Genetics

# Nuclear Gene Mutations in *Endothia* (*Cryphonectria*) *parasitica* that Affect Morphology and Virulence

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**ABSTRACT**

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Crossing and progeny analyses showed that five deeply pigmented hypovirulent strains contain single nuclear genes that confer the morphology characteristic of a class of European hypovirulent strains formerly called Jaune Régénéré or Pigmentato. These single nuclear genes also determine restricted virulence in host chestnut trees. Such strains arise only from dsRNA-containing strains and may be the result of integration of cytoplasmic genetic material into the nuclear genome.

*Additional key words*: American chestnut, biological control, chestnut blight, hypovirulence.

Strains of *Endothia parasitica* (Murr.) And. (*Cryphonectria parasitica* (Murr.) Barr) that are hypovirulent (11) and curative have been found in Europe and the United States. They are being tested for potential use in biological control of chestnut blight, a lethal disease of American chestnut trees (*Castanea dentata* (Marsh.) Borkh.) (5,13). The most common curative strains from Europe have little or no pigment (white H). These grow more slowly than normal virulent strains (V) at 25–27 C on agar media in the laboratory (8) and grow very little in the chestnut host. Slow-growing, white H strains produce uninucleate conidia that can grow into normal V, white H, intermediate white H, or deeply pigmented H strains (11,15,16). This cytoplasmic segregation has been linked to the presence of double-stranded RNA (dsRNA) in H strains (18). The deeply pigmented H strains present an anomaly because although they contain dsRNA, their conidia grow into colonies that all resemble their progenitor. These have little aerial mycelium (therefore "flat") and produce conidia on short, curved branches directly on the vegetative mycelium instead of in pycnidia, which are typical of *E. parasitica*. Strains with this flat morphology

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were called JR (Jaune Régénéré) and P (Pigmentato) by earlier workers (11,15,16). Grente (14) remarked that it is noteworthy that such strains have never been obtained from natural cankers in more than 12,000 isolations. He suggested that they are artifacts of laboratory culture. The frequency of recovery of flat strains from conidia of white H strains in the laboratory can be manipulated by changing the physical conditions during growth and conidiogenesis. This indicates that they might be mutants of some kind.

I used five flat H strains as male parents in crosses with genetically marked strains in the laboratory and examined the progeny. Because the flat morphology segregated as if determined by a single nuclear gene, some of the progeny were saved for more tests.

If white or flat H strains are paired with V strains, the morphology of the V strains can be converted to that of white H following hyphal anastomoses and transfer of cytoplasm (including dsRNA) (9,14). All flat H strains used as male parents in these crosses contained dsRNA and were able to convert V strains to white H morphology. Because dsRNA has not been reported to pass into ascospore progeny, selected progeny were tested for their ability to convert V strains to H, and none did. They were paired with H strains and acquired the cytoplasmic determinants that allowed them to convert V to H. They were then tested for virulence and dsRNA content.

If the flat morphology were the result of mutation at a highly mutable locus, back-mutations to normal morphology should occasionally occur. Therefore, two strains were examined for ability to revert to normal by isolating single germinated conidia and noting the morphology of the isolates.

If mutation cannot be shown to be the source of flat strains from white H strains, some other explanation must be sought.

## MATERIALS AND METHODS

My use of the word "strain" means "clonally propagated culture" and does not imply "physiologic race." An "isolate" is the first pure culture from a host or from a single spore. *E. parasitica* strains used are listed in Table 1.

The abnormal morphology, which has been called JR or P, segregated as a single nuclear gene in all crosses and will be called *flat*. The following description translated from Grente (14) fits the morphology of all the *flat* types in this study.

"The mycelium is at first hyaline with cylindrical hyphae; on the third day short, swollen braches appear, with unusual strong refraction and protoplasm packed with yellow-orange granules; often lateral vesicles appear which also have a large number of yellow-orange granules in the cytoplasm. These short branches develop rapidly, resulting in a mass of isodiametric elements. Starting on the eighth day, spores form on the surface [of these short branches] by budding. They are identical to the conidia formed in the pycnidia of normal [*E. parasitica*] strains."

