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MINIREVIEW

Fatal Attraction: Nonself Recognition and Heterokaryon Incompatibility in Filamentous Fungi

N. Louise Glass* and Isao Kaneko

Department of Plant and Microbial Biology, University of California, Berkeley, California 94720

Vegetative incompatibility is a common phenomenon in filamentous fungi, including ascomycete, basidiomycete, and zygomycete fungi (27, 70, 80). A subset of vegetative incompatibility reactions includes events that require hyphal fusion and heterokaryon formation, whereby genetically different nuclei coexist in a common cytoplasm. Nonself recognition leading to rejection of heterokaryon formation is referred to as "heterokaryon incompatibility." Heterokaryon incompatibility is a genetically regulated process and most often results in death of the hyphal fusion cell (Fig. 1). This review will focus on recent developments in our understanding of the molecular mechanisms of nonself recognition and downstream effectors of death during heterokaryon incompatibility. The recent release of the *Neurospora crassa* genome sequence has allowed the evaluation of the conservation of genes involved in heterokaryon incompatibility in filamentous fungi and their possible relationship to programmed cell death (PCD) in other multicellular eukaryotes.

HETEROKARYON INCOMPATIBILITY IS A FUNGAL NONSELF RECOGNITION SYSTEM

Filamentous fungi grow by tip extension, branching, and hyphal fusion to form a tridimensional hyphal network (12). Different individuals are also capable of undergoing hyphal fusion with each other to form a vegetative heterokaryon (Fig. 1). Heterokaryon formation in filamentous ascomycete fungi has potential benefits of functional diploidy and mitotic genetic exchange during the parasexual cycle (61). Heterokaryon formation can also be used to increase biomass for cooperation in resource exploitation (18). Although there are apparent benefits associated with heterokaryon formation, heterokaryosis by hyphal fusion is believed to be virtually excluded in nature by genetic differences at *het* (heterokaryon incompatibility) loci (14, 52, 55, 63). Heterokaryon incompatibility has been shown to reduce the risk of transmission of infectious cytoplasmic elements such as virus-like double-stranded RNAs (16, 19) and exploitation by aggressive genotypes (18). In some cases, DNA polymorphisms associated with *het* allele specificity show transspecies polymorphisms (82), indicating that these loci are subject to balancing selection. Transspecies polymorphisms are also found at loci in other organisms that confer nonself recognition, such as the *S* locus in plants (15) and the major histocompatibility complex loci in animal systems (36).

HETEROKARYON INCOMPATIBILITY RESULTS IN HYPHAL COMPARTMENTATION AND DEATH

The triggering of hyphal compartmentation and death following hyphal fusion between *het*-incompatible individuals is morphologically similar among different fungi (1–3, 8, 25, 53). Hyphal fusion between compatible individuals (identical specificity at all *het* loci) leads to stable heterokaryon formation and is often associated with dramatic changes in cytoplasmic flow (30) (Fig. 2A). Hyphal fusion between *het*-incompatible individuals results in rapid compartmentation and death of the hyphal fusion cell and often surrounding cells (Fig. 2B). Cytoplasmic granules form a few minutes after hyphal fusion, and the septal pores which bracket the heterokaryotic cell (and often subtending cells) become occluded (see inset, Fig. 2B). Vacuolization of the cytoplasm is a prominent feature of heterokaryon incompatibility, and bursting of these vacuoles is apparent (see open arrows in Fig. 2B). Vacuoles in filamentous fungi contain numerous proteases and degradative enzymes, which are released into the cytoplasm upon lysis of the vacuoles. Destruction of the heterokaryotic cell can be complete within 30 min after hyphal fusion. The similarity in microscopic phenotype suggests that heterokaryon incompatibility mediated by different *het* loci and between different fungal systems might share common cell death machinery. Other results, such as heterologous expression of *N. crassa het-c* in *Podospora anserina*, also support this hypothesis (71). Ultrastructural studies of *het*-incompatible partial diploids in *N. crassa* show organelle degeneration, shrinkage of the plasma membrane, and septal plugging (34). It has been suggested that the ultrastructural and microscopic phenotypes associated with destruction of heterokaryotic hyphal compartments may share some features with PCD in multicellular metazoans (13, 34, 37, 40). In support of this hypothesis, it has been reported that nuclear degradation, a prominent feature of apoptosis, also occurs during heterokaryon incompatibility in *N. crassa* (S. Marek, J. Wu, N. L. Glass, D. G. Gilchrist, and R. M. Bostock, unpublished results).

