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Programmed cell death correlates with virus transmission in a filamentous fungus

Silvia Biella[1,2], Myron L. Smith[3], James R. Aist[1], Paolo Cortesi[2] and Michael G. Milgroom[1*]

[1]*Department of Plant Pathology, Cornell University, Ithaca, NY 14853, USA*
[2]*Istituto di Patologia Vegetale, Università degli Studi di Milano, 20133 Milan, Italy*
[3]*Department of Biology, Carleton University, Ottawa, Ontario, Canada K1S 5B6*

Programmed cell death (PCD) is an essential part of the defence response in plants and animals against pathogens. Here, we report that PCD is also involved in defence against pathogens of fungi. Vegetative incompatibility is a self/non-self recognition system in fungi that results in PCD when cells of incompatible strains fuse. We quantified the frequency of cell death associated with six vegetative incompatibility (*vic*) genes in the filamentous ascomycete fungus *Cryphonectria parasitica*. Cell death frequencies were compared with the effects of *vic* genes on transmission of viruses between the same strains. We found a significant negative correlation between cell death and virus transmission. We also show that asymmetry in cell death correlates with asymmetry in virus transmission; greater transmission occurs into *vic* genotypes that exhibit delayed or infrequent PCD after fusion with an incompatible strain. Furthermore, we found that virus infection can have a significant, strain-specific, positive or negative effect on PCD. Specific interactions between *vic* gene function and viruses, along with correlations between cell death and transmission, strongly implicate PCD as a host-mediated pathogen defence strategy in fungi.

Keywords: asymmetric programmed cell death; vegetative incompatibility; heterokaryon incompatibility; horizontal transmission; chestnut blight fungus; *Cryphonectria parasitica*

1. INTRODUCTION

Programmed cell death (PCD) is a genetically controlled process that functions during normal cellular development and defence against pathogen attack in plants and animals (Roulston *et al.* 1999; Lam *et al.* 2001). Although the mechanisms of PCD may differ in detail, some of the genes and pathways responsible for PCD are conserved among diverse organisms (Aravind *et al.* 1999). In contrast to the accumulating data on PCD in plants and animals, little is known about PCD in fungi. In fungi, a form of PCD occurs when cells of vegetatively incompatible conspecific strains fuse (Leslie & Zeller 1996; Jacobson *et al.* 1998; Glass *et al.* 2000). Cells involved in incompatible fusions proceed through a series of recognizable stages that include cytoplasmic granulation, vacuolization and condensation, shrinkage of the plasma membrane away from the cell wall and, finally, cell death. This series of stages leading to cell death as a result of incompatible cell fusions has been described in diverse fungi, indicating that a form of PCD is operating and is conserved (Glass *et al.* 2000), even though the signalling pathways and mechanisms leading to PCD remain virtually unknown (Glass *et al.* 2000; Saupe 2000; Saupe *et al.* 2000).

PCD in fungi appears to be involved in pathogen defence, as it is in plants and animals (Roulston *et al.* 1999; Lam *et al.* 2001), although it may largely be triggered by vegetative (*vic*) or heterokaryon (*het*) incompatibility gene products instead of by the recognition of pathogen elicitors. One perspective states that vegetative incompatibility is an 'evolutionary accident' without any beneficial role in defence (Saupe *et al.* 2000). Alternatively, vegetative incompatibility may have evolved as a cellular defence mechanism by limiting the transmission, via cell fusions, of infectious genetic elements among individuals (Caten 1972; Hartl *et al.* 1975). Rapid localized PCD after the fusion of incompatible cells prevents the formation of heterokaryons and restricts infectious elements from spreading into uninfected fungal colonies. However, the restriction in transmission of infectious genetic elements between incompatible strains is not absolute. Plasmids, mitochondria, supernumerary chromosomes and viruses can be transmitted between incompatible strains, although usually at reduced frequencies compared with compatible interactions (reviewed in Cortesi *et al.* 2001).

The role of vegetative incompatibility in the horizontal transmission of viruses has been studied extensively in the chestnut blight fungus *Cryphonectria parasitica*, because of the potential for biological control (Van Alfen *et al.* 1975; Nuss 1992). As in other fungi, transmission of viruses and plasmids between vegetatively incompatible *C. parasitica* isolates is restricted, but not entirely (Anagnostakis & Day 1979; Anagnostakis 1983; Huber 1996; Liu & Milgroom 1996; Baidyaroy *et al.* 2000; Cortesi *et al.* 2001). Cortesi *et al.* (2001) quantified the effect of alleles at each of six *vic* loci on virus transmission in *C. parasitica* and found significant variation in transmission associated with the different loci. Furthermore, incompatibility associated with some *vic* genes resulted in markedly asymmetric virus transmission in which the frequency of transmission depended on which *vic* allele was present in the donor or recipient.

* Author for correspondence (mgm5@cornell.edu).

Variation in virus transmission associated with different *vic* genotypes raises some intriguing questions. In studies on the effect of vegetative incompatibility on the transmission of infectious elements in fungi, the implicit, but as yet to our knowledge untested, assumption is that a reduction in transmission is due to an increased rate or frequency of PCD triggered by *vic* or *het* genes (Anagnostakis & Day 1979). A similar phenomenon may occur in plant defences; for example, delayed PCD in transgenic tobacco plants allowed tobacco mosaic *virus* to spread from cell to cell, evading plant defences (E. Lam, personal communication). On the basis of this model, we would expect variation in virus transmission, including asymmetric transmission, associated with different *vic* loci to be correlated with the rate and frequency of PCD. In other words, strains that exhibit more rapid PCD are less likely to become infected following cell fusion with an incompatible virus-infected strain. Conversely, efficient virus transmission between individuals is expected when PCD is infrequent or delayed.

