

Heterokaryon incompatibility function of barrage-associated vegetative incompatibility genes (*vic*) in *Cryphonectria parasitica*

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Abstract: Six vegetative incompatibility (*vic*) loci have been identified in *Cryphonectria parasitica* based on barrage formation during mycelial interactions. We used hygromycin B- and benomyl-resistance as forcing markers in *C. parasitica* strains to test whether heteroallelism at each *vic* locus prevents heterokaryon formation following mycelial interactions. Paired strains that had allelic differences at any of *vic1*, 2, 3, 6 or 7 but not *vic4* displayed heterokaryon incompatibility function, as recognized by slow growth or aberrant morphology. While clearly forming barrages in mycelial interactions, paired strains with different alleles at *vic4* formed stable heterokaryons. With examples from other fungi, this inconsistency at *vic4* suggests that barrage formation and heterokaryon incompatibility are not different manifestations of the same process. Rather, the evidence indicates that heterokaryon incompatibility represents a component of a vegetative incompatibility system that may also use cell-surface or extracellular factors to trigger programmed cell death to modulate nonself recognition in fungi.

Key words: chestnut blight fungus, programmed cell death, virus transmission

INTRODUCTION

In fungi, nonself recognition can occur during the sexual and vegetative (asexual) stages of the life cycle (reviews in: Fraser and Heitman 2003, Glass et al 2000, Micali and Smith 2005, Saupe et al 2000). The ubiquitous occurrence of vegetative incompatibility systems among filamentous fungi may reflect strong selection to restrict the transmission of parasitic elements (Buss 1982, Caten 1972, Debets and Griffiths 1998). Vegetative nonself recognition between conspecific fungi has been identified in two

ways. One method is based on a mycelial incompatibility assay, in which the formation of barrages (also referred to as demarcation or zone lines) may occur where two genetically distinct mycelia meet. Nonself recognition also is studied by testing for incompatibility using heterokaryon or partial diploid analyses. Strains that carry different nuclear types (heterokaryons) or strains that are partially diploid (having a chromosome or chromosome segment duplicated) will grow poorly or not at all if they contain incompatible alleles at one or more heterokaryon incompatibility (*het*) loci. Barrage formation and heterokaryon incompatibility often are regarded as different manifestations of the same vegetative incompatibility function. This is reasonable given that cell death due to heterokaryon incompatibility would also result in a line of demarcation associated with dead cells in the fusion zone between two incompatible colonies. However, the relationship between barrage formation and heterokaryon incompatibility has been examined in relatively few fungi, and while there is concordance in *Podospora anserina* (Rizet 1952), this is not the case in all species. For example, in *Neurospora crassa* barrage formation is not apparently associated with differences at *het-6*, *un-24*, *het-c* or *mat*, four genes with well-characterized heterokaryon incompatibility function (Micali and Smith 2003). Similarly, heterokaryon and mycelial incompatibility responses are not correlated perfectly in *Sclerotinia sclerotiorum* (Ford et al 1995). Independence of these two incompatibility systems would suggest that more than one pathway is involved in nonself recognition during the vegetative phase, while concordance would indicate that heterokaryon and mycelial incompatibility have common biochemical and genetic bases.

Cryphonectria parasitica, the causal agent of chestnut blight, provides a unique opportunity to examine the relationship between genetically well-defined mycelial incompatibility loci and heterokaryon incompatibility and to refine our understanding about the role incompatibility systems play in restricting the spread of infectious elements. *C. parasitica* has six known vegetative incompatibility (*vic*) loci, *vic1*, 2, 3, 4, 6 and 7 (Cortesi and Milgroom 1998); allelic differences at one or more *vic* loci result in a barrage in the region of mycelial contact associated with programmed cell death in fusion cells (Biella et al 2002). Previous studies indicated that some *vic* loci may restrict heterokaryon formation in *C. parasitica*.