* Corresponding author. Mailing address: Department of Plant and Microbial Biology, The University of California, Berkeley, CA 94720. Phone: (510) 643-2399. Fax: (510) 642-4995. E-mail: Lglass@uclink.berkeley.edu.

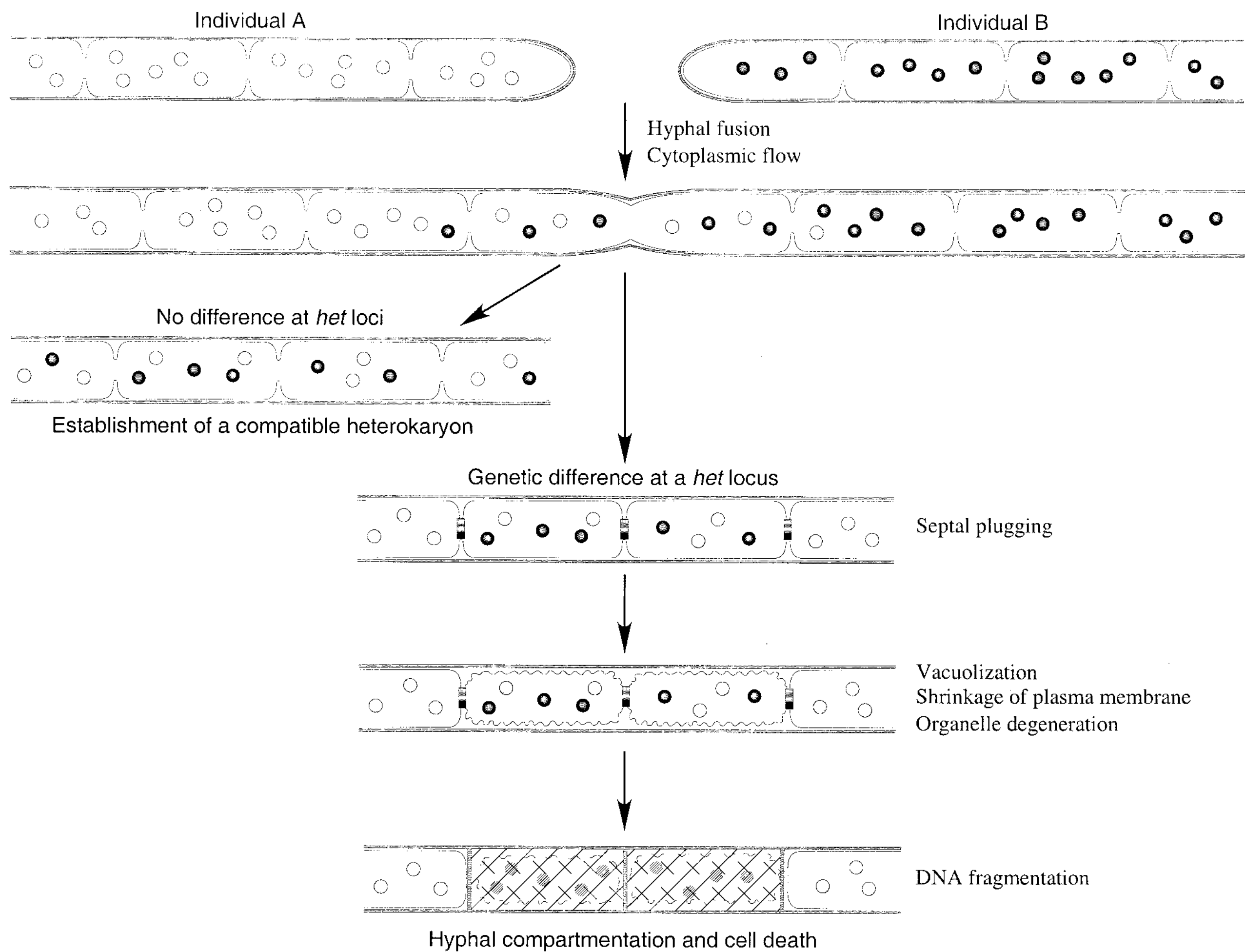

FIG. 1. Schematic diagram of the consequences of hyphal fusion between two fungal individuals. If the two fungal individuals are identical in allelic specificity at all *het* loci, a vigorous heterokaryon is formed that is indistinguishable in phenotype from a wild-type homokaryotic colony. However, if the two fungal individuals differ in allelic specificity at a *het* locus, the septa in the hyphal fusion cell are occluded and the hyphal fusion cell (and often surrounding cells) die.

THE PRODUCTS OF *HET* LOCI ARE DIVERSE

Genetic analyses in ascomycete fungi show that the number of *het* loci in a particular species is between 5 and 11 (27, 70). In species where they have been genetically examined, *het* loci are distributed among linkage groups. With few exceptions, there are generally only two to four allelic specificities at each *het* locus. Even though the number of allelic specificities at a particular *het* locus is low, the fact that a particular species possesses a number of unlinked *het* loci greatly increases the number of potentially incompatible *het* genotypes in an outbreeding species. The fact that these nonself recognition loci are unlinked is predicted to affect mechanisms of selection; mathematical models of *het* allele selection indicate that trans-species polymorphisms are not an essential feature of balancing selection acting on these loci (50).