This research focused on the relationship between PCD and the probability of virus transmission between vegetatively incompatible isolates of *C. parasitica*. We had four specific objectives in this study: (i) to describe the effect of incompatibility associated with different *vic* genes on the cytology of cell fusion and PCD; (ii) to test whether variation in virus transmission associated with specific *vic* loci is related to the frequency of PCD; (iii) to test whether asymmetry of virus transmission correlates with asymmetry of PCD; and (iv) to test whether virus infection changes the PCD response associated with vegetative incompatibility. This last objective follows from several examples in animal systems, in which parasites modify host behaviour, and enhance their own transmission to a new or alternative host (Poulin 2000).

2. MATERIAL AND METHODS

(a) *Isolates of* Cryphonectria parasitica

We organized 28 isolates of *C. parasitica* into 24 pairs (table 1); these are the same pairs as used previously to estimate the effects of *vic* genes on horizontal virus transmission (Cortesi *et al.* 2001). As in most ascomycetes, two individuals of *C. parasitica* are vegetatively incompatible if they have different alleles (heteroallelic) at one or more *vic* loci. To date, only six *vic* loci (*vic1*, *vic2*, *vic3*, *vic4*, *vic6*, *vic7*) have been clearly identified in *C. parasitica* (Cortesi & Milgroom 1998); no other *vic* loci are known to be polymorphic in the isolates used in this study. Of the 24 isolate pairs, three involve single isolates (i.e. self-pairing), three are genetically distinct but vegetatively compatible isolates (i.e. the same *vic* genotype), and 18 are incompatible because isolates are heteroallelic at a single *vic* locus. The incompatible isolate pairs were selected to obtain three independent replicates heteroallelic at one of each of the six *vic* loci. For simplicity, we refer to isolate groups self, non-self compatible, heteroallelic at *vic1*, *vic2*, *vic3*, *vic4*, *vic6* and *vic7*, as groups S, N, 1, 2, 3, 4, 6 and 7, respectively. Within each group we named each of the three pairs as a, b or c, and we indicate allele *1* or allele *2* at the heteroallelic *vic* locus in each isolate with 1 and 2, respectively (table 1). For example, isolate pair P20-2/P32-4 is called Na and isolate P20-2 is Na-1. During this study, isolate names were randomly coded so the observer did not know the *vic* genotypes of isolates until after each experiment was completed. Reciprocal virus transmission data for each pair of isolates (table 1) were taken from Cortesi *et al.* (2001).

(b) *Preparation of specimens for microscopy*

Many fungi produce filamentous cells called hyphae, which form networks that are collectively called a mycelium. Specimens for observing cell fusions between pairs of isolates were prepared according to Kohn *et al.* (1990), with minor modifications. All isolates were cultured in Petri dishes on malt agar medium ($10\,g\,l^{-1}$ malt extract, $20\,g\,l^{-1}$ bacto agar, Difco, Detroit, MI) and incubated in the dark at 24 °C. Sterile 7.5 cm × 2.5 cm glass microscope slides were coated with a thin layer of gelrite ($10\,g\,l^{-1}$, Sigma, St Louis, MO) dissolved in water, by dipping into molten medium (*ca.* 80 °C). Each coated slide was then placed within a sterile glass Petri dish, on a bent glass rod subtended by two layers of filter paper (Whatman No. 1, Maidstone, Kent) wetted with 3 ml of sterile distilled water. Each slide was inoculated with an isolate pair. Inoculum for each isolate consisted of two blocks of agar (each 0.5 cm × 0.75 cm) containing mycelium from the margin of a colony that had been growing for less than 5 days on malt agar. Inoculum blocks for each isolate were placed side by side, mycelium-side down 3 mm from the other isolate in the pair, with growing hyphal tips intact and pointing towards each other. After inoculation, the Petri dishes were wrapped loosely in plastic bags to maintain adequate moisture but allowing for gas exchange, and incubated for 24–48 h in the dark at 24 °C.

(c) *Microscopy*

We used light microscopy to observe inter-pair fusions between compatible and incompatible isolates and to describe the cytology of the vegetative incompatibility reactions. Slides were examined with Nomarsky differential interference contrast microscopy using a Zeiss IM35 inverted microscope with Planapo ×40 and ×63 objectives. The interaction zone between two isolates was examined in order to observe cell fusions. Areas with dense mycelium in which individual hyphae could not be easily identified were avoided for these observations. Hyphae were traced visually from fusion cells in the interaction zone back to the colony origins to verify that the fusions were between the two isolates in each pair and not between hyphae from the same isolate. Video recordings of cell fusions between selected isolates were made using a Hamamatsu C2400 video camera fitted to the microscope and connected to a Sony 5800H U-matic VCR. The frames for micrographs were captured from the video recording using ImageSavant software (IO Industries, Inc., London, Ontario) and processed using Adobe Photoshop v. 5.0 (Adobe Systems, San Jose, CA).

In this report, cell deaths observed following fusion between isolates are considered to be PCDs. Cytoplasmic shrinkage was chosen as the criterion with which to identify dying cells because it was the least ambiguous and the most easily detected stage of the progression to cell death. Cells were recorded as dead whenever cytoplasmic shrinkage was observed in hyphae of one or both isolates at the point of fusion.