TABLE I. Strains used in this study

Isolate ^a	<i>vic</i> genotype ^b	EU-type ^b	origin ^c
22.1H	2211-22	EU-5 (vc40)	<i>hyg</i> ^R of EP155-2 from A. Churchill
EP155B	2211-22	EU-5 (vc40)	ATCC#38755 from A. Churchill
P1-11B	2211-22	EU-5 (vc40)	PC17 × PC7
P10-2B	2211-22	EU-5 (vc40)	VO29 × PC7
P1-5B	1 211-22	EU-31	PC17 × PC7
P11-23B	1 211-22	EU-31	P1-11 × P3-3
P1-6B	2 1 11-22	EU-6	PC17 × PC7
P42-7B	2 1 11-22	EU-6	P1-11 × LJ13
P74-3B	2 2 21-22	EU-60	P1-11 × P50-16
P78-6B	2 2 21-22	EU-60	P50-4 × P74-7
P4-4B	22 1 2-22	EU-1 (vc10)	VO54 × PC7
P5-1B	22 1 2-22	EU-1 (vc10)	VO54 × VO64
P10-18B	2211- 1 2	EU-21	VO29 × PC7
MJ1-3-20B	2211- 1 2	EU-21	P17-8 × JA17
P24-33B	2211- 2 1	EU-18	P4-4 × SA26
P32-35B	2211- 2 1	EU-18	PC39 × VA35

^aOriginal strain number transformed to hygromycin (H) or benomyl (B) resistance. Strain numbers beginning with “P” or “MJ” are ascospore progeny from crosses reported in Cortesi and Milgroom (1998).

^bGenotypes for vegetative incompatibility loci and the vegetative incompatibility group (EU-type designations) are based on Cortesi and Milgroom (1998); known correspondence to vc type designations of Anagnostakis (1988) is given in parentheses. Alleles (1 or 2) are given for each *vic* locus in the order *vic1*, 2, 3, 4-, 6, 7. Alleles that differ from those in strain 22.1H are indicated in bold type.

^cA. Churchill, Boyce Thompson Institute, Ithaca, New York; ATCC = American Type Culture Collection; cross designations are from Cortesi and Milgroom (1998).

Using spontaneous drug-resistance, Vannacci et al (1997) found low protoplast fusion frequencies of ~0.6% between two incompatible strains compared to about 1.5% with two compatible strains. Rizwana and Powell (1995) used protoplast fusion to force heterokaryons of auxotrophic strains and investigated the effects of UV-light treatment on the frequency of heterokaryon formation. They detected heterokaryons following fusion of protoplasts that differed at *vic1* or at *vic2* but not when strains differed at two or more *vic* loci. The frequency of heterokaryon formation was approximately the same or 100-fold less with strains that differed at *vic1* or *vic2*, respectively, than when *vic*-compatible isolates were fused. Interestingly, treatment of protoplasts with UV light immediately prior to protoplast fusions increased the frequency of heterokaryon formation between incompatible strains by 100- to 1000-fold. In an unpublished study, Huber (1996) used complementation of conidial color mutants to demonstrate heterokaryon formation following hyphal fusion between compatible strains and strains that were heteroallelic at *vic4*. Color complementation was not observed when strains differed at any of *vic1*, *vic2* or *vic3*.

In addition to heterokaryon formation, *vic* genes also affect virus transmission between *C. parasitica* strains. Cortesi et al (2001) reported that the trans-

mission of double-stranded RNA (dsRNA) hypoviruses was unimpeded (100% successful transmission) between strains with no *vic* differences or with *vic4* differences only. In contrast, transmission occurred on average in 56%, 25%, 83%, 47% and 78% of pairings between strains that differed at one of *vic1*, 2, 3, 6 or 7, respectively. In spite of providing no barrier to virus transmission, heteroallelism at *vic4* resulted in only slightly lower frequencies (~0.41) of programmed cell death in fusion-cells compared to strains that differed at one of *vic1*, 2, 3, 6 or 7 (0.47, 0.60, 0.44, 0.58 and 0.73, respectively; Biella et al 2003).

