Preliminary Investigations on the Effect of Individual *vic* Genes Upon the Transmission of dsRNA in *Cryphonectria parasitica*

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ABSTRACT. The effect of two, and possibly three, different *vic* genes upon the transmission of dsRNA was examined *in Cryphonectria parasitica.* The segregation of progeny from sexual crosses between several of the v-c types in this study confirm that the strains differ from each other at two loci, vicl and *vic2.* A difference may also be present at a putative third locus, *vic3,* although present evidence suggests that this gene could be a third allele of *vicl.* Preliminary evidence suggests that the *vicl* locus is associated with a directional exclusion of the transmission of dsRNA. Recipient strains with the allele *vicl-2* would not readily receive dsRNA from a *vicl-1* donor, but transmission in the reciprocal pairing was unimpeded. The *vic2* locus was found to prevent dsRNA transmission although in a conditional manner. Whereas the effect of the vicl locus was independent of the *vic2* alleles (as long as both donor and recipient were homogenic for *vic2),* the exclusionary effect of the vic2 locus was reduced when the allele *vicl-1* occurred in the recipient mycelium. The putative *vic3* locus did not prevent transmission and is indistinguishable in its effects from *vicl-1.* In addition, this study showed that a two gene difference can be more permissive to dsRNA transmission in some cases than a particular one gene difference.

The transmission of fungal viruses and other cytoplasmic genetic elements between fungal mycelia is dependent upon the establishment of cytoplasmic continuity between the mycelia (9, 10). Consequently, processes that limit cellular fusions between different mycelia should limit the spread of cytoplasmic genetic elements. In many ascomycetes, the ability of hyphae from different individuals to fuse and maintain a heterokaryotic condition is regulated by a vegetative (or heterokaryon) incompatibility system. It could be expected that the processes that produce the vegetative incompatibility reactions also may regulate the transmission of other cytoplasmic genetic elements, although the genetics and cellular basis for intermycelial cytoplasmic transmission are not well known (11).

Vegetative incompatibility in *Cryphonectria parasitica* (Murr.) Barr is determined by a heterogenic, allelic system that functions by requiring the two interacting individuals to have identical alleles at each corresponding vegetative compatibility (v-c) locus, whereas differences between individuals at any v-c locus results in an incompatible reaction (1, 3, 5). Stable, vegetative cell fusions are produced when strains have compatible *vic* genes, but transient cell fusions and cell death can be the result of incompatible *vic* gene interactions (18). The genetic basis of the vegetative incompatibility reactions in *C. parasitica* and the potential

contribution of specific *vic* genes to the incompatibility reaction and their effect upon cytoplasmic transmission between cells is poorly understood.

Even though vegetative incompatibility in *C. parasitica* is poorly understood, incompatibility has been attributed to be the limiting factor in the spread of dsRNAs in North America. (1, 4, 6, 7). Anagnostakis (4) showed that vegetatively incompatible strains were found to prevent the transmission of dsRNA while compatible strains freely permitted transmission. In addition, a less developed incompatible reaction termed a "weak" barrage did not prevent the transmission of dsRNA. Other studies have shown that some vegetative compatibility groups do not behave consistently with regard to dsRNA transmission (6,16). An exclusionary effect has been associated with incompatibility reactions that is apparently dependent upon the number of v-c loci by which two strains differ: dsRNA transmission was thought to be increasingly restricted between strains that differed at more v-c loci (7). However, the potential effects of individual *vic* genes upon dsRNA transmission and the possibility of epistatic interactions between *vic* genes has not been addressed. This study represents preliminary work toward the goal of understanding how individual *vic* genes affect dsRNA transmission and vegetative cell fusions during vegetative incompatibility reactions in *C. parasitica.*

MATERIALS AND METHODS

Strains. Strains of *C. parasitica* used in this study are listed in Table 1. Strains obtained from the Connecticut Agricultural Experiment Station collection, courtesy of S. Anagnostakis, are Ep388, Ep389, Ep289, American Type Culture Collection (ATCC) 22508, Ep2001 and Ep243. The dsRNA used in the transmission experiments originated from strain 80-2 that was obtained from the West Virginia University collection, courtesy of William MacDonald.

Vegetative incompatibility and dsRNA transmission tests. Vegetative incompatibility tests were performed as described by Anagnostakis (5) except that the medium used was potato dextrose agar (Difco) without nutritional supplements. Red dye (FD&C #40, Miesel Co, Detroit, Mich.) was added to the medium (40 drops per liter) to help visually observe barrage formation. DsRNA transmission tests were performed by pairing agar plugs (about 5mm x 5mm) of a dsRNA-containing donor strain and a recipient strain about 2 or 3 mm apart near one edge of a petri dish. For the transmission test to be considered valid, the amount of physical contact between the two growing strains was arbitrarily chosen to be equal to two-thirds the diameter of the plate. This allowed for differences in growth rate of the two strains, and permitted substantial opportunity for hyphae of the two strains to fuse. Transmission could occur between some strains so rapidly that the "two-thirds" rule was not required.