"The thallus of JR types grows slowly (50 to 60% of the growth rate of white hypovirulent strains); the edge of the thallus is very irregular, especially at the first transfer after isolation. Aerial mycelium is rare or absent, but sporulation is abundant."

Mycelium was grown and stored refrigerated on Difco potato-

TABLE 1. *Endothia parasitica* strains used

| Strain | ATCC no. | Source | Morphology | dsRNA | Genotype |
|---|---|---|---|---|---|
| V strains | | | | | |
| 67 | 38753 | SC,[a] Italy | Normal pigment, virulent | − | *a*, v-c 10 |
| 42 | 38751 | MI,[b] Connecticut | Normal pigment, virulent | − | *A*, v-c 5 |
| 155 | 38755 | MI, Connecticut | Normal pigment, virulent | − | *A*, v-c 40 |
| 323 | | SA,[c] laboratory cross | Cream-colored, temperature-sensitive[d] | − | *A, cre-1,*[e] *ts-1,*[e] v-c 5[f] |
| 329 | | SA, laboratory cross | Cream-colored, temperature-sensitive | − | *a, cre-1, ts-1*, v-c 5 |
| 339 | | SA, laboratory cross | Normal pigment, temperature-sensitive | − | *a, ts-1*, v-c 5 |
| 390 | 38981 | SA, laboratory cross | Cream-colored | − | *A, cre-1*, v-c 5 |
| H strains | | | | | |
| 420 | | SC, Mt. Amiata, Italy | Flat[g] | + | *a*, [I],[h] *flat* |
| 504 | | SC, Mercogliana, Italy | Flat | + | *a*, [I], *flat* |
| 166 | 38762 | SC, Italy | Flat | + | *A*, [I], *flat* |
| 167 | 38761 | SC (sibling of 166), Italy | Flat | + | *A*, [I], *flat* |
| 4 | 38769 | SC, Collobrieres, France | Flat | + | *A*, [F],[h] *flat* |
| 748 | 52572 | 67 convert; cytoplasm, Mt. Amiata, Italy | White H | + | *a*, [I], v-c 10 |
| 752 | 52573 | 42 convert; cytoplasm, Mt. Amiata, Italy | White H | + | *A*, [I], v-c 5 |
| 747 | 52575 | 155 convert; cytoplasm, Mt. Amiata, Italy | White H | + | *A*, [I], v-c 40 |
| Progeny | | | | | |
| 501-3 | 52570 | SA, 420o × 323o | Flat, orange | − | *flat* |
| 501-2 | 52569 | SA, 420o × 323o | Flat, cream-colored | − | *flat, cre-1* |
| FP-10 | 52567 | SA, 504o × 323o | Flat, orange | − | *flat* |
| FP-11 | 52568 | SA, 504o × 323o | Flat, orange | − | *flat, ts-1* |
| FP-8 | 52565 | SA, 166o × 329o | Flat, orange | − | *flat* |
| FP-9 | 52566 | SA, 166o × 329o | Flat, orange, temperature-sensitive | − | *flat, ts-1* |
| FP-7 | 52564 | SA, 4o × 339o | Flat, orange | − | *flat* |
| Converts of progeny | | | | | |
| FP-4 | 52561 | FP-10, [I] from 747 | Flat, orange | + | *flat*, [I] |
| FP-5 | 52562 | FP-10, [I] from 752 | Flat, orange | + | *flat*, [I] |
| FP-6 | 52563 | FP-11, [I] from 752 | Flat, orange, temperature-sensitive | + | *flat, ts-1*, [I] |
| FP-2 | 52559 | FP-8, [I] from 752 | Flat, orange | + | *flat*, [I] |
| FP-3 | 52560 | FP-9, [I] from 752 | Flat, orange, temperature-sensitive | + | *flat, ts-1*, [I] |
| FP-1 | 52558 | FP-7, [I] from 747 | Flat, orange | + | *flat, [I]* |

