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THE GENETICS OF HYPHAL FUSION AND VEGETATIVE INCOMPATIBILITY IN FILAMENTOUS ASCOMYCETE FUNGI

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■ **Abstract** Filamentous fungi grow as a multicellular, multinuclear network of filament-shaped cells called hyphae. A fungal individual can be viewed as a fluid, dynamic system that is characterized by hyphal tip growth, branching, and hyphal fusion (anastomosis). Hyphal anastomosis is especially important in such nonlinear systems for the purposes of communication and homeostasis. Filamentous fungi can also undergo hyphal fusion with different individuals to form heterokaryons. However, the viability of such heterokaryons is dependent upon genetic constitution at heterokaryon incompatibility (*het*) loci. If hyphal fusion occurs between strains that differ in allelic specificity at *het* loci, vegetative incompatibility, which is characterized by hyphal compartmentation and cell lysis, is induced. This review covers microscopic and genetic analysis of hyphal fusion and the molecular and genetic analysis of the consequence of hyphal fusion between individuals that differ in specificity at *het* loci in filamentous ascomycetes.

CONTENTS

INTRODUCTION

In filamentous ascomycetes, an individual hypha (a multinucleate, multicellular filament with incomplete crosswalls, or septa) grows by hyphal tip extension and branching. Hyphal tip growth is believed to be mediated by delivery of the components for cell wall extension to hyphal tips by a vesicle supply center, or "Spitzenkörper" (43, 95). Numerous mutants have been identified that affect hyphal tip growth and branching (87), including mutants in signaling pathways (69) and the cytoskeleton (114). As filamentous fungi grow, fusions between hyphae are continuously formed (a process called anastomosis), yielding a network of interconnected hyphae, or mycelium, that makes up the fungal individual. Networked hyphae are presumably important in intra-hyphal communication and homeostasis in an individual colony during growth and reproduction. Growth in filamentous fungi thus consists of three balanced processes: hyphal tip extension, branching, and fusion. Of these, the molecular, genetic, and physiological control of hyphal anastomosis is the least understood. Hyphal fusions are a way to increase protoplasmic flow restricted by septa, which may be important in influencing hyphal pattern formation in the mycelium (22, 31, 94). Rayner (94), in his discussions of "mycelial interconnectedness,"argues that such complex mycelial dynamics cannot be easily described by current linear growth models for filamentous fungi (30, 31).

Different fungal individuals are capable of undergoing hyphal fusion with each other to form a heterokaryon, whereby genetically different nuclei may coexist in a common cytoplasm. Heterokaryon formation has potential benefits of functional diploidy and mitotic genetic exchange ("parasexual cycle") (89) or may increase biomass for cooperation in physiological efforts such as resource exploitation or asexual/sexual reproduction (22, 48). Although there are obvious benefits to filamentous fungi to form heterokaryons, a genetic mechanism exists that restricts heterokaryon formation between two genetically different individuals. If individuals differ in specificity at one or more *het* (*het*erokaryon incompatibility; also called *vic* for *v*egetative *in*compatibility) loci, heterokaryotic fusion cells are usually compartmentalized and undergo death by a lytic process (42), a phenomenon called vegetative incompatibility [or sometimes referred to as heterogenic, heterokaryon, or somatic incompatibility (39, 45, 70)]. Heterokaryosis by

hyphal fusion is believed to be virtually excluded in nature by the action of *het* genes (24, 80, 84, 93). Vegetative incompatibility reduces the risks of transmission of infectious cytoplasmic elements, such as virus-like dsRNAs (24, 33), and of exploitation by aggressive genotypes (32); this self-defense mechanism may be important in filamentous fungi because they lack cellular compartmentation.

This review focuses on the (*a*) process of hyphal anastomosis and (*b*) its consequences when hyphal fusion occurs between individuals that differ in *het* constitution. We describe the hyphal fusion process based on microscopic studies and initial genetic analyses and integrate these studies with genetic and molecular analysis of vegetative incompatibility. We also draw upon other systems and analogous processes to provide insight into pathways that may be relevant to hyphal fusion and vegetative incompatibility and thus provide avenues and components for future study.

HYPHAL ANASTOMOSIS

Microscopic Analyses of Hyphal Anastomosis

Hyphal anastomosis is comparable to cell fusion events in other organisms, such as fertilization between egg and sperm, and muscle differentiation. In fungi that outcross, fusion between opposite mating-type cells is a prerequisite for entry into sexual reproduction (25). Although vegetative hyphal fusion has been observed in filamentous fungi since the earliest days of mycology, Buller (22) was the first to outline the process of anastomosis from start to finish; surprisingly little has been added to Buller's framework in the past 60 or so years (48, 94). Mechanistically, it is useful to view hyphal anastomosis as three distinct physiological states of participating hyphae: pre-contact, post-contact, and post-fusion (Figure 1). Hyphae at the leading edges of the colony exhibit negative autotropism, or avoidance, which keeps a suitable distance between growing tips (90). The mechanism of avoidance is unclear, although suggestions of both a negative response to accumulating metabolites and positive chemotropism to oxygen levels have been made [reviewed in (46)]. Behind the growing colony margin, hyphae involved in anastomoses exhibit positive autotropism or attraction of hyphal tips (14, 22, 42, 76). The pre-contact initiation of new tips (very short specialized fusion branches called pegs) and re-direction of hyphal growth are most likely due to diffusible chemical signals (46). Only a few exceptions to the rule of fusion between hyphal tips have been reported, most notably hyphae-to-spore fusions in the nematode trapping fungi *Arthrobotrys conoides* and *A. cladodes* (51).