Two types of genetic systems that regulate heterokaryon incompatibility have been described, allelic and nonallelic. In allelic systems, heterokaryon incompatibility is triggered when individuals that contain alternative allelic specificities at a single *het* locus undergo hyphal fusion (24, 63, 65). Strains that contain alternative *het* alleles within a single nucleus (either by the introduction of an alternative *het* gene by transformation or by crosses with translocation strains to form partial diploids) show greatly inhibited growth and hyphal compartmentation and death (51, 59, 73, 79). Because heterokaryon incompatibility is usually expressed only during vegetative growth, heterothallic (outbreeding) individuals with numerous allelic *het* differences can undergo sexual reproduction. In nonallelic systems, an interaction between specific alleles at two different *het* loci results in heterokaryon incompatibility. Nonallelic incompatibility can affect sexual reproduction (9, 70). In pseudohomothallic fungal species, such as *P. anserina* and *Neurospora tetrasperma*, opposite mating-type nuclei are compartmentalized in a single ascospore. If parental strains differ in allelic specificity at either allelic or nonallelic *het* loci, nuclei with incompatible *het* alleles are compartmentalized within a single ascospore. In this case, reproductive dysfunction can occur or progeny display heterokaryon incompatibility (62, 64, 70). It has been suggested that heterokaryon incompatibility may play a role in reproductive isolation in some species of filamentous fungi (22, 33, 64, 70).