[a] Strain derived from a single (uninucleate) conidium.
[b] Strain derived from a mass isolate from a natural canker.
[c] Strain derived from a single (uninucleate) ascospore.
[d] The progenitor strain 32 (ATCC 22507) has light cream-colored mycelium and pycnidia and is temperature-sensitive (grows at 20 C but not at 35 C, osmotically repairable but nutritionally irrepairable), and is virulent.
[e] These segregate as single loci (6,17).
[f] Vegetative compatibility type (1,6).
[g] Little aerial mycelium, conidia produced on conidiophores directly on the hyphae instead of in pycnidia (see text for full description).
[h] Cytoplasmic genes are enclosed in square brackets: [I] = cytoplasmic genes for hypovirulence from Italy, [F] = cytoplasmic genes for hypovirulence from France.

dextrose agar with methionine (100 mg/L) and biotin (1 mg/L) (PDAmb).

Crosses were made by growing the V strain intended as the female parent on autoclaved chestnut stem segments supported by 2% water agar with methionine (100 mg/L) in deep plastic petri dishes (2). After 2 wk at 25–27 C with 12 hr/day of white fluorescent light, the strains had covered the stems with fluffy mycelial growth and formed many pycnidia. The H strains to be used as male parents were grown on PDAmb at 25–27 C with 12 hr/day of white fluorescent light. After 7 days, conidia were washed from the surface of the cultures with sterile demineralized water and filtered through sterile Miracloth (Chickopee Mills Inc., Milltown, NJ) in small funnels. The conidial suspensions were pipetted onto the female-parent mycelium and the plates were gently tilted to distribute conidia across the stroma in the chestnut bark. Excess liquid was decanted and plates were incubated at 20 C with 8 hr/day of white fluorescent light. Perithecia were formed 2–4 wk later.

Single germinated ascospores were isolated as described previously (17). Two progeny from each of crosses 504♂ × 323♀, 166♂ × 329♀, and 4♂ × 329♀ and four from the cross 420♂ × 323♀, all with the flat morphology of the male parents, were paired with V strains 67, 42, and 155 on cellophane over PDAmb (9) to see if the V strains would be changed to H morphology.

The same 12 progeny were then paired with H strains 748, 752, and 747 in eight replicates on cellophane over PDAmb (9) and the 12 progeny were reisolated from the outside edge of mycelium after 5 days of growth. Like-isolates from the eight replicates were all inoculated together in the center of a PDAmb plate and incubated for 7 days. These 36 cultures were paired in four replicates with V strains 67, 42, and 155. Five progeny (FP 7–11) and their isogenic counterparts that had acquired the cytoplasmic determinants for converting V strains to H (ie, the converts of the progeny) (FP 1–6) were tested for virulence and for dsRNA content.

Virulence tests were made in five healthy American chestnut sprouts 8–14 cm diameter as described previously (8,17). Strains were inoculated seven per side and randomized east/west (five replicates total). Three well-studied V strains, 67, 42, and 155 (8), were included as controls. Final canker diameters were confirmed by cutting away the bark after 91 days. Samples were then taken from all cankers for isolation and confirmation of fungal strain properties.

The dsRNA extraction methods used were described elsewhere (4,12). The dsRNA extracted from about 4 g (wet weight) of mycelium of each strain was purified and then suspended in 150 μl Tris (80 mM) boric acid (40 mM) and EDTA (2 mM) buffer; 30 μl bromphenol blue in buffer was added to each as a tracker dye. Samples of 10–19 μl were placed in wells in 1.2% agarose slab gels containing 5 μg/ml of ethidium bromide. Gels were 6 mm deep, and electrophoresis was in a model MPH horizontal electrophoresis apparatus (International Biotechnologies Co., New Haven, CT) at 120V for 3.5 hr. The slab tray was occasionally removed and the gel examined with ultraviolet light to note progress of the dsRNA through the gel. Strains 748, 752, and 747 served as controls in these tests.

Back-mutation tests were done with strains FP 4 and FP 10 by plating conidia from PDAmb cultures on complete medium (17) and isolating single germinated conidia, 10 per plate, to PDAmb. The plates were incubated at 25–27 C with 12 hr/day of white fluorescent light.

## RESULTS

Of 2,681 single-ascospore progeny examined from these crosses, 1,373 had *flat* morphology. Segregation details are given in Table 2. The *flat* progeny from different male-parent strains may not all be mutants at the same locus. Linkage tests for the genes segregating in these crosses are shown in Table 3.

