis identifying a new locus *vic3*. A cross was performed between EP29 and tester strain A1.10 *cre nic* (Table 6). The vc genotype of A1.10 was known to be *vic1-2*, *vic2-1*, *vic4-1*, *vic5-1* (the status of *vic3* was not known at the time of the cross). The ascosporic progeny from this cross segregated into eight compatibility groups plus nine progeny that were incompatible with these eight groups and four progeny that did not give clear reactions in the wood/agar vc assay. The eight compatibility groups included two parental groups, three unknown nonparental groups, and three nonparental groups compatible with tester genotypes *vic1-2*, *vic2-1*, *vic4-1*, *vic5-1*; *vic1-2*, *vic2-1*, *vic4-1*, *vic5-2*; and *vic1-2*, *vic2-2*, *vic4-1*, *vic5-1*. These tester strains indicated that EP29 must carry the alleles *vic2-2* and *vic5-2*. In addition, a *br* tester strain of genotype *vic1-1*, *vic2-1*, *vic4-1*, *vic5-1* was not compatible with any *cre* progeny indicating that *vic1-1* was not present in EP29. The presence of eight predominant vc groups among the progeny suggested the presence of one unidentified strong incompatibility gene segregating in this cross. Therefore, two additional crosses were performed in an attempt to separate the strong *vic* gene from *vic5-2* and any other weak *vic* genes that might be present.

To identify the new strong *vic* gene present in EP29, ascosporic progeny K2.30 *cre*, derived from cross EP29 X A1.10 *cre nic*, was chosen as a potential carrier of the new *vic* gene (*vic3-2*) because it formed a weak barrage with the parental strain EP29 and strong barrages with the other identified compatibility types from this cross. Therefore, K2.30 was postulated to carry the unidentified strong *vic* gene from parent EP29, and to be homoallelic with respect to the weak *vic* genes carried by this parent.

The first cross tested whether the putative new gene *vic3-2* in K2.30 was allelic with, or linked to, *vic1*. This was accomplished by crossing K2.30 *cre* with J2.69 *br nic* (Table 7). Strain J2.69 has the genotype *vic1-1*, *vic2-2*, *vic3-1*, *vic4-1*, *vic5-1*. These two strains were hypothesized to be heteroallelic at only *vic1* and the new strong *vic* locus designated *vic3*. The progeny from this cross segregated into two parental and two nonparental compatibility groups in a 1:1:1:1 ratio using the PDA heterokaryon assay, demonstrating that *vic3-2* is not allelic with locus *vic1* (Table 7). In order to provide additional confirmation that these four vegetative compatibility groups did not contain other segregating, weak incompatibility genes, 54 strains representing each of the four vc groups were also subjected to compatibility tests using the wood/agar vc assay. The wood/agar vc assay verified that all of the strains within each of the four groups were compatible with each other, demonstrating that no weak *vic* genes segregated in this cross.

The second cross tested whether or not the putative *vic3-2* gene was allelic with, or linked to, *vic4*. Strain K2.30 *cre*, with the postulated genotype *vic1-2*, *vic2-2*, *vic3-2*, *vic4-1*, *vic5-1*, was crossed with strain J2.23 *br nic* known to have the genotype *vic1-2*, *vic2-2*, *vic3-1*, *vic4-2*, *vic5-1*. (This cross was already referred to above). These two strains were hypothesized to be heteroallelic at only *vic3* and *vic4*. The progeny from this cross segregated into two parental and two nonparental compatibility groups in a 1:1:1:1

Table 6. Number of progeny in each vegetative compatibility group that segregated from cross EP29 X A1.10 *cre nic*.

	Vegetative compatibility genotypes/groups					
	vic1-2,2-1,3-1,4-1,5-1[a]	*vic1-2,2-2,3-1,4-1,5-1*	*vic1-2,2-1,3-2,4-1,5-1*	*vic1-2,2-2,3-2,4-1,5-1*	*vic1-2,2-1,3-1,4-1,5-2*	
	F3.4[b,c]	J2.57	K1.43	K2.30	F3.15	
number of progeny	10	8	2	6	12	
	K1.9	K1.32/K2.39[d]	EP29	Incompatible with testers	Ambiguous vc reactions	total progeny
number of progeny	5	11	13	9	4	80

[a] For brevity vegetative incompatibility genotypes have been written as *vic1-1,2-1* rather than as *vic1-1*, *vic2-1*.

[b] Strains used in the vegetative compatibility testing. Where known the vc genotype is placed above the tester strain. Vegetative incompatibility testing was done using the wood/agar vc assay.

[c] Strain F3.4 *br* is compatible with parent A1.10 *cr*.

[d] Strains K1.32 and K2.39 are vegetatively compatible. Both were used in vc typing.

Table 7. Number of progeny in each vegetative compatibility genotype that segregated from crosses K2.30 *cre* X J2.69 *br nic* and K2.30 *cre* X J2.23 *br nic*.

	Vegetative compatibility genotypes						
	vic1-1,2-2,3-1,4-1,5-1[a]	*vic1-2,2-2,3-1,4-1,5-1*	*vic1-1,2-2,3-2,4-1,5-1*	*vic1-2,2-2,3-2,4-1,5-1*	*vic1-2,2-2,3-1,4-2,5-1*	*vic1-2,2-2,3-2,4-2,5-1*	
	A1.13/J2.69[b]	A1.8/J2.57	M1.6/M1.5	K2.30/M1.21/L1.8[c]	L1.6/J2.23	L1.17/L1.39	total progeny
K2.30 X J2.69	23	29	19	24			95
K2.30 X J2.23		13		8	13	14	48

[a] For brevity vegetative incompatibility genotypes have been written as *vic1-1,2-1* rather than as *vic1-1, vic2-1*.

[b] Pairs of *cre* and *br* strains used in the vegetative compatibility testing for the genotype indicated above. Vegetative incompatibility testing was done using the heterokaryon assay. Strains within each vc genotype from cross K2.30 X J2.69 were then subjected to the wood/agar vc assay to further confirm that no weak incompatibility genes had segregated in the cross.

[c] Strain M1.21 *br* was used in the compatibility testing with cross K2.30 X J2.69, and strain L1.8 *br* was used in compatibility testing with cross K2.30 X J2.23.

ratio (Table 7). The independent segregation of the putative gene *vic3-2* and *vic4-1* demonstrated that *vic3-2* is not allelic with locus *vic4*.

A third cross was performed to identify the nonparental vc genotype of ascosporic progeny K1.43. Strain K1.43 *cre* was crossed with F4.13 *br* (*vic1-1*, *vic2-1*, *vic3-1*, *vic4-1*, *vic5-1*) (Table 8). The progeny from this cross would have assorted into four classes if only *vic1* and *vic3* segregated, or eight classes if *vic5* also segregated. The ascosporic progeny were found to segregate into only four classes: two parental, one nonparental type compatible with *vic1-2*, *vic2-1*, *vic3-1*, *vic4-1*, *vic5-1*, and one additional nonparental type. Additional verification that weak compatibility genes did not segregate in this cross was provided by subjecting all of the progeny in each of the four vegetative compatibility groups to the wood/agar vc assay. This test confirmed that all of the strains in each of the four groups were compatible with each other, and that no weak *vic* genes had segregated in this cross. Consequently, K1.43 was shown to carry allele *vic5-1*. This cross also provided further confirmation that *vic1* and *vic3* are distinct loci because of the independent segregation of the alleles at these loci.

Cross EP29 X A1.10 *cre nic* also provided confirmation that the gene previously designated *vic5-2* represents a new *vic* locus. Gene *vic5-2* was found to be present in EP29 because it segregated in the nonparental progeny class compatible with the tester genotype *vic1-2*, *vic2-1*, *vic3-1*, *vic4-1*, *vic5-2*. The presence of this nonparental vc genotype, as well as the others identified above, demonstrate that *vic5* segregates independently of both *vic2* and *vic3*. This cross thereby provides the final confirmation that gene *vic5-2*, originally identified in cross 389.7 *cre nic* X 80-2c *br*, represents a new

Table 8. Number of progeny in four different vegetative compatibility genotypes that segregated from cross F4.13 *br* X K1.43 *cre*.

	Vegetative compatibility genotypes				Total progeny
	vic1-1,2-1,3-1,4-1,5-1[a]	*vic1-2,2-1,3-1,4-1,5-1*	*vic1-1,2-1,3-2,4-1,5-1*	*vic1-2,2-1,3-2,4-1,5-1*	
	389.7/F4.13[b]	A1.10/F3.4	N1.9/N1.19	K1.43/N1.39	
number of progeny	18	13	13	4	48

[a] For brevity vegetative incompatibility genotypes have been written as *vic1-1,2-1* rather than as *vic1-1*, *vic2-1*.

[b] Pairs of *cre* and *br* strains used in the vegetative compatibility testing for the genotype indicated above. Vegetative incompatibility testing was done with the heterokaryon assay. Strains within each vc genotype were then subjected to the wood/agar vc assay to further confirm that no weak incompatibility genes had segregated in the cross.

vic locus.

The preceeding crosses have shown that the gene designated *vic3-2*, derived from strain EP29, is not allelic with *vic1*, *vic2*, *vic4*, or *vic5*, and should therefore be given a new locus name. The new locus is here named *vic3* because it is the strong, unnamed *vic* locus that Anagnostakis (1981) observed to be segregating in her analysis.

Linkage analysis. Linkage analysis was conducted on the five *vic* loci, the two auxotrophic mutations, and the two pigmentation mutations using several crosses (Table 9). These nuclear genes were tested for Mendelian segregation using chi-square analysis with one degree of freedom. The color mutations *cre* (cream) and *br* (brown) were found to be tightly linked. One thousand forty four progeny were pooled from five crosses between different *cre* and *br* parents. Of these combined progeny three recombinants (0.3% recombination frequency) were found that displayed the orange wildtype coloration. Anagnostakis (1982) had crossed *cre* and *br* strains but did not find any recombinant orange progeny. In the study by Anagnostakis fewer progeny were counted so that tight linkage could have been missed. Also, it is not known if the *br* mutation in this study is the same genetic locus as the *br* mutation in the study by Anagnostakis; the *cre* mutations in both studies are the same. The brown pigmentation mutation segregated as a single locus when crossed with the wild type orange strain EP155 (111 *br* progeny; 122 orange progeny; $X^2 = 0.52$; P = not significant). The nicotinamide requirement mutation (*nic*) also segregated as a single genetic locus. The two auxotrophic markers, nicotinamide and methionine (*met*) requirements, were not linked to each other or to *cre* and *br*. The *nic* mutation was not linked to any of the *vic* loci. The *met* mutation was not linked to *vic1*

Table 9. Linkage analysis of the loci used in this study.

Loci	Cross	Progeny numbers		Recombination frequency	X^2[a]
		Parental	Recombinant		
nic : *met*	389.7 X 22508	28	34	54.8	0.58
nic : *cre*		33	29	46.8	0.26
met : *cre*		35	27	43.5	1.03
vic1 : *nic*		33	29	46.8	0.26
vic1 : *met*		33	29	46.8	0.26
vic1 : *cre*		30	32	51.6	0.06
vic2 : *nic*		32	30	48.4	0.06
vic2 : *met*		28	34	54.8	0.58
vic1 : *vic2*		35	27	43.5	1.03
vic2 : *cre*	combined crosses: 389.7 X 22508, 389.7 X EP289	58	44	43.1	1.92
cre : *br*	combined crosses: 389.7 X 80-2c, F3.16 X A1.13, K2.30 X J2.69, K2.30 X J2.23, F4.13 X K1.43	1044	3[b]	0.29	1035
vic4 : *br*	389.7 X 80-2c	84	81	49.1	0.05
vic5 : *br*		83	82	49.7	0.006
vic4 : *nic*		53	72	57.6	2.89
vic5 : *nic*		64	61	48.8	0.07
vic4 : *vic5*		68	97	58.8	5.10*
vic1 : *vic4*		71	94	57.0	3.21
vic1 : *vic5*		86	79	47.9	0.30
vic3 : *vic4*	K2.30 X J2.23	21	27	56.3	0.75
vic2 : *vic4*	F3.16 X A1.13	34	56	62.2	5.38*
vic3 : *nic*	K2.30 X J2.69	31	28	47.5	0.15
vic3 : *cre*		45	50	52.6	0.26
vic1 : *vic3*		47	48	50.5	0.01

[a] The null hypothesis for the chi-square test is that two loci segregate in a 1:1 ratio.

[b] Recombinant phenotype was orange.

* Significantly different from 1:1 at $P < 0.05$.

or *vic2*; its linkage to the other three *vic* loci was not tested. Anagnostakis (1982) had previously shown that *cre* was not linked to *vic1* or to *met*. The present study has found that *cre* is also not linked to *vic2*, *vic3*, *vic4*, or *vic5*. The loci *cre* and *vic2* demonstrated unlinked segregation using the combined progeny from crosses 389.7 *cre nic* X 22508 *met* and 389.7 *cre nic* X EP289 *met*. These loci also tested as unlinked from cross 389.7 *cre nic* X EP289 *met* alone (parental type = 19, recombinant type = 21; $X^2 = 0.10$; P = not significant). However, in cross 389.7 *cre nic* X 22508 *met* these loci deviate from 1:1 segregation at a significance level of $P < 0.05$ (parental type = 39, recombinant type = 23; $X^2 = 4.13$). The deviation from 1:1 segregation in cross 389.7 *cre nic* X 22508 *met* is considered to be the result of random variation.

Chi-square analysis indicates that *vic1* is not linked to *vic2*, *vic3*, *vic4* or *vic5*. *Vic3* and *vic4* are also not linked. However, *vic4* and *vic5* failed to segregate in a 1:1 ratio ($P < 0.05$). Loci *vic2* and *vic4* also did not segregate in a 1:1 ratio ($P < 0.05$). The apparent deviation from 1:1 segregation of these two pairs of *vic* loci may indicate either loose linkage or random variation such as observed with the loci *cre* and *vic2* described above.

The linkage of *vic2*, *vic3* and *vic5* to each other could not be determined from cross EP29 X A1.10 *cre nic* because some of the nonparental vc genotypes could not be determined. However, the independent segregation of the alleles at loci *vic2*, *vic3*, and *vic5* was demonstrated by the identifiable vc genotypes of one of the parental strains and four of the nonparental classes. Although linkage analysis could not be done, it is evident from segregation ratios that these three loci are not closely linked to each other (Table 6).

Discussion

This study sought to identify new *vic* loci that control vegetative cell fusions in *C. parasitica*, to test whether heterokaryon formation occurs in this species, and to test the effects of individual *vic* loci upon heterokaryon formation.

Identification of three new *vic* loci. The present study identified three new *vic* loci designated *vic3*, *vic4*, and *vic5* in *Cryphonectria parasitica*. These new *vic* loci were detected and characterized using a combination of barrage development on chestnut phloem/cortex tissue and complementary pigmentation mutations as a nonselective assay to detect heterokaryons. Prior to this study only two *vic* loci had been identified in *C. parasitica* by genetic analysis (Anagnostakis, 1980, 1982, 1988). The new locus *vic5* was undetected in two previous studies (Huber and Fulbright, 1994; Anagnostakis, 1988) where the segregation of this locus through sexual recombination is now known to have occurred. All five *vic* loci have two known alleles that produce incompatibility only through allelic interactions; vegetative incompatibility resulting from nonallelic gene interactions has not been found in this species. This study has also shown that all five *vic* loci strictly prevent heterokaryon formation on autoclaved chestnut tissue even though each locus permits varying degrees of cytoplasmic transmission of hypoviruses and a senescence-inducing agent (chapters 4 and 5). Therefore, vegetative incompatibility in *C. parasitica*, as defined by barrage formation in the wood/agar vc assay, is equivalent to heterokaryon incompatibility.

Anagnostakis (1988) assigned tentative genotypes to five different vc types (5, 8, 10, 39, 71) based upon sexual crosses of three of the genotypes (8, 10, 39) with vc type 5.

My genetic analysis concurs with that of Anagnostakis (1988) and demonstrates that vc types 5, 8, 39, and 71 represent all four combinations of the alleles at loci *vic1* and *vic2*. However, Anagnostakis also tentatively assigned to these four genotypes genes designated *vic3-1*, *vic4-1*, *vic5-1*, *vic6-1*, and *vic7-1*. These other five genes were assigned because vc types 5 and 10 were thought to be heteroallelic at seven *vic* loci although none of these loci were actually identified. Therefore, in order to name new *vic* genes without creating an alternative nomenclature, I am here considering the designations for *vic3*, *vic4*, *vic5*, *vic6*, and *vic7* found in Anagnostakis (1988) to be superseded by this study while retaining the identities of *vic1* and *vic2* because they have been genetically defined (Anagnostakis 1982, 1988). In addition, in keeping with the nomenclatural precedent set by Anagnostakis (1988), the *vic* genes present in vc type 5 are considered to represent the "1" allele for each new locus identified. This revision does not affect the argument by Anagnostakis (1982, 1988) as to the degree of genetic differentiation between vc types 5 and 10, but does mean that vc types 5 and 10 must be considered to be heteroallelic at presently unidentified *vic* loci.