Taken together, variation in the effects of *vic* loci on heterokaryon formation, virus transmission and cell death frequency suggest that the biological function of *vic* genes may vary among loci. Our goal was to determine whether or not each of the six known *vic* loci in *C. parasitica* has heterokaryon incompatibility function. Furthermore, the relative effect of barrage formation and heterokaryon incompatibility systems on virus transmission is discussed in view of our findings.

MATERIALS AND METHODS

Strains and growth conditions.—Strains used in this study (TABLE I) were grown on potato-dextrose agar (PDA,

Difco, Detroit, Michigan) at ~21 C. Strains transformed with benomyl-resistance or hygromycin B-resistance plasmids are designated (TABLE I) by the original strain number followed by "B" or "H", respectively. We obtained strain 22.1H from A. Churchill (Boyce Thompson Institute, Ithaca, New York) as a hygromycin-resistant transformant derived from EP155-2. All other benomyl-resistant strains were obtained through transformation, as explained below, for use in double-drug resistance assays. PDA medium was amended with 30 µg/mL hygromycin B (382 units/mg, Calbiochem, San Diego, California) for selection of hygromycin-resistant strains and with 0.3 µg/mL benomyl (Bonide Products, Yorkville, New York) for benomyl-resistant strains. These media are referred to below as PDA+hyg and PDA+ben, respectively.

Transformation of C. parasitica.—*C. parasitica* strains were transformed to benomyl resistance by the method of Providenti et al (2004) with either one of two plasmids, pSV50 (Vollmer and Yanofsky 1986) or pMYX2 (Campbell et al 1994), obtained from the Fungal Genetics Stock Center (FGSC, U. Missouri, Kansas City, Missouri). Conidia from one- to two-week-old cultures were allowed to germinate overnight at 25 C with shaking (100 rpm) in potato-dextrose broth. The germlings were collected by centrifugation (1000 × g, 5 min), washed twice and resuspended in 1 M sorbitol. The cells were treated with 5 mg/mL Novozyme 234 (Interspex, Foster City, California) 40 min at 25 C with gentle shaking (100 rpm), centrifuged (800 × g, 10 min), washed twice and resuspended at ~1 × 10⁸ cells/mL with ice-cold 1 M sorbitol. Plasmid vector (~0.5 µg) was added to 100 µL of the cell suspension prior to electroporation with a Gene Pulser (Bio-Rad, Hercules, California). Electroporation conditions were 1.5 kV, 25 µF, 800 Ω in 1-mm cuvettes. Electroporated cells were immediately placed in 1 mL of ice-cold 1 M sorbitol and left on ice 20 min before being plated onto 20 mL PDA. The plates were incubated 12–16 h at ~21 C before the addition of 10 mL of ~45 C top agar (0.5 × PDA, 0.8 µg/mL benomyl). Transformed colonies were evident after one to two weeks incubation at 24 C. To verify the stability of benomyl resistance in these transformants, four serial transfers of hyphae from the colony margin were made to selective medium (PDA+ben), followed by four serial transfers to PDA before examining for growth on selective medium again. About 8–20 stable benomyl-resistant transformants/µg DNA were obtained using this procedure.

Mycelial incompatibility and heterokaryon incompatibility tests.—Mycelial incompatibility tests were done as previously described on agar medium containing the pH indicator dye bromocresol green (Cortesi et al 1996, Powell 1995). Incompatibility reactions were evident as a dark (bromocresol green) line at the confluence of two colonies growing on agar medium. Those strains that did not form a barrage were considered *vic*-compatible.