Hyphal tips show growth arrest after physical contact, prior to hyphal fusion. There are both temporal and spatial aspects to the fusion event: Initially, the cell wall is broken down, presumably by the delivery of hydrolytic enzymes to the contact point, and then a new cell wall bridge is formed between the two hyphae, presumably by the delivery of cell wall material to this area. Galun et al (40)

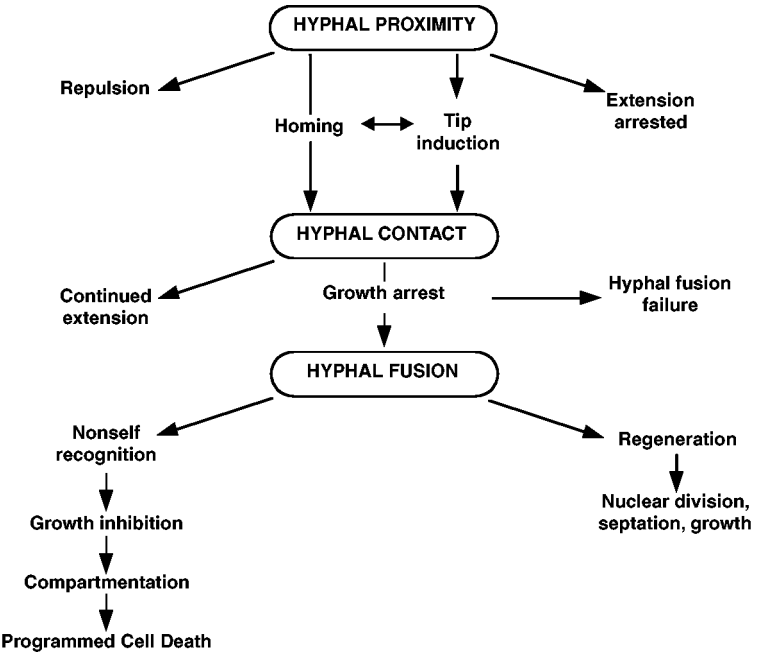

Figure 1 Flow diagram of the major steps in vegetative hyphal fusion. Recognition events between hyphae are apparent at all three physiological stages: pre-contact, pre-fusion, and post-fusion. Adapted from (1).

reported that chitin could be visualized in the anastomosis bridges, in peg-like projections, as well as in hyphal tips. Post-contact events of hyphal fusion, involving plasma membrane fusion and cytoplasmic mixing, are also virtually uncharacterized (Figure 1), although ultrastructural studies on anastomoses in *Cryphonectria parasitica* showed numerous microtubules and mitochondria across newly formed cytoplasmic bridges (81).

Genetic Control of Anastomosis

Only a few mutants have been reported that fail to undergo "self" hyphal fusions (termed heterokaryon self-incompatible or *hsi*). These mutants may or may not fail to undergo hyphal fusions with other individuals to form heterokaryons. Generally, such mutations do not affect other traits, such as branching or virulence (26, 57), although exceptions have been reported (52, 111). Correll et al (26) determined that heterokaryon self-incompatibility in *Gibberella fujikuroi* was controlled by a single segregating gene (*hsi-1*). Similarly, a *h*yphal *a*nasto*m*osis mutant, *ham-1* was identified in *Neurospora crassa* that failed to undergo both self and nonself hyphal fusions (111).

Since anastomosis always appears to be preceded by branch initiation and hyphal tip growth, it is possible that mutants affecting colony morphology may

have pleiotropic qualitative or quantitative effects on anastomosis. In *N. crassa*, over 150 loci have been described where mutations affect morphology; the vast majority can be complemented in heterokaryons with wild type (87). Although the growth and branching phenotypes of these mutants have been described, a systematic microscopic survey of the effect of these mutations on self-anastomosis has not been done.

Possible Relationship Between Hyphal Anastomosis and Cell Fusion During Mating in *Saccharomyces cerevisiae*

Although mechanisms involved in anastomosis are not understood in filamentous fungi, fusion during mating has been well characterized in the yeast *Saccharomyces cerevisiae* (6, 107). Many of the processes believed to be required for fusion of vegetative hyphae in filamentous fungi are also required by yeast cells during mating, i.e. signaling by diffusible substances, redirected growth of fusion cells, attachment of the two cell types to one another, production and targeting of hydrolytic enzymes to the attachment site, fusion of the plasma membrane, and reformation of the cell wall to form a contiguous bridge between the two cells. Although the mechanism of pheromone signaling is well characterized in *S. cerevisiae* (107), regulatory and functional mechanisms associated with cell wall breakdown at the contact site are not as well understood (41). Database searches of the *Neurospora* genome (http://www.mips.biochem.mpg.de/proj/neurospora/) and ESTs (http://unm.edu/~ngp and http://www.genome.ou.edu/fungal.html) with genes involved in cytoskeletal polarization (e.g. *CDC42*, *RVS167*, and *RSR1*), bud-site selection (e.g. *BUD6*) and the mating pheromones signal transduction pathway (e.g. *STE20*, *FUS3*, *KSS1*) have revealed a number of potential homologs in *N. crassa* (16, 63; H Ledford & NL Glass, unpublished results). *N. crassa* genes encoding proteins with amino acid similarity to proteins involved in the yeast pheromone signal transduction pathway, Ste11p and Fus3p/Kss1p, have been molecularly characterized (16, 63). The *N. crassa nrc-1* (putative Ste11p MEKK homolog) and *mak-2* (putative Fus3p/Kss1p MAPK homolog) mutants, in addition to being female sterile, also show defects in hyphal fusion during vegetative growth (16; Q Xiang & NL Glass, unpublished results). These observations suggest that some of the machinery involved in yeast cell fusion during mating may be required for hyphal anastomosis in filamentous fungi.