The new locus *vic3* has not been previously identified even though the allele name *vic3-2* has been used in reference to the vc genotype of EP29 (Rizwana and Powell, 1992, 1995). The use of the designation *vic3-2* by Rizwana and Powell was in reference to Anagnostakis (1988) where a cross between vc types 5 and 16 were stated to produce only parental vc types. Two points need to be made in reference to this statement. First, Anagnostakis (1988) did not resolve whether a *vic* gene reported to segregate in a cross between vc 5 and vc 16 represented a new locus, which could have been named *vic3*, or a new allele at *vic1*. Therefore, the use of the name *vic3-2* by Rizwana and Powell (1992, 1995) in reference to the genotype of EP29 was invalid. Secondly, Anagnostakis (1981) began a genetic analysis of EP29 that demonstrated the presence of *vic2-2* in that strain. This also was not incorporated by Rizwana and Powell (1992, 1995) into their vc genotype for EP29. Consequently, the report by Rizwana and Powell (1992, 1995) that vc 16 carries *vic1-1* and *vic2-1* is incorrect. My study has shown that EP29 (vc type 16) carries genes *vic1-2*, *vic2-2*, *vic3-2*, *vic4-1*, and *vic5-2*. The presence of more than eight vegetative incompatibility groups in cross EP29 X A1.10 is probably due to the segregation of alleles at an additional heteroallelic *vic* locus (or loci). Additional genetic analysis is needed to verify this.

How many polymorphic *vic* loci are in the North American population of *C. parasitica*? The frequently quoted estimate of five to seven polymorphic loci was based upon a cross between vc types 5 and 10 where more than 100 vc types were found among the progeny (Anagnostakis, 1982). Virus transmission tests between a vc 10 strain and several vc genotypes representing differences at *vic1*, *vic2*, and *vic3* indicate that none of the 32 vc genotypes representing the recombination of *vic1* through *vic5* could be compatible with vc 10 (chapter 4). Therefore, if vc 5 and vc 10 differ at seven *vic* loci, which I believe is a reasonable estimate, then the North American population would be polymorphic at more than seven *vic* loci. A further consideration is that *vic5* was not readily detectable with the traditional PDA vc assay which suggests that other weak incompatibility loci may also be present thereby further inflating the number of potentially polymorphic *vic* loci in North America.

Heterokaryon formation under nonselective conditions. The utilization of independent methods (barrage development and heterokaryon formation) to identify vegetatively compatible individuals has clarified the relationship between vegetative compatibility and heterokaryosis in *C. parasitica*. Previous work had shown that heterokaryons could form between nutritional mutants and morphological mutants, but the heterokaryons did not form easily nor were they maintained (Puhalla and Anagnostakis, 1971). The authors concluded that heterokaryons would not readily occur in nature even between vegetatively compatible strains. Anagnostakis (1981) later found a strain that was heterokaryotic with regard to mating type. My study has shown that heterokaryons always formed between vegetatively compatible individuals without the imposition of selective growth conditions, and that these heterokaryons can be maintained through serial subculturing.

Heterokaryons in Ascomycetes have been found to be either confined to the anastomosed cells, which limits proliferation of the heterokaryon (e.g. *Gibberella zeae*) (Adams et al., 1987), or to be characterized by the presence of both nuclear types in the hyphal tips which permits proliferation (e.g. *Neurospora crassa*) (Beadle and Coonradt, 1944). Hyphal tips of *C. parasitica* were found to harbor both nuclear types at variable ratios and to be maintained upon serial subculturing. Interestingly, the ratios of the two nuclear types in the hyphal tips corresponded to the color of the mycelium that grew from the hyphal tips: a greater proportion of *cre* nuclei resulted in a lighter orange mycelium, whereas relatively more *br* nuclei produced a darker orange-brown mycelium. Both the brown hyphal tip subculture and the sixth serial mass transfer of heterokaryotic mycelium yielded only *br* conidia. The collection of a brown hyphal tip subculture demonstrates that particular hyphae from a heterokaryotic mycelium may have greatly skewed nuclear ratios, or may even be composed of a single nuclear type. The finding of only *br* conidia from the sixth serial mass transfer may also be due to the progressive skewing of the ratio of *br* to *cre* nuclei through prolonged growth.

Differences in the ratios of nuclear types in hyphal tips have also been observed in *Neurospora* (Prout et al., 1953; Pittenger and Atwood, 1956). Pittenger and Atwood (1956) found that the hyphae at the colony front did not grow autonomously, but grew at a relatively constant rate determined by the mycelium rather than according to the nuclear ratios of individual hyphae. This example of the physiological integration of the mycelium could explain why the uniformly orange heterokaryotic mycelium in *C. parasitica* could produce hyphal tips with variable nuclear ratios and corresponding differences in color.

The effects of *vic* genes upon heterokaryon formation. Some evidence was found to suggest that the effectiveness of individual *vic* genes at preventing heterokaryon formation varies, and is associated with growth substrate. Unusual mycelial interactions suggestive of heterokaryosis sometimes occurred between strains heteroallelic at only *vic4* or *vic5* when grown on PDA. Although conidia derived from hyphal tip subcultures taken from these abnormal orange mycelial interactions did not show both nuclear types, both *cre* and *br* conidia were collected from the abnormal orange sectors, and the orange mycelium was maintained upon subculturing suggesting that both nuclear types were present though perhaps segregated into different hyphal tips or at greatly skewed ratios. Previous studies have also noted differences in incompatibility interactions in *C. parasitica* by describing strong and weak barrages (Anagnostakis 1988). Morphological differences in the interactions of conspecifics due to incompatibility genes have also been observed in *Ophiostoma ulmi* (Brasier 1984). One possible effect of growth substrate upon heterokaryosis may be that the frequency of hyphal anastomoses varies on the different substrates as has been found in the Basidiomycete *Corticium vellereum* (Bourchier, 1957).

The unusual mycelial interactions associated with heteroallelism at *vic4* and *vic5* were always prevented whenever strains were also heteroallelic at *vic1*, *vic2*, or *vic3*. This indicates that heteroallelism at *vic1*, *vic2*, and *vic3* is epistatic over the mycelial interactions associated with *vic4* and *vic5*. In addition, when strains were heteroallelic at both *vic4* and *vic5*, the unusual mycelial interactions never occurred, indicating that *vic4* and *vic5* apparently have an additive effect that is similar to the other three loci. To my knowledge, evidence for effects upon heterokaryon formation due to epistasis between genes involved in vegetative incompatibility have only been reported previously by Coenen et al. (1994) and Holloway (1955). Additive effects between incompatibility genes have been reported in *A. nidulans* (Dales et al.,1983; Coenen et al.,1994).

The weak nature of the incompatibility elicited by *vic5*, and to a lesser extent *vic4*, may be comparable to the *partial-het* genes described by Coenen et al. (1994), *hetA* described by Dales et al. (1983), and the genes affecting incompatibility described by Holloway (1955). Heteroallelism at a single *partial-het* locus in *A. nidulans* permitted some heterokaryotic growth under selective conditions, but heteroallelism at two *partial-het* loci prevented heterokaryotic growth. Dales et al. (1983) found that *hetA* in *A. nidulans* prevented heterokaryon formation under nonselective conditions but often permitted normal appearing heterokaryons under selective conditions. Holloway (1955) described interesting interactions among four loci which limited the growth of heterokaryotic mycelia in *N. crassa*. He found that the formation of heterokaryons under selective growth conditions was dependent upon heteroallelism at three loci (*W*, *X*, *Y*) while the growth rate and the time of growth initiation of heterokaryons depended upon loci *X*, *Y*, and *Z*. Under natural growth conditions the *partial-het* genes and some of the genes described by Holloway may prevent heterokaryon formation just as *vic5* prevents it on natural substrate.

The function of vegetative incompatibility genes. The biological function of vegetative incompatibility genes is not presently understood. The presence of large numbers of vegetative incompatibility groups in Ascomycete species in nature, especially among sexually reproducing species, suggests that incompatibility has some selective advantage. However, hypotheses concerning the biological function of vegetative incompatibility are complicated by the many effects associated with incompatibility genes. Several hypotheses have been proposed including the prevention of somatic cell parasitism (Buss 1987), the prevention of the infectious transmission of parasitic genetic elements (Caten 1972; Nauta and Hoekstra 1994), the development of reproductive organs (Bernet 1992, and references therein), and ecological competition (Rayner et al., 1984; Rayner 1991).

The presence and consequences of heterokaryon formation in ascomycetous fungi in nature is an open question. Few studies have recovered heterokaryons from nature or have tested the effects of heterokaryosis upon fitness. Examples are known where heterokaryons are less aggressive than the component homokaryons (Grindle and Pittenger, 1968); other examples are known where the heterokaryon is more aggressive (e.g. Jinks, 1952a, 1952b). The ability of *vic* loci to prevent heterokaryon formation in *C. parasitica*, while permitting cytoplasmic transmission of genetic elements, suggests that there may be a selective disadvantage to the formation of heterokaryons in nature. Of course, vegetative incompatibility systems do permit heterokaryon formation between genetically different individuals although these compatible individuals would share significant genetic similarities. Clearly, the determination of the biological significance of vegetative incompatibility genes must take into account the effects of individual incompatibility genes upon the transmission of nuclei and genetic elements, the potential for pleiotropy, and the environmental growth conditions of the mycelium.

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Chapter 4

The Effects of Vegetative Incompatibility Genes upon the Horizontal Transmission of Viruses in the Chestnut Blight Fungus, *Cryphonectria parasitica*, are Locus Specific and Modified by Epistasis

Abstract

The effects of specific vegetative incompatibility (*vic*) loci upon the horizontal (cytoplasmic) transmission of dsRNA hypoviruses in the chestnut blight pathogen, *Cryphonectria parasitica*, were examined. Two characterized hypoviruses and one uncharacterized dsRNA element were used in the transmission studies. Five *vic* loci were examined for their effects upon transmission. Differences in the effects of these five *vic* loci can be categorized as differences in the efficiency of transmission, reciprocality, and epistatic effects. Homoallelism at all *vic* loci always permitted transmission between donor and recipient strains. Strains heteroallelic at *vic1* exhibited nonreciprocality in transmission. A *vic1-2* recipient strain was infrequently infected by a *vic1-1* donor strain while the reciprocal situation (*vic1-2* donor, *vic1-1* recipient) always permitted infection. Locus *vic3* also showed nonreciprocality in transmission but was more leaky than *vic1*. Heteroallelism at either *vic4* or *vic5*, or both, did not prevent transmission. The effect of *vic2* was subject to epistasis. Heteroallelism at *vic2* prevents transmission when the allele *vic1-2* is also present in the recipient. But when the allele *vic1-1* occurs in conjunction with the allele *vic2-1* in the recipient, the transmission barrier created by *vic2* becomes leaky. These data suggest that *vic1-1* is epistatic over *vic2-1* when both occur in the recipient, and corresponds to the nonreciprocality due to *vic1*. Other genetic background effects also caused the *vic2* barrier to become unilaterally leaky. The effects of heteroallelism at *vic1* and *vic2* upon transmission are epistatic over the effects of *vic4* and *vic5*; *vic1* is epistatic over *vic3*.

Fungi are hosts to an array of viruses, plasmids, and transposable elements (Buck 1986; Kistler and Miao 1992; Nuss and Koltin 1990; Griffiths, 1995). The horizontal mobility of these parasitic elements has been demonstrated in numerous laboratory transmission studies (Anagnostakis and Day, 1979; Brasier, 1984; Griffiths et al., 1990; Collins and Saville, 1990; Debets et al., 1994). Recent work highlighting the widespread occurrence of viruses and plasmids in natural populations of fungi suggests that horizontal transmission may also be a frequent occurrence in nature. For example, two recent surveys of the distribution of plasmids in *Neurospora* species have shown that some plasmids have worldwide distributions and are present in several species (Arganoza et al., 1994a; Yang and Griffiths, 1993). The horizontal transmission of transposable elements has also been demonstrated by the movement of the transposon *Tad* between nuclei of a heterokaryon in *N. crassa* (Kinsey 1990), and has been inferred from the localized distribution of the *grh* retroelement in a subgroup of the rice blast pathogen, *Magnaporthe grisea* (Dobinson et al. 1993). The most frequently observed horizontally mobile genetic elements in filamentous fungi are viruses and double-stranded (ds) RNA elements. Viruses and dsRNA elements are common in fungi and have been shown to be infectious in nature in several species, notably, *Ophiostoma ulmi* (Brasier 1983) and *Cryphonectria parasitica* (Van Alfen et al. 1975). Concerted attention on *C. parasitica*, in particular, has uncovered a rich diversity of dsRNA viruses (Nuss 1992; Enebak et al., 1994; Polashock and Hillman, 1994; Chung et al., 1994; Smart and Fulbright, 1996), some of which have significant effects upon host fitness (Elliston 1985).

Among the important factors defining host/parasite relationships is mode of transmission. Mode of transmission may determine the extent of the infectious spread of a parasite as well as the coevolutionary direction of the host/parasite relationship (May and Anderson 1983). Although little information exists as to the impact of most fungal parasitic genetic elements upon their hosts, the spread of dsRNA viruses in populations of the pathogens *O. ulmi* and *C. parasitica* is thought to have had significant effects upon fungal virulence (Brasier 1990). The transmission of viruses through fungal populations occurs through both horizontal (infectious) and vertical (intergenerational) routes, although vertical transmission is generally restricted to asexual spores rather than to meiotic offspring (Buck 1986). The horizontal transmission of viruses in filamentous fungi is dependent upon direct cytoplasmic contact from hyphal fusions between mycelia because extracellular infection is not possible (Buck 1986).

The viability of intermycelial vegetative cell fusions in Ascomycetes is controlled by incompatibility systems which effectively mediate the formation of heterokaryons and provide a barrier to free parasite movement. Such systems in Ascomycetes can discriminate between conspecifics due to the presence of incompatibility loci (Glass and Kuldau 1992; Leslie 1993). Compatibility requires homoallelism at each of the incompatibility loci. When one or more of the compatibility loci of two individuals are heteroallelic, an incompatible reaction occurs which generally terminates the hyphal fusion by killing the fused cells and sometimes neighboring cells (Garnjobst and Wilson 1956; Newhouse and MacDonald 1991). One of the consequences of these vegetative cell compatibility systems is to maintain the genetic and physiological integrity of the individual (genotype) while permitting viable cell fusions to occur between some, though not all, close relatives. Vegetative incompatibility systems have been attributed to be a cellular defense mechanism for resisting infection by parasites and other suppressive cytoplasmic genetic elements (Caten 1972; Debets et al. 1994; Nauta and Hoekstra 1994).

Cryphonectria parasitica (Murr.) Barr., the chestnut blight pathogen, is responsible for the chestnut blight pandemic which spread through the *Castanea dentata* (American chestnut) population in eastern North America, and the *Castanea sativa* population in Europe (reviewed in Anagnostakis 1987; Fulbright et al. 1988; Griffin 1986; Heiniger and Rigling 1994). This pathogen/host system has been of particular interest because of the appearance of less virulent (hypovirulent) forms of the fungus in the North American and European populations which have acted as a natural biological control. The most frequent cause for the hypovirulent phenotypes of the fungus has been found to be novel dsRNA viruses which have recently been recognized as a new family, the Hypoviridae (Nuss 1992; Hillman et al. 1995). Although these viruses are horizontally transmissible by means of intraspecific hyphal fusions (anastomoses), their localized distribution in North America suggests that barriers to infection are present (MacDonald and Fulbright 1991). Recently, other infectious agents requiring intermycelial hyphal fusions for transmission, and unassociated with detectable dsRNA, have been found in nature and are also capable of causing transmissible hypovirulence (Fulbright 1985; Mahanti et al. 1993; Huber et al. 1994).

What are the rules governing the horizontal transmission of cytoplasmic genetic elements in fungi? The picture of horizontal transmission in *C. parasitica* that has emerged from studies of natural isolates has been complex and confusing; the presence of discernible rules governing transmission has been obscured by curious exceptions to apparent patterns. Virus transmission has been found to be prevented, permitted, limited to varying degrees, or to occur in nontransitive steps between different vegetative incompatibility genotypes (e.g. Anagnostakis 1983; Kuhlman et al. 1984), suggesting that individual incompatibility genes have different effects upon horizontal transmission.