(d) *Variation in cell death among groups of isolates heteroallelic at different* vic *loci*

We determined the frequency of PCD in paired isolates as the number of cell deaths divided by the number of hyphal contacts observed between isolates. Four replicate slides for each of the 24 pairs were examined, each on different days. For each slide,

Table 1. Frequency of virus transmission and cell deaths in *Cryphonectria parasitica*.

heteroallelic *vic* locus[a]	pair[b]	allele *1* isolate[c]	allele *2* isolate[c]	% transmission[d]		number of contacts	number of cell deaths in:			mean (± s.e.)[e]	asymmetry[f]		
				allele *1* in recipient	allele *2* in recipient		both isolates	allele *1* isolate	allele *2* isolate		within pairs (± s.e.)	within groups (± s.e.)	*p*-value
none	Sa	P20-2	P20-2	100[g]	100	70	0	5	4		0.031 ± 0.130		
(self)	Sb	P32-4	P32-4	100	100	67	0	0	0		0.000 ± 0.000		
	Sc	P16-1	P16-1	100	100	58	1	2	1	0.07 ± 0.04	0.009 ± 0.018	0.013 ± 0.020	0.20
none[h]	Na	P20-2	P32-4	100	100	57	2	6	20		−0.263 ± 0.271		
(non-self)	Nb	P16-1	P19-1	100	100	56	0	0	1		−0.013 ± 0.026		
	Nc	P8-3	P9-1	100	100	63	0	0	1	0.17 ± 0.16	−0.025 ± 0.050	−0.100 ± 0.054	0.42
vic1	1a	P19-4	P26-1	95	2.5	47	8	0	13		−0.263 ± 0.192		
	1b	P30-9	P35-3	100	0	57	1	3	27		−0.460 ± 0.260		
	1c	P20-2	P32-3	97.5	0	53	2	1	20	0.47 ± 0.03	−0.352 ± 0.395	−0.358 ± 0.081	0.02
vic2	2a	P1-2	P1-5	5	15	62	17	16	6		0.171 ± 0.138		
	2b	P1-6	P1-11	17.5	5	63	18	14	3		0.151 ± 0.131		
	2c	P2-4	P4-4	40	7.5	58	12	19	5	0.60 ± 0.02	0.202 ± 0.317	0.175 ± 0.056	0.02
vic3	3a	P19-4	P25-27	52.5	75	45	11	2	15		−0.235 ± 0.262		
	3b	P16-1	P16-7	67.5	72.5	66	6	11	6		0.072 ± 0.059		
	3c	P26-1	P25-6	65	75	60	11	3	7	0.44 ± 0.09	−0.055 ± 0.128	−0.073 ± 0.059	0.50
vic4	4a	P25-27	P16-7	100	100	48	12	11	7		0.074 ± 0.213		
	4b	P25-6	P16-2	100	100	57	1	4	9		−0.078 ± 0.174		
	4c	P10-18	P5-2	100	100	68	4	5	15	0.41 ± 0.11	−0.152 ± 0.149	−0.052 ± 0.055	0.45
vic6	6a	P16-1	P17-2	45	20	64	9	33	4		0.488 ± 0.348		
	6b	P9-13	P1-4	47.5	5	73	17	5	15		−0.100 ± 0.173		
	6c	P9-2	P1-16	4.3	12.5	40	11	6	4	0.58 ± 0.07	0.063 ± 0.125	0.150 ± 0.07	0.56
vic7	7a	P16-1	P9-13	42.5	100	53	7	23	4		0.336 ± 0.170		
	7b	P26-1	P35-3	28.3	97.5	65	19	22	8		0.191 ± 0.266		
	7c	P32-3	P1-6	10	100	41	15	14	4	0.73 ± 0.05	0.241 ± 0.231	0.256 ± 0.05	0.03

[a] *vic* loci at which isolates within each pair have different alleles.
[b] Group and pair abbreviations (see § 2).
[c] Isolates are from crosses (Cortesi & Milgroom 1998) and were used for virus transmission studies (Cortesi *et al.* 2001). Allele *1* and allele *2* isolates refer to isolates with these alleles at the heteroallelic *vic* locus. Full *vic* genotypes are in Cortesi *et al.* (2001).
[d] Percentage of trials in which virus was observed to be transmitted between isolates; data from Cortesi *et al.* (2001).
[e] Mean cell death frequency within each group is the number of deaths divided by the number of contacts.
[f] Asymmetry is defined as the difference in deaths in allele *1* and *2* isolates, divided by the number of contacts.
[g] Transmission is assumed to be 100% for self pairings (Cortesi *et al.* 2001).
[h] Independent isolates with the same *vic* genotypes.

six areas, 0.4 mm long × 0.16 mm wide, within and along the length of the interaction zone between the two isolates, were randomly chosen. Observations were made within each of the six areas from each slide 30 h after the inoculation. For each pair of isolates, we recorded: (i) the number of hyphal contacts between the two isolates, (ii) the number of cell fusions where PCD occurred in both isolates, and (iii) the number and identity of the isolate undergoing PCD when it occurred in one isolate. The number of contacts between isolates was recorded as an estimate of the total number of cell fusions, although we do not have cytological evidence to establish which contacts were actually fusions. We estimated asymmetry as the difference in the number of cell deaths in isolates 1 and 2 divided by the total number of contacts. This index varies theoretically from 0 when there is no asymmetry, to 1 or −1 when all contacts result in cell death exclusively in isolate 1 or 2, respectively.