Extraction and detection of dsRNA. The extraction of the dsRNA was accomplished on single or double cellulose columns following the technique of Day et al. (14) as modified by Fulbright et al. (15). Mycelium for dsRNA extraction was either grown on Endothia complete medium (14) or on PDA overlain with cellophane. One or more dsRNA extractions were done on nearly every different v-c genotype pairing. Where questionable instances of dsRNA transmission occurred, due to unclear color reactions or lack of morphological change, dsRNA extraction was performed.

RESULTS

Vegetative incompatibility. Vegetative incompatibility tests were conducted on every combination of strains Ep388, Ep389, Ep289, 22508, Ep243 and Ep2001. Each of the six strains were compatible with themselves but incompatible with each of the others as judged by the formation of a barrage. Vegetative interaction between strains Ep389 and Ep243 demonstrated a weaker barrage than observed with any of the other incompatible combinations.

Genetic evidence for v-c types. Genetic support for establishing v-c genotypes of the strains used in this study was obtained from sexual crosses between several of the strains (Table 2). When strain Ep389 was crossed with strain 22508, four v-c types were produced that were compatible with either of the parental strains or with Ep388 or Ep289. The production of these four v-c types supports the interpretation that Ep389 and Ep22508 differ by two loci

Table 1. Strains of *Cryphonectria parasitica* used in this study. V-c genotypes are from Anagnostakis (5) except for Ep243, which is our interpretation of data presented here and data published elsewhere (5).

strain	ATCC#	phenotype	v-c type	v-c genotype
Ep 389	ATCC 38980	cre, ts, A	v-c 5	*vic*1-1,2-1,3-1,4-1,5-1,6-1,7-1
Ep 388	ATCC 38979	met, a	v-c 39	*vic*1-2,2-1,3-1,4-1,5-1,6-1,7-1
Ep 289		a	v-c 71	*vic*1-1,2-2,3-1,4-1,5-1,6-1,7-1
22508	ATCC 22508	met, a	v-c 8	*vic*1-2,2-2,3-1,4-1,5-1,6-1,7-1
Ep 243		a	v-c 56	*vic*1-1,2-1,3-2,4-1,5-1,6-1,7-1*
Ep 2001	ATCC 60589	met, a	v-c 10	*vic*1-2,2-2,3-2,4-2,5-2,6-2,7-2
80-2			unknown	

*The v-c 56 genotype is the same as that reported in Rizwana and Powell (19) for Ep29 v-c 16, although the v-c genotypes of Ep243 and Ep29 are not the same since they are incompatible with each other. Their work appeared in publication after this paper was submitted. Both Ep243 and Ep29 differ from v-c 5 by one gene, but it is not known how they differ from each other. The v-c genotype of Ep243 may represent a third allele at *vic1*, *vic2* or *vic3*, or a new locus; additional genetic analysis is needed to establish the correct genotype.

Table 2. Vegetative compatibility types of the progeny from *C. parasitica* sexual crosses involving different v-c types.

		Number of progeny in v-c types				
	v-c type	5	39	71	8	56
Cross	v-c genotype	*vic*1-1,2-1	*vic*1-2,2-1	*vic*1-1,2-2	*vic*1-2,2-2	vic1-1,2-1,3-2
Ep389(v-c5) × 22508(v-c8)		7	8	8	11	
Ep389(v-c5) × Ep388(v-c39)		9	11			
Ep389(v-c5) × Ep289(v-c71)		7		12		
Ep389(v-c5) × Ep243(v-c56)*		8				4

*Ep389 and Ep243 form a weak barrage; the v-c genotype of Ep243 may be *vic*1-3 instead of *vic3-2*.

(2, 3). To verify that Ep389 and Ep388 differed by one locus, these two strains were crossed. Each of the progeny tested were compatible with either of the parents, indicating segregation at one locus. Ep389 also was crossed with Ep289 and each of the tested progeny were compatible with one or the other parent. Ep388 has not been crossed with Ep289, which would verify that these strains differ at two loci rather than a single locus. Instead, we found each of the progeny from the cross Ep389 x 22508 to be compatible with either one or the other of the parents, or with Ep388 or Ep289, and none of the progeny were incompatible with all four testers. Therefore, Ep388 and Ep289 must differ by the same two loci as Ep389 and strain 22508 (Table 1). Ep389 also was crossed with Ep243 and each of the tested progeny was compatible with one or the other parent, indicating segregation at one locus. Since Ep243 is vegetatively incompatible with strains Ep388, Ep389, Ep289 and 22508, which represent every combination of the alleles at vicl and *vic2,* Ep243 may represent either a third locus or a new allele at *vicl* or *vic2.* Work in progress will identify the genotype of Ep243.