This study addressed the question of how specific vegetative incompatibility (*vic*) genes affect the horizontal cytoplasmic transmission of hypoviruses in *C. parasitica*. The effects of heteroallelism at five *vic* loci as well as interactions between these genes were examined for their influence upon transmission. Individual loci were found to vary considerably in their effect upon transmission, and to be influenced by *vic* alleles at other loci. This study presents the first evidence of epistatic effects between specific vegetative incompatibility genes on virus transmission. Epistatic interactions were found to either decrease or increase the efficiency of cytoplasmic transmission.

MATERIALS AND METHODS

***C. parasitica* strains and culture conditions:** The strains used in this study and their sources are listed in Table 1. Two characterized dsRNA hypoviruses and one uncharacterized dsRNA genetic element were utilized in the horizontal transmission tests. Hypovirus CHV1-713 is the most extensively characterized of the Cryphonectria hypoviruses (Nuss, 1992). CHV1-713 was transferred sequentially into the transmission tester strains by the methods described below. This virus was first transmitted from Ep713 (source strain) to strain Ep78, and then from Ep78 to strain P1.9, and finally to strain 389.7. Hypovirus CHV3-GH2 was originally obtained from a nonlethal canker on a chestnut tree in Grand Haven, Michigan (Fulbright et al., 1983). Hypovirus CHV3-GH2 was transferred from strain GH2 to Ep289. The uncharacterized dsRNA-80-2 was obtained from strain 80-2 which was collected in West Virginia by William MacDonald (West Virginia University). This dsRNA may be of Italian origin as *C. parasitica* isolates containing Italian dsRNAs were released into field plots for studies in West Virginia (William MacDonald, personal communication).

Subcultures of the strains used in the transmission tests were taken from cultures grown on Petri plates containing potato dextrose agar (PDA, Difco). A single plate from which subcultures were drawn for the transmission tests was sometimes used for up to three months stored at room temperature. This storage or longer term storage on PDA plates at 4° C did not cause loss of dsRNA.

Transmission tests: Tests for transmission of virus were conducted by placing a subculture of a virus-containing donor strain about 1 cm away from a subculture of a virus-free strain on PDA near one edge of a 100 X 15 mm Petri plate. Stacks of three to five Petri plates were kept under cool white flourescent lights at room temperature (about 25° C) and were rotated by placing the top plate on the bottom of the stack every other day while the cultures were growing. The donor and recipient strains grew across the Petri plates, making contact as they grew. Occasionally paired strains would grow close together but not make contact. When this occurred, the trial was rejected and repeated.

Each of the three viruses used in the horizontal transmission tests produced a distinct, visible phenotype in the fungal strains which carried them. The transmission of a virus into a recipient strain was detectable by a distinct change in morphology of the recipient. The presence of hypoviruses in each of the donor strains was verified by dsRNA extraction using established protocols (Morris and Dodds, 1979; Fulbright et al., 1983). Each strain was also analyzed for the presence of dsRNA prior to infection to establish that unknown dsRNAs were not present. The reliability of morphological changes as an indicator of hypovirus presence was thereby verified for every strain.

In order to consider a virus transmission test as negative (no transmission), donor and recipient strains were required to grow in contact on the surface of the PDA for a distance of 2/3 of the plate diameter. That is, if transmission did not occur between strains that grew in contact with each other for a distance less than 2/3 plate diameter (because one strain grew faster than the other) this transmission test was rejected as invalid and repeated. No attempt was made to specify the amount of time that the strains had been in contact before transmission was observed.

Hypovirus transmission tests were also conducted on live chestnut tissue. The method used was adapted from the Cryphonectria virulence assay developed by Lee et al.(1992). Chestnut stems about 2 cm in diameter were cut into lengths 4 to 5 cm long. Each section was split longitudinally and the bark/phloem outer layer was peeled off the underlying secondary xylem. This test was limited to stems collected during the growing season as the outer layer would become strongly attached to the secondary xylem during late fall and winter and be impossible to remove intact. The transmission test consisted of placing 5 mm^3 plugs of fungal subcultures of actively growing donor and recipient strains in contact (side by side) in the center of the inside of the bark. The live bark tissue with the fungus was placed into sterile Petri dishes with a damp piece of Whatman filter paper and sealed with parafilm. The cultures were kept in the dark at room temperature (about 25° C) for one week. Hypovirus transmission into a recipient strain was observed by the dramatic reduction in the growth of the recipient as compared to the growth rate of controls of uninfected recipient strains on live chestnut tissue.

Table 1. Strains used in this study.

Strain	Genetic markers[a]	Vegetative incompatibility genotype[b]	Hypovirus/ dsRNA	Source[c]
389.7	*cre, nic, MAT1-1*	*vic1-1,2-1,3-1,4-1,5-1*		Mutagenesis of EP389
389.7(713)			CHV1-713	this study
389.7(GH2)			CHV3-GH2	this study
389.7(80-2)			80-2	this study
F2.36	*br*			cross 389.7 X 80-2c
F2.36(713)			CHV1-713	this study
F2.36(GH2)			CHV3-GH2	this study
F2.36(80-2)			80-2	this study
EP388	*met, MAT1-2*	*vic1-2,2-1,3-1,4-1,5-1*		ATCC 38979
EP388(713)			CHV1-713	this study
EP388(GH2)			CHV3-GH2	this study
EP388(80-2)			80-2	this study
F2.17	*br*			cross 389.7 X 80-2c
F2.17(713)			CHV1-713	this study
F2.17(80-2)			80-2	this study
EP289	*met, MAT1-2*	*vic1-1,2-2,3-1,4-1,5-1*		Conn. Agri. Exp.
EP289(713)			CHV1-713	this study
EP289(GH2)			CHV3-GH2	this study
EP289(80-2)			80-2	this study
J2.43	*br*			cross F3.16 X A1.13
J2.43(713)			CHV1-713	this study
J2.43(80-2)			80-2	this study
22508	*met, MAT1-2*	*vic1-2,2-2,3-1,4-1,5-1*		ATCC 22508
22508(713)			CHV1-713	this study
22508(GH2)			CHV3-GH2	this study
22508(80-2)			80-2	this study
J1.27	*br, met*			cross F3.16 X A1.13
J1.27(713)			CHV1-713	this study
J1.27(80-2)			80-2	this study
N1.25	*br*	*vic1-1,2-1,3-2,4-1,5-1*		cross F4.13 X K1.43
N1.25(80-2)			80-2	this study

Table 1 (cont'd).

M1.5	*br, nic*	*vic1-1,2-2,3-2,4-1,5-1*		cross K2.30 X J2.69
M1.5(713)			CHV1-713	this study
M1.5(80-2)			80-2	this study
N1.39	*br*	*vic1-2,2-1,3-2,4-1,5-1*		cross F4.13 X K1.43
N1.39(80-2)			80-2	this study
N1.36	*cre*			cross F4.13 X K1.43
N1.36(80-2)			80-2	this study
M1.14	*br, nic*	*vic1-2,2-2,3-2,4-1,5-1*		cross K2.30 X J2.69
M1.14(80-2)			80-2	this study
F3.26	*br, nic*	*vic1-1,2-1,3-1,4-2,5-1*		cross 389.7 X 80-2c
F3.26(713)			CHV1-713	this study
F3.26(GH2)			CHV3-GH2	this study
F3.16	*br, nic*	*vic1-2,2-1,3-1,4-2,5-1*		cross 389.7 X 80-2c
F3.16(713)			CHV1-713	this study
F3.16(80-2)			80-2	this study
J2.31	*br, nic*	*vic1-1,2-2,3-1,4-2,5-1*		cross F3.16 X A1.13
J2.31(713)			CHV1-713	this study
J2.23	*br, nic*	*vic1-2,2-2,3-1,4-2,5-1*		cross F3.16 X A1.13
J2.23(713)			CHV1-713	this study
J2.23(80-2)			80-2	this study
F3.10	*br, nic*	*vic1-1,2-1,3-1,4-1,5-2*		cross 389.7 X 80-2c
F3.10(713)			CHV1-713	this study
F3.10(GH2)			CHV3-GH2	this study
F3.15	*br, nic*	*vic1-2,2-1,3-1,4-1,5-2*		cross 389.7 X 80-2c
F3.15(713)			CHV1-713	this study
F3.15(80-2)			80-2	this study
F3.39	*br, nic*	*vic1-1,2-1,3-1,4-2,5-2*		cross 389.7 X 80-2c
F3.39(713)			CHV1-713	this study
F3.39(80-2)			80-2	this study
F2.2	*br, nic*	*vic1-2,2-1,3-1,4-2,5-2*		cross 389.7 X 80-2c
F2.2(713)			CHV1-713	this study
80-2c	*br*			conidium of strain 80-2
80-2c(80-2)			80-2	this study

Table 1 (cont'd).

EP155	*MAT1-2*	unknown		ATCC 38755
EP713	*MAT1-2*	unknown	CHV1-713	ATCC 52571
EP2001	*met, MAT1-2*	unknown		ATCC 60589
EP2001(713)			CHV1-713	this study
GH2		unknown	CHV3-GH2	MSU collection[e]
EP78		unknown		ATCC 38752
P1.9		unknown		cross EP155 X 80-2c

[a] Each genetic marker is also present in the hypovirus-infected derivative of each strain.

[b] For brevity vegetative incompatibility genotypes have been written as *vic1-1,2-1* rather than as *vic1-1*, *vic2-1*. Each vc genotype applies to each strain in the column beneath the printed genotype until a new printed vc genotype appears.

[c] The crosses, mutagenesis of EP389, and derivation of strain 80-2c were described in Chapter 3.

[d] Strain provided by S. L. Anagnostakis, Connecticut Agricultural Experiment Station.

[e] Strain provided by Dennis W. Fulbright, Michigan State University collection.

Results

Hypovirus effects upon fungal morphology, and the detection of transmission. Fungal strains were infected with two characterized hypoviruses, CHV1-713 and CHV3-GH2, and one uncharacterized dsRNA element, 80-2. Each of the infectious elements produced a characteristic morphological change in the host mycelium (Figures 1 and 2).

The uncharacterized dsRNA 80-2 causes a loss of pigmentation and a reduction of conidiation in all infected strains. Mycelial growth vigor on PDA was not obviously impaired by infection, although rarely a subculture from an infected strain would show an abnormal morphology and grow poorly. Strain J2.43(80-2) was consistently more debilitated as evidenced by growth cessation prior to reaching the Petri plate margin.

Hypovirus CHV3-GH2 causes the infected mycelium on PDA to grow appressed with fewer aerial hyphae and reduced mycelial vigor so that the growth front does not reach the margin of the Petri dish. Neither pigmentation nor conidiation were obviously reduced in infected strains. All of the strains infected with CHV3-GH2 exhibited similar effects upon growth morphology.

Hypovirus CHV1-713 causes a loss of pigmentation, reduction of conidiation, and reduced growth vigor (see also Nuss, 1992). The effects upon mycelial growth could be variable both within a single strain and between strains although the range of variability within a single strain was itself characteristic of this hypovirus. For example, the mycelial growth front generally would stop before reaching the edge of the Petri plate although in some subcultures of a particular strain growth would proceed to the edge. In some strains, CHV1-713 caused a more severe debilitation characterized as dense mycelium with an

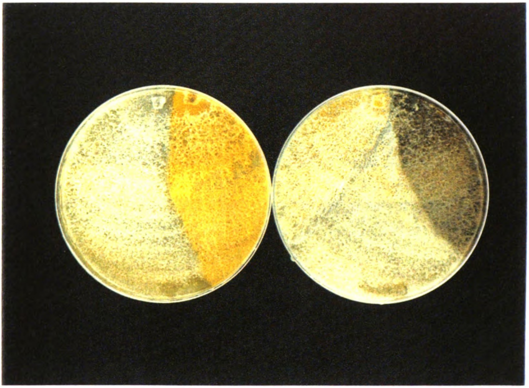

Figure 1. Nonreciprocal transmission of 80-2 dsRNA between donor and recipient strains caused by heteroallelism at *vic1*. The strain on the left of each plate is the donor (infected with dsRNA); the strain on the right is the recipient (dsRNA-free). The recipient has become infected in the pairing in the right plate but not in the left plate. The left plate contains strains F2.36(80-2) and EP388. The right plate contains strains EP388(80-2) and F2.36.

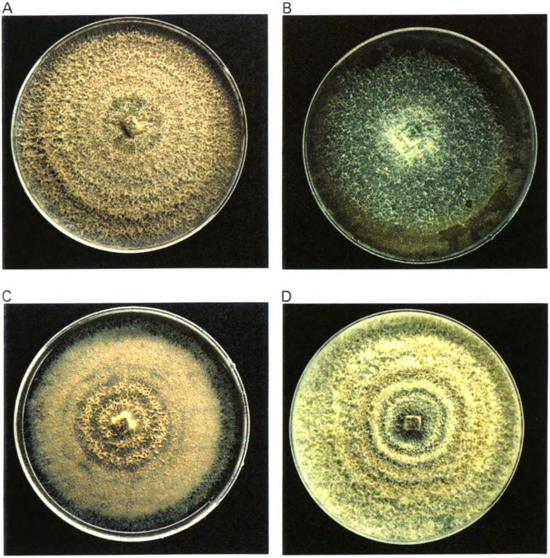

Figure 2. Phenotypic changes in strain F2.36 caused by hypovirus infection. A. uninfected strain F2.36; B. strain F2.36(713) infected with CHV1-713; C. strain F2.36(GH2) infected with CHV3-GH2; D. strain F2.36(80-2) infected with dsRNA 80-2.

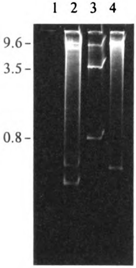

Figure 3. Banding patterns of dsRNA isolated from the infected strains of *C. parasitica* shown in Figure 2 electrophoresed in a 5% polyacrylamide gel and stained with ethidium bromide. Lanes: 1, F2.36; 2, F2.36(713); 3, F2.36(GH2); lane 4, F2.36(80-2). Sizes of dsRNA molecules present in strain F2.36(GH2) are indicated in kilobase pairs (kb).

irregular growth front that did not extend beyond about half the plate diameter. In other strains, particular subcultures of infected mycelium would grow with either the less dense, vigorous growth form or the dense, slow growing, more debilitated growth form. Similar variability in the effects of dsRNA upon growth morphology has been described by Anagnostakis (1981). Because the transmission assay required strains to grow in contact for a distance equal to two-thirds of the Petri plate diameter (about 6 cm), the more extremely debilitated strains or subcultures of strains were not used in transmission tests because they could not meet this growth requirement.

The observation of hypovirus/dsRNA transmission between mycelia was dependent upon the morphological change in the recipient mycelium accompanying infection. All three dsRNAs transferred laterally through the recipient mycelium more rapidly than the mycelium grew (see also Martin and Van Alfen 1991). The donor and recipient mycelia were positioned on the medium surface so that they were not in immediate contact when growth commenced, thereby permitting the recipient to produce a portion of morphologically normal growth before dsRNA infection was possible through mycelial contact. Therefore, when infection occurred, the recipient mycelium exhibited a characteristic and easily observed laterally spreading morphological change that clearly differed from normal growth (Figure 1).

The time between contact of the growing strains and the visible detection of virus transmission could be quite variable. Compatible strains would normally permit transmission as soon as vegetative contact was made, whereas incompatible strains could permit transmission any time during which the actively growing mycelia were in contact which might last up to ten days. Every strain used as a donor was also tested for dsRNA after initial infection to verify that the morphological change was caused by the presence of the hypovirus. Figure 2 shows the morphological changes in the fungus caused by each hypovirus, and Figure 3 shows the hypoviral genomes as they appear on a polyacrylamide gel. Occasionally, the morphology of the recipient strain was ambiguous. In these cases the recipient was either subcultured for further observation of its morphology or a dsRNA extraction was performed to verify hypovirus infection.

The effect of genetic background upon transmission when all *vic* loci are homoallelic. Comparisons of the effects of different genes upon phenotype are best considered in isogenic strains. Unfortunately, the long reproductive cycle of *C. parasitica* in culture precluded the feasibility of creating near-isogenic strains through backcrossing for this study. Instead, the effects of individual *vic* genes were examined both among some strains which shared several unlinked genetic markers as well as among strains with quite different genetic backgrounds to test whether the observed effects upon transmission were genotype specific. A number of the strains used are progeny from cross 389.7 X 80-2c or descendants from strain 389.7 (Table 1). Where possible, strains were used that carried the genetic markers *br* and *nic*. These two markers are not linked to loci *vic1*, *vic2*, *vic3*, *vic4*, or *vic5* (chapter 3). Hypovirus/dsRNA transmission was found to be unimpeded in all reciprocal tests between strains homoallelic with respect to several combinations of the alleles of the five *vic* loci, indicating that the genetic background did not have a discernible effect (Table 2). Of course, genes whose effect would be to reduce the transmission barrier caused by *vic* loci would remain unobserved in this test.