VEGETATIVE INCOMPATIBILITY

Morphological and Biochemical Aspects Associated with Vegetative Incompatibility

As filamentous fungi grow in nature, they are likely to come into physical contact with genetically different individuals of the same species. One possible outcome of this contact is hyphal fusion between two individuals. Fungal species differ in

the extent to which the resulting heterokaryons proliferate; two main types have been described. In the first type, in species such as *Verticillium dahliae* (92) and *G. fujikuroi* (93), heterokaryons are limited only to actual fusion cells and nuclei do not migrate between cells. Heterokaryons are continually reformed by repeated fusion events within the mycelium. In the second type, in species such as *N. crassa* and *Podospora anserina*, heterokaryotic cells proliferate and almost all cells within the mycelium are heterokaryotic.

Hyphal fusion between genetically different strains most often results in vegetative incompatibility due to genetic differences at *het* loci. Differences in how heterokaryotic cells proliferate also affect how vegetative incompatibility is ascertained in different species [reviewed in (45, 70)]. Two auxotrophic strains may fail to form a viable heterokaryon, or a "barrage" zone consisting of dead hyphal segments may be observed in the contact region between incompatible strains (39, 45, 70). In *N. crassa*, loci involved in vegetative incompatibility have also been identified using partial diploids (aneuploids); strains that are heterozygous for a *het* locus show growth inhibition, hyphal compartmentation, and death (Figure 2, see color plate) (79, 83, 85). Microscopic and ultrastructural features associated with vegetative incompatibility have been examined in only a few species (42, 80, 81). Common features include septal plugging, presumably to compartmentalize dying hyphal segments, vacuolization of the cytoplasm, organelle degradation, and shrinkage of the plasma membrane from the cell wall (56) (Figure 2). These ultrastructural changes in dying cells are consistent with features associated with programmed cell death (PCD) in multicellular eukaryotes (62). DNA fragmentation, a hallmark feature of early PCD, was also observed by TUNEL (terminal deoxyribonucleotidyl transferase) assays on incompatible transformants and heterokaryons (74). Other biochemical features found to be correlated with the incompatibility reaction include a decrease in RNA production (66), appearance of new proteins (20), and increase in proteolytic and other enzymatic activities such as phenoloxidases, malate/NADH dehydrogenase, proteases, and amino acid oxidase (9, 18).

The similarity in vegetative incompatibility phenotypes among different *het* interactions and among different species suggests that pathways mediating the morphological manifestations of vegetative incompatibility may have common genetic or biochemical features. The comparative genetic and molecular analysis of *het* loci in different species now under way will provide answers to these questions.

Genetic and Molecular Analysis of *het* Loci

Genetic studies in several ascomycetes show that there are a number of *het* loci in each species: at least 11 in *N. crassa* (45), 9 in *P. anserina* (10), 8 in *Aspergillus nidulans* (2, 29), and 7 in *C. parasitica* (27, 53). Two types of genetic systems have been described that regulate vegetative incompatibility, allelic and nonallelic. In allelic systems, anastomosis between individuals that contain alternative

specificities at a single *het* locus triggers vegetative incompatibility. In nonallelic systems, an interaction between specific alleles at two different loci mediates incompatibility (10, 45, 70). In *N. crassa* and *A. nidulans* only allelic systems have been described (86), whereas in *P. anserina* both allelic and nonallelic systems have been characterized (39). However, recent results suggest that incompatibility mediated by differences at *het-6* in *N. crassa* may be due to a nonallelic interaction between closely linked genes [(106) and see below].

Molecular characterization of vegetative incompatibility has been initiated in *N. crassa* and *P. anserina.* Alleles at three *het* loci have been characterized in *N. crassa* (mating type (*mat*), *het-c*, and *het-6*; Table 1). In *P. anserina*, the allelic incompatibility locus, *het-s*, and the nonallelic *het* loci, *het-c* and *het-e*, have been characterized (Table 1) (*het-c* in *N. crassa* has no relationship to *het-c* in *P. anserina*). It is clear that *het* loci encode very different gene products. For example, the *mat* locus in *N. crassa* functions as a *het* locus; fusion of opposite mating-type hyphae during vegetative growth results in vegetative incompatibility. The genes required to confer mating identity, *mat A-1* and *mat a-1*, encode proteins with similarities to known transcription factors (44, 108) (Table 1). Mutations in *mat A-1* and *mat a-1* result in strains that fail to mate and that will also form vigorous heterokaryons with either mating type (49). The *P. anserina het-c* locus encodes a putative glycolipid transfer protein [GLTP; hereafter referred to as *het-c*(GLTP)], first identified from pig brains (97); disruption of *het-c*(GLTP) drastically impairs ascospore maturation. The *N. crassa un-24* gene [which is involved in *het-6* incompatibility; (106)] encodes the large subunit of ribonucleotide reductase (RNR) (105) (Table 1), which is essential for DNA replication (59).

het loci encode different gene products, and yet three regions of similarity have been detected between predicted products of the *het-6* locus of *N. crassa* (106), the *tol* locus (which encodes a mediator of mating-type incompatibility in *N. crassa*, see below) (104) and the predicted product of the *het-e* locus in *P. anserina* (98). These regions are distinct from the GTP-binding domain and the β-transducin-like WD repeats in HET-E and the LRR and the coiled-coil domain in TOL, and thus may represent domains necessary for vegetative incompatibility mediated by all three *het* interactions (106). Disruption of *het-e*, *het-s*, or *N. crassa het-c* (Table 1) does not result in an obvious vegetative or sexual phenotype, (38, 103, 109). However, as with *mat* and *P. anserina het-c*(GLTP) mutants (above), *het-e*, *het-s*, and *N. crassa het-c* mutants lose the capacity for nonself recognition and consequently form vigorous heterokaryons with formerly incompatible strains.