When 12 progeny representing four *flat* male parents were paired with V strains 67, 42, and 155, there were no changes in the morphology of either member of the pair in any combination. Pairing *flat* progeny of 504, 166, and 4 (FP 7–11 plus four others) with R strains 748, 752, and 747 resulted in no changes in morphology of either member of the pair. Three other progeny (of 420) were also paired and changed in morphology in 1 of 27, 1 of 27, and 8 of 27 pairings, respectively. The unchanged flat cultures were isolated from the margin of flat mycelium opposite the H mycelium, cultured on PDAmb for 7 days, and these 36 strains (12 *flat* strains paired with 3 H strains) were paired with V strains 67, 42, and 155. As a result of these pairings, the morphology of the V strains was changed to that of slow-growing white H strains: strain 67, 9 times in 144 pairings; strain 42, 12 times in 144 pairings; and strain 155, 8 times in 144 pairings.

Five *flat* strains that had acquired the ability to convert V strains to H without changes in flat morphology were then studied further (FP 1–6) along with their nonconverting *flat* progenitors (FP 7–11).

The extractions and electrophoresis in agarose gels of dsRNA from strains 748, 752, and 747 resulted in ethidium bromide fluorescent bands similar to but much less distinct from those found before with polyacrylamide gel electrophoresis of these strains (4,12). The flat progeny, strains FP 7–11, contained no dsRNA; however, strains FP 1–6 did contain dsRNA and the bands migrated in the same region as those of 748, 752, and 747.

In the virulence tests, the *flat* strains produced smaller cankers than the V control strains. Cankers produced by the *flat* strains that contained dsRNA were similar (Table 4). At the end of the test, bark was removed from all the cankers—those initiated by V controls, progeny, and converts of progeny. *E. parasitica* was not isolated from all of these bark samples (because of contamination) but those recovered were grown on PDAmb and their morphology noted (Table 4). In a few cases, morphology of isolates was not the

TABLE 2. Segregation of *flat* morphology among single ascospore progeny from crosses of *Endothia parasitica*

| Strains crossed[a] | Total no. of ascospores | *flat*[b] | *flat*[+] |
|---|---|---|---|
| *flat* from 420 | | | |
| 420♂ × 323♀ | 363 | 178 | 185 |
| 420♂ × 390♀ | 344 | 198 | 146 |
| 501-2♂ × 42♀ | 262 | 148 | 114 |
| 501-4♂ × 42♀ | 134 | 62 | 72 |
| 501-3♂ × 42♀ | 91 | 50 | 41 |
| *flat* from 504 | | | |
| 504♂ × 323♀ | 93 | 46 | 47 |
| *flat* from 166 | | | |
| 166♂ × 329♀ | 159 | 79 | 80 |
| *flat* from 4 | | | |
| 4♂ × 339♀ | 603 | 312 | 291 |
| 4♂ × 329♀ | 215 | 71 | 144 |
| *flat* from 167 | | | |
| 167♂ × 329♀ | 417 | 229 | 188 |
| Total | 2,681 | 1,373 | 1,308 |

[a] In each cross, the male parent had *flat* morphology.
[b] Little aerial mycelium, conidiophores formed directly on the hyphae instead of in pycnidia (see text for full description).

TABLE 3. Linkage tests for the genes segregating in the crosses of *Endothia parasitica* listed in Table 2

| Nuclear genes | Source of *flat* strain | Parental | Nonparental | Recombinant (%) |
|---|---|---|---|---|
| *cre*[a]:*flat*[b] | 420 | 566 | 539 | 49 |
| | 504 | 54 | 39 | 42 |
| | 166 | 79 | 80 | 50 |
| | 4 | 60 | 64 | 52 |
| *ts*[c]:*flat* | 504 | 46 | 47 | 51 |
| | 166 | 75 | 84 | 53 |
| | 4 | 368 | 362 | 50 |
| *ts*:*cre* | ··· | 190 | 186 | 49 |

[a] Cream-colored mycelium and pycnidia (6,17).
[b] Little aerial mycelium, condiophores formed directly on the hyphae instead of in pycnidia (see text for full description).
[c] Temperature-sensitive, grows at 20 C but not at 35 C, osmotically repairable but nutritionally irrepairable (6).

same as that of the strains inoculated. The 54 isolates were paired with V strains 67, 42, and 155 to see if their conversion ability had changed. Their ability to convert strains to H morphology is also recorded in Table 4.