Table 2. Transmission of dsRNAs where donor and recipient strains are homoallelic at all *vic* loci (=vegetatively compatible).[a]

Donor		Recipient		Transmission of dsRNA		
vic[b] *1 2 3 4 5*	strain	*vic 1 2 3 4 5*	strain	CHV1-713	CHV3-GH2	80-2
1 1 1 1 1	389.7	*1 1 1 1 1*	F2.36	8/8	10/10	8/8
1 1 1 1 1	F2.36	*1 1 1 1 1*	389.7	10/10	10/10	6/6
2 1 1 1 1	EP388	*2 1 1 1 1*	F2.17	10/10	7/7	8/8
2 1 1 1 1	F2.17	*2 1 1 1 1*	EP388	5/5	NT[c]	6/6
1 2 1 1 1	EP289	*1 2 1 1 1*	J2.43	9/9	8/8	8/8
1 2 1 1 1	J2.43	*1 2 1 1 1*	EP289	8/8	NT	5/5
2 2 1 1 1	22508	*2 2 1 1 1*	J1.27	8/8	8/8	8/8
2 2 1 1 1	J1.27	*2 2 1 1 1*	22508	5/5	NT	6/6
1 1 1 2 2	EP243	*1 1 1 2 2*	F3.39	NT	NT	8/8
1 1 1 2 2	F3.39	*1 1 1 2 2*	EP243	NT	NT	6/6

[a] Virus transmission is given as the number of times that the virus was detected in the recipient mycelium per number of times that the donor and recipient strains were grown together. Reciprocal pairings of donor and recipient strains are grouped as couplets in rows.

[b] For brevity *vic* loci are designated only by their number as a column heading with alleles for each locus listed in the column beneath the appropriate locus. Only two alleles, designated as *1* or *2*, are known for each *vic* locus.

[c] NT = not tested.

The effects of *vic4* and *vic5* upon transmission. Table 3 shows reciprocal transmission tests using strains which are heteroallelic at *vic4*, or *vic5*, or both loci. To test for possible epistatic effects due to other *vic* alleles, strains with alternate combinations of alleles at *vic1* and *vic2* were examined. Neither *vic4* nor *vic5* were found to impede the horizontal transmission of hypovirus/dsRNA. Simultaneous heteroallelism at both *vic4* and *vic5* also showed no diminution of transmission indicating that no additive effect occurred. The potential additive effects of *vic4* and *vic5* were also tested in conjunction with heteroallelism at *vic1* and *vic2* (Tables 4, 5, and 6), and will be described below.

The effect of *vic1* upon transmission. The effects of heteroallelism at *vic1* upon horizontal transmission were tested using reciprocal pairings of thirteen different combinations of donor and recipient strains (Table 4). Reciprocal testing showed that the effects of *vic1* depended upon which allele of *vic1* was present in the donor strain and which was in the recipient (Table 4). When *vic1-2* was in the recipient strain and *vic1-1* was in the donor strain, transmission of hypoviruses/dsRNA was prevented or greatly hindered. Most frequently, the infection of the *vic1-2* strain did not occur at all. However, when the recipient carried *vic1-1* and the donor carried *vic1-2*, infection occurred in every test. Therefore, *vic1* has a nonreciprocal or unidirectional effect upon the horizontal transmission of hypoviruses.

The potential epistatic effects of other *vic* genes upon heteroallelism at *vic1* were also tested by incorporating different *vic* alleles into the donor and recipient strains. First, the effects of heteroallelism at *vic1* were examined when donor and recipient strains were homoallelic for *vic2-2* (pairing EP289 and 22508) to test for potential epistatic effects

Table 3. Transmission of dsRNAs where the donor and recipient strains are heteroallelic at vegetative incompatibility loci *vic4* and *vic5*.[a]

Donor		Recipient		transmission of dsRNA		
vic[b] *1 2 3 4 5*	strain	*vic 1 2 3 4 5*	strain	CHV1-713	CHV3-GH2	80-2
1 1 1 1 1	389.7	*1 1 1 2 1*	F3.26	8/8	8/8	6/6
1 1 1 2 1	F3.26	*1 1 1 1 1*	389.7	6/6	5/5	NT[c]
2 1 1 1 1	EP388	*2 1 1 2 1*	F3.16	10/10	NT	NT
2 1 1 2 1	F3.16	*2 1 1 1 1*	EP388	10/10	NT	NT
1 2 1 1 1	EP289	*1 2 1 2 1*	J2.31	10/10	NT	NT
1 2 1 2 1	J2.31	*1 2 1 1 1*	EP289	9/9	NT	NT
2 2 1 1 1	22508	*2 2 1 2 1*	J2.23	10/10	NT	NT
2 2 1 2 1	J2.23	*2 2 1 1 1*	22508	10/10	NT	NT
1 1 1 1 1	389.7	*1 1 1 1 2*	F3.10	10/10	10/10	NT
1 1 1 1 2	F3.10	*1 1 1 1 1*	389.7	8/8	5/5	NT
2 1 1 1 1	EP388	*2 1 1 1 2*	F3.15	10/10	NT	NT
2 1 1 1 2	F3.15	*2 1 1 1 1*	EP388	7/7	NT	NT
1 1 1 1 1	389.7	*1 1 1 2 2*	F3.39	8/8	6/6	6/6
1 1 1 2 2	F3.39	*1 1 1 1 1*	389.7	9/9	6/6	6/6
2 1 1 1 1	EP388	*2 1 1 2 2*	F2.2	10/10	NT	NT
2 1 1 2 2	F2.2	*2 1 1 1 1*	EP388	10/10	NT	NT

[a] Virus transmission is given as the number of times that the virus was detected in the recipient mycelium per number of times that the donor and recipient strains were grown together. Reciprocal pairings of donor and recipient strainsare grouped as couplets in rows.

[b] For brevity *vic* loci are designated only by their number as a column heading with alleles for each locus listed in the column beneath the appropriate locus. Only two alleles, designated as *1* or *2*, are known for each *vic* locus.

[c] NT = not tested.

Table 4. **Transmission of dsRNAs where donor and recipient strains are heteroallelic at vegetative incompatibility locus *vic1*. The presence of additive and epistatic effects between *vic* genes was tested by varying the alleles present at *vic4* and *vic5*.[a]**

Donor		Recipient		Transmission of dsRNA		
vic[b]*1 2 3 4 5*	strain	*vic 1 2 3 4 5*	strain	CHV1-713	CHV3-GH2	80-2
1 1 1 1 1	389.7	*2 1 1 1 1*	EP388	0/8	0/9	2/15
2 1 1 1 1	EP388	*1 1 1 1 1*	389.7	10/10	8/8	16/16
1 1 1 1 1	F2.36	*2 1 1 1 1*	EP388	0/10	NT[c]	NT
2 1 1 1 1	EP388	*1 1 1 1 1*	F2.36	10/10	NT	NT
1 1 1 1 1	389.7	*2 1 1 1 1*	F2.17	0/10	NT	NT
2 1 1 1 1	F2.17	*1 1 1 1 1*	389.7	10/10	NT	NT
1 1 1 1 1	F2.36	*2 1 1 1 1*	F2.17	0/10	NT	NT
2 1 1 1 1	F2.17	*1 1 1 1 1*	F2.36	10/10	NT	NT
1 2 1 1 1	EP289	*2 2 1 1 1*	22508	0/10	0/13	1/12
2 2 1 1 1	22508	*1 2 1 1 1*	EP289	10/10	9/9	15/15
1 1 1 1 1	389.7	*2 1 1 2 1*	F3.16	1/8	NT	NT
2 1 1 2 1	F3.16	*1 1 1 1 1*	389.7	10/10	NT	NT
1 2 1 1 1	EP289	*2 2 1 2 1*	J2.23	2/9	NT	NT
2 2 1 2 1	J2.23	*1 2 1 1 1*	EP289	10/10	NT	NT
1 1 1 2 1	F3.26	*2 1 1 1 1*	EP388	0/7	NT	NT
2 1 1 1 1	EP388	*1 1 1 2 1*	F3.26	10/10	NT	NT
1 2 1 2 1	J2.31	*2 2 1 1 1*	22508	0/6	NT	NT
2 2 1 1 1	22508	*1 2 1 2 1*	J2.31	10/10	NT	NT

Table 4
(cont'd).

1 1 1 1 1	389.7	*2 1 1 1 2*	F3.15	3/10	NT	NT
2 1 1 1 2	F3.15	*1 1 1 1 1*	389.7	5/5	NT	NT
1 1 1 1 2	F3.10	*2 1 1 1 1*	EP388	0/10	NT	NT
2 1 1 1 1	EP388	*1 1 1 1 2*	F3.10	10/10	NT	NT
1 1 1 1 1	389.7	*2 1 1 2 2*	F2.2	1/7	NT	NT
2 1 1 2 2	F2.2	*1 1 1 1 1*	389.7	10/10	NT	NT
2 1 1 1 1	EP388	*1 1 1 2 2*	F3.39	10/10	NT	NT
1 1 1 2 2	F3.39	*2 1 1 1 1*	EP388	0/7[d]	NT	0/8

[a] Virus transmission is given as the number of times that the virus was detected in the recipient mycelium per number of times that the donor and recipient mycelia were grown together. Reciprocal pairings of donor and recipient strains are grouped as couplets in rows.

[b] For brevity *vic* loci are designated only by their number as a column heading with alleles for each locus listed in the column beneath the appropriate locus. Only two alleles, designated as *1* or *2*, are known for each *vic* locus.

[c] NT = not tested

[d] Donor and recipient strains did not grow in contact for the minimum required distance because the donor grew poorly.

associated with allele *vic2-2* (Table 4). This alteration in vc genotype had no effect upon transmission. Secondly, the possibility that heteroallelism at either *vic4* or *vic5*, or both loci, in conjunction with *vic1* would decrease the transmission associated with *vic1* was also tested (Table 4). No additive effects due to the combined activity of *vic1*, *vic4* and *vic5* could be found whereby the receptivity of the *vic1-1* strain was diminished, nor was the nonreceptivity of the *vic1-2* strain obviously relaxed although each of the four recipient strains carrying *vic4-2* or *vic5-2* were infrequently infected.

The effect of *vic2* upon transmission. To determine the effects of heteroallelism at *vic2* upon transmission, the first test used donor and recipient strains homoallelic for *vic1-2* to preclude possible effects due to the nonreciprocal receptivity associated with *vic1-1*. Hypovirus transmission between strains EP388 and 22508 was found to be virtually prevented by *vic2* (Table 5). Transmission was then examined between strains 389.7 and EP289 which were homoallelic for *vic1-1* to test whether *vic1-1* would have any effect upon the *vic2* transmission barrier (Table 5). In this case, the *vic2* barrier became unidirectionally less restrictive where the *vic1-1*, *vic2-1* recipient (389.7) became much more receptive to hypovirus infection. That is, when the recipient strain carried the allele *vic2-2* and the donor carried *vic2-1*, transmission was prevented or occurred infrequently irrespective of whether both strains were homoallelic for *vic1-1* or *vic1-2*. But when the recipient strain carried *vic1-1* along with *vic2-1*, the recipient became more receptive to virus infection. This demonstrates that locus *vic2* prevents horizontal transmission in a conditional manner whereby increased cytoplasmic transmission results from the epistasis of *vic1-1* over *vic2-1* when both occur in the recipient.

The epistatic nature of the relationship between *vic1-1* and *vic2-1* was demonstrated by pairing strains 389.7 and EP388 (Table 4). If *vic1-1* is epistatic over *vic2-1*, then *vic2-1* should not have a reciprocal effect upon *vic1-1* whereby the receptivity of *vic1-1* (when *vic1* is heteroallelic) is diminished. This pairing shows that the presence of *vic2-1* in the recipient does not diminish the receptivity of *vic1-1*. Therefore the relationship between these genes can be considered epistatic.

The effect of heteroallelism at *vic2* was also tested where the genetic background consisted of strains homoallelic for *vic1-1*, and heteroallelic for *vic4*, or *vic5*, or both loci. Three pairs of strains represent these genotypes: EP289 paired with F3.26, F3.10, and EP243 (Table 5). In all three of these pairs transmission occurred with a prominent nonreciprocal bias where the recipient that carried *vic1-1* and *vic2-1* was frequently infected. Heteroallelism at *vic4*, *vic5*, or both loci did not eliminate the epistasis of *vic1-1* over *vic2-1*.

The epistasis of *vic1-1* over *vic2-1* was further tested using strains that were simultaneously heteroallelic at both *vic1* and *vic2* (Table 6). Transmission was found to be prevented in reciprocal tests with strains EP289 and EP388, but transmission occurred unidirectionally between strains 389.7 and 22508 when the recipient carried both *vic1-1* and *vic2-1* (Table 6). Concurrent heteroallelism at *vic4* and *vic5* also did not diminish viral transmission below levels associated with *vic1* and *vic2*, nor was the unidirectional transmission associated with the epistasis of *vic1-1* over *vic2-1* prevented (Table 6). In summary, every test for the epistasis of *vic1-1* over *vic2-1* in recipients showed the same relaxation of the *vic2* transmission barrier.

Table 5. Transmission of dsRNAs where donor and recipient strains are heteroallelic at vegetative incompatibility locus *vic2*. The presence of additive and epistatic effects between *vic* genes was tested by varying the alleles at *vic1*, *vic4*, and *vic5*.[a]

Donor		Recipient		Transmission of dsRNA		
vic[b] *1 2 3 4 5*	strain	*vic 1 2 3 4 5*	strain	CHV1-713	CHV3-GH2	80-2
1 1 1 1 1	389.7	*1 2 1 1 1*	EP289	0/8	1/9	0/13
1 2 1 1 1	EP289	*1 1 1 1 1*	389.7	5/11	6/9	13/21
2 1 1 1 1	EP388	*2 2 1 1 1*	22508	1/10	0/8	0/13
2 2 1 1 1	22508	*2 1 1 1 1*	EP388	0/8	0/10	0/12
1 1 1 1 1	389.7	*1 2 1 2 1*	J2.31	4/9	NT[c]	NT
1 2 1 2 1	J2.31	*1 1 1 1 1*	389.7	3/8	NT	NT
1 1 1 2 1	F3.26	*1 2 1 1 1*	EP289	0/8	NT	NT
1 2 1 1 1	EP289	*1 1 1 2 1*	F3.26	7/9	NT	NT
1 2 1 1 1	EP289	*1 1 1 1 2*	F3.10	7/8	NT	NT
1 1 1 1 2	F3.10	*1 2 1 1 1*	EP289	1/8	NT	NT
2 1 1 1 1	EP388	*2 2 1 2 1*	J2.23	6/14	NT	8/19
2 2 1 2 1	J2.23	*2 1 1 1 1*	EP388	0/4	NT	0/21
2 1 1 2 1	F3.16	*2 2 1 1 1*	22508	0/9	NT	0/10
2 2 1 1 1	22508	*2 1 1 2 1*	F3.16	6/14	NT	9/10
2 1 1 1 2	F3.15	*2 2 1 1 1*	22508	0/12	NT	0/10
2 2 1 1 1	22508	*2 1 1 1 2*	F3.15	3/7	NT	9/10
1 2 1 1 1	EP289	*1 1 1 2 2*	EP243	NT	NT	8/12
1 1 1 2 2	EP243	*1 2 1 1 1*	EP289	NT	NT	1/13

Table 5
(cont'd)

2 1 1 2 2	80-2c	*2 2 1 1 1*	22508	NT	NT	0/9
2 2 1 1 1	22508	*2 1 1 2 2*	80-2c	NT	NT	1/7

[a] Virus transmission is given as the number of times that the virus was detected in the recipient mycelium per number of times that the donor and recipient mycelia were grown together. Reciprocal pairings of donor and recipient strains are grouped as couplets in rows.
[b] For brevity *vic* loci are designated only by their number as a column heading with alleles for each locus listed in the column beneath the appropriate locus. Only two alleles, designated as *1* or *2*, are known for each *vic* locus.
[c] NT = not tested.