Although *het-s* mutants have no phenotype, other than promiscuous vegetative compatibility, several lines of evidence indicate that the *het-s* gene product behaves as a prion analog (28, 96, 110). A prion is an abnormal conformational state of a normal cellular protein capable of "infecting" and converting the normal form (PrPC) into the infectious form (PrPSc) (91). Hyphal anastomosis between *het-s* and the neutral *het-s** strain results in the cytoplasmic transmission and infectious propagation of the [Het-s] phenotype. Strains exhibiting the [Het-s] character spontaneously sector into the [Het-s*] phenotype, and crosses between *het-s* and *het-s**

TABLE 1 Molecularly characterized genes involved in vegetative incompatibility (inc.)

Species and gene	Class of gene	Nature of the protein and its functional motifs	References
Neurospora crassa			
mat A-1	Allelic *het* gene	Mating-type transcriptional regulator; α-domain (Matα1p)	44
mat a-1	Allelic *het* gene	Mating-type transcriptional regulator; HMG box	108
tol	Mediator, *mat* inc.	Coiled-coil, leucine-rich repeat, region with similarity to HET-6 and HET-E	104
het-c	Allelic *het* gene	Signal peptide, variable domain (specificity), glycine-rich region	102, 103
un-24	Nonallelic *het* gene	Ribonucleotide reductase; allosteric activity site, variable domain (specificity)	105, 106
het-6	Nonallelic *het* gene	Region with similarity to TOL and HET-E	106
Podospora anserina			
het-s	Allelic *het* gene	Prion analog; single amino acid differences alters allelic specificity	28, 109
het-e	Nonallelic *het* gene	GTP-binding domain, WD repeat, region with similarity to TOL and HET-6	38, 98
het-c	Nonallelic *het* gene	glycolipid transfer protein; amphipathic α-helix, single amino acid differences alters allelic specificity	97
mod-D	Modifier, *het-C/E* inc.	α-subunit of G protein; GTP-binding site	73
mod-E	Modifier, *het-R/V* inc.	Heat-shock protein (Hsp90 family)	72
idi-2	Induced by *het-R/V* inc.	Signal peptide	21
mod-A	Modifier, nonallelic inc.	SH3-binding domain	7
idi-1, idi-3	Induced by nonallelic inc.	Signal peptide	21
pspA	Induced by nonallelic inc.	Serine (vacuolar) protease	100

strains show non-Mendelian inheritance of the [Het-s] and [Het-s*] phenotypes. Although [Het-s] acts as prion, the relationship of the capacity of [Het-s] to convert [Het-s*] and vegetative incompatibility, which is mediated by an interaction between *het-s* and *het-S*, is unclear.

A *het* locus in one species may or may not function to confer vegetative incompatibility in a different species. For example, the *mat* locus in *P. anserina* encodes transcription factors very similar to those of *N. crassa* (34). However, the *P. anserina mat* genes do not confer vegetative incompatibility in *P. anserina*, nor do they when introduced into *N. crassa* (3), even though heterologous mating activity is conferred. Nonetheless, the association of mating-type and vegetative incompatibility is not restricted to *N. crassa*, but has been reported in other species, such as *Ascobolus stercorarius* (15), *A. heterothallicus* (64), and *Sordaria brevicollis* (J Bond, personal communication). The *N. crassa het-c* locus encodes a glycine-rich protein that displays some similarities to cell wall proteins (103) (Table 1). A gene that shows a high degree of sequence similarity to *N. crassa het-c* has also been identified in *P. anserina* (101). DNA sequence analysis of the *P. anserina het-c homolog* (*hch*) among 11 *P. anserina* isolates that differed at all other known *het* loci did not reveal any polymorphisms, suggesting that *hch* does not function as a *het* locus in *P. anserina*. However, the introduction of *N. crassa het-c* alleles into *P. anserina* triggered growth inhibition, hyphal compartmentation, and death (101), with a phenotype very similar to *het-c* incompatibility in *N. crassa*. Predicted ORFs that have significant similarity to *P. anserina* HET-C(GLTP) and HET-E are present in *N. crassa* (http://www.mips.biochem.mpg.de/proj/neurospora/), but it is unclear whether these genes represent any of the previously identified *het* loci in *N. crassa* or if they encode genes required for cellular functions other than vegetative incompatibility. Thus, a species may contain loci that have the capacity to function as a *het* locus, but whether they do or not is dependent on the presence or absence of polymorphisms within populations.

The Molecular Basis of Allelic Specificity

DNA sequence analyses of various *het* loci in *N. crassa* and *P. anserina* have revealed that alleles conferring alternative specificities are polymorphic. For example, *P. anserina* alternative allele proteins, HET-S and HET-s, differ by 12 amino acids (109) (Table 1). All surveyed *het-s* isolates were identical in *het-s* sequence and all *het-S* isolates had identical *het-S* sequences, indicating that these polymorphisms were maintained in populations. Analysis of *het-S*/*het-s* chimeric constructs and site-directed mutagenesis indicated that a single amino-acid change is sufficient to switch *het-S* specificity to *het-s* (35). In *P. anserina het-c*(GLTP) (Table 1), 16 polymorphic positions (distributed throughout the ORF) were identified in a comparison between four HET-C proteins (99). As with *het-s*, a single amino acid change in HET-C(GLTP) can effectively change its allelic specificity; some *het-c* chimeric constructs conferred novel allelic specificities. The nonallelic partner of *P. anserina het-c*(GTLP), *het-e*, encodes a predicted protein with

similarity to the β-subunit of transducin, which is characterized by a repeat sequence (WD) in the carboxyl terminal region of the protein (Table 1). Although the vegetative incompatibility function of HET-E required the WD region, allelic specificity was apparently not defined by the number of repeats (98).