Heterokaryon incompatibility tests were done by placing ~1-mm³ agar blocks, or conidia suspended in water, of

a benomyl-resistant strain and a hygromycin-resistant strain 1–2 mm apart on a cellophane disk on the surface of PDA. After growth and contact of the two mycelia (~30 h), the cellophane disk was transferred to the selective medium consisting of PDA with 0.3 µg/mL benomyl and 30 µg/mL hygromycin B (hereafter referred to as PDA+ben+hyg). Controls consisted of each strain being inoculated separately and handled exactly as strain combinations. Immediately after transfer to the selective medium, the colony margins were marked on the bottom of the petri dish. Growth was monitored at 21 C on selective medium at daily intervals. Outgrowths were verified as heterokaryotic by transferring hyphal tips to PDA+ben+hyg and subsequently, by examining nuclear proportions as described below.

Heterokaryon nuclear proportions.—In the following sections, heterokaryons that are heteroallelic for *vic4* are referred to as *vic4*-incompatible while those with no differences at *vic* loci are called *vic*-compatible. Nuclear proportions in *vic4*-incompatible (strains P5-1B + 22.1H and P4-4B + 22.1H) and *vic*-compatible (EP155B + 22.1H) heterokaryons were estimated by testing cultures derived from single conidia for benomyl and hygromycin resistance. In *C. parasitica*, single nuclei can be sampled effectively in this way because more than 99.5% of conidia are uninucleate (McGuire et al 2004). For analysis of conidia, a small section (~1 mm²) of mycelium was taken from the colony margin of a heterokaryotic outgrowth and transferred to PDA+ben+hyg. After ~3 wk, conidia were removed from the colony, diluted in water, plated onto PDA and incubated overnight at room temperature. Germinated conidia were excised under a dissecting microscope and transferred to PDA for growth at room temperature for 2–3 d. Mycelium of each resulting single-spore-derived colony (between 15 and 70 isolates for each heterokaryon sector examined) was transferred separately to each of PDA+ben and PDA+hyg and after 2 d the presence or absence of growth was recorded.

Nuclear sorting in heterokaryons grown under non-selective conditions.—Drug-resistant homokaryotic isolates (five replicates for each of 22.1H, EP155B, P4-4B and P5-1B), *vic*-compatible heterokaryons (nine replicates for EP155B+22.1H) and *vic4*-incompatible heterokaryons (seven replicates for P4-4B+22.1H; 11 replicates for P5-1B+22.1H) were each inoculated onto the end of separate ~1 × 7.5 cm strips of sterile cellophane overlaid on PDA in 9-cm petri dishes. The cultures were incubated at ~21 C and each day the colony margins were marked on the bottom of each petri dish. After 12 d, the cellophane with growing mycelium was aseptically transferred to PDA+ben+hyg. The regions along the mycelial strip where growth into the selective medium occurred were monitored over a period of 8 d. The location along the cellophane strip closest to the colony origin at which no growth occurred on PDA+ben+hyg was interpreted as the point where either the benomyl- or hygromycin-resistant nuclear type was lost. The corresponding location on the PDA petri dish, with daily growth marks, was then used to determine the day

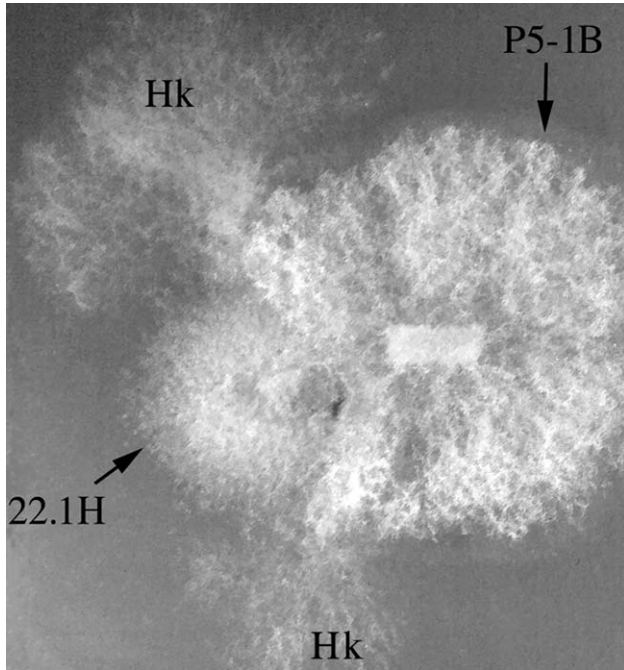


FIG. 1. Heterokaryotic sectors (Hk) arising from the zone of confluence between *vic4*-incompatible strains 22.1H and P5-1B two days after transfer to PDA+ben+hyg medium.