Table 6. Transmission of dsRNAs where donor and recipient strains are heteroallelic at both vegetative incompatibility loci *vic1* and *vic2*. The presence of additive or epistatic effects between *vic* genes was also tested by incorporating heteroallelism at *vic4* and *vic5*.[a]

Donor		Recipient		transmission of dsRNA		
vic[b]*1 2 3 4 5*	strain	*vic 1 2 3 4 5*	strain	CHV1-713	CHV3-GH2	80-2
2 1 1 1 1	EP388	*1 2 1 1 1*	EP289	0/10	0/11	0/12
1 2 1 1 1	EP289	*2 1 1 1 1*	EP388	0/10	0/11	0/13
1 1 1 1 1	389.7	*2 2 1 1 1*	22508	0/8	0/8	0/13
2 2 1 1 1	22508	*1 1 1 1 1*	389.7	7/8	5/8	5/15
2 1 1 1 1	EP388	*1 2 1 2 1*	J2.31	3/11	NT[c]	NT
1 2 1 2 1	J2.31	*2 1 1 1 1*	EP388	0/7	NT	NT
2 1 1 2 1	F3.16	*1 2 1 1 1*	EP289	0/8	NT	NT
1 2 1 1 1	EP289	*2 1 1 2 1*	F3.16	0/10	NT	NT
2 1 1 1 2	F3.15	*1 2 1 1 1*	EP289	0/8	NT	NT
1 2 1 1 1	EP289	*2 1 1 1 2*	F3.15	0/8	NT	NT
2 1 1 2 2	80-2c	*1 2 1 1 1*	EP289	NT	NT	0/7
1 2 1 1 1	EP289	*2 1 1 2 2*	80-2c	NT	NT	0/6
1 1 1 1 1	389.7	*2 2 1 2 1*	J2.23	0/9	NT	NT
2 2 1 2 1	J2.23	*1 1 1 1 1*	389.7	2/7	NT	NT
1 1 1 2 1	F3.26	*2 2 1 1 1*	22508	0/10	NT	NT
2 2 1 1 1	22508	*1 1 1 2 1*	F3.26	4/9	NT	NT
1 1 1 1 2	F3.10	*2 2 1 1 1*	22508	0/8	NT	NT
2 2 1 1 1	22508	*1 1 1 1 2*	F3.10	3/9	NT	NT

Table 6
(cont'd)

1 1 1 2 2	EP243	*2 2 1 1 1*	22508	NT	NT	0/13
2 2 1 1 1	22508	*1 1 1 2 2*	EP243	NT	NT	5/16

[a] Virus transmission is given as the number of times that the virus was detected in the recipient mycelium per number of times that the donor and recipient mycelia were grown together. Reciprocal pairings of donor and recipient strains are grouped as couplets in rows.

[b] For brevity *vic* loci are designated only by their number as a column heading with alleles for each locus listed in the column beneath the appropriate locus. Only two alleles, designated as *1* or *2*, are known for each *vic* locus.

[c] NT = not tested.

Other genetic background effects upon the transmission barrier caused by *vic2*. In the course of testing the epistasis of *vic1-1* over *vic2-1*, the genotypes were further modified by incorporating heteroallelism at the weak incompatibility loci *vic4* and *vic5* in conjunction with heteroallelism at *vic1* and *vic2*. This revealed additional genetic background effects that also resulted in the unidirectional reduction of the transmission barrier caused by *vic2*. First, strains were paired that were homoallelic for *vic1-2*, heteroallelic for *vic2*, and heteroallelic for either *vic4* or *vic5*: EP388 was paired with J2.23, and 22508 was paired with both F3.16 and F3.15 (Table 5). In these three reciprocal pairings transmission was still inhibited when *vic1-2* and *vic2-1* occurred together in the recipient, but the reciprocal pairings resulted in an increased level of transmission relative to the other tests of *vic2* described above. This higher receptivity of strains J2.23, F3.15 and F3.16 was associated with the presence of either *vic4-2* or *vic5-2*. It is possible that these genes are responsible for the unidirectional relaxation of the *vic2* transmission barrier.

To further test the putative effects of *vic4-2* and *vic5-2* upon heteroallelism at *vic2*, three additional tests were conducted. Strains 389.7 and J2.31 were paired, and again the transmission barrier caused by *vic2* was relaxed in the recipient carrying *vic4-2* (Table 5). Strains Ep388 and J2.31 were also tested where both *vic1* and *vic2* were heteroallelic (Table 6). Here, a small reduction of the *vic2* transmission barrier may be present but is not clear. Lastly, strains 22508 and 80-2c were used to test the effects of simultaneous heteroallelism at *vic2*, *vic4* and *vic5* (Table 5). This test resulted in the inhibition of transmission in reciprocal tests rather than a unidirectional relaxation of the *vic2* barrier.

Modifications of transmission due to these gene associations require further testing.

The effect of *vic3* upon transmission, and modifications due to epistasis. Heteroallelism at *vic3* was found to cause nonreciprocal transmission (Table 7). This analysis also indicated that locus *vic1* may exhibit two distinct epistatic effects upon *vic3*. In one case, allele *vic1-1* appears to reduce the transmission barrier caused by *vic3* when both *vic1-1* and *vic3-1* are present in a recipient. In the other case, the unidirectional inhibition due to heteroallelism at *vic1* was epistatic over the unidirectional transmission caused by *vic3*. Some evidence was also found that suggests that the unidirectional transmission of *vic3* may be epistatic over *vic2* in an allele specific manner. Not all of the combinations of the alleles at *vic1*, *vic2*, and *vic3* were tested, nor were multiple genetic backgrounds with each vc genotype tested.

As with the previous *vic* loci, the effects of *vic3* were first tested with homoallelism at the other *vic* loci. Since *vic1-1* was known from previous studies to have an epistatic influence upon another *vic* gene, the first tests of *vic3* were performed in pairs of strains homoallelic for *vic1-2*. Transmission tests between the pairs of strains 22508/M1.14 and EP388/N1.39 showed that *vic3* is also associated with nonreciprocal transmission (Table 7). Recipients with allele *vic3-2* were infected by *vic3-1* donors in most tests while the reciprocal relationship (recipient *vic3-1*, donor *vic3-2*) resulted in much less or no transmission.

The potential epistatic modification of the *vic3* transmission barrier by either allele *vic1-1* or heteroallelism at *vic1* was tested as follows. First, the effect of *vic1* on *vic3* was examined by testing the effects of both *vic1* alleles upon *vic3* alleles. The barrier to

Table 7. Transmission of dsRNAs where donor and recipient strains are heteroallelic at *vic3*. The presence of additive and epistatic effects between *vic* genes was tested by varying the alleles at *vic1* and *vic2*.[a]

Donor		Recipient		Transmission of dsRNA	
vic[b] *1 2 3 4 5*	strain	*vic 1 2 3 4 5*	strain	CHV1-713	80-2
2 1 1 1 1	EP388	*2 1 2 1 1*	N1.39	NT[c]	10/10
2 1 2 1 1	N1.39	*2 1 1 1 1*	EP388	NT	0/10
1 1 1 1 1	389.7	*1 1 2 1 1*	N1.25	5/6	13/13
1 1 2 1 1	N1.25	*1 1 1 1 1*	389.7	NT[d]	8/16
1 2 1 1 1	EP289	*1 2 2 1 1*	M1.5	9/10	NT
1 2 2 1 1	M1.5	*1 2 1 1 1*	EP289	1/7	NT
2 2 1 1 1	22508	*2 2 2 1 1*	M1.14	10/10	17/17
2 2 2 1 1	M1.14	*2 2 1 1 1*	22508	NT[d]	0/9
1 1 1 1 1	389.7	*1 2 2 1 1*	M1.5	NT[d]	2/12
1 2 2 1 1	M1.5	*1 1 1 1 1*	389.7	0/5	1/14
2 2 1 1 1	22508	*2 1 2 1 1*	N1.39	NT	5/9
2 1 2 1 1	N1.39	*2 2 1 1 1*	22508	NT	0/10
1 2 1 1 1	EP289	*1 1 2 1 1*	N1.25	1/3	1/4
1 1 2 1 1	N1.25	*1 2 1 1 1*	EP289	0/6	0/8
1 1 1 1 1	389.7	*2 1 2 1 1*	N1.39	NT[d]	1/13
2 1 2 1 1	N1.39	*1 1 1 1 1*	389.7	NT	13/14
1 1 1 1 1	389.7	*2 1 2 1 1*	N1.36	NT	3/8
2 1 2 1 1	N1.36	*1 1 1 1 1*	389.7	NT	6/6

Table 7
(cont'd)

1 1 1 1 1	389.7	*2 2 2 1 1*	M1.14	NT	0/10
2 2 2 1 1	M1.14	*1 1 1 1 1*	389.7	NT	3/10

[a] Virus transmission is given as the number of times that the virus was detected in the recipient mycelium per number of times that the donor and recipient strains were grown together. Reciprocal pairings of donor and recipient strains are grouped as couplets in rows.

[b] For brevity *vic* loci are designated only by their number as a column heading with alleles for each locus listed in the column beneath the appropriate locus. Only two alleles, designated as *1* or *2*, are known for each *vic* locus.

[c] NT = not tested.

[d] Transmission tests were attempted but the donor strain grew too poorly to meet minimum assay growth requirements.

transmission caused by *vic3* was found to be reduced when the recipient carried alleles *vic3-1* and *vic1-1*, and did not require heteroallelism at *vic1* (pair 389.7/N1.25; Table 7). This indicates that *vic1-1* may be epistatic over allele *vic3-1* in a similar manner to *vic2-1*. Another test of this putative epistatic relationship was conducted using strains EP289 and M1.5 which were homoallelic for *vic2-2* (Table 7). This test, however, did not show the same reduction in the transmission barrier.

Secondly, the effect of heteroallelism at *vic1* upon heteroallelism at *vic3* was tested by using strains where the unidirectional transmission orientations of *vic1* and *vic3* were opposed (pairings 389.7/N1.39 and 389.7/N1.36) (Table 7). In both tests transmission was found to have a unidirectional bias where strain 389.7 was more frequently infected. This indicated that the unidirectional inhibition of *vic1* was epistatic over the unidirectional transmission of *vic3*. To determine whether this epistatic relationship was due to epistasis between specific alleles (*vic1-2* and *vic3-2*) in the recipient or due to the masking of the heteroallelic *vic3* reaction by heteroallelism at *vic1*, strains EP388 and N1.39 were paired in reciprocal transmission tests (Table 7). Transmission was again found to be unidirectional, demonstrating that the presence of *vic1-2* and *vic3-2* in the recipient was insufficient to stop transmission, and consequently showing that the elimination of receptivity in the *vic3-2* recipient required heteroallelism at *vic1*.

Is the receptivity of allele *vic3-2* epistatic over *vic2-1* in a similar manner to the epistasis of *vic1-1* over *vic2-1*? A reciprocal transmission test between strains 22508 and N1.39 provided evidence that the unidirectional transmission of *vic3* is epistatic over the transmission barrier caused by *vic2* (Table 7). Evidence that this epistatic interaction is allele specific was provided by pairing strain M1.5 with 389.7 (Table 7). An additional test of allele specificity using strains Ep289 and N1.25 was inconclusive because of an insufficient number of successful transmission tests (Table 7). Further testing is needed to establish the presence of epistatic interactions between the alleles of *vic2* and *vic3*.

Prevention of transmission due to undescribed incompatibility genes. The transmission barrier imposed by the incompatibility genotype of strain EP2001 (vc type 10) was evaluated with several tester strains representing differences at each of the five *vic* loci (Table 8). Strain EP2001 represents the most divergent vegetative compatibility genotype known relative to vc type 5 (*vic1-1*, *vic2-1*, *vic3-1*, *vic4-1*, *vic5-1*) (Anagnostakis, 1982). In addition, EP713 was tested as a donor and recipient with EP2001. Vegetative compatibility type 40 (EP713) is reported to differ from vc type 10 (EP2001) at a single *vic* locus (Anagnostakis, 1988), but it is not known which *vic* alleles EP2001 shares with any of the tester strains. Hypovirus transmission occurred freely between EP713 and EP2001 indicating that EP2001 was competent as both a donor and a recipient. However, none of the seven tester strains was capable of infecting EP2001. Six of seven tester strains could not be infected by EP2001(713), but 389.7 was infected (Table 8). Based upon the transmission profiles representing most combinations of the alleles of the strong *vic* loci, *vic1-vic3*, this unusual result may also be due to epistatic interactions between unidentified *vic* genes.

Horizontal transmission efficiencies of different dsRNA genomes. Two characterized dsRNA hypoviruses, CHV1-713 and CHV3-GH2, and one uncharacterized dsRNA element, 80-2, were used in transmission tests to see if different cytoplasmic

Table 8. Transmission of dsRNAs where the *vic* gene differences between vegetatively incompatible donor and recipient strains are not known.[a]

Donor		Recipient		Transmission of dsRNA	
vic[b] *1 2 3 4 5*	strain	*vic 1 2 3 4 5*	strain	CHV1-713	80-2
1 1 1 1 1	389.7	unknown	EP2001	0/5	0/8
unknown	EP2001	*1 1 1 1 1*	389.7	8/8	NT[c]
2 1 1 1 1	EP388	unknown	EP2001	0/5	0/8
unknown	EP2001	*2 1 1 1 1*	EP388	0/7	NT
1 2 1 1 1	EP289	unknown	EP2001	0/5	0/7
unknown	EP2001	*1 2 1 1 1*	EP289	0/8	NT
2 2 1 1 1	22508	unknown	EP2001	0/7	0/7
unknown	EP2001	*2 2 1 1 1*	22508	0/8	NT
2 2 2 1 1	M1.14	unknown	EP2001	0/7	NT
unknown	EP2001	*2 2 2 1 1*	M1.14	0/5	NT
2 1 1 2 2	80-2c	unknown	EP2001	NT	0/8
unknown	EP2001	*2 1 1 2 2*	80-2c	NT	NT
unknown	EP713	unknown	EP2001	5/5	NT
unknown	EP2001	unknown	EP713	5/5	NT

[a] Virus transmission is given as the number of times that the virus was detected in the recipient mycelium per number of times that the donor and recipient strains were grown together. Reciprocal pairings of donor and recipient strains are grouped as couplets in rows.

[b] For brevity *vic* loci are designated only by their number as a column heading with alleles for each locus listed in the column beneath the appropriate locus. Only two alleles, designated as *1* or *2*, are known for each *vic* locus.

[c] NT = not tested.

Table 9. Transmission of hypovirus CHV1-713 between donor and recipient strains conducted on live chestnut tissue.[a]

Donor		Recipient		transmission of virus
vic[b] *1 2 3 4 5*	strain	*vic 1 2 3 4 5*	strain	CHV1-713
1 1 1 1 1	389.7	*1 1 1 1 1*	F2.36	4/4
1 1 1 1 1	389.7	*2 1 1 1 1*	F2.17	1/6[c]
2 1 1 1 1	F2.17	*1 1 1 1 1*	389.7	6/6
1 1 1 1 1	389.7	*1 2 1 1 1*	J2.43	+[d]
1 1 1 1 1	389.7	*1 1 2 1 1*	N1.25	2/3
1 1 1 1 1	389.7	*1 1 1 1 2*	F3.10	4/4
1 1 1 1 1	389.7	*1 1 1 2 1*	F3.26	4/4
1 1 1 1 1	389.7	*1 1 1 2 2*	F3.39	4/4

[a] Virus transmission is given as the number of times that the virus was detected in the recipient mycelium per number of times that the donor and recipient strains were grown together. See materials and methods for an explanation of procedures.

[b] For brevity *vic* loci are designated only by their number as a column heading with alleles for each locus listed in the column beneath the appropriate locus. Only two alleles, designated as *1* or *2*, are known for each *vic* locus.

[c] This single record of infection is uncertain because of the presence of contaminants on the wood. Two other possible infections of F2.17 were also observed, but are not recorded because of more severe contamination in those trials.

[d] One pairing between 389.7(713) and J2.43 showed infection of 2.43 upon subculturing although contaminants were present. Other pairings were also done, but contaminants obscured the results.

elements transmit with different efficiencies. In most tests all three cytoplasmic elements were transmitted with equivalent efficiency. Because CHV1-713 causes a more severe debilitation in the fungus, transmission tests sometimes had to be repeated several times in order to obtain pairings that met the growth standards set for this study. This only had to be done when the donor strain differed from the recipient at *vic1* or *vic2* because transmission between compatible strains or those heteroallelic at *vic4* and *vic5* occurred rapidly so that the distance of growth contact before infection was not significant.

Horizontal transmission on live chestnut tissue. Because the effects of vegetative incompatibility upon heterokaryon formation were found to vary depending upon whether strains were grown on PDA or chestnut tissue (Chapter 3), hypovirus transmission was also tested on live chestnut tissue using a limited number of vc genotypes (Table 9). Viral transmission was unimpeded between strains homoallelic at all *vic* loci. Heteroallelism at *vic1*, *vic4*, and *vic5* were also found to have similar effects upon transmission when strains were grown on live chestnut compared to those grown on PDA. Transmission was found to occur when *vic2* was heteroallelic although this represents only a single observation because other tests were destroyed by contaminants. *vic3* was found to limit, but not prevent, transmission. Additional testing on live chestnut tissue is necessary to determine the efficiency of transmission through the *vic2* and *vic3* incompatibility barriers. Ideally, these transmission tests should also be conducted by infecting live trees with these fungal strains so that mycelial interactions can be observed under natural conditions.