(e) *Effect of virus infection on cell death*

We compared cell death frequency between virus-free and virus-infected pairs to assess whether or not the presence of the virus increased or decreased PCD. Four of the 24 isolate pairs used above were chosen because we observed more cell death than expected based on high levels of virus transmission (see § 3). *Cryphonectria hypovirus 1* was previously transmitted into each of these eight isolates (Cortesi *et al.* 2001). Isolates were paired in the following combinations: virus-free/virus-free (designated O/O, for isolates 1 and 2, respectively), virus-infected/virus-free (V/O), virus-free/virus-infected (O/V) and virus-infected/virus-infected (V/V). Four replicate slides for each of the 16 pairs were examined as described above. The results for each combination were arcsine square-root transformed and subjected to two-tailed *t*-tests to determine whether virus infection changed the cell death frequency in isolates 1 or 2. Asymmetric cell death was estimated as described above.

3. RESULTS

(a) *Cell death associated with fusion between vegetatively incompatible isolates*

Cell fusions were commonly observed between both compatible (figure 1*a*) and incompatible isolates (figure 1*b–d*). Fusions between incompatible isolates were characterized by a degenerative process of the fused cells, often extending into a neighbouring cell(s), for all the pairs of isolates, representing heteroallelism at each of the six *vic* loci. In contrast, this degenerative process was observed rarely (less than 10%) following fusion between isolates that were vegetatively compatible, i.e. the same alleles at all *vic* loci. The progression of the degenerative process could be observed through time, or spatially by scanning along the lengths of hyphae involved in incompatible fusions (figure 1*d*). The degenerative process was first evident as an increase in the number and size of vacuoles (figure 1*b*,*d*) and by an increase in the granulation of cytoplasm in the inter-vacuolar region. Following vacuolization and granulation, the cytoplasm of the affected cells progressively shrank (figure 1*b*) and became more opaque (condensation), until it was finally observed as a small mass of material inside the cell wall (shrinkage; figure 1*c*). The appearance of incompatible reactions was similar regardless of which *vic* locus was heteroallelic.

(b) *Variation in cell death among groups of isolates heteroallelic at different* vic *loci*

The frequency of cell deaths per contact in self-pairings (S) and between vegetatively compatible, but genetically distinct, pairs (N) was significantly less than for vegetatively incompatible pairs ($p < 0.003$; table 1). Cell death frequencies were significantly negatively correlated with virus transmission frequencies into recipient isolates in the 24 pairs of isolates ($r = -0.64$, $n = 48$, $p < 0.001$; figure 2). Significant asymmetry in cell death was observed in groups 1, 2 and 7; the degree of asymmetry varied, however, with strong asymmetry in groups 1 and 7 and relatively weak asymmetry in group 2 (table 1; figure 3). Asymmetric cell death was significantly correlated with virus transmission asymmetry observed in this study ($r = 0.60$, $n = 24$, $p < 0.002$; figure 3) and previously ($r = 0.81$, $n = 7$, $p < 0.02$; from γ_i in table 2 of Cortesi *et al.* (2001)).

(c) *Effect of virus infection on PCD*

The partial correlation between cell death and virus transmission (figure 2) and variable cell death frequencies among replicates for specific pairings in virus-free isolates prompted us to investigate whether or not virus infection affects cell death. We selected four isolate pairs for this experiment: Na, Nb, 3a and 4a. Unlike other pairs in their respective groups, Na, 3a and 4a were characterized by highly variable and relatively high mean cell death frequencies in independent experiments, and by a high probability of virus transmission in both directions. We hypothesized that viruses might reduce the frequency of cell deaths, thus allowing more virus transmission. For comparison, we chose pair Nb (*vic*-compatible) that had consistently low cell death frequencies and 100% virus transmission in both directions. Virus infection influenced the frequency of cell deaths in allele *1* or allele *2* isolates, taken separately (figure 4; electronic Appendix A, available on The Royal Society's Publications Web site). In pair Nb, when virus is present in Nb-1 (V/O) and in both Nb isolates (V/V), we observed increased cell death frequency in both Nb-1 and Nb-2. A more interesting situation is evident in pair 3a in which virus infection reduced the frequency of cell death. Virus in 3a-1 (V/O) is associated with a significant decrease in cell death in the virus-free isolate 3a-2 and a strong trend ($p = 0.08$) to increased cell death in 3a-1. Conversely, virus in 3a-2 (O/V) and in both 3a-1 and 3a-2 (V/V) is associated with a significant reduction in the cell death frequency in 3a-2, and a non-significant trend ($p \leqslant 0.2$) towards fewer cell deaths in 3a-1 (figure 4). The effect of virus in 3a-2 appears to be dominant over the effect of virus in 3a-1 based on pairings where both 3a-1 and 3a-2 contain the virus. In pair 4a, we observed that virus in 4a-1 (V/O) resulted in a reduction in cell death by 4a-1, similar to results for isolate 3a-2. Note that isolates 3a-2 and 4a-1 are actually the same isolate (P25-27) used in groups 3 and 4 to represent heteroallelism at different loci (table 1). In 4a pairings, virus-infected isolates exhibited a reduction in *vic4*-mediated PCD, a result consistent with the lack of inhibition of virus transmission between *vic4*-heteroallelic isolates (Cortesi *et al.* 2001).