In *N. crassa*, a highly variable domain in alternative *het-c* alleles was necessary and sufficient to confer allelic specificity (102, 103). Allelic specificity of *het-c* correlated with unique insertion and deletion pattern within the variable domain. The *N. crassa un-24* and *het-6* loci are closely linked and recombination between these loci has not been detected in laboratory crosses, nor in a survey of wild-type isolates (78). Preliminary results suggest that *het-6*–mediated vegetative incompatibility may be due to a nonallelic interaction between *un-24*, *het-6*, and other loci within the nonrecombining region (106). A variable region in the carboxyl-terminal domain in ribonucleotide reductase (UN-24) correlated with allelic specificity; alternative alleles at the *het-6* locus encode predicted proteins that are only 68% identical, with polymorphic positions scattered throughout the ORFs (106).

Molecular Basis of Recognition

A single allelic or nonallelic genetic difference at a *het* locus is sufficient to trigger growth inhibition, hyphal compartmentation, and death. Alternative alleles at *het* loci are polymorphic, suggesting that structural differences in HET proteins mediate nonself recognition. These observations suggest that either alternative HET proteins physically interact to mediate nonself recognition, or that they modify products that physically interact. Physical interaction between mating-type proteins that mediate nonself recognition during the sexual cycle are well documented in fungi, for example, heterodimerization of **a**1-α2 in *S. cerevisiae* (47) and heterodimerization of bW and bE in *Ustilago maydis* (60). An interaction via yeast two-hybrid analysis has been detected between the *N. crassa* mating-type polypeptides, MAT A-1 and MAT a-1 (5); mutations in *mat a-1* that abolish vegetative incompatibility also disrupt MAT a-1–MAT A-1 interaction. Heterocomplex formation has been reported between alternative *N. crassa* HET-C polypeptides (112), but homocomplexes of HET-C polypeptides were not detected. Variations in the specificity domain affect the capacity of alternative HET-C polypeptides to form a heterocomplex (112), although the molecular mechanism of how this is achieved in unclear. An interaction between HET-s/HET-S (S/S, s/s, and S/s) proteins (monomers and multimers) has also been detected by yeast two-hybrid and Western analysis (28). Based on genetic evidence, it has also been proposed that *P. anserina* HET-C(GLTP) and HET-E physically interact (38). Thus, heterocomplex formation between alternative HET polypeptides may be the "recognition" complex and the formation of this complex may act as the "trigger" to mediate the biochemical and morphological aspects of vegetative incompatibility. Alternatively, the formation of a HET heterocomplex may function to poison the cell and thus may directly mediate growth inhibition and death (10).

BIOCHEMICAL AND MOLECULAR ANALYSIS OF MEDIATORS OF VEGETATIVE INCOMPATIBILITY

Mutations that suppress vegetative incompatibility of particular *het* interactions have been isolated and characterized in both *N. crassa* and *P. anserina*. A recessive mutation, *tol*, (for *tol*erant) suppresses mating-type associated vegetative incompatibility in *N. crassa* such that *tol*; *A* and *tol*; *a* strains form vigorous heterokaryons (82, 104) (Table 1). Not all species of *Neurospora* exhibit mating-type associated vegetative incompatibility. In the pseudohomothallic species *N. tetrasperma*, lack of mating-type incompatibility is mediated by a natural variant allele of *tol*, tol^T (55). The *tol* mutation does not suppress vegetative incompatibility mediated by differences at *het* loci other than the mating-type locus (71). Expression studies indicate that *tol* is not regulated by the *mat* transcriptional regulators, but may rather interact with MAT A-1 and MAT a-1 to form a heterocomplex that mediates vegetative incompatibility (104).

In *P. anserina*, mutations at *mod-C* (for *mod*ifier), suppress both growth inhibition and lysis of an incompatible *het-R/V* strain (67). By contrast, mutations at the *mod-A* locus (encoding a putative SH3 binding protein; Table 1) suppress growth inhibition of all nonallelic interactions (*het-C/D*, *het-C/E*, and *het-R/V*) (7, 11, 13), but lysis of hyphal compartments is still apparent. Allelic incompatibility systems are not affected by *mod-A* or *mod-C* mutations. Lysis in incompatible strains with the *mod-A1* allele is suppressed by a second mutation at the *mod-B* locus (12, 65) (Table 1). Although a mutant phenotype was not observed for single *mod-A1* or *mod-B1* mutants, a *mod-A1 mod-B1* double mutant shows defects in protoperithecia formation and is female sterile (12, 13, 17, 19). A mutation at *mod-D* (encoding a putative Gα subunit; Table 1) suppresses lysis in a *het-C/E mod-A1* incompatible strain (37, 68); *mod-D* mutants show defects in aerial hyphal growth, protoperithecial formation, ascospore germination, and secondary hyphal ramifications (68, 73).

The *mod-E1* mutation was selected on its ability to restore growth renewal from the quiescent stage in a *mod-D* mutant (*mod-D2*); the *mod-E1* allele suppresses all developmental defects of the *mod-D2* mutant (36). MOD-E is a member of the Hsp90 family and is responsive to heat-shock conditions (72). The fact that *mod-D* and *mod-A mod-B* mutants show defects in protoperithecial development has led to the model that genes involved in nonallelic incompatibility are involved in a starvation pathway that regulates the subsequent development of protoperithecia (19, 100). Based on the *mod-E1* mutant phenotype and the fact that other Hsp90 proteins are implicated in cell cycle control, MOD-E could be involved in negative control of the cell cycle (e.g. by association with a protein kinase) that may affect both sexual development and vegetative incompatibility.