Discussion

The effects of five *vic* loci upon the horizontal cytoplasmic transmission of hypoviruses in *C. parasitica* have been found to be quite variable. The different types of effects can be categorized as differences in the efficiency of the transmission barrier, presence or absence of reciprocality in transmission, and modifications in transmission due to epistasis between *vic* genes.

Individual *vic* genes have different effects upon horizontal transmission. This study found that the effectiveness of vegetative incompatibility polymorphisms in *C. parasitica* as a barrier to horizontal transmission is quite variable due to the unique effects of individual *vic* genes (summarized in Table 10). Heteroallelism between conspecifics at loci *vic4* and *vic5*, individually and in concert, did not prevent hypovirus and dsRNA element transmission. In contrast, heteroallelism at *vic1*, *vic2*, and *vic3* can prevent the transmission of these cytoplasmic parasites although their effectiveness is dependent upon either reciprocality (*vic1* and *vic3*) or upon epistasis by other *vic* genes (*vic2* and *vic3*).

The permissiveness to hypovirus transmission of each of the five *vic* loci, and the epistatic modifications of transmission at two of the *vic* loci, stand in sharp contrast to the restrictiveness of the same loci with regard to horizontal nuclear transmission. Heterokaryosis was previously found to be strictly prevented by each of the *vic* loci when mycelia were grown on chestnut tissue. On an agar substrate *vic1*, *vic2*, and *vic3* were also found to prevent heterokaryon formation although *vic4*, and especially *vic5*, were associated with abnormal mycelial interactions suggestive of limited nuclear exchange (Chapter 3). In contrast, *vic4* and *vic5* always permitted hypovirus transmission on both

Table 10. Summary of the effects of each *vic* locus upon the horizontal (cytoplasmic) transmission of hypoviruses in *Cryphonectria parasitica*.

***vic* locus**[a]	**Effect upon transmission**[b]	**Exceptions (due to epistasis or genetic background)**
vic1	nonreciprocal *vic1-1* ← *vic1-2*	none
vic2	reciprocal inhibition *vic2-1* →|← *vic2-2*	1. Unidirectional transmission where allele *vic1-1* is epistatic over allele *vic2-1* in the recipient: <u>donor</u> <u>recipient</u> *1-2,2-2* → *1-1,2-1* *1-1,2-2* → *1-1,2-1* 2. Unidirectional transmission associated with *vic4-2* and *vic5-2* in recipient. 3. Unidirectional transmission where *vic3-2* may be epistatic over *vic2-1*.[c]
vic3	nonreciprocal *vic3-1* → *vic3-2*	1. Heteroallelism at *vic1* is epistatic over heteroallelism at *vic3*: *1-1,3-1* ← *1-2,3-2* 2. Allele *vic1-1* may be epistatic over allele *vic3-1*.[c]
vic4	no inhibition *vic4-1* ↔ *vic4-2*	none
vic5	no inhibition *vic5-1* ↔ *vic5-2*	none

[a] Each *vic* locus has two known alleles designated *1* or *2*, and written as *vic1-1* and *vic1-2*, etc.

[b] Arrows indicate direction of hypovirus transmission. →|← indicates no (or little) transmission in either direction.

[c] Requires further testing.

PDA and live chestnut tissue. Both the study of hypovirus transmission and heterokaryosis are congruent, though, in so far as they demonstrate that *vic4* and *vic5* are less restrictive to cytoplasmic exchange than *vic1*, *vic2* and *vic3*. Interestingly, while simultaneous heteroallelism at *vic4* and *vic5* showed an additive effect that prevented the formation of abnormal mycelial interaction zones, they did not cumulatively inhibit hypovirus transmission. The differences between viral and nuclear transmission for all five *vic* loci may indicate that small cytoplasmic elements are able to breach some incompatibility barriers that entirely prevent the horizontal movement of the larger, and less abundant nuclei.

Strains of *C. parasitica* previously have been found whose ability to infect several different vegetative incompatibility genotypes was considered novel (Kuhlman et al. 1984; Kuhlman and Bhattacharyya 1984). At least two types of genetic explanations could account for this behavior. Suppressor mutants have been found in *Neurospora crassa* (Arganoza et al. 1994b) and in *Podospora anserina* (Bernet 1992b) that suppress specific vegetative incompatibility genes. It is possible that some *C. parasitica* strains that can transmit dsRNA to multiple vc types contain similar suppressor mutations. Alternatively, those strains capable of receiving dsRNA from a broad donor may simply differ from the donor at loci such as *vic4* and *vic5* which cause incompatibility but do not stop cytoplasmic transmission of dsRNA. For example, the present genetic analysis of the vc genotype *vic1-2*, *vic2-2*, *vic3-1*, *vic4-1*, *vic5-1* has shown that it could infect at least 20 of the 32 vc groups derived from the recombination of alleles at *vic1* through *vic5*, and possibly more. This genotype could certainly be referred to as having broad infection capabilities, but its transmission behavior is determined by the particular complement of *vic* alleles that it and the recipient strains possess, and not by suppressor mutations or other unique characteristics. Therefore, without specific genetic evidence for the suppression of incompatibility, the capacity to broadly infect need only be considered a function of the particular *vic* alleles present in a population.

What effects will *vic* gene polymorphisms have upon virus transmission in natural populations of *C. parasitica*? This study has shown that the effects of vegetative incompatibility upon horizontal transmission will depend upon both the particular loci that are polymorphic in a population as well as the complement of *vic* alleles present in each individual. *C. parasitica* populations polymorphic at only loci such as *vic4* and *vic5* would be expected to experience much horizontal transmission. Polymorphisms at other loci such as *vic2* may pose a significant barrier to transmission although epistasis may mitigate the restrictiveness of particular loci such as *vic2*. Nonreciprocal transmission may also become important depending upon which genotypes are acting as donors and recipients in a population. The nonreciprocal transmission found between genotypes vc 5 and vc 10, which probably differ at seven *vic* loci, likely represents an outstanding example of the effects of epistasis, and well illustrate the difficulty in predicting transmission efficiencies without prior knowledge of the genotypes involved. Field studies are needed to test whether these *vic* genes limit virus transmission in a similar manner in natural populations (Double, 1982).

Other studies have also found that individual vegetative incompatibility genes in Ascomycetes have different effects upon the horizontal transmission of cytoplasmic agents and nuclei. Handley and Caten (1973) observed that *hetA* and *hetB* have different capacities to hinder transmission of the cytoplasmic *vgd* mutation in *Aspergillus amstelodami*. In *Ophiostoma ulmi*, a single incompatibility locus was found to limit the transmission of the d-factor more than several other loci (Brasier 1984). Recently, Debets et al. (1994) found that *het* loci in *N. crassa* can have different effects upon the cytoplasmic transmission of plasmids and senescence. Coenen et al. (1994) and Dales et al. (1983) have found differences in the effects of vegetative incompatibility loci upon heterokaryon formation.

Nonreciprocal transmission caused by *vic* loci. Unexpectedly, nonreciprocal (unilateral) horizontal transmission was found to be produced by *vic1* and *vic3* and in conjunction with some epistatic modifications of *vic2*. The pronounced nonreciprocality caused by *vic1* and *vic3* was always associated with particular alleles at these loci and was never found to be reversed. The inhibitory effects of *vic2* were also found to be decreased nonreciprocally due to the epistasis of *vic1-1* over *vic2-1* and to other genetic background effects that are probably attributable to the presence of alleles *vic4-2* and *vic5-2*. Liu and Milgroom (1996) have also found nonreciprocal transmission. The present study is the first demonstration of nonreciprocal horizontal transmission associated with particular vegetative incompatibility loci in an Ascomycete.

Other types of unilateral effects associated with incompatibility reactions between the somatic cells of fungi and plasmodial protists are known. The Ascomycete *Podospora anserina* exhibits unilateral cell death with respect to a nonallelic interaction between particular alleles of the incompatibility loci *r* and *v* (Bernet 1992a and references therein).

Bernet has suggested that the *r* locus produces a transfusable hormone-type signal while *v* produces its receptor. Directional cell death responses also occur in some somatic cell reactions between incompatible plasmodia of the cellular slime mold *Didymium* (Clark and Collins 1973). It is not known whether similar mechanisms underlie these processes.

Evidence that epistasis between *vic* genes modifies cytoplasmic transmission. This study has provided evidence for three different types of epistatic interactions between *vic* genes. The first type of epistasis occurred between specific alleles at different *vic* loci and decreased the barrier to cytoplasmic transmission caused by one of the loci. The clearest example of this type of epistasis was the effect of the *vic1-1* allele upon the *vic2-1* allele where the *vic2* transmission barrier was reduced when both alleles occurred in the recipient. No other epistatic relationships were found among the other combinations of *vic1* and *vic2* alleles. It is also undoubtedly significant that this epistatic relationship was associated with the unidirectional transmission caused by *vic1* and that it was present whether or not *vic1* was heteroallelic; that is, epistasis did not depend upon the presence of heteroallelism at both *vic1* and *vic2*. Therefore, this interaction cannot be described as an additive effect produced by two separate incompatibility reactions, but rather is evidence for the epistatic modification of one *vic* gene by another.

The second type of epistatic relationship occurred when the effect of heteroallelism at one *vic* locus was epistatic over the effect of heteroallelism at a different *vic* locus. The barriers to transmission caused by *vic1*, *vic2*, and *vic3* were epistatic over the uninhibited transmission associated with *vic4* and *vic5*. Interestingly, although both *vic1* and *vic3* were found to cause unidirectional transmission, the effects of *vic1* were epistatic over *vic3*, indicating that there are differences in the action of these two phenotypically similar loci. The nonreciprocal transmission observed between vc types 5 and 10 is particularly striking because these two genotypes probably differ at seven *vic* loci (Anagnostakis 1982), and may also be due to epistasis. Another example of epistasis between vegetative incompatibility loci is in *O. ulmi* where transmission inhibition of the *w* locus is epistatic over the less restrictive barriers of several other incompatibility loci (Brasier 1984).

Less conclusive evidence was found for a third type of epistatic relationship. The *vic2* incompatibility barrier was also relaxed unilaterally by other genetic background effects associated with the presence of alleles *vic4-2* and *vic5-2* when they occurred in the recipient. In this case, the apparent epistatic association was between alleles *vic4-2* or *vic5-2* and locus *vic2*, where the *vic2* transmission barrier was reduced irrespective of which *vic2* allele was present in the recipient. That all modifications of transmission resulted in an unusual unilateral relaxation of the transmission barrier suggests that a similar cause may be responsible for the effect in each case. If so, the other unilateral alterations of the *vic2* barrier may be due to the presence of alleles *vic4-2* and *vic5-2* which were always present when the effects were observed. Although *vic4* and *vic5* do not prevent cytoplasmic transmission, the effects of these loci may be similar to that of *vic1* but undetected by the virus transmission assay. However, this putative epistatic relationship requires further testing.

The epistasis of *vic1-1* over *vic2-1* is quite different from the nonallelic incompatibility found in *Podospora anserina* because the nonallelic interactions in *P. anserina* cause an incompatibility reaction whereas epistasis in *C. parasitica* does not. In fact, the epistasis between *vic* genes in *C. parasitica* actually acted to reduce the effectiveness of the transmission barrier caused by the *vic2* and *vic3* allelic incompatibility reactions. Significantly, this study has demonstrated that epistatic interactions can increase cytoplasmic transmission even when the number of heteroallelic *vic* loci increases. This is the first evidence for epistasis between vegetative incompatibility genes that decreased the barrier to cytoplasmic transmission imposed by a particular vegetative incompatibility locus in an Ascomycete.

Several authors have provided evidence that the transmission barrier imposed by a vegetative incompatibility reaction in *C. parasitica* becomes incrementally greater with each additional heteroallelic incompatibility locus; that is, the effects of vegetative incompatibility loci are additive (Anagnostakis and Waggoner 1981; Liu and Milgroom, 1996). The recent work by Liu and Milgroom is especially significant because they examined a series of strains from nature that differed by 0, 1, 2, and more than 2 *vic* loci, and found that generally the frequency of transmission between unknown genotypes was successively decreased by increasing the number of heteroallelic *vic* loci. However, because the vc genotypes of their strains were unknown it is not possible to strictly attribute the differences in transmission to the successive addition of new genes rather than the individual effects of particular genes. For example, some of their vc genotypes that differed at two *vic* loci did not exhibit decreases in transmission relative to other single *vic* gene differences, indicating that transmission is influenced by particular genes as well as by additive effects among the genes.

In contrast, I have found that heteroallelism at a single *vic* locus (*vic1* or *vic2*) can be more prohibitive to transmission than a particular four locus difference. Although untested, certain five locus differences should also be less prohibitive than *vic1* and *vic2*. It is important to note that these permissive four or five gene differences include heteroallelism at *vic1* and *vic2*. In other words, because of the combined effects of nonreciprocality and epistasis, transmissibility even depends upon the total complement of *vic* alleles the donor and recipient carry (vc genotype) and not simply on heteroallelism at a certain locus. It is likely that the strains examined by Liu and Milgroom (1996) which may demonstrate additive effects represent different *vic* genes than those studied here. An important corollary arising from the transmission genetics is that genetic relatedness between vc genotypes cannot be directly inferred from the degree of cytoplasmic transfer because of epistasis and the widely different effects of individual *vic* loci.

The implications of cytoplasmic transmission variability for *vic* gene action. The cytoplasmic transmission of viruses has proven to be a sensitive assay for the subtle differences in the effects of individual *vic* genes. Although the mechanism of *vic* gene action is not known, it has been suggested that the gene products produced by allelic *vic* genes may form either homomultimeric or heteromultimeric complexes that function to trigger cell death through either the induction of incompatibility or through the blocking of inhibitors of the reaction (Arganoza et al. 1994b; Begereut et al. 1994). It is especially intriguing that every genetic alteration of the incompatibility reaction resulted in a nonreciprocal (unilateral) effect upon hypovirus transmission. The epistasis between particular *vic* alleles suggests that these genes, or gene products, are interacting with each other or with a common site of action in an antagonistic manner. The phenomena described here are reminiscent of the effects of some of the suppressor mutations recently studied by Arganoza et al. (1994b) that affect one allele but not both alleles at a *het* locus, although in *C. parasitica*, of course, suppression is associated with epistasis between *vic* genes. The nonreciprocal transmission and epistasis between *vic* genes found in this study indicate that the interactions of the *vic* genes (*vic* gene products) are more complex than previously recognized.

Presumably, viral transmission is prevented by the death of the heterokaryotic cells and the concomitant plugging of the septal pores that form a boundary for the cell death reaction (Garnjobst and Wilson 1956; Newhouse and MacDonald 1991). Conceivably, the cytoplasmic transmission of viruses could be limited by the rate of the cell death reaction that terminates the cytoplasmic pathway, by the extent of cell death, or by some other effect upon the cytoplasm that affects viral particle movement. Variability in the rate and extent of cell death have been observed in *N. crassa* (Garnjobst and Wilson 1956). As mentioned above, unilateral cell death has been found in nonallelic incompatibility interactions in *P. anserina* (Labarere et al. 1974). I have observed barrages in *C. parasitica* produced by *vic1* on PDA to be more extensive in the *vic1-2* mycelium suggesting that the unilateral cytoplasmic transmission may be due to a unilateral cell death response.

Since nothing is known about the molecular interactions of the *vic* gene products in allelic incompatibility, or how this interaction is translated into cell death, it is difficult to speculate on how the allelic interactions can be epistatically modified as observed in this study. However, any model of allelic incompatibility must take into account the following phenomena. First, *vic* loci have been found to have three basic effects upon cytoplasmic transmission: reciprocal inhibition, nonreciprocal (unilateral) inhibition, and no inhibition. Secondly, incompatibility caused by some *vic* loci can be modified by epistasis between specific *vic* alleles as well as between heteroallelic *vic* loci. Lastly, every genetic modification of transmission occurred when the respective genes were present in the same nucleus, and did not occur when the same genes were placed together in the transient heterokaryotic state that follows hyphal fusions between incompatible strains.

Transmission efficiences of different viruses. Do different types of cytoplasmic genetic elements transfer with different efficiencies through hyphal fusions or through vegetative cell incompatibility reactions? The two characterized hypoviruses, CHV1-713 and CHV3-GH2, and the uncharacterized dsRNA-80-2 transferred with comparable efficiencies through the vegetative incompatibility barriers imposed by the five *vic* loci. A limited examination of the transmission profile of a non-dsRNA-induced senescence phenotype in *C. parasitica* has shown that it transmits cytoplasmically with similar efficiency to the hypoviruses (Chapter 5).