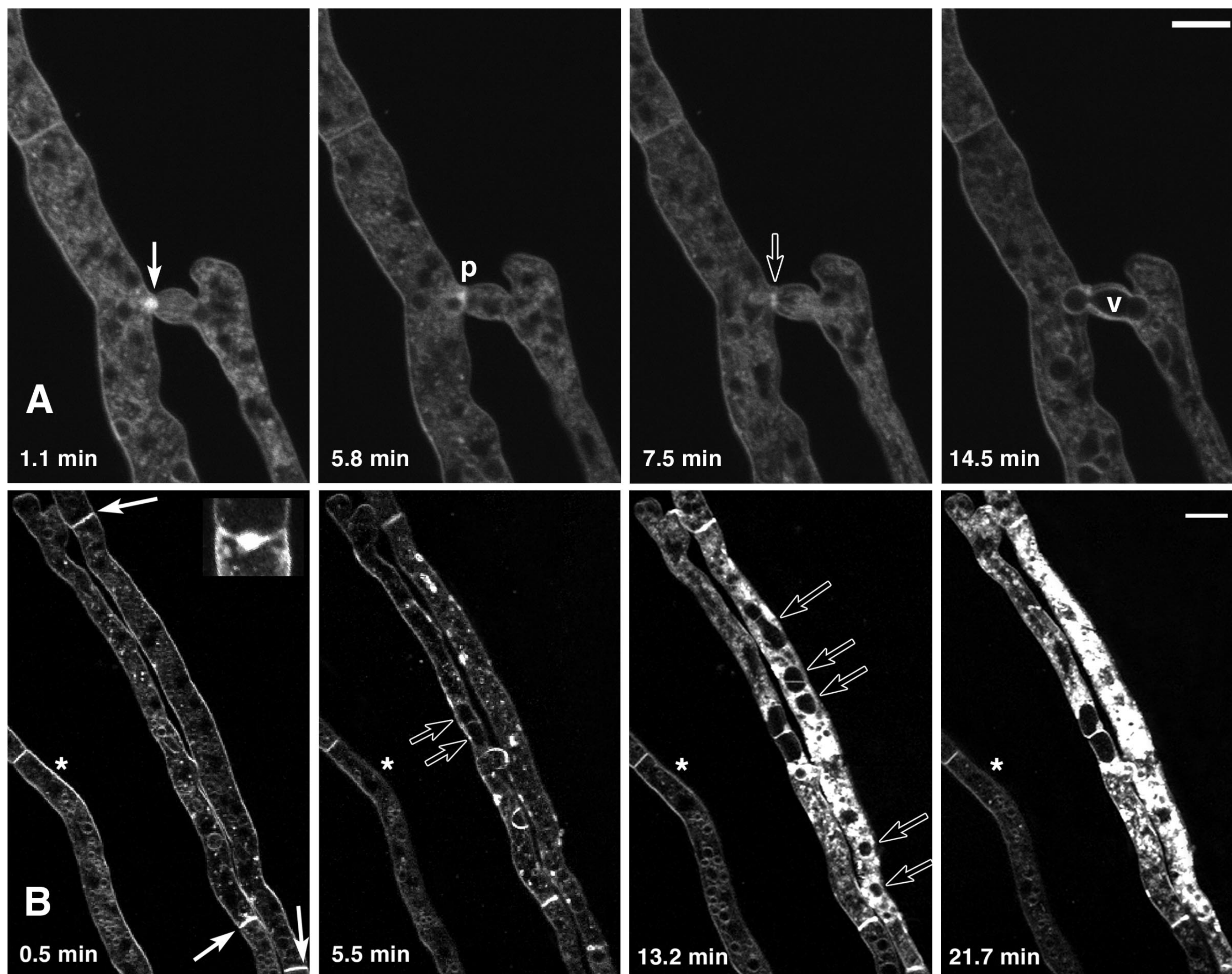

FIG. 2. A time course of compatible and incompatible hyphal fusion in *N. crassa*. The figure shows confocal images showing different stages of the hyphal fusion process at the times indicated after staining with the membrane-selective fluorescent dye FM4-64 (30). FM4-64 initially stains the plasma membrane and subsequently becomes visible in internal membranes (23). (A) Compatible hyphal fusion between two *N. crassa* strains that have identical specificity at all *het* loci. Initially, two Spitzenkörper (solid arrow; 1.1 min) are present on either side of the region where pore formation will subsequently occur (p; 5.8 min). A persistent ring of fluorescence (open arrow; 7.5 min) is present around the fusion pore after it has formed. Cytoplasmic flow is associated with movement of organelles through the pore, including nuclei and large vacuoles (v; 14.5 min). Note that the FM4-64 dye has become slightly photobleached in these cells during the time course. Bar = 10 μm. Time-lapse movies of hyphal fusion are available at the Fungal Genetics and Biology website (30) and www.Neurospora.org. (B) Incompatible hyphal fusion between two *N. crassa* strains that differ in allelic specificity at *het-c*. Heterokaryotic cells become compartmentalized by occlusion of septa (solid arrows and inset; 0.5 min). Increased cytoplasmic staining is due to the permeabilization of the plasma membrane and nonspecific incorporation of FM4-64 into internal membranes. Compare the incompatible fusion cells with the relatively constant level of staining of nearby healthy hypha (*) (the healthy hypha becomes slightly photobleached over time). Large vacuoles form within incompatible fusion cells (open arrows; 5.5 and 13.2 min) but eventually burst as cell death proceeds (21.7 min). Bar = 10 μm. Images are courtesy of D. J. Jacobson (University of California—Berkeley) and N. D. Read (University of Edinburgh).