Tests for reversion to normal morphology were done with strains FP 4 and FP 10, which have the same nuclear genotype and different cytoplasmic genotype, and gave different results. When 1,277 isolates from single conidia of FP 10 were examined for morphology, all resembled FP 10 on PDAmb. However, among 1,303 single-conidial isolates of FP 4, there were seven that resembled *cre-1, flat* strains; they had cream-colored mycelium, conidia formed on conidiophores on the surface of the hyphae (not in pycnidia), and little aerial mycelium (*cre-1* is described in 6 and 7). The two progenitors had been grown under conditions identical to those for the conidial isolates. These "*cre*" *flat* strains were stable when mass-transferred.

## DISCUSSION

A special mating method was needed to make these crosses because our normal method of simply growing male and female parents together (2) might have resulted in conversion of the potential female parent to H. The H strains tested so far have failed to serve as female parents, presumably because of a lack of production of protoperithecia (S. L. Anagnostakis, *unpublished*).

The designation *flat* was chosen for this genotype because of the sparse aerial mycelium produced by these strains. We cannot assume that the several *flat* types recovered from curative strains have morphologies determined by alleles at the same gene locus just because they look similar in culture. They are separated in Tables 2 and 3 by origin, but crosses would have to be made between these types to see whether a single locus is involved.

The segregation of *flat* to *flat*$^+$ in most crosses was close enough to 1:1 to assume control by a single gene. Because the percentage of recombinants in each comparison (Table 3) is close to 50%, there is probably no linkage of any *flat* with the pigment locus *cre-1* or the locus for temperature sensitivity, *ts-1*, and there was no evidence for linkage between *cre-1* and *ts-1* consistent with previous results (3,6).

One consequence of this single-gene change in morphology is that it changes the anamorph from a member of the Sphaeropsidales to a member of the Moniliales. Fungi in the Sphaeropsidales produce their asexual spores in pycnidia, whereas those in the Moniliales produce asexual spores on conidiophores formed on the vegetative mycelium. Because this criterion is used by taxonomists to distinguish Deuteromycetes (which form anamorphs but no teleomorphs), this change determined by a single gene is very important.

It was interesting that there was no evidence of a "cross-feeding" phenomenon in the pairings of *flat* with *flat*$^+$ V strains. Neither type was altered in morphology in the area where the mycelia were in contact, indicating that the morphology of *flat* could not be restored to normal by the transfer of metabolic products or cytoplasmic genes. Neither could the morphology of *flat*$^+$ strains be changed to *flat* by similar transfer.

The results indicate that *flat* strains are capable of being invaded by the cytoplasmic determinants for hypovirulence and that the [I] genes (Italian hypovirulence determinants) could multiply in *flat* strains without affecting their morphology. Introduction of [I] genes into normal strains causes a large modification of morphology (4,12). The [I] determinants in *flat* strains could then be passed on to *flat*$^+$ V strains, converting them to typical H morphology.

Relatively few pairings of *flat* V strains with *flat*$^+$ H strains and *flat* H strains with *flat*$^+$ V strains resulted in conversion. This might be attributable to some specific property of *flat* but more likely is simply the result of vegetative incompatibility (1). The strains used in the crosses were not all typed for vegetative compatibility (v-c) type, and segregation for quite disparate types may have occurred among the progeny because v-c is controlled by several loci (6). Transfer of hypovirulence determinants between strains in different v-c groups does occur but with varying frequency (7,9,10).

The effect of this phenotype on virulence is striking, but further studies are needed to determine whether reduced growth rate in general can explain virulence reduction or whether there is a direct effect of this genome on products necessary for virulence. The slow expansion of cankers that resulted from inoculation of *flat* strains made it hard to see a clear distinction between expansion rates of *flat* and *flat*$^+$ [I] strains, even though the presence of [I] usually causes a significant reduction in canker expansion rate (4).