Primary function of vegetative incompatibility genes. What is the primary function of vegetative incompatibility genes? Is their function to restrict the infectivity of parasitic cytoplasmic elements? What other possible primary functions might these genes have? Several hypotheses have been proposed. The most widely considered hypothesis is that vegetative incompatibility functions as a protective mechanism to prevent an individual (genet) from infection by cytoplasmically restricted parasitic genetic elements, including viruses, plasmids, and mutant mitochondria (Caten 1972; Nauta and Hoekstra 1994). A second hypothesis, similar to the first, considers vegetative incompatibility to protect against cellular parasitism which could occur if a less fit nuclear genotype proliferates in a cytoplasm along with a more fit genotype (Hartl et al. 1975; Buss 1987). A third hypothesis regards vegetative incompatibility to be the pleiotropic effect of mutant genes whose normal function lies elsewhere, such as in development (Bernet 1992a, and references therein). Other proposed functions for incompatibility genes are the maintenance of the developmental and/or physiological independence of the mycelium (Rayner and Coates 1987) and ecological competition (Rayner et al., 1984; Rayner, 1991).

I have shown in chapter 3 that all five *vic* loci in *C. parasitica* effectively prevent heterokaryosis, while the present study has shown that none of the five loci totally prevent viral transmission, and two of the loci, *vic4* and *vic5*, do not appear to provide any hindrance to transmission. Therefore, while particular vegetative incompatibility polymorphisms have been found to prevent horizontal viral transmission, no single *vic* locus has been found to prevent transmission irrespective of genetic background. These results suggest that the effects of vegetative incompatibility genes are more important for maintaining the nuclear integrity of the individual (genet). Considerations of the evolutionary origin and ecological impact of vegetative incompatibility must take into account these highly variable effects.

The variety of biological effects associated with vegetative incompatibility genes has made discernment of a primary function difficult and well illustrates the inherent problems in identifying adaptations (Gould and Lewontin 1979). However, an important distinction can be made between primary function and associated effect (*sensu* Williams 1966). If molecular studies show that vegetative incompatibility genes do have a primary or normal function in some process other than protecting the individual from cytoplasmic invasion, self protection may still be of great ecological and evolutionary importance. Indeed, the abundance of cytoplasmic genetic elements in fungi, and their restriction to intracellular transmission routes, implicates vegetative incompatibility systems as important determinants of the coevolution of these host-parasite relationships.

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Chapter 5

The Transmission and Phenotypic Effects of a Senescence Syndrome in *Cryphonectria parasitica*

Abstract

A severely debilitating senescence phenotype has been identified in *Cryphonectria parasitica* strain KFC9 collected in southwestern Michigan. The syndrome includes several of the phenotypic characteristics of senescence found in other filamentous Ascomycetes. Mycelium shows a progressive loss of growth potential such that subcultures taken from increasingly distal regions of a colony show increasingly debilitated growth and morphology. The senescence agent is horizontally (cytoplasmically) transmissible to other mycelia, and causes a rapid degeneration in the recipient mycelium. The effects of *vic* loci upon transmission were found to be similar to their effects upon hypovirus transmission. Transmission occurs between strains homoallelic at all *vic* loci and between strains heteroallelic at only *vic4*. Loci *vic2* and *vic1* (only *vic1-2* was tested as recipient) prevent transmission; *vic3* and *vic5* were not tested. Senescing mycelia exhibited a pronounced decline in virulence (aggressiveness). Conidia from senescing mycelia exhibited varying degrees of senescence ranging from normal growth to death soon after germination. Senescence was characterized by elevated levels of respiration via the alternative oxidase. Collectively, these characteristics are indicative of mitochondrial dysfunction and are similar to the suppressiveness quality of the *Neurospora* senescence syndrome.

Diminished aggressiveness in the chestnut blight fungus (*Cryphonectria parasitica*), termed hypovirulence, has been found to be caused by cytoplasmically transmissible genetic elements. The best characterized of these elements are double-stranded RNA viruses (hypoviruses) that have been collected from strains of *C. parasitica* from healing chestnut cankers in numerous locations in Europe and eastern North America (MacDonald and Fulbright, 1991; Nuss, 1992; Heiniger and Rigling, 1994). A second type of hypovirulence phenotype has also been discovered in fungal strains from healing chestnut cankers in Michigan that are not associated with dsRNA (Fulbright, 1985; Mahanti et al., 1993; Huber et al., 1994). These preliminary studies of the dsRNA-free type of hypovirulence have shown that it is comparable, phenotypically, to senescence in *Neurospora*, *Podospora*, and *Aspergillus* (reviewed in Griffiths, 1992).

The causal agent of this new, senescence-like form of hypovirulence is not yet known. Physiological characterization of several dsRNA-free hypovirulent strains by Mahanti et al. (1993) found that the hypovirulence was associated with elevated levels of cyanide-resistant respiration, which is an indication of mitochondrial energy metabolism dysfunction. Extensive work in *Neurospora* has shown that its senescence syndrome includes the induction of high levels of cyanide-resistant respiration through an alternative oxidase following from any of several different types of mitochodrial DNA mutations (reviewed in Griffiths, 1992). To test whether mitochondrial DNA mutations are capable of reducing virulence and eliciting senescence in *C. parasitica*, Monteiro-Vitorello et al. (1995) induced mutations in the mitochondrial chromosome. They found that induced mitochondrial mutations could reduce virulence, thereby providing additional evidence that the new senescence-like form of hypovirulence could have a mitochondrial etiology. However, the nature of the naturally occurring type of senescence in *C. parasitica* is still not known, nor is its potential for cytoplasmic transmission through vegetative incompatibility barriers understood.

Hypovirulent strains of *C. parasitica* which lack detectable levels of dsRNA have been found in healing cankers of American chestnut trees in the Kellogg Forest near the Michigan State University Kellogg Biological Station (Fulbright, 1985; Mahanti et al., 1993). This study presents a preliminary characterization of another dsRNA-free strain from this site that exhibited severe senescence-like characteristics. The hypovirulence phenotype of this mutant strain has been found to be associated with elevated levels of the alternative oxidase, is cytoplasmically transmissible, and produces a progressive degeneration of the mycelium that culminates in death. These characteristics are suggestive of the suppressive mitochondrial mutations of *Neurospora* although the molecular basis of this syndrome is not yet known. However, the nature of this mutant phenotype indicates that it can be termed senescent in keeping with the use of this term in other fungi (Griffiths, 1992). A preliminary report of this work has been published (Huber et al., 1994).

MATERIALS AND METHODS

C. parasitica strains and culture conditions: The strains of *Cryphonectria parasitica* used in this study and their sources are listed in Table 1. Strain KFC9 was collected from healing cankers on American chestnut trees from the Kellogg Forest, Michigan State University. All of the strains used in this study were grown on potato dextrose agar (PDA; Difco Laboratories) at room temperature (about 25° C) under cool white flourescent lights (34 watt). Endothia complete medium (Puhalla and Anagnostakis, 1971) was used to grow mycelia prior to the respiration assay.

Conidia were collected from strains infected with the senescence agent by scraping a small amount of conidiating mycelium from the surface of a PDA plate and placing it in sterile water. After a serial dilution, the conidia were spread onto PDA plates. When the conidia had germinated (at about 24 hours) they were individually cut out of the PDA

Table 1. Strains used in this study.

Strain	Vegetative incompatibility genotype[a]	Senescence agent	Source[b]
KFC9	unknown	present	Kellogg Forest, MI
389.7	*vic1-1,2-1,3-1,4-1,5-1*	none	this study (chap. 2)
EP388	*vic1-2,2-1,3-1,4-1,5-1*	none	ATCC 38979
22508	*vic1-2,2-2,3-1,4-1,5-1*	none	ATCC 22508
A1.18		none	389.7 X 22508
EP289	*vic1-1,2-2,3-1,4-1,5-1*	none	Conn. Agri. Exp. Sta.[c]
EP289(KFC9)		KFC9 source	infection of EP289
A1.13		none	389.7 X 22508
C1.3		none	389.7 X EP289
C1.20		none	389.7 X EP289
C1.20(KFC9)		KFC9 source	infection of C1.20
C2.8		none	389.7 X EP289
C2.10		none	389.7 X EP289
F3.2	*vic1-1,2-1,3-1,4-2,5-1*	none	389.7 X 80-2c
F3.13	*vic1-2,2-1,3-1,4-2,5-1*	none	389.7 X 80-2c
J2.31	*vic1-1,2-2,3-1,4-2,5-1*	none	F3.16 X A1.13
J2.31(KFC9)		KFC9 source	infection of J2.31
EP155	unknown	none	ATCC 38755
EP2001	unknown	none	ATCC 60589

[a] For brevity, vegetative incompatibility genotypes have been written as *vic1-1,2-1* rather than as *vic1-1*, *vic2-1*.

[b] The sexual crosses from which certain strains were derived are described in chapter 3.

[c] Strain provided by S. L. Anagnostakis, Connecticut Agricultural Experiment Station.

under a dissecting scope (40X) and placed onto new PDA plates.

Strain KFC9 was tested for the presence of double stranded RNA (dsRNA) using the protocol of Morris and Dodds (1979) as modified by Fulbright et al. (1983).

Transmission assay: Tests for the horizontal (cytoplasmic) transmission of the senescence-inducing agent were conducted by placing a 5 mm^3 subculture of a senescing donor strain on PDA near one edge of 100 X 15 mm Petri plate. The prospective recipient strain was placed on the PDA about 1 cm from the donor. The two cultures made contact with each other as they grew across the Petri plate. Because the senescent strains grew much less vigorously than the wild type strains, the senescent donor strain was allowed to grow for five to seven days before the recipient strain was added to the medium so that it could attain a diameter of about 2 cm. Care was taken to observe continued mycelial growth in the senescent culture following contact between the two mycelia in order to be certain that the senescent strain was still viable. The infection of the recipient strain with the senescence agent was easily observed as a severe change in morphology of the recipient.

Virulence assays: The virulence assay used follows that described by Lee et al. (1992). Chestnut stems about 2 cm in diameter were cut into sections about 5 cm long. Each section was bisected longitudinally and the outer layer of bark was peeled away from the underlying secondary xylem. A mycelial plug (5 mm^3) cut from cultures grown on PDA was placed on the inside center of each bark tissue piece with the mycelial side down. The cultures were then incubated in petri dishes on top of damp Whatman filter paper, sealed by Parafilm, and stored in the dark for five days at 25° C. Only the outer tissue layer consisting of living bark/cortex/phloem was used for the virulence assays. The underlying secondary xylem was not used for the virulence assays as described by Lee et al. (1992) because the fungus did not grow well on this tissue. The mycelial plugs taken from PDA cultures to inoculate the chestnut tissue were taken a few millimeters behind the leading edge of cultures that had been growing for one to two weeks. This zone consisted of mycelium which showed a more advanced development of senescence compared to the center of the colony.

Alternative oxidase respiration assay: The procedure used for the alternative oxidase assay follows that of Mahanti et al. (1993) and Monteiro-Vitorello et al. (1995). These procedures were adapted from Lambowitz and Slayman (1971). Strains which were tested for the presence of alternative oxidase were first grown on PDA. Small plugs (5 mm^3) of PDA with mycelium from near the actively growing margin of a colony were placed into a stationary culture of 50 ml of Endothia complete broth (Puhalla and Anagnostakis, 1971) in a 125 ml Erhlenmeyer flask for two or more days. The number of mycelial plugs added to a flask and the number of days given for growth depended upon the rate of growth of the particular strain. Nonsenescent strains were grown in stationary culture for two days using three or four mycelial plugs per flask. Senescent strains were grown in stationary culture for five or six days using five or six mycelial plugs per flask. After this time period, both senescent and nonsenescent cultures had roughly comparable amounts of growth (measurements of the mass of the cultures were not taken). The liquid cultures were then shaken at 200 rpm overnight. The mycelium was homogenized with a Biospec Products, Inc. Tissue Tearer homogenizer and placed into 6 ml of Vogel's medium

(Vogel, 1956). Three ml of the Vogel's/mycelial slurry were added to a chamber of YSI 5300 biological oxygen monitor. Glucose (200 μl of 20%) was also added to the chamber. The mycelial slurry was aerated for 20-30 seconds with an aquarium pump to saturate the solution. Oxygen consumption was measured by inserting a Clark electrode into the reaction chamber maintained at 25° C and mixed continuously with a magnetic stirring bar.

The contributions to respiration provided by the cytochrome chain and the alternative oxidase pathways were measured using inhibitors of each of the two pathways. Potassium cyanide (KCN) was used as the inhibitor of the cytochrome chain, and salicylhydroxamic acid (SHAM) was used as an inhibitor of the alternative oxidase pathway. KCN was added to the mycelial slurry in the respiration chamber to a final concentration of 1.0 mM from a 0.1 M solution in 10mM Tris-HCl, 5 mM EDTA, pH 7.2. SHAM was added to the reaction chamber to a final concentration of 4.16 mM from a 50 mg ml^{-1} solution in 95% ethanol. The inhibitors were added in succession to the reaction chambers. The order of the inhibitors was reversed for each of the 3 ml aliquots of the Vogel's solution/mycelial slurry. The second inhibitor was not added until the oxygen consumption became approximately linear following the addition of the first inhibitor.

RESULTS

The senescence phenotype. Strain KFC9 showed a progressive degeneration of the mycelium as it grew on PDA (Figure 1). A subculture of the mycelium taken from the center of a senescing culture produced normal appearing mycelium as it began to grow.

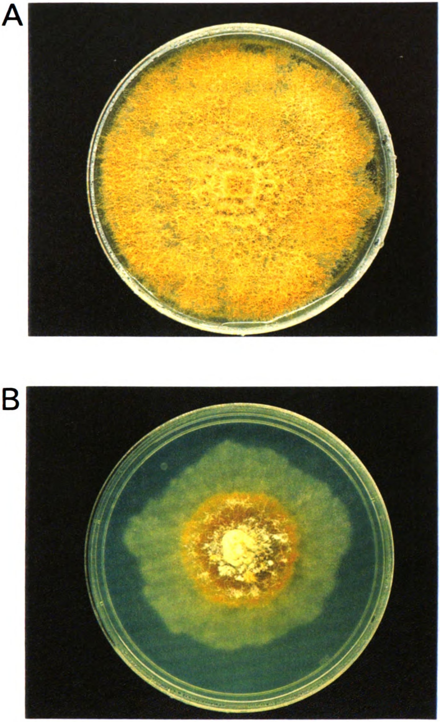

Figure 1. Growth phenotypes of (A) strain EP289 and (B) strain EP289(KFC9). Strain EP289(KFC9) was infected with the senescence agent from strain KFC9 and exhibits the senescence growth form.

Then as the mycelium advanced it lost aerial growth and became progressively thinner. Before reaching the edge of the Petri dish, the mycelium became very thin and only grew within, rather than on the surface of, the medium. Growth ceased before the edge of the petri dish was reached. Those senescent strains with aerial mycelium at the center of the colony produced exceptional amounts of conidia, visible as globules on top of the mycelium at the center of the colony, but no conidia in the region where growth became very thin and ceased. Subcultures from a senescent mycelium grown on PDA were found to resume growth according to the stage of senescence present in the sampled region of mycelium. That is, subcultures taken from the center of the senescent culture where the mycelium appeared normal exhibited the same pattern of growth and degeneracy as the parental culture, while subcultures taken progressively closer to the growth front showed increasingly advanced stages of senescence. Mycelium subcultured from the leading edge of the colony exhibited extremely poor growth often requiring a microscope to observe.

Conidia collected from KFC9 showed different degrees of senescence. The type of growth observed from conidia ranged from normal mycelium to thin, appressed growth to only germination. The range of degeneracy exhibited by the conidia was similar to the levels of senescence shown by subcultures taken from senescent mycelia as described above. For example, some conidia produced a small amount of growth (anywhere from 3cm or less maximum colony diameter) that never included normal mycelial growth. Conidia collected from a strain converted by KFC9, EP289(KFC9), also showed the presence of senescence in some of the resulting colonies, thereby demonstrating that the effect of the senescence agent upon conidia is also transmitted between strains.

Strain EP289(KFC9), which exhibits pronounced senescence, was subjected to dsRNA extraction using established protocols. No dsRNA was found in EP289(KFC9).

Serial transmission of the senescence phenotype. Transmission of the senescence phenotype between donor and recipient mycelia resembled the transmission of the hypovirulent phenotype caused by hypovirus infection in *C. parasitica*. The leading edge of the recipient mycelium became very debilitated with appressed, thin mycelium morphologically similar to the degenerative phenotype of the leading edge of the donor mycelium. This degeneration then spread laterally through the leading edge of the recipient mycelium in the same manner as hypovirus infection. Transmission of the senescence phenotype was accomplished serially thereby demonstrating that infected recipients can also act as donors. The serial transmission of the senescence agent was conducted in this order: KFC9 to EP289 to A1.13 to J2.31 (Table 2). EP289 was also used as donor with several other strains (see below).