It is apparent that *het* loci encode very different gene products (Table 1). Some *het* loci have defined roles in addition to heterokaryon incompatibility (65, 67, 76). Mutational analysis of other *het* loci has not revealed functions other than their role in heterokaryon incompatibility (73, 78). However, all the *het* locus mutants identified so far have lost the capacity for nonself recognition and will form a stable heterokaryon with strains with which they were formerly incompatible (29, 67, 68, 73, 78).

Recently, it has been shown that the *het-s* locus in *P. anserina* also acts as a prion (17). Two alternative allelic specificities occur at the *het-s* locus, *het-s* and *het-S*. The prion form [Het-s] is required to elicit heterokaryon incompatibility when a *het-s* strain fuses with a *het-S* strain. A strain containing the genetically identical, nonprion [Het-s*] form is neutral and will form vigorous heterokaryons with both *het-s* and *het-S* strains. Overexpression of *het-s* resulted in the formation of amyloid-like fibrils in *Escherichia coli* (20), and HET-s aggregation in *P. anserina* was induced by biolistic introduction of HET-s protein aggregates into a [HET-s*] strain (45). Although [HET-s] acts

TABLE 1. Genes involved in vegetative incompatibility and PCD and their homologs in *N. crassa*

Gene[a]	Characteristics of gene[c]	Homolog in *N. crassa*		Reference(s)
		NCU no. (e value)[a,b]	Linkage group	
N. crassa				
het genes				
mat A-1 (NCU01958.1)	Mating-type type gene, transcriptional factor, α domain	ND		26
mat a-1	Mating-type type gene, transcriptional factor, HMG box	NCU03481.1 (1e-12)	Unknown	77
het-c (NCU03493.1)	Allelic *het* gene, signal peptide, transmembrane domain, glycine-rich region	NCU03125.1 (e-104)	IR	73
het-6 (NCU03533.1)	Allelic *het* gene, TOL–HET-6–HET-E domain	NCU09045.1 (1e-71)	Unknown	76
un-24 (NCU03539.1)	Allelic *het* gene, ribonucleotide reductase large subunit	ND		75, 76
Suppressors				
tol (NCU04453.1)	Coiled-coil, leucine-rich repeat, TOL–HET-6–HET-E domain	NCU03015.1 (7e-91)	IR	74
vib-1 (NCU03725.1)	Nuclear localization sequence	NCU04729.1 (1e-39)	VIL	83
P. anserina				
het genes				
het-c	Nonallelic *het* gene against *het-d* and *het-e*, glycolipid transfer protein	NCU07947.1 (3e-70)	IV	67
het-d	Nonallelic *het* gene against *het-c*, GTP-binding, WD repeat, TOL–HET-6–HET-E domain	NCU00794.1 (5e-51) NCU06205.1 (5e-50)	Unknown III	21
het-e	Nonallelic *het* gene against *het-c*, GTP-binding, WD repeat, TOL–HET-6–HET-E domain	NCU00794.1 (7e-53) NCU06205.1 (2e-52)	Unknown III	21, 68
het-s	Allelic *het* gene, prion analog	NCU08705.1 (1e-11)	IIR	17
Vegetative incompatibility-related genes				
idi-1	Induced by *het-c/e* and *r/v* incompatibility, signal peptide	ND		11
idi-2	Induced by *het-r/v* incompatibility, signal peptide	ND		11
idi-3	Induced by *het-c/e* and *r/v* incompatibility, signal peptide	ND		11
mod-A	Modifier of *het-c/e, c/d*, and *r/v* incompatibility, SH3-binding motif	NCU07121.1 (e-135)	VIL	6
mod-D	Modifier of *het-c/e* incompatibility, G protein α subunit	NCU05206.1 (e-176)	IVR	43
mod-E	Modifier of *het-r/v*, HSP90	NCU04142.1 (0.0)	VR	44
pspA	Induced by *het-c/e* and *r/v* incompatibility, subtilisin-like serine protease	NCU00673.1 (0.0)	IR	56
		NCU07159.1 (7e-67)	Unknown	
S. cerevisiae apoptosis-like PCD genes				
ATP4	F_0F_1-ATPase	NCU00502.1 (7e-54)	IR	49
CDC48	Cell division cycle, AAA ATPase, fusion of ER-derived vesicles	NCU00018.1 (0.0)	Unknown	46
HEL10 (Ynl208w)	Unknown	ND		42
HEL13 (Yor309c)	Unknown	ND		42
NSR1	rRNA processing	NCU03092.1 (3e-41)	IR	42
PPA1	Vacuolar H^+-ATPase	NCU09747.1 (2e-47)	I	42
SAR1	ER to Golgi transport	NCU00381.1 (7e-77)	Unknown	42
STM1	Suppressor of *pop2* and *tom2*	NCU00225.1 (1e-13)	Unknown	42
YCA1 (*MCA1*, Yor197w)	Metacaspase	NCU09882.1 (2e-90) NCU02400.1 (7e-89)	Unknown VIIR	48
Ygl129c	Mitochondrial small ribosomal subunit	NCU08120.1 (3e-18)	IL	48