The presence of high-molecular-weight dsRNA molecules in strains that convert V strains to H morphology supports the correlation noted earlier between the characteristics of H strains and presence of dsRNA (12). However, this is still only circumstantial evidence that all of the genes determining these characteristics are on or associated with the dsRNA.

Grente (14) presented a table with the effects of culture conditions on the kinds of conidial isolates from a white H strain. As noted before, such single-conidial isolates usually fall into one of four general morphological groups. Grente noted that the percentage of each type could be varied by changing the conditions of culture of the white H strain and the location (superficial or submerged) of the pycnidium yielding the conidia. The lowest percentage of flat isolates came from conidia isolated from superficial pycnidia formed in continuous light. However, 14-day-old superficial pycnidia developed in total darkness yielded conidial isolates that all had flat morphology.

This high rate of recovery of flat isolates is hard to explain by spontaneous gene mutation. Even a selection process favoring the replication of rare mutant (*flat*) nuclei during growth of the mycelium is not a very plausible explanation. Because flat types by definition do not form pycnidia, the *flat* genotype could not have exclusively occupied the mycelium forming the pycnidia. *E. parasitica* forms heterokaryons only with great difficulty without auxotrophic forcing markers (17), so testing the dominance of *flat* in combination with *flat*$^+$ nuclei will be difficult. Therefore, we cannot speculate about the relative number of *flat*$^+$ nuclei necessary in a cell to allow pycnidium initiation.

There are two possible explanations for the origin of *flat* that provide rather interesting speculation. If certain culture conditions allow an increase in the mutation rate at the *flat* locus in developing conidia, then the flat morphology should be found among single-conidial isolates from both H and V strains. However, Grente (14) said that in making sequential single-conidial isolations from a mass isolate of the white H type, each "generation" of isolates gave rise to 30–60% flat types. After four steps of cloning a normal V-type single-conidial isolate (100 isolates in each step), no isolates with flat morphology were found. These V types had the same

TABLE 4. Virulence tests of *Endothia parasitica flat* strains and results of reisolation from the host

| Strain | Avg. canker diameters (mm) 91 days | Morphology of isolates from replicate infections (number/total number recovered) | No. of isolates able to convert V strains |
|---|---|---|---|
| FP 1 | 18 | Flat (2/2) | 2 |
| FP 2 | 21 | Flat (2/4), normal (1/4), white (1/4) | 2 |
| FP 3 | 28 | Flat (4/4) | 3 |
| FP 4 | 39 | Flat (5/5) | 0 |
| FP 5 | 25 | Flat (4/4) | 4 |
| FP 6 | 24 | Flat (1/1) | 1 |
| FP 7 | 23 | Flat (4/4) | 0 |
| FP 8 | 22 | Flat (3/3) | 0 |
| FP 9 | 23 | Flat (5/5) | 0 |
| FP 10 | 39 | Flat (5/5) | 0 |
| FP 11 | 41 | Flat (5/5) | 2 |
| 67 | 78[a] (30)[b] | Normal (3/4), white (1/4) | 0 (white lost) |
| 42 | 87 (36) | Normal (2/4), white (2/4) | 1 |
| 155 | 94 (32) | Normal (4/4) | 0 |

[a] Average of four cankers.

[b] One canker (not all on the same tree); these data indicate spread of H from other cankers.

nuclear genotypes as their sibling H types after the first step of cloning, so the potential mutation rate should have been the same. Perhaps the dsRNA or other cytoplasmic genes in H strains induce instability at the *flat* locus.

Another possibility is that *flat* is the result of integration of cytoplasmic genetic material into nuclear genetic material under the influence of certain culture conditions. If this were the case, experiments exposing *flat* strains to stress might cause excision (in analogy with integrated bacterial plasmids), yielding *flat*$^{+}$ H or *flat* H cultures.

This study has provided basic information about the nature of this unusual class of curative hypovirulent strains. Even if they are as Grente (14) says, "an artifact of laboratory culture," they may provide useful tools for further studies on the behavior of cytoplasmic determinants in *E. parasitica*.

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