None of the four pairs of isolates show significant asymmetry in cell death without virus infection (table 1;

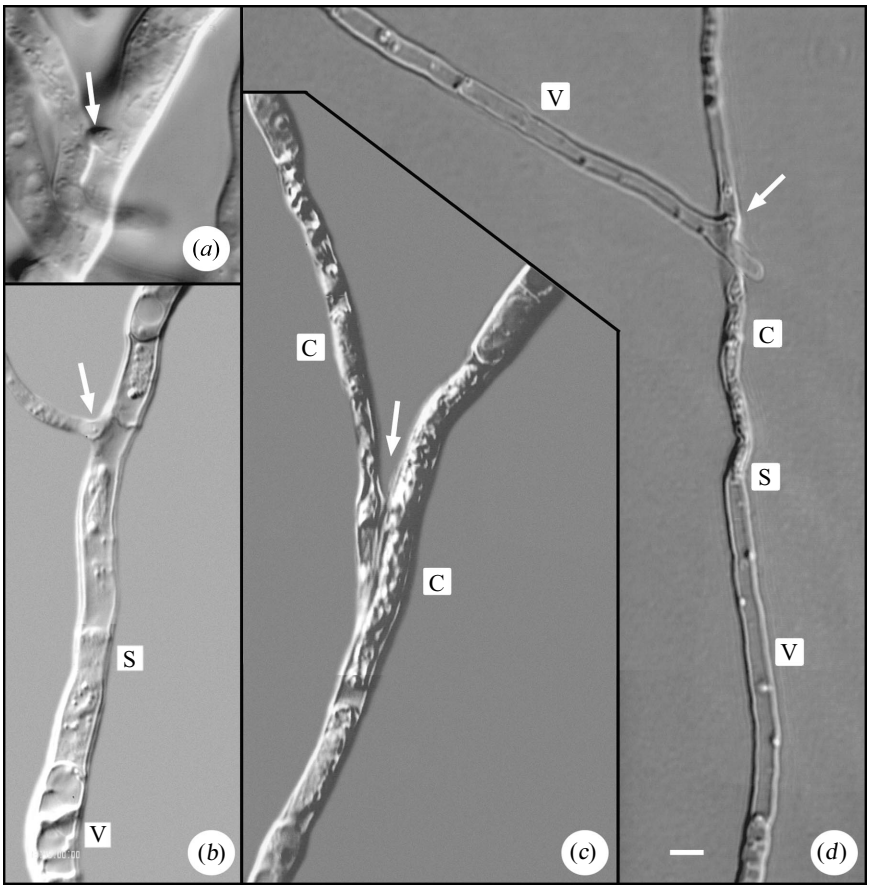

Figure 1. Cell fusions between *Cryphonectria parasitica* isolates (see arrows); magnification, ×63; scale bar (in *d*), 5 µm. (*a*) Cell fusion between vegetatively compatible isolates Na-1 (on the left) and Na-2 (on the right). (*b*) Cytoplasmic shrinkage of isolate 1b-2 near the point of fusion with *vic1*-incompatible isolate 1b-1. (*c*) Incompatible fusion between isolates 1b-1 (on the left) and 1b-2 (on the right) with fusion cells of both isolates showing collapse. (*d*) Incompatible fusion between isolates 1b-1 (on the left) and 1b-2 (on the right) with cells of one isolate (1b-2) showing progressive stages of cell death typical of incompatible reactions. (V, vacuolization; S, cytoplasmic shrinkage; C, collapsed cell contents.)

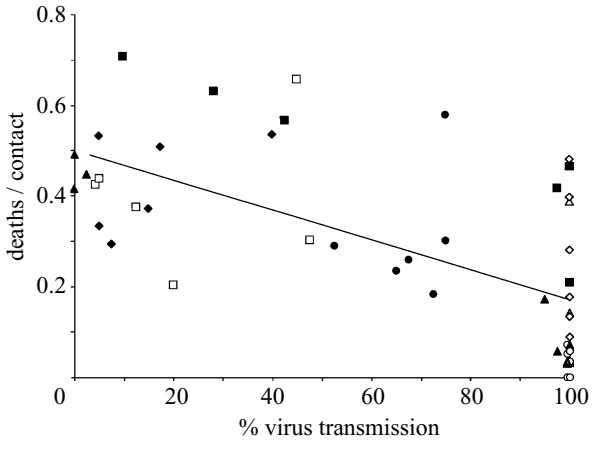

Figure 2. Correlation between cell death frequency (deaths/contact) and percentage virus transmission ($r = -0.64$, $p < 0.001$). Each data point represents the frequency of cell death and the frequency of virus transmission (data from Cortesi *et al.* (2001)) into a recipient isolate, i.e. there are two data points for each isolate pair. Points represent pairings of single isolates (i.e. self-pairings, open circles), genetically distinct but *vic*-compatible isolates (open triangles), and isolates heteroallelic at *vic1* (filled triangles), *vic2* (filled diamonds), *vic3* (filled circles), *vic4* (open diamonds), *vic6* (open squares), or *vic7* (filled squares).