[a] NCU numbers represent the numbers of annotated genes in the *N. crassa* genome database (http://www-genome.wi.mit.edu/annotation/fungi/neurospora/).
[b] The SEG filter was used for the Blastp search. ORFs with low scores (<e-10) were eliminated. The high-scoring homologs for each protein are shown in this list. Blastp searches for HET-6, TOL, HET-D, HET-E, MOD-D, PSPA, CDC48, NSR1, SAR1, and YCA1 detected 10, 27, 66, 67, 3, 4, 21, 11, 7, and 2 proteins with e values of <e-10, respectively. ND, not detected.
[c] ER, endoplasmic reticulum.

as a prion to convert [Het-s*] to [Het-s], the relationship among prion activity, aggregation, and heterokaryon incompatibility is unclear. For diseases associated with formation of amyloid fibrils, such as Alzheimer's and Parkinson's diseases, it has been suggested that oligomeric intermediates, rather than the fibrils themselves, are toxic (28). Aggregation of HET-s when it is overexpressed in *P. anserina* might be a manifestation of a similar protective phenomenon.

MOLECULAR MECHANISMS ASSOCIATED WITH NONSELF RECOGNITION

A genetic difference at a *het* locus (either allelic or nonallelic) between two strains is sufficient to trigger growth inhibition, hyphal compartmentation, and death. Alleles conferring alternative *het* specificity are polymorphic (62, 69, 72, 76), suggesting that structural differences in HET proteins mediate nonself recognition. These observations suggest either that alternative HET proteins physically interact to mediate nonself recognition or that they modify products that physically interact. Recently, Sarkar et al. (66) have detected a physical interaction between alternative *N. crassa* HET-C proteins. In *N. crassa*, three allelic specificities occur at *het-c* (32, 72). A polymorphic region of 34 to 48 amino acids that is different among the three alternative HET-C proteins is sufficient to confer allelic specificity (72, 81). A HET-C heterocomplex, which consists of HET-C proteins encoded by *het-c* alleles of alternative specificity, localized specifically to the plasma membrane of dead hyphal compartments in *N. crassa*. Localization of the HET-C heterocomplex to the plasma membrane was essential for triggering typical heterokaryon incompatibility (66). These data suggest that the HET-C specificity domain may mediate protein-protein interactions between alternative HET-C proteins. Similarly, an interaction between the *P. anserina* HET-s/HET-S has been reported from yeast two-hybrid experiments (17), and genetic analyses suggest a physical interaction between *P. anserina* HET-C and HET-D/HET-E (21). Presumably, a HET heterocomplex may act as a "trigger" to mediate the downstream biochemical and morphological aspects of heterokaryon incompatibility. Alternatively, the formation of a HET heterocomplex may function to poison the cell and thus may directly mediate growth inhibition and death.