at which double resistance was lost between zero days (no growth) and 12 d (growth at the colony margin on PDA+ben+hyg medium).

RESULTS

Mycelial incompatibility assays were performed by confronting strains (TABLE I) in all pair-wise combinations on PDA containing bromocresol green (a pH indicator dye). All mycelial interactions were consistent with *vic*-genotype designations for the study strains (TABLE I, Cortesi and Milgroom 1998); thin, dark (bromocresol green) zone lines were evident within ~1 wk after colony contact in all pairings with differences at *vic1*, 2, 3, 4, 6 or 7, and no zone line was evident in pairings between strains with the same *vic* genotype. The dark lines associated with incompatible pairings are presumably due to cell death and lysis after fusion, with the localized bromocresol green color development in association with the release of acidic cytoplasmic contents (Powell 1995).

Heterokaryon compatibility was assayed between hygromycin-resistant strain 22.1H and each of 15 benomyl-resistant strains that differed at none or one of each of the six *vic* loci. Putatively heterokaryotic sectors were observed to grow out from the zone of contact in all pairings that did not differ at any of the six *vic* loci. Similar putative heterokaryotic sectors were visible for those pairings that differed only at

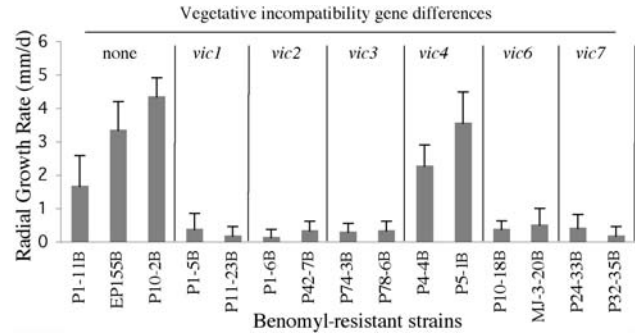


FIG. 2. Mean growth rates (+SD, $n \geq 4$) of potential heterokaryons synthesized with a hygromycin resistant strain, 22.1H, and each of the benomyl-resistant strains given across the bottom. Differences at specified *vic* genes are given across the top.

vic4 (FIG. 1). Outgrowths were not observed for strain pairs that differed at any of *vic1*, 2, 3, 6 or 7. A ~1-mm² section of hyphae was taken from the contact zone between strains of each pairing and transferred to fresh PDA+ben+hyg medium and growth rates were recorded daily for 1 wk (FIG. 2). Subcultures from pairings that differed at *vic1*, 2, 3, 6 or 7 had radial growth of <1 mm/day, suggesting that these *vic* loci inhibit heterokaryon formation and/or growth. In contrast, sustained growth of >2 mm/day on PDA+ben+hyg by subcultures from *vic*-compatible pairings and pairings in which strains differed only at *vic4* was observed, consistent with heterokaryosis. Thus, mycelial incompatibility appears correlated with heterokaryon incompatibility function in *C. parasitica* at all *vic* loci except for *vic4*.