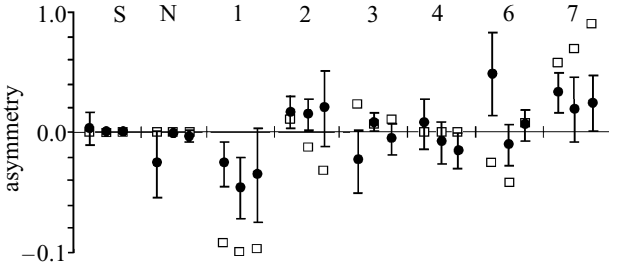

Figure 3. Asymmetry in cell death (solid circles ± s.e.) and virus transmission (open squares) in replicate pairs of *Cryphonectria parasitica* isolates. Three replicate pairs are shown of vegetatively compatible (S and N) and incompatible isolates heteroallelic at six *vic* loci (groups 1, 2, 3, 4, 6 and 7). Cell death asymmetry is estimated as the difference in number of cell deaths in allele *1* and allele *2* isolates divided by the number of contacts. Virus transmission asymmetry is estimated as the difference in transmission into allele *2* and allele *1* isolates. Note that the signs are reversed between cell death and virus transmission asymmetries to show a correlation visually.

figure 4). By contrast, there were three cases of significant asymmetric cell death when one of the isolates was infected. In Na, in which asymmetry in virus-free isolates is marginally significant ($p = 0.07$), virus infection of either

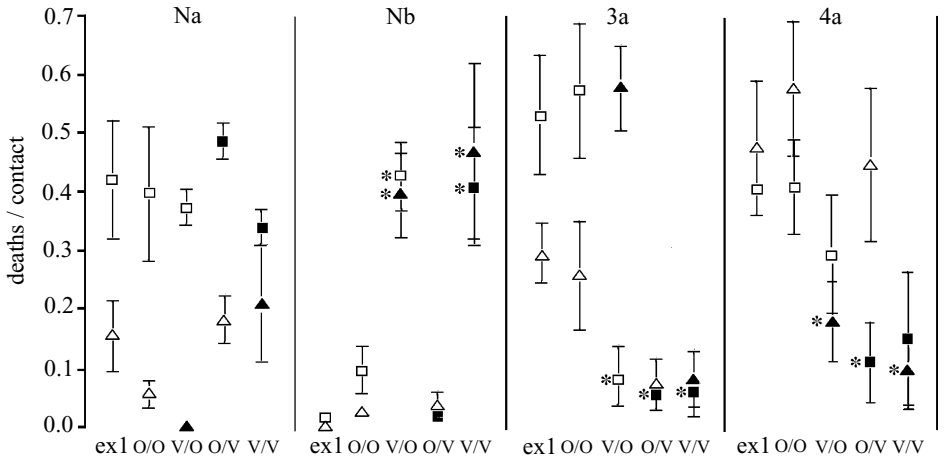

Figure 4. Effect of virus infection on cell death frequencies within each of four pairs of isolates (Na, Nb, 3a and 4a; see electronic Appendix A). Isolates 1 and 2 in each pair are denoted by triangles and squares, respectively. Virus-free isolates are indicated by open symbols; virus-infected isolates are indicated by filled symbols, and by the designations at the bottom of each panel, where virus in neither isolate is denoted O/O, in isolate 1 only (V/O), in isolate 2 only (O/V) and in both isolates (V/V). Each data point represents the mean (± s.e.) of four replications. Asterisks indicate significant differences in cell death frequencies of each isolate compared to virus-free controls from a previous experiment (ex1, table 1) and from this one (O/O).

isolate, but not both, resulted in significant asymmetric cell death ($p \leqslant 0.01$). This effect of virus on asymmetry was largely due to a reduction in the variance about cell death frequency in the V/O and O/V treatments compared with the virus-free control (O/O). A clear effect on asymmetry is shown in pair 3a where virus in 3a-1 alone (V/O) resulted in asymmetric cell death of 3a-1 ($p < 0.01$) primarily, and a dramatically different cell death pattern in comparison with the virus-free controls.

4. DISCUSSION

From our cytological investigations of cell fusions between vegetatively incompatible *C. parasitica* isolates, we observed progressive stages leading to cell death that were characterized by granulation, vacuolization and cytoplasm condensation. Cell death in *C. parasitica* is similar in appearance regardless of which *vic* locus is heteroallelic. This result indicates that all six *vic* genes known for *C. parasitica* trigger a common PCD pathway. The progression of stages in PCD in *C. parasitica* is similar to those observed after incompatible fusions in *Neurospora crassa* (Garnjobst & Wilson 1956; Jacobson *et al.* 1998) and *Sclerotinia sclerotiorum* (Kohn *et al.* 1990), consistent with the hypothesis that the pathways involved in cell death may be conserved in fungi (Glass *et al.* 2000); similar to the conservation of PCD pathways among plants and animals (Aravind *et al.* 1999; Lam *et al.* 2001). Further evidence for vegetative incompatibility as part of a conserved PCD pathway is the recent finding that a gene involved in PCD in *Arabidopsis* (Brodersen *et al.* 2002) is homologous to the *het-c* gene in the ascomycete *Podospora anserina* (Saupe *et al.* 1994).

Overall, we observed a higher frequency of PCD, both symmetric and asymmetric, in contacts between vegetatively incompatible pairings than in self- or non-self compatible pairings. Combining data from all heteroallelic loci, we found a significant negative correlation between cell death and virus transmission into recipient isolates heteroallelic at single *vic* loci. This negative correlation is consistent with the hypothesis that cell death prevents virus transmission. We also found significant asymmetric PCD in isolates that were heteroallelic at *vic1*, *vic2* and *vic7*. Cell death asymmetries have also been observed between incompatible strains in *P. anserina* (Labarère *et al.* 1974), *S. sclerotiorum* (Kohn *et al.* 1990) and *N. crassa* (Wilson *et al.* 1961) and in the cellular slime mould *Didymium iridis* (Clark & Collin 1973). Our results for *C. parasitica* further showed that asymmetric cell death correlates with asymmetric virus transmission and that both types of asymmetry can be attributed to specific alleles occurring at well-defined *vic* loci. In other words, the probability of virus transmission in *C. parasitica* is greater into a recipient isolate that tends to exhibit delayed cell death or does not die as frequently upon fusion with an incompatible infected donor. Thus, we provide evidence that cell death reactions associated with *vic* incompatibility are correlated with restriction in the transmission of infectious genetic elements in fungi, as hypothesized previously (Anagnostakis & Day 1979).