Biochemical features of the *P. anserina het-R/V* incompatibility reaction can be studied since lethality of the incompatibility reaction is temperature sensitive (65). By taking advantage of this temperature-sensitive lethality, three genes *i*nduced

*d*uring *i*ncompatibility (*idi*) were isolated, which encode small proteins [201, 157, and 196-aa] with putative signal peptides (21). Expression of two *idi* genes, *idi-1* and *idi-3*, is restricted to nonallelic systems; induction is eliminated in mutants that suppress nonallelic incompatibility (*het-R/V mod-A1 mod-B1* and *het-R/V mod-C1*). The IDI proteins may localize to the septa during incompatibility (J Bégueret, personal communication). Plugging of septal pores is a hallmark of dying hyphal compartments (8), presumably to confine the disintegrating cells. The *idi* genes are possible candidates that may cause either cell disintegration or hyphal compartmentalization during vegetative incompatibility.

Even though a number of *het* loci have been molecularly characterized, it is not yet possible to outline a comprehensive model for how any *het* interaction mediates vegetative incompatibility. The *het* loci encode very different gene products, suggesting that the initial events associated with vegetative incompatibility differ, although a common mechanism of heterocomplex formation between alternative *het* polypeptides may mediate recognition. The identification of genes downstream of *het* loci have also not illuminated molecular mechanisms, although a link between events associated with sexual development and vegetative incompatibility has been postulated in *P. anserina.* Although phenotypic events associated with hyphal compartmentation and death are similar between *het* interactions, it is still unclear whether each *het* interaction causes growth inhibition, hyphal compartmentation, and death by distinct or similar biochemical and genetic mechanisms.

EVIDENCE FOR SELECTION MECHANISMS ACTING ON *het* LOCI

Vegetative incompatibility is believed to function as a nonself recognition system to limit the spread of infectious elements (23), to prevent exploitation by nonadaptive nuclei (50), and/or to prevent resource plundering during sexual reproduction (32). Such hypotheses would require that selection acts on *het* loci to maintain polymorphisms in fungal populations for nonself recognition functions. Other nonself recognition systems such as mating-type loci (75, 88), the MHC (major histocompatibility complex) (4) loci in mammals and *S* (self-incompatibility) (54) locus in plants show polymorphisms that are maintained by balancing selection through multiple speciation events, so-called trans-species polymorphisms (61). Evidence for loci subject to balancing selection include: (*a*) trans-species polymorphisms, (*b*) the presence of a large number of alleles in a population, (*c*) allele frequencies that are approximately equal within populations, and (*d*) the rate of nonsynonymous substitution per site in polymorphic regions in excess of the rate of synonymous substitution, suggesting that selection is maintaining diversity.

The best evidence for balancing selection at any *het* locus (other than *mat*) appears in the *het-c* gene of *N. crassa*. Allelic specificity is mediated by a variable

domain of 34–48 amino acids in HET-C; swapping this domain between alleles switches *het-c* specificity. The *het-c* allelic specificity region exhibits trans-species polymorphisms across different species and genera within the Sordariaceae (113) (Figure 3); i.e. DNA sequences of the *het-c* specificity region fell into groups by *het-c* allelic type rather than according to species. Allelic polymorphisms at *het-c* were therefore generated in an ancestral species and have been maintained during multiple speciation events. The allelic specificity domain of *N. crassa het-c* also shows an excess of nonsynonomous substitutions, consistent with the hypothesis that selection is maintaining diversity at this locus and alleles were equally frequent within populations (113). In *P. anserina*, alternative alleles at *het-s* and *het-c* (98, 109) also show an excess of nonsynonymous over synonymous substitutions. Data from population studies suggest that balancing selection may also be acting at the *het-6-un-24* region of *N. crassa*. In a population of 40 isolates of *N. crassa*, the *het-6-un-24* region was apparently inherited as a block (see above) and the two allele combinations were equally frequent (78). Genetic analysis of *het* allele frequencies in populations of *C. parasitica* did not support frequency- dependent selection acting at a number of *het* loci because *het* allele frequencies differed substantially from 1:1 (77). Thus, whether polymorphisms at a particular *het* locus are subject to balancing selection and selected for within populations may be dependent upon the *het* locus.

In addition to its postulated role in nonself recognition, vegetative incompatibility may also play a role in speciation in filamentous fungi. In fungi that are self-fertile, such as pseudohomothallic species like *P. anserina* and *N. tetrasperma*, vegetative incompatibility may provide an effective barrier to outbreeding and thus favor speciation (39, 55, 58). By contrast, vegetative incompatibility is apparently not a barrier to crosses between individuals in heterothallic species. In these species, vegetative incompatibility must be suppressed during sexual reproduction to allow opposite mating-type nuclei to proliferate in a common cytoplasm prior to karyogamy. How this is accomplished in unclear, but, at least for *tol*, transcription is apparently suppressed in sexual tissue (104).

It is possible that polymorphisms at some *het* loci are not under selection, but simply reflect genetic divergence between isolates. Heterokaryon formation places these polymorphic gene products in a common cytoplasm, which may have detrimental effects on vegetative growth and thus act as a "poison" complex to mediate vegetative incompatibility (10, 100). Field studies indicate that differences at *het* loci effectively exclude the formation of heterokaryons in nature (24, 80, 84, 93), which may in turn prevent or severely limit the transfer of infectious cytoplasmic elements, as demonstrated in laboratory studies (33, 53). However, whether each *het* locus functions in nonself recognition to limit transfer of cytoplasmic elements or prevent resource plundering, or is simply a reflection of genetic divergence between isolates, is a difficult question to address on an experimental level and requires both molecular data on *het* loci and population genetic analyses.