DOWNSTREAM EFFECTORS OF HETEROKARYON INCOMPATIBILITY

In *P. anserina*, heterokaryon incompatibility is associated with the synthesis of new polypeptides, including laccases, dehydrogenases, an amino acid oxidase, and two specific proteases (7, 10). An aspartyl protease, the *papA* product, is induced by glucose starvation (57). Although disruption of *papA* does not affect heterokaryon incompatibility, a heterokaryon incompatibility suppressor mutant, the *mod-A1* mutant, reduced the expression of *papA*. These data suggest a relationship between starvation and heterokaryon incompatibility. A subtilisin-like serine protease gene, *pspA*, is induced by heterokaryon incompatibility mediated by differences at the *P. anserina* nonallelic *het* loci, *het-r*/*het-v* and *het-c*/*het-e* (56). PSPA is the ortholog of protease B of *Saccharomyces cerevisiae*, which is a vacuolar protease involved in autophagy (56). A *P. anserina* ortholog (*idi-7*) of an *S. cerevisiae* gene involved in autophagy, *AUT7*, is also induced during incompatibility mediated by genetic differences at *het-r*/*het-v*. The presence of autophagic bodies in vacuoles, concomitant with relocalization of IDI-7, is associated with heterokaryon incompatibility in *P. anserina* (60). These observations have led to the hypothesis that heterokaryon incompatibility may be similar to autophagic PCD (13, 60).

Phylogenetic and genetic analyses suggest that PCD is an ancient process, some parts of which evolved in bacteria (4, 38). A full genome analysis of *N. crassa* (Whitehead Institute, http://www-genome.wi.mit.edu/annotation/fungi/neurospora/; MIPS, http://www.mips.biochem.mpg.de/proj/neurospora/) showed that *N. crassa* lacks the central components of the metazoan apoptosis pathway, such as the caspases, Bcl-2/Bax, and TNF receptor family genes, similar to what has been reported from analysis of the *S. cerevisiae* and *Schizosaccharomyces pombe* genomes (38). However, expression of genes in *S. cerevisiae* that induce apoptosis in mammals, such as *Bax*, results in an apoptosis-like phenotype (41, 85). The apoptosis-like phenotype in *S. cerevisiae* is suppressed by expression of mammalian inhibitors of apoptosis, such as *Bcl-X$_L$* (41). Mutations in *S. cerevisiae CDC48* (46) and overexpression of *HEL10*, *HEL13*, *NSR1*, *PPA1*, *SAR1*, *STM1*, and *YCA1* result in cell death in *S. cerevisiae* with a typical apoptotic phenotype (42, 47, 48). These results have led to the hypothesis that *S. cerevisiae* is capable of undergoing apoptosis under certain stress or age-related conditions (35, 39, 47). *YCA1* encodes a metacaspase, which is believed to be evolutionarily related to caspases in multicellular eukaryotes. *N. crassa* has two predicted open reading frames (ORFs) that show high similarity to YCA1, in addition to *CDC48* and *SAR1* homologs (Table 1). An *ATP4* homolog, which is required for Bax-mediated killing in *S. cerevisiae*, is also found in the *N. crassa* genome (49). An AP-ATPase and a NACHT-GTPase, which are associated with PCD in vertebrates, also exist in *N. crassa* but have not been identified in yeasts (38). Interestingly, *P. anserina* HET-E belongs to the NACHT family of proteins. The role of any of these other genes in heterokaryon incompatibility is unknown.

Three genes involved in mediating heterokaryon incompatibility in *P. anserina*, *mod-A*, *mod-D*, and *mod-E*, also have homologs in the *N. crassa* genome (Table 1). MOD-A contains an SH3-binding domain (6), which is a domain known to be involved in protein-protein interactions. The *mod-D* gene encodes the Gα subunit of heterotrimeric G protein (43), and *mod-E* encodes a protein which belongs to the Hsp90 family (44) of protein chaperones (58). MOD-D (Gα) and MOD-E (HSP90) are highly conserved components of signal transduction pathways and have additional functions in the life cycle besides heterokaryon incompatibility (43, 44).