DsRNA transmission tests. Ability to transmit dsRNA was tested between every combination of strains Ep388, Ep389, Ep289, 22508 and Ep243 with each strain being tested as both the donor and recipient of the cytoplasmic-borne 80-2 dsRNA molecules. Most of the possible combinations of the alleles of *vicl,* 2 and 3 were represented in these reciprocal transmission tests. Strain Ep2001 (v-c 10), which may differ from the other strains by five or more genes, was only tested as a recipient because 80-2 dsRNA could not be transmitted into it. Every transmission test between a donor and recipient of the same v-c type resulted in transmission of the dsRNA to the dsRNA-free clone (Table 3). The transmission of dsRNA from strain 80-2 could be seen by the abrupt loss of pigmentation and a minor morphological change in the mycelium of the recipient strain.

The transmission of dsRNA was inhibited in a unidirectional manner when the interacting strains had allelic differences only at *vicl* (Table 3). When the strain with the allele *vicl-2* was the donor and the recipient was *vicl-1,* dsRNA transmission occurred in every trial. In contrast, when the strain acting as the donor carried allele *vicl-1* and the recipient strain carried vicl-2, dsRNA transfer occurred infrequently. This directional exclusion was independent of the alleles occurring at *vic2* as long as both strains were homogenic at this locus.

The locus vic2 was found to prevent dsRNA transmission, although the effect was dependent upon which alleles were present at *vic 1* (Table 3). When both strains carried the allele vicl-2 and differed from each other at vic2, dsRNA transmission was entirely inhibited. This was true regardless of which allele of *vic2* was carried by the recipient. However, when both strains carried the allele *vicl-1,* the effect of the difference at *vic2* became unidirectional. That is, when the recipient was genotype *vic2-2,* transmission was prevented, but when the recipient was *vic2-1,* dsRNA transmission occurred in more than 60% of the tests. This suggested that *vicl-1* may be partially epistatic over *vic2-1* when both alleles occur in the recipient.

Differences in the transmissibility of dsRNA between strains that differed simultaneously at both *vic* 1 and *vic* 2 also was apparently dependent upon which alleles co-occur in the recipient (Table 3). When the donor has the genotype *vicl-2,* 2-1 and the recipient is *vicl-1,* 2-2, transmission is entirely prevented. This also is true when the donor and recipient strains are reversed. When the donor is *vicl-1,* 2-1 and the recipient is *vicl-2,* 2-2, transmission is prevented. However, when these donor and recipient strains are reversed, dsRNA transmission occurred in 30% of the tests. This situation again shows a possible epistatic effect of *vicl-1* upon *vic2-1* when both occur in the recipient strain.

The putative *vic3* locus is associated with a dsRNA transmission pattern that is indistinguishable from the pattern associated with Ep389. Ep243 and Ep389 that differ only at locus vic3 produce a weak barrage and allow unimpeded dsRNA transmission between them (Table 3). When Ep243 was paired in reciprocal combinations with Ep388, Ep289 and 22508, the transmission of dsRNA in each case was like that seen when Ep389 was interacting with each of these strains. This could suggest that the effect of the *vic3* locus upon dsRNA transmission is negligible compared to *vicl* and *vic2,* or vic3 could be a third allele at *vicl.* Further genetic analysis is necessary to establish the identity of vic3.

DISCUSSION

This study has presented evidence that individual *vic* genes in *C. parasitica* may have specific effects upon the transmission of cytoplasmically carried dsRNA genetic elements. Although each of the loci, vicl, vic2 and the putative *vic3,* are capable of producing a vegetative incompatibility reaction, each are associated with different ef-

Table 3. DsRNA transmission tests. Transmission frequency data (number of times dsRNA transmission was detected per number of pairings with the potential recipient) for a donor (containing 80-2 dsRNA) and a dsRNA-free recipient arranged in reciprocal pairings.