No significant differences were observed between *vic4*-incompatible and *vic*-compatible heterokaryons with respect to growth rates (FIG. 2). Each *vic4*-incompatible heterokaryon also appeared to have wildtype morphology; colony margins were more or less even, conidiation abundant and pigmentation well-developed. Differences were observed, however, in nuclear proportions of *vic4*-incompatible and *vic*-compatible heterokaryons. Nuclear proportions of *vic4*-incompatible heterokaryons tended to be skewed toward one or the other nuclear type, but in all cases both nuclear types were detected. In contrast, a more even distribution of nuclear ratios was observed for heterokaryons with no *vic* differences (FIG. 3). This observation suggested a possible means by which *vic4* incompatibility may be avoided; under severely skewed nuclear proportions, relatively few cells may be truly heterokaryotic, and therefore the heterokaryon may not be stable in the long run. We tested this possibility by examining nuclear sorting patterns in *vic4*-incompatible and *vic*-compatible heterokaryons grown under nonselective conditions

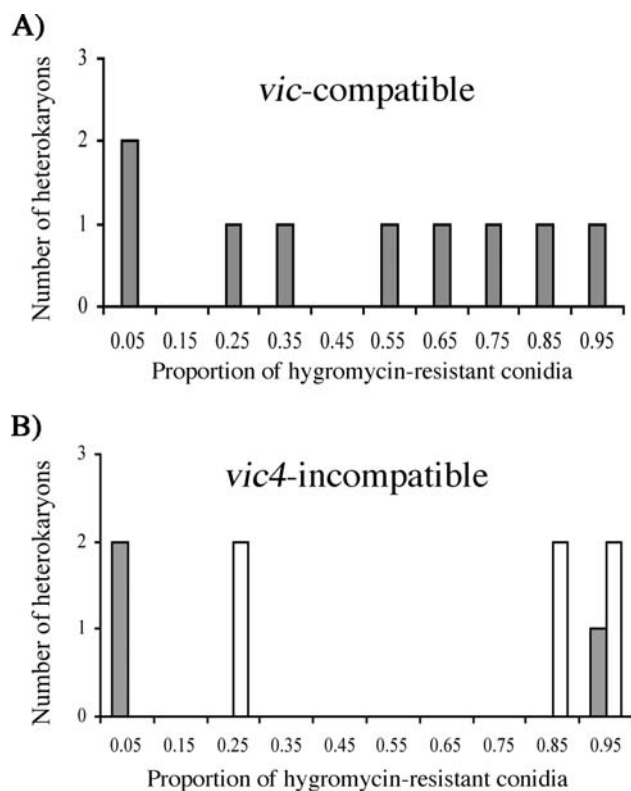


FIG. 3. Nuclear proportions (hygromycin-resistant conidia/total conidia) estimates from *vic*-compatible heterokaryons (A, EP155B+22.1H, $n = 9$) and *vic4*-incompatible heterokaryons (B, dark bars = P4-4B+22.1H, $n = 3$; light bars = P5-1B+22.1H, $n = 6$).

(FIG. 4). As expected in control experiments, growth of separate homokaryons 22.1H, EP155B, P4-4B and P5-1B was not observed after transfer to PDA+ben+hyg ($n = 5$ for each, data not shown). Surprisingly, there was no significant difference in nuclear sorting rates between *vic*-compatible and *vic4*-incompatible heterokaryons, although the latter, contrary to expectations, had a tendency to maintain both nuclear types longer under nonselective conditions than did the *vic*-compatible heterokaryons we tested.

DISCUSSION

Mycelial incompatibility in *C. parasitica* has been of special interest because of its hypothesized role in impeding the transmission of dsRNA hypoviruses (Carbone et al 2004, Cortesi et al 2001) and, therefore, in limiting the effectiveness of hypovirus-mediated biological control of chestnut blight in North America (Anagnostakis 1982, Milgroom and Cortesi 2004). The six known *vic* loci in *C. parasitica* were identified originally using classical genetics by association with barrage formation in mycelial interactions (Cortesi and Milgroom 1998). We found that

heteroallelism at any of *vic1*, 2, 3, 6 or 7 also is correlated with heterokaryon incompatibility function. In the exception, heteroallelism at *vic4* results in barrage formation but not heterokaryon incompatibility. Heterokaryons that are heteroallelic at *vic4* were observed to grow at rates comparable to those of *vic*-compatible heterokaryons and at significantly higher rates than strain combinations that were heteroallelic at *vic1*, 2, 3, 6 or 7. We verified, again after our heterokaryon compatibility tests, that barrages were associated with *vic4* differences in our strain combinations.