The relationship between cell death and virus transmission for *vic2*-incompatible isolates is less clear. Virus transmission between isolates in group 2 is not significantly asymmetric ($p = 0.44$; table 1), although cell death is ($p = 0.02$; table 1), with allele *1* isolates dying more frequently. However, the magnitude of the cell death asymmetry is relatively small (0.175 ± 0.056; table 1; figure 3) in this and additional experiments (data not shown). For a larger number of *vic2*-incompatible pairs, the magnitude of transmission asymmetry was small and transmission into recipient isolates carrying allele *1* tended to be greater. Although cell death asymmetry associated with *vic2* appears to contradict the correlation between frequent and/or rapid cell death and restricted virus transmission, both types of asymmetry were small for *vic2* compared with observations for *vic1* and *vic7*.

Although results from different experiments are generally reproducible (results not shown), variation in cell death frequencies and asymmetry among different pairs of isolates heteroallelic at the same *vic* locus indicates that

other factors contribute to cell death in *C. parasitica* in addition to the known major *vic* genes. One possible explanation is that some of these isolate pairs are heteroallelic at additional weak *vic* loci, e.g. *vic5* (Huber 1996), which is not detectable with our assay (Cortesi & Milgroom 1998). Partial heterokaryon incompatibility genes in *Aspergillus nidulans* (Coenen *et al.* 1994) have been shown to have an additive effect even though individually they do not prevent heterokaryon formation or horizontal transmission of mitochondria. Although *vic* genes with partial effects have not been identified in *C. parasitica* (except perhaps *vic5*), such genes might be a source of unexplained variance in these studies. As more is discovered about signalling pathways leading to PCD in fungi, additional types of genes might also be identified as contributing to this variation.

Cell death frequencies in some isolate pairs did not correlate closely to virus transmission frequencies. For this reason, we hypothesized that virus infection may be a significant factor affecting the frequency of cell death. Although this part of the study is preliminary, because cell death was studied in only four pairs of isolates, we found that virus infection can have significant positive or negative effects on cell death frequencies, depending on the isolates tested. A simple explanation for an increase in total cell death frequency is that there is an additive negative effect of virus and vegetative incompatibility. However, it should be noted that both cases in which we observed a significant increase in total cell death frequencies associated with virus infection were in pair Nb, a *vic*-compatible pairing (figure 4). This result indicates that factors in addition to the six defined *vic* genes may be involved in triggering cell death in response to viruses, or that a virus itself may elicit PCD. Reductions in cell death frequencies were associated with virus infection in other isolate pairs. For example, the presence of the virus in 3a and 4a pairings resulted in significant reductions in mean cell deaths per contact. In pair 3a (heteroallelic at *vic3*), we observed a significant reduction in cell death frequency in the virus-free recipient isolates. As shown in figure 4, when 3a-1 and/or 3a-2 is virus-infected, cell death occurs significantly less often in 3a-2 than in the virus-free controls.

In animal systems, there are many examples of parasitized individuals having different behaviours or phenotypes compared with uninfected conspecifics (Moore 1995; Poulin 2000). Some of these behavioural alterations appear to be caused by the parasite and result in increases in transmission. In other cases, behavioural changes are attributed to defence reactions by the host. Alternatively, inhibition and stimulation of PCD by viruses in animal systems are hypothesized to represent a balance between maintaining a living cell within which viral replication can occur, and promoting cell lysis to allow for viral dissemination, respectively (Roulston *et al.* 1999). We speculate that decreases in cell death associated with virus infection in *C. parasitica* may depend on specific host–pathogen interactions where the virus may suppress the incompatibility response of the recipient isolate, thus promoting its own transmission. We further speculate that the fungus may react by increasing the rate or frequency of PCD in response to the presence of viruses in order to prevent infection, roughly analogous to hypersensitive responses in plants (Lam *et al.* 2001) when confronted with pathogens, including fungi. This type of increased rate of cell death in fungi would only be beneficial, however, if recipient individuals could detect the presence of virus in donors soon after cell fusion and respond rapidly.

In this study, we carried out a comprehensive microscopic examination of cell death associated with all known *vic* genes in a fungus. We demonstrated that cell death associated with vegetative incompatibility in *C. parasitica* is correlated with a reduction in transmission of infectious elements. Furthermore, virus infection modified, in some cases, the frequency of cell death of the host, strongly implicating PCD as a host-mediated pathogen defence strategy in fungi. Further studies are needed to identify fungus and/or virus factors involved in specific host–virus interactions in influencing the cell death response of vegetative incompatibility. Remarkable progress has been made in understanding the role of PCD in relation to host defences against pathogens in plants and animals (Roulston *et al.* 1999; Lam *et al.* 2001), yet virtually nothing is known about PCD in fungi. The well-characterized *vic* genotypes and viruses in *C. parasitica* present an excellent system in which to understand the regulation of vegetative incompatibility in response to virus infection in fungi.

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REFERENCES

Anagnostakis, S. L. 1983 Conversion to curative morphology in *Endothia parasitica* and its restriction by vegetative compatibility. *Mycologia* **75**, 777–780.

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

Aravind, L., Dixit, V. M. & Koonin, E. V. 1999 The domains of death: evolution of the apoptosis machinery. *Trends Biochem. Sci.* **24**, 47–53.