Donor	Recipient	Transmission frequency
*vic*1-1, 2-1	*vic*1-1, 2-1	8/8
*vic*1-2, 2-1	*vic*1-2, 2-1	8/8
*vic*1-1, 2-2	*vic*1-1, 2-2	8/8
*vic*1-2, 2-2	*vic*1-2, 2-2	7/7
*vic*1-1, 2-1, 3-2	*vic*1-1, 2-1, 3-2	7/7
	one gene difference	
*vic*1-1, 2-1	*vic*1-2, 2-1	2/15
*vic*1-2, 2-1	*vic*1-1, 2-1	16/16
*vic*1-1, 2-1	*vic*1-1, 2-2	0/13
*vic*1-1, 2-2	*vic*1-1, 2-1	13/21
*vic*1-1, 2-2	*vic*1-2, 2-2	1/12
*vic*1-2, 2-2	*vic*1-1, 2-2	15/15
*vic*1-2, 2-1	*vic*1-2, 2-2	0/13
*vic*1-2, 2-2	*vic*1-2, 2-1	0/12
*vic*1-1, 2-1, 3-1	*vic*1-1, 2-1, 3-2	9/9
*vic*1-1, 2-1, 3-2	*vic*1-1, 2-1, 3-1	7/7
	two or three genes difference	
*vic*1-1, 2-1	*vic*1-2, 2-2	0/13
*vic*1-2, 2-2	*vic*1-1, 2-1	5/15
*vic*1-2, 2-1	*vic*1-1, 2-2	0/12
*vic*1-1, 2-2	*vic*1-2, 2-1	0/13
*vic*1-2, 2-1, 3-1	*vic*1-1, 2-1, 3-2	13/13
*vic*1-1, 2-1, 3-2	*vic*1-2, 2-1, 3-1	1/13
*vic*1-1, 2-2, 3-1	*vic*1-1, 2-1, 3-2	8/12
*vic*1-1, 2-1, 3-2	*vic*1-1, 2-2, 3-1	1/13
*vic*1-2, 2-2, 3-1	*vic*1-1, 2-1, 3-2	5/16
*vic*1-1, 2-1, 3-2	*vic*1-2, 2-2, 3-1	0/13
	several genes difference	
*vic*1-1, 2-1, 3-1	v-c 10	0/8
v-c 10	*vic*1-1, 2-1, 3-1	NP*
*vic*1-2, 2-1, 3-1	v-c 10	0/8
v-c 10	*vic*1-2, 2-1, 3-1	NP
*vic*1-1, 2-2, 3-1	v-c 10	0/7
v-c 10	*vic*1-1, 2-2, 3-1	NP
*vic*1-2, 2-2, 3-1	v-c 10	0/7
v-c 10	*vic*1-2, 2-2, 3-1	NP
*vic*1-1, 2-1, 3-2	v-c 10	0/9
v-c 10	*vic*1-1, 2-1, 3-2	NP

*NP = not possible

fects upon the transmission of dsRNA between mycelia. Differences also have been found in the effects of two heterokaryon incompatibility loci upon the cytoplasmic transmission of a suppressive mutation in *Aspergillus amstelodami* (12). In this ascomycete, the locus hetB was attributed with completely blocking the transmission of the cytoplasmic mutation while the locus hetA only reduced the frequency of the transmission.

A directional exclusion to the transmission of dsRNA was found associated with the *vic1* locus and conditionally with the *vic2* locus. This directional exclusion of dsRNA may be the result of different manifestations of the incompatibility reaction in the two interacting mycelia although this has not been shown. Vegetative incompatibility reactions are known in other fungi that are produced asymmetrically in the interacting mycelia. *Podospora anserina* has an asymmetric type of incompatibility reaction resulting from the non-allelic interaction of the loci R and V (17). In this case, cell death occurs predominantly in the strain with the R allele, and is thought to be the consequence of a diffusable V gene product from the other strain. A directional effect also is known in the Myxomycete, *Didymium iridis,* where the dominant mycelium will kill the recessive mycelium in an incompatible reaction (13).

Some evidence has suggested that the barriers to cytoplasmic transmission in *C. parasitica* become more exclusive as the number of v-c loci that differ between strains increases *(7).* The same type of effect has been suggested for *Aspergillus amstelodami* (11) and *Ceratocystis ulmi* (8). While this might be true for *C. parasitica* in general, these results suggest that the particular alleles present in an individual may be as important. This study showed that a two gene difference can be more permissive to dsRNA transmission in some cases than a particular one gene difference. The presence of *vic1-1* appears to be partially epistatic over *vic2-1* when both occur in the recipient, thereby allowing dsRNA transmission in more than 30% of the pairings even though the donor and recipient differ by two genes. Anagnostakis (3, 5) has suggested that non-allelic interactions may be present because of the high numbers of v-c types found in a sexual cross between v-c types 5 and 10. However, evidence for epistatic interactions derived from cytoplasmic transmission assays using known v-c genotypes has not been reported previously. Epistatic interactions between vegetative incompatibility genes are also probable in *Ceratocystis ulmi* (8).

It should be emphasized that the relationship here between *vic* genes and dsRNA transmission is correlative since isogenic strains were not used. Further confirmation is necessary to demonstrate whether the transmission of dsRNA is determined by these *vic* genes or by other physiological determinants that are unique to these strains. This consideration as well as the genetic status of *vic3* are currently being addressed.

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