Considering the lack of correlation between mycelial and heterokaryon incompatibility associated with *vic4* differences, we observed that *vic*-compatible heterokaryons had more uniformly distributed nuclear proportions than did *vic4*-incompatible heterokaryons, which tended to be skewed toward one or the other nuclear type. A similar even distribution pattern of nuclear types was found in *N. crassa* compatible heterokaryons (Beadle and Coonradt 1944, Pittenger and Atwood 1956). Furthermore, change in nuclear proportions throughout growth was associated with *het-1* incompatibility in *N. crassa* (Pittenger and Brawner 1961). Nuclei carrying *het-1* are thought to replicate at a greater relative rate and inhibit replication of *het-i* nuclei under certain conditions. However, different division rates of nuclei carrying allele *vic4-1* compared to allele *vic4-2* cannot explain the skewed nuclear proportions observed in our experiments because one nuclear type was not a consistent majority in *vic4*-incompatible heterokaryons (FIG. 3). Nevertheless, if *vic4*-incompatible heterokaryons were a mosaic of homokaryotic and heterokaryotic hyphal segments, then relatively few cells may be truly heterokaryotic. Therefore, we hypothesized that the proportion of minority nuclei may be small enough to avoid an incompatibility reaction but large enough to confer antibiotic resistance to the colony. Such mosaics of heterokaryotic and homokaryotic sections were observed previously in *N. crassa* heterokaryons (Prout et al 1953, Atwood and Mukai 1955). To examine whether their rate of nuclear sorting differs, *vic*-homoallelic and *vic4*-heteroallelic heterokaryons were grown on nonselective medium, and a radially-oriented mycelial sample was transferred to selective medium to test the maintenance of double resistance. Loss of double resistance is presumably due to loss in hyphal tips of one nuclear type and the associated antibiotic resistance. However, our nuclear sorting experiment clearly indicated that *vic4*-incompatible heterokaryons are as, or more, stable than *vic*-compatible controls.