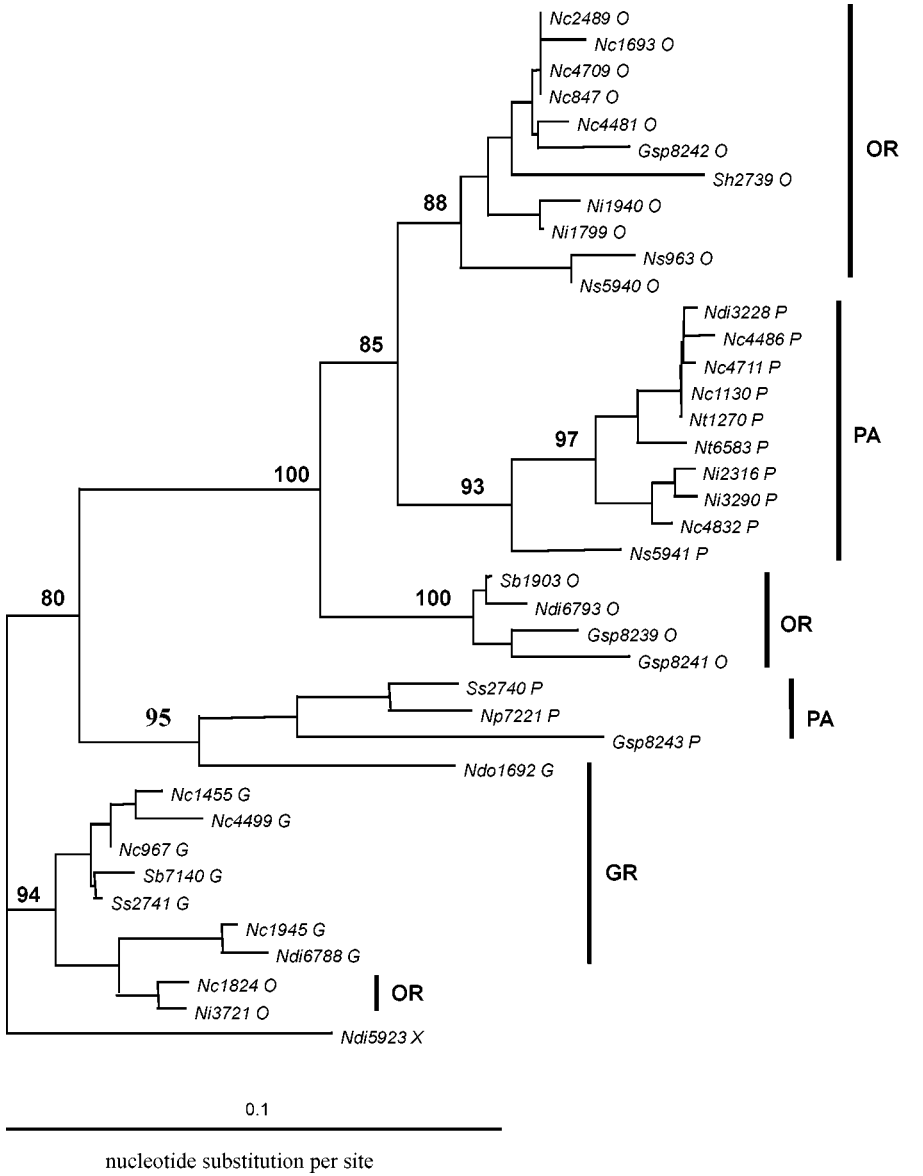

Figure 3 The *het-c* specificity domain shows trans-species polymorphisms. Unrooted neighbor-joining tree from the DNA sequence (390 nucleotides) alignment in the *het-c* specificity region (adapted from 113; reprinted with permission). Bootstrap support is shown by the percentage out of 500 replicates. Branch lengths are proportional to genetic distances. The designation OR, PA and GR refer to the reference allelic specificity of *het-c* in *N. crassa. Nc, Neurospora crassa; Nt, N. tetrasperma; Ni, N. intermedia; Ns, N. sitophila; Ndi, N. discreta; Np, N. pannonica; Ndo, N. dodgei; Gsp, Gelasinospora* sp.; *Sb, Sordaria brevicollis; Ss, S. sclerogenia; Sh, S. heterothallis.*

FUTURE DIRECTIONS

The processes of tip growth, branching, septum formation, and hyphal fusion interact to form the architecture of the filamentous fungal mycelium. Unraveling how the capacity for hyphal fusion allows a fungal individual to integrate and respond to information from the environment is a difficult proposition. Rayner (94) promotes an extreme view of mycelial architecture as exhibiting "hyperepigenetic variation,"which is extremely sensitive to conditions of the local environment. Phenotype (mycelial patterns) would be effectively uncoupled from genotype by autocatalytic processes. Mathematical models that integrate these ideas into growth and development of the fungal mycelia are currently being developed (e.g. 30, 31). Parallel studies to understand the genetic control of each growth process will be required to separate the genetic from epigenetic components.

Genetic or epigenetic mechanisms involved in the hyphal fusion process are not well understood in any filamentous fungus. Mycelial growth requires the polarized secretion of cell wall material and enzymes to the hyphal tip. It is probable that some of the machinery involved in yeast cell mating, cytoskeletal polarization, and secretion will be conserved in filamentous fungi and may also play a role in hyphal fusion during vegetative growth. A combination of mutational analyses and a rational reverse genetic approach using *S. cerevisiae* homologs that are known to be involved in yeast cell mating fusion are feasible avenues to begin to dissect the requirements for hyphal anastomosis. The molecular characterization of hyphal fusion mutants, such as *ham-1* in *N. crassa* (111), and the identification of additional mutants that affect self and nonself hyphal fusion, such as *nrc-1* (63) and *mak-2* (16), can be used as starting points to dissect the genetic regulation of the hyphal fusion process. Epigenetic effects in such mutants could be subsequently ascertained by assessing responses to environmental cues that affect mycelial growth patterns.