Baidyaroy, D., Glynn, J. M. & Bertrand, H. 2000 Dynamics of asexual transmission of a mitochondrial plasmid in *Cryphonectria parasitica*. *Curr. Genet.* **37**, 257–267.

Brodersen, P., Petersen, M., Pike, H. M., Olszak, B., Skov, S., Ødum, N., Jørgensen, L. B., Brown, R. E. & Mundy, J. 2002 Knockout of *Arabidopsis ACCELERATED-CELL-DEATH11* encoding a sphingosine transfer protein causes activation of programmed cell death and defense. *Genet. Dev.* **16**, 490–502.

Caten, C. E. 1972 Vegetative incompatibility and cytoplasmic infection in fungi. *J. Gen. Microbiol.* **72**, 221–229.

Clark, J. & Collin, O. R. 1973 Directional cytotoxic reactions between incompatible plasmodia of *Didymium iridis*. *Genetics* **73**, 247–257.

Coenen, A., Debets, F. & Hoekstra, R. F. 1994 Additive action of partial heterokaryon incompatibility (*partial-het*) genes in *Aspergillus nidulans*. *Curr. Genet.* **26**, 233–237.

Cortesi, P. & Milgroom, M. G. 1998 Genetics of vegetative incompatibility in *Cryphonectria parasitica*. *Appl. Environ. Microbiol.* **64**, 2988–2994.

Cortesi, P., McCulloch, C. E., Song, H., Lin, H. & Milgroom, M. G. 2001 Genetic control of horizontal virus transmission in the chestnut blight fungus, *Cryphonectria parasitica*. *Genetics* **159**, 107–118.

Garnjobst, L. & Wilson, J. F. 1956 Heterocaryosis and protoplasmic incompatibility in *Neurospora crassa*. *Proc. Natl Acad. Sci. USA* **42**, 613–618.

Glass, N. L., Jacobson, D. J. & Shiu, P. K. T. 2000 The genetics of hyphal fusion and vegetative incompatibility in filamentous ascomycete fungi. *A. Rev. Genet.* **34**, 165–186.

Hartl, D. L., Dempster, E. R. & Brown, S. W. 1975 Adaptive significance of vegetative incompatibility in *Neurospora crassa*. *Genetics* **81**, 553–569.

Huber, D. H. 1996 Genetic analysis of vegetative incompatibility polymorphisms and horizontal transmission in the chestnut blight fungus *Cryphonectria parasitica*. PhD thesis, Michigan State University, East Lansing, MI.

Jacobson, D. J., Beurkens, K. & Klomparens, K. L. 1998 Microscopic and ultrastructural examination of vegetative incompatibility in partial diploids heterozygous at *het* loci in *Neurospora crasssa*. *Fungal Genet. Biol.* **23**, 45–56.

Kohn, L. M., Carbone, I. & Anderson, J. B. 1990 Mycelial interactions in *Sclerotinia sclerotiorum*. *Exp. Mycol.* **14**, 255–267.

Labarère, J., Bégueret, J. & Bernet, J. 1974 Incompatibility in *Podospora anserina*: comparative properties of the antagonistic cytoplasmic factors of a non-allelic system. *J. Bacteriol.* **120**, 854–860.

Lam, E., Kato, N. & Lawton, M. 2001 Programmed cell death, mitochondria and the plant hypersensitive response. *Nature* **411**, 848–853.

Leslie, J. F. & Zeller, K. A. 1996 Heterokaryon incompatibility in fungi: more than just another way to die. *J. Genet.* **75**, 415–424.

Liu, Y.-C. & Milgroom, M. G. 1996 Correlation between hypovirus transmission and the number of vegetative incompatibility (*vic*) genes different among isolates from a natural population of *Cryphonectria parasitica*. *Phytopathology* **86**, 79–86.

Moore, J. 1995 The behavior of parasitized animals. *Bioscience* **45**, 89–96.

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

Poulin, R. 2000 Manipulation of host behaviour by parasites: a weakening paradigm? *Proc. R. Soc. Lond.* B **267**, 787–792. (DOI 10.1098/rspb.2000.1072.)

Roulston, A., Marcellus, R. C. & Branton, P. E. 1999 Viruses and apoptosis. *A. Rev. Microbiol.* **53**, 577–628.

Saupe, S. J. 2000 Molecular genetics of heterokaryon incompatibility in filamentous ascomycetes. *Microbiol. Mol. Biol. Rev.* **64**, 489–502.

Saupe, S. J., Descamps, C., Turcq, B. & Béguret, J. 1994 Inactivation of the *Podospora anserina* vegetative incompatibility locus *het-c*, whose product resembles a glycolipid transfer protein, drastically impairs ascospore production. *Proc. Natl Acad. Sci. USA* **91**, 5927–5931.

Saupe, S. J., Clavé, C. & Béguret, J. 2000 Vegetative incompatibility in filamentous fungi: *Podospora* and *Neurospora* provide some clues. *Curr. Opin. Microbiol.* **3**, 608–612.

Van Alfen, N. K., Jaynes, R. A., Anagnostakis, S. L. & Day, P. R. 1975 Chestnut blight: biological control by transmissible hypovirulence in *Endothia parasitica*. *Science* **189**, 890–891.

Wilson, J. F., Garnjobst, L. & Tatum, E. L. 1961 Heterocaryon incompatibility in *Neurospora crassa*. Micro-injection studies. *Am. J. Bot.* **48**, 299–305.

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