One explanation that might account for the lack of

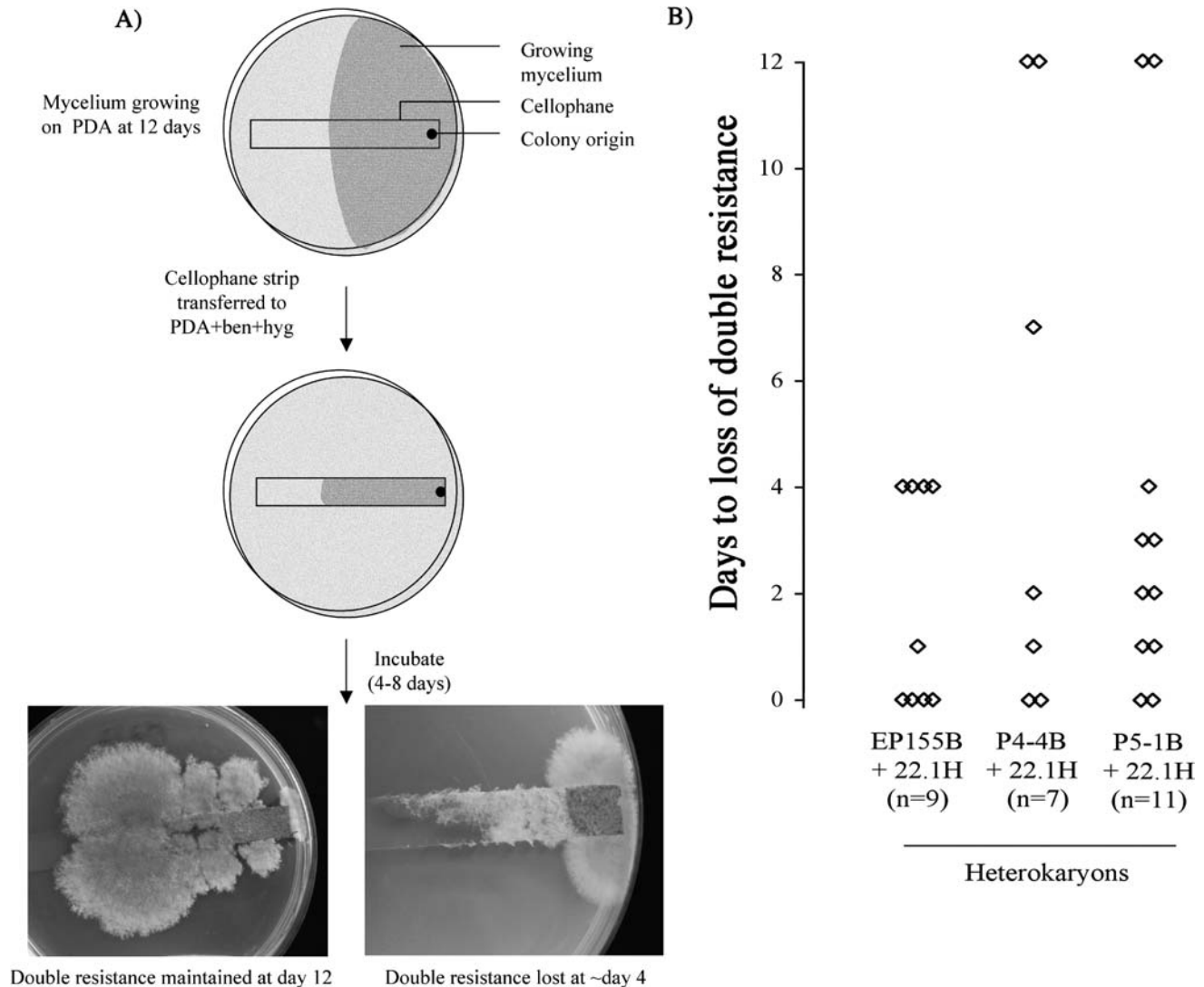


FIG. 4. A. Schematic of protocol to examine nuclear sorting rates. B. Days that *vic*-compatible heterokaryons (EP155B+22.1H) and *vic4*-incompatible heterokaryons (P4-4B+22.1H and P5-1B+22.1H) grown on nonselective PDA lose the ability to grow on PDA+ben+hyg.

correlation between mycelial and heterokaryon incompatibility associated with *vic4* is the possibility that the locus encodes an extracellular factor toxic to individuals carrying the other *vic4* allele. Such an extracellular incompatibility factor has not been identified to our knowledge. However, the observations of Rizwana and Powell (1995) indicate that *vic1* and *vic2* heterokaryon incompatibility function can be partially evaded when protoplasts are fused, suggesting that cell surface molecules may be involved in eliciting a heterokaryon incompatibility response associated with some *vic* loci. These observations may be relevant to *C. parasitica* in nature. Heterokaryons of *C. parasitica* that are heteroallelic at the mating-type (*MAT*) locus have been found in several natural populations (McGuire et al 2004). These *MAT*

heterokaryons also exhibit highly skewed ratios of nuclei with different mating type. In one clonal population, heterokaryon formation appears to have occurred between vegetatively incompatible clones (McGuire et al 2005). The mechanism for this putative heterokaryon formation between incompatible individuals is not known, but considering that UV light may modulate heterokaryon incompatibility and that heterokaryon incompatibility is circumvented during the sexual cycle, it is possible that illegitimate heterokaryons can form under certain conditions, such as environmental stress or aborted matings.

A more pronounced lack of correlation between heterokaryon and mycelial incompatibility systems is noted in some of the limited sets of fungi investigated to date. Whereas five of six loci associated with

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