The effects of vegetative incompatibility loci upon transmission of senescence. The cytoplasmic transmissibility of the senescence phenotype was tested by growing strain KFC9 on PDA plates along with potential recipient strains so that their hyphae would have the opportunity to anastomose. KFC9 was paired with six tester strains which have known vegetative compatibility genotypes relative to each other (389.7, EP388, EP289, 22508, F3.2, and F3.13) and to two strains with unknown vc genotypes (EP155 and EP2001) (Table 2). Strains EP155 and EP2001 are unable to transmit or to receive dsRNA from these four tester strains. These eight genotypes were chosen because they represent a broad range of vegetative incompatibility genotypes thereby increasing the chances that one

Table 2. Transmission of the senescence-inducing agent between donor and recipient strains that differ at several vegetative incompatibility (*vic*) loci.

Donor		Recipient		Transmission of senesence agent
vic[a] *1 2 3 4 5*	strain	*vic 1 2 3 4 5*	strain	
unknown	KFC9	*1 1 1 1 1*	389.7	-
unknown	KFC9	*2 1 1 1 1*	EP388	-
unknown	KFC9	*1 2 1 1 1*	EP289	+
unknown	KFC9	*2 2 1 1 1*	22508	-
unknown	KFC9	*1 1 1 2 1*	F3.2	-
unknown	KFC9	*2 1 1 2 1*	F3.13	-
unknown	KFC9	unknown	EP155	-
unknown	KFC9	unknown	EP2001	-
1 2 1 1 1	EP289	*1 2 1 1 1*	C1.3	+
1 2 1 1 1	EP289	*1 2 1 1 1*	C1.20	+
1 2 1 1 1	EP289	*1 2 1 1 1*	C2.8	+
1 2 1 1 1	EP289	*1 2 1 1 1*	C2.10	+
1 2 1 1 1	EP289	*1 2 1 1 1*	A1.13	+
1 2 1 1 1	EP289	*2 2 1 1 1*	A1.18	-
1 2 1 1 1	A1.13	*1 2 1 2 1*	J2.31	+

[a] For brevity, *vic* loci are designated only by their number as a column heading, rather than as *vic1*, *vic2*, etc. The alleles for each locus that are present in a particular strain are listed in the column beneath the appropriate *vic* locus. Only two alleles, designated as *1* or *2*, are known for each *vic* locus.

would be a competent recipient of the senescence agent in KFC9. Transmission of the senescence phenotype only occurred into strain EP289. Strains 389.7, EP388, EP289 and 22508 represent all the combinations of the alleles at *vic1* and *vic2*. Therefore, although the vegetative incompatibility genotype of KFC9 is not known, this transmission pattern suggests that KFC9 carries the alleles *vic1-2* and *vic2-2* since hypovirus transmission would be limited in this same manner if the unknown genotype was so constituted. However, since KFC9 is vegetatively incompatible with EP289 these strains may only differ at weak incompatibility loci, or alternatively epistatic effects or unidirectional loci could also be involved.

Transmission of the senescence agent occurred from donor strain EP289(KFC9) into five other recipient strains which are homoallelic at all *vic* loci (Table 2). The senescence agent freely moved between vegetatively compatible strains in contrast to the lack of transmission associated with the previous incompatibility tests. The effect of *vic4* upon transmission was tested by pairing the donor A1.13(KFC9) with recipient strain J2.31. This test demonstrated that transmission of the senescence agent is not noticeably inhibited by vegetative incompatibility caused by *vic4*. These limited tests indicated that transmission of the senescence agent is affected by *vic* genes similarly to hypoviruses.

Respiration, senescence and reduced virulence. To determine whether the agent causing the transmissible senescence in *C. parasitica* might be causing mitochondrial dysfunction such as occurs in *Neurospora*, respiration was assayed. Mitochondrial dysfunction in fungi, such as cytochrome deficient mutants of *Neurospora*, has been found to induce the activity of an alternative terminal oxidase (Lambowitz and Zannoni, 1978).

The determination of the presence of the two pathways and the contribution of each to total respiratory activity can be accomplished by using inhibitors specific for each pathway. Electron transport through the cytochrome electron transport system can be inhibited at the cytochrome oxidase step by KCN which blocks the terminal electron acceptor. The pathway to the alternative oxidase can be blocked by SHAM. Therefore, mycelia with normal levels of cyanide-sensitive respiration will show a dramatic reduction in respiration when exposed to KCN, but not to SHAM. Conversely, respiration due primarily to the cyanide-insensitive alternative oxidase will be little affected by KCN, but greatly affected by SHAM.

The percent of total respiration attributable to the alternative oxidase in wildtype strains EP289, C1.20, and J2.31 was in the range of about 10 to 25% (Table 3). This is consistent with previous work which took baseline readings of alternative oxidase in nonsenescent *C. parasitica* (Mahanti et al., 1993; Monteiro-Vitorello et al., 1995). The field-collected senescent strain KFC9 was found to have 78% of total respiration due to the alternative oxidase. Each of the three strains infected with the senescence agent showed significantly elevated levels of cyanide-insensitive respiration relative to the isogenic uninfected progenitors (Table 3). EP289 showed 23% of respiration that was cyanide insensitive, whereas EP289(KFC9) showed 60% of its respiration due to the cyanide-insensitive alternative oxidase. Strain C1.20 showed a larger change in respiration: C1.20 had 14% of total respiration attributable to the alternative oxidase, while C1.20(KFC9) had 61% so attributable.

Virulence of the wildtype and senescent strains was measured as growth on live

Table 3. Alternative oxidase as percent of total respiration, virulence, and senescence phenotypes of strains that have been cytoplasmically infected with the senescence inducing agent from strain KFC9.

Strains	Phenotype	Virulence (cm^2)	Alt. Oxd. as % of total resp.
KFC9	S	ND	78 ± 10.4
EP289	N	4.17 ± 0.16	23 ± 3.1
EP289(KFC9)	S	0.23 ± 0.08	60 ± 7.0
C1.20	N	7.01 ± 0.13	14*
C1.20(KFC9)	S	1.29 ± 0.49	61*
J2.31	N	6.36 ± 0.42	12*
J2.31(KFC9)	S	0.29 ± 0.15	ND

S, senescent growth; N, normal growth. ND, no data. Values are means ± SE.
*, percent alternative oxidase based upon one sample. Virulence was determined by growth on live chestnut bark as described in the materials and methods.

chestnut tissue. The virulence of each of the strains was dramatically reduced when they were infected with the senescence agent (Table 3). In fact, the growth of EP289(KFC9) and J2.31(KFC9) was extremely debilitated and may not have continued if the inoculations on the live chestnut tissue were observed for a longer time.

DISCUSSION

Filamentous fungi capable of producing anastomoses with genotypically different mycelia are inherently vulnerable to infection by cytoplasmically borne genetic agents. Infectious cytoplasmic agents have been found in several plant pathogenic fungi that reduce their aggressiveness (Buck, 1986), in particular, hypoviruses in *C. parasitica* (Van Alfen et al. 1975, MacDonald and Fulbright, 1991) and d-factor in *Ophiostoma ulmi* (Brasier 1983). Recently, hypovirulent strains of *C. parasitica* have been found that lack detectable levels of dsRNA viruses (Fulbright, 1985; Mahanti et al., 1993; Huber et al., 1994). This study has shown that one type of nonviral hypovirulence occurring in strains from nature causes infectiously transmissible senescence. The senescence phenotype was found to be transmissible through hyphal fusions, includes a respiratory deficiency characterized by high levels of respiration via an alternative oxidase, and exhibits a progressive degeneration during vegetative growth. These characteristics are similar to the transmissible senescence syndrome in *Neurospora* and implicate mitochondrial dysfunction in *C. parasitica* senescence.

In *Neurospora*, senescence has been found to result from energy metabolism deficiencies that are due to mitochondrial dysfunction. Mitochondrial dysfunction can occur as the result of several naturally occurring mutations in mitochondrial DNA that inhibit mitochondrial protein synthesis, including deletions (Bertrand et al., 1980), point mutations (Mannella and Lambowitz, 1978), and the integration of plasmids into the mitochondrial chromosome (Bertrand et al., 1986; Akins et al., 1986). The disruption of mitochondrial protein synthesis impairs the function of the cytochrome-mediated electron transport chain which thereby reduces oxidative phosphorylation, and consequently growth.

One of the diagnostic physiological symptoms of senescence in *Neurospora* is the induction of high levels of respiration via an alternative oxidase rather than cytochrome oxidase (Griffiths, 1992; Bertrand, 1983; Lambowitz and Zannoni, 1978). Cellular respiration in some fungi and plants has been found to consist of the conventional cytochrome-mediated electron transport pathway plus an alternate branch (beginning at ubiquinone) where electrons can be transferred to an alternative terminal oxidase. The induction of high levels of respiration through the alternative oxidase in *Neurospora* occurs when the cytochrome system is not functioning properly which can occur due to the disruption of mitochondrial protein synthesis by naturally occurring mutations or by the use of chemical inhibitors. Under normal physiological conditions, less than 10% of the total respiration in *Neurospora* is contributed by the alternative oxidase. However, in senescent mutants the alternative oxidase can contribute the majority of respiration.

An outstanding characteristic of transmissible senescence in *C. parasitica* is the rapidity of degeneration of the recipient mycelium, recalling the suppressiveness quality of the *Neurospora* senescence syndrome. Suppressiveness in *Neurospora* refers to the replacement of normal mitochondria by mutant mitochondria resulting in degeneration of the mycelium. Several models have been suggested to explain this unusual phenomenon in the filamentous fungi (reviewed in Bertrand, 1995). One model suggests that mutant mitochondrial chromosomes are able to replicate more efficiently than wild type chromosomes due to their smaller size and more efficient origins of replication (Blanc and Dujon, 1980; Almasan and Mishra, 1988). However, this model does not adequately account for the induction of suppressiveness by point mutations and insertions (Bertrand et al. 1986).

Recently, an alternative model has been presented by Bertrand (1995) that more cogently accounts for the unusual characteristics of suppressiveness. Bertrand notes that suppressiveness is associated with those mitochondrial mutations that inhibit electron transport and thereby adversely affect oxidative phosphorylation. These senescing fungal strains have been found to have increased numbers of mutant mitochondria. It also has been shown that the number of mitochondria will increase in a cell as a result of the chemical inhibition of oxidative phosphorylation. Taken together, this evidence suggests that mitochondria with inhibited oxidative phosphorylation are induced to proliferate more rapidly than those with normal oxidative phosphorylation. This response has been named the OXPHOS stress response by Bertrand. The senescence model presented by Bertrand therefore considers the suppressive quality of senescence in *Neurospora* to be the result of the natural proliferation of mitochondria resulting from induction of the OXPHOS stress response in individual mitochondria. The induction of high levels of alternative oxidase in the senescent strains of *C. parasitica* suggests that a similar replacement of metabolically normal mitochondria by metabolically abnormal mitochondria may be occurring in this species also.

Cytoplasmically transmissible agents capable of causing mitochondrial dysfunction have been found in several filamentous Ascomycetes. The diseased state of *O. ulmi* occurs after infection of mitochondria with dsRNA elements (d-factor) and is characterized by the loss of cytochrome aa_3 (Rogers et al., 1987). Another diseased state in *O. ulmi* has been found that is not associated with dsRNA, but is infectiously transmissible, and causes the generation of plasmids derived from the mitochondrial DNA of the recipient strain (Charter et al., 1993). In *Neurospora*, the linear plasmids, *kalilo* and *maranhar*, are cytoplasmically transmissible and integrate into the mitochondrial genome, thereby causing the induction of senescence (Bertrand et al., 1986; Griffiths, 1992). The cytoplasmic mutation *vgd* in *Aspergillus amstelodami* is also transmissible through hyphal fusions and may affect mitochondrial function although the molecular basis of its effect has not been elucidated (Caten, 1972). Several similar mutants termed ragged (*rgd*) have also been identified in *A. amstelodami* that show the generation of head-to-tail concatamers of specific regions of mitochondrial DNA (Lazarus et al., 1980). The horizontal mobility of the senescence phenotype has also been shown in *Podospora* (Marcou and Schecroun, 1959). Senescence in *Podospora* is associated with the appearance of circular DNA molecules termed senDNA that are composed of head-to-tail multimers of specific regions of the mitochondrial chromosome (reviewed in Griffiths, 1992).

Cytoplasmic transmission in fungi is not limited to parasitic genetic elements. The transmission of mitochondrial chromosomes has also been shown to occur in *C. parasitica* and several other fungi. Gobbi et al. (1990) reported that the mitochondrial chromosome of *C. parasitica* would not transfer between strains through hyphal fusions. However, Mahanti and Fulbright (1995) and Monteiro-Vitorello et al. (1995) have shown that the mitochondrial chromosome is capable of lateral transfer under laboratory conditions. Whether such transmission through hyphal fusions is a regular occurrence between compatible strains of *C. parasitica* in nature is not known. Transmission of the mitochondrial chromosome of *Neurospora* has been found to occur even during incompatible vegetative cell fusions when strains were placed under nutrient selection for heterokaryotic growth (Collins and Saville, 1990). Again the significance of this observation for populations growing under natural conditions has not been studied. In the Basidiomycetes, recent studies of cytoplasmic mixing following mating have shown that heteroplasmons form in a restricted region of hyphal fusions between the parents under laboratory conditions (Baptista-Ferreira et al., 1983; Hintz et al., 1988; May and Taylor, 1988; Smith et al., 1990). However, Smith et al. (1990) did not find evidence for heteroplasmy in natural populations of *Armillaria* even though heteroplasmy occurred in laboratory matings suggesting that natural conditions may be more restrictive of cytoplasmic exchange.

Different types of cytoplasmic genetic elements exist in filamentous fungi. Are all of these genetic elements infectiously transmitted between strains with the same efficiency? Viral transmission in *C. parasitica* has been shown to be highly responsive to specific *vic* genes (chapter 4). Two *vic* genes whose effects upon the horizontal transmission of hypoviruses are known were tested for their effects upon the transmission of the senescence agent in *C. parasitica*. Each of the *vic* loci examined were found to have the same effect upon senescence transmission as they had upon hypovirus transmission: transmission occurred between strains homoallelic at all *vic* loci and between strains heteroallelic at only *vic4*; transmission was prevented by heteroallelism at *vic1* (recipient *vic1-2*). The effects of *vic3* and *vic5* upon transmission, as well as the reciprocality of *vic1*, were not tested. To my knowledge, this is the first time that the efficiency of transmission of viruses has been compared to nonviral cytoplasmic agents.

The presence of these various instances of horizontally transmissible diseases that specifically affect mitochondria indicates that the populations of mitochondria in coenocytic fungal cells are susceptible to infectious agents. Caten (1972) suggested that the purpose of vegetative incompatibility systems was to limit cytoplasmic infection. The original suggestion by Caten (1972) was applied to the *vgd* cytoplasmic mutation in *Aspergillus* which is presumed to affect the mitochondria. Caten's work and subsequent studies demonstrated that the transmission of the *vgd* mutation could be limited by vegetative incompatibility. The efficiency of cytoplasmic transmission of the *vgd* mutation was found to vary according to whether strains were heteroallelic at *hetA* or *hetB* (Handley and Caten, 1973), or whether strains were heteroallelic at one or two *het* genes (Caten, 1972). Recently, the effects of vegetative incompatibility upon the transmission of the senescence-inducing linear plasmids in *Neurospora* has been examined. Using nonselective conditions, Debets et al. (1994) found that while cytoplasmic transmission of *kalilo* and two other plasmids occurred between strains of *N. crassa* that differed at *hetD* and *hetE*, transmission was usually prevented by *hetC*. With the application of forcing conditions, however, the *kalilo* plasmid has been transmitted between different species of *Neurospora* (Griffiths et al., 1990). The infectious cytoplasmic spread of these agents through populations in nature has not been studied. The extent of the spread of the senescence agent in *C. parasitica* populations is also not known. Several American chestnut trees in the vicinity of the KFC9 collection site have healing cankers which may be due to infection with the senescence agent, but this has not been investigated.

The cytoplasmically transmissible senescence phenotype in *C. parasitica* is of particular interest as a potential biocontrol agent. The cytoplasmically infectious hypoviruses have functioned effectively as a biocontrol agent in European *C. parasitica* populations and locally in North American populations. It is possible that the senescence agent may also prove to be effective in this manner. Presently, only a few healing cankers can be attributed to this agent so its potential for population level infection is not known. The severity of the disease may be a limitation in its spread. However, the abundance of conidia produced by diseased mycelia under laboratory conditions and their (unproven) potential for sexual inheritance may significantly improve their transmission through populations, and hence, effectiveness as a biocontrol.

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