The relationship between hyphal anastomosis and vegetative incompatibility is unclear. Some of the machinery involved in the hyphal fusion process per se may be involved or affected by vegetative incompatibility. For example, the *N. crassa het-c* locus encodes a protein that has similarity to other cell wall proteins, although the cellular localization of HET-C is not known. A mutation that suppresses *het-c* vegetative incompatibility, *vib-1* (for *v*egetative *i*ncompatibility *b*locked; Q Xiang & NL Glass, unpublished) results in a strain that cannot undergo self or nonself hyphal anastomosis (suppression of *het-c* incompatibility can be shown by partial diploid analysis and by transformation experiments). These observations suggest that a link between the hyphal fusion process and vegetative incompatibility may occur in at least some *het* interactions.

Although *het* loci and their suppressors are now being identified and characterized, much remains to complete our understanding of vegetative incompatibility. If heterocomplex formation between alternative HET proteins is a common recognition theme for vegetative incompatibility, how does the formation of this

complex ultimately result in hyphal compartmentation and death? Do the different *het* interactions converge on a common "death"pathway or do each cause hyphal compartmentation and death by a distinct mechanism? Are there genetic similarities between programmed cell death in multicellular eukaryotes and vegetative incompatibility in filamentous fungi? Do all *het* loci have a cellular function in addition to vegetative incompatibility, or are some solely nonself recognition loci? The phenotype of the *P. anserina mod* mutants suggests that the normal cellular function of these genes is involved in the link between environmental sensing of starvation and induction of female reproductive structures (100). These observations suggest that either vegetative incompatibility is an "accident" that occurs when polymorphic HET proteins are present in a common cytoplasm, or that vegetative incompatibility is a secondary function of these loci. How do *het* loci evolve and how are they selected for in populations? Different mating strategies, such as pseudohomothallism versus heterothallism, may affect selection and evolution of *het* loci. Both allelic and nonallelic incompatibility systems function as barriers to limit outcrossing in pseudohomothallic fungi, irrespective of any role in vegetative incompatibility. And finally, how important is vegetative incompatibility in populations and to the maintenance of fungal individuality? As fundamental properties of filamentous fungal growth, both hyphal fusion and post-fusion recognition events have important impacts on the mycelium, and are critical to developing a more complete understanding of the complex relationship of the fungal individual with its environment.

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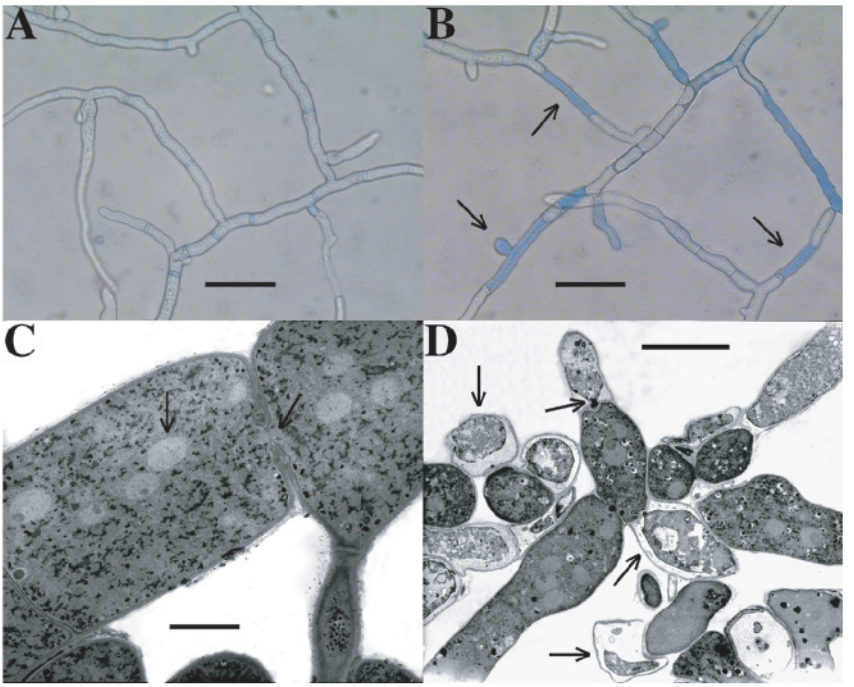

Figure 2 Microscopic features of vegetative incompatibility. *A* and *B*. Hyphae of *N. crassa* wild type and a partial diploid strain containing antagonistic *het-c* alleles, respectively. Hyphae were stained with Evans blue, which is excluded by cells with intact plasma membranes; dead or dying cells take up the stain (56). Note stained, dying hyphal compartments in B (*arrows*). Bar, 10 μm. *C* and *D*. Transmission electron micrographs of *N. crassa* wild type and a partial diploid strain containing antagonistic *het-c* alleles, respectively. *C*. Normal ultrastructure of wild-type *N. crassa*. Note multinuclear hyphal compartments separated by incomplete septa (*arrows*). Bar, 1 μm. *D*. Ultrastructure of dying, hyphal compartments (*arrows*). Features of vegetative incompatibility include plugging of septal pores, nucleolar release, extensive organelle degradation and plasmolysis (56). Bar, 5 μm.