Downstream effectors of heterokaryon incompatibility in *N. crassa*, *tol* and *vib-1*, have also been characterized. The *tol* mutant suppresses mating-type-associated heterokaryon incompatibility (54, 74). A surprising result of *N. crassa* genome searches is the number of predicted ORFs with similarity to TOL (Table 1; included in cluster 9 at http://www.mips.biochem.mpg.de/proj/neurospora/). Twenty-six predicted ORFs show similarity to TOL in the *N. crassa* genome (e values between e-10 and e-42). One additional predicted ORF is highly similar to TOL (Table 1). It was previously reported that TOL shows three regions of similarity to three other HET proteins, HET-6 from *N. crassa* and HET-E and HET-D from *P. anserina* (21, 76). In both HET-E and HET-D, the region of similarity to TOL is separate from the GTP binding site and WD repeat domain. The region of similarity of these 27 *N. crassa* TOL-like proteins also lies within the TOL–HET-6–HET-E domain (31). Whether or how this domain is relevant to heterokaryon incompatibility in *N. crassa* is unclear.

The *N. crassa vib-1* mutant was isolated as a suppressor of

het-c-associated heterokaryon incompatibility (83). Mutations at *vib-1* also partially suppress mating-type heterokaryon incompatibility. VIB-1 shows similarity to PHOG, an *Aspergillus nidulans* ORF annotated as a putative nonrepressible acid phosphatase (5), and Ndt80p, a transcription factor involved in regulating meiosis in *S. cerevisiae* (84). *vib-1* does not encode the structural gene for nonrepressible acid phosphatase (83), but VIB-1 may rather regulate its activity. Both *N. crassa het-c* and *vib-1* also have additional homologs in the *N. crassa* genome (Table 1). The *N. crassa het-c* and *het-c-like* genes are highly conserved in a number of ascomycete and basidiomycete species but are absent from the genomes of *S. cerevisiae* and *S. pombe* (T. A. J. vanderLee, personal communication).

The conservation of *P. anserina* genes involved in heterokaryon incompatibility in *N. crassa* suggests that *N. crassa* has the genetic potential to utilize these loci for nonself recognition and heterokaryon incompatibility. In support of this hypothesis, the introduction of *N. crassa het-c* into *P. anserina* triggered growth inhibition, hyphal compartmentation, and death, with a phenotype very similar to that for *het-c* incompatibility in *N. crassa* (71). *P. anserina* possesses a homolog of *N. crassa het-c*, called *hch* (*het-c* homolog). However, DNA sequence analysis of *hch* among nine *P. anserina* isolates that were different at all other known *het* loci did not reveal polymorphisms, suggesting that *hch* may not function as a *het* locus in *P. anserina*. In *N. crassa*, nonallelic incompatibility can be triggered by the introgression of genes linked to *tol* from a related species, *N. tetrasperma* (33). Thus, a species may contain loci that have the capacity to function as a *het* locus and thereby trigger heterokaryon incompatibility, but whether they do or not may be dependent on the presence or absence of polymorphisms within fungal populations.

CONCLUDING REMARKS

Heterokaryon incompatibility serves as a nonself recognition system in filamentous fungi, which presumably evolved because of their ability to form vegetative heterokaryons. Analyses of *het* loci in *N. crassa* and *P. anserina* have revealed molecular mechanisms of allelic specificity and nonself recognition. The results of several studies suggest that heterokaryon incompatibility is mediated by conserved cellular machinery among filamentous fungi. Further analyses of PCD-associated genes in filamentous fungi will define the relationship among autophagy, apoptosis-like phenomena, and heterokaryon incompatibility. Although downstream effectors of death may be identified by comparative genomic analyses, a central and critical unknown component of our understanding of heterokaryon incompatibility is how recognition triggers entry into the cell death pathway.

The evolution of *het* loci is an intriguing phenomenon. How does the ability to recognize nonself evolve? Apparently filamentous fungi utilize genetic differences found in populations to mediate nonself recognition. The molecular analysis of *het* loci has not revealed a common principle for defining a *het* gene, even though genetic differences at a *het* locus may activate a common pathway that leads to heterokaryon incompatibility. The availability of complete filamentous fungal genomes will facilitate the identification of additional *het* loci and will hopefully reveal underlying principles of the evolution of nonself recognition systems.

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