MINIREVIEW

Fatal Attraction: Nonself Recognition and Heterokaryon Incompatibility in Filamentous Fungi

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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).

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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 transpecies 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 (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. 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. 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. 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. 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.
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
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.

### Table 1. Genes involved in vegetative incompatibility and PCD and their homologs in N. crassa

<table>
<thead>
<tr>
<th>Gene</th>
<th>Characteristics of gene</th>
<th>Homolog in N. crassa</th>
<th>Reference(s)</th>
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<tbody>
<tr>
<td>N. crassa</td>
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<tr>
<td>het genes</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>mat A-1</td>
<td>Mating-type type gene, transcriptional factor, (\alpha) domain</td>
<td>ND</td>
<td>26</td>
</tr>
<tr>
<td>mat a-1</td>
<td>Mating-type type gene, transcriptional factor, HMG box</td>
<td>NCU03481.1 (1e-12)</td>
<td>Unknown</td>
</tr>
<tr>
<td>het-c (NCU03493.1)</td>
<td>Allelic het gene, signal peptide, transmembrane domain, glycine-rich region</td>
<td>NCU03125.1 (e-104)</td>
<td>IR</td>
</tr>
<tr>
<td>het-6 (NCU03533.1)</td>
<td>Allelic het gene, TOL–HET-6–HET-E domain</td>
<td>NCU09045.1 (1e-71)</td>
<td>Unknown</td>
</tr>
<tr>
<td>un-24 (NCU03539.1)</td>
<td>Allelic het gene, ribonucleotide reductase large subunit</td>
<td>ND</td>
<td>75, 76</td>
</tr>
<tr>
<td>Suppressors</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>tol (NCU04453.1)</td>
<td>Coiled-coil, leucine-rich repeat, TOL–HET-6–HET-E domain</td>
<td>NCU03015.1 (7e-91)</td>
<td>IR</td>
</tr>
<tr>
<td>vib-1 (NCU03725.1)</td>
<td>Nuclear localization sequence</td>
<td>NCU04729.1 (1e-39)</td>
<td>VIL</td>
</tr>
<tr>
<td>P. anserina</td>
<td></td>
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<tr>
<td>het genes</td>
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<td></td>
<td></td>
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<tr>
<td>het-c</td>
<td>Nonallelic het gene against het-d and het-e, glycolipid transfer protein</td>
<td>NCU07947.1 (3e-70)</td>
<td>IV</td>
</tr>
<tr>
<td>het-d</td>
<td>Nonallelic het gene against het-c, GTP-binding, WD repeat, TOL–HET-6–HET-E domain</td>
<td>NCU00794.1 (5e-51)</td>
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<tr>
<td>het-e</td>
<td>Nonallelic het gene against het-c, GTP-binding, WD repeat, TOL–HET-6–HET-E domain</td>
<td>NCU06205.1 (5e-50)</td>
<td>III</td>
</tr>
<tr>
<td>het-s</td>
<td>Allelic het gene, prion analog</td>
<td>NCU08705.1 (1e-11)</td>
<td>IIR</td>
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<td>Vegetative incompatibility-related genes</td>
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<tr>
<td>idi-1</td>
<td>Induced by het-c/e and r/v incompatibility, signal peptide</td>
<td>ND</td>
<td>11</td>
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<tr>
<td>idi-2</td>
<td>Induced by het-r/v incompatibility, signal peptide</td>
<td>ND</td>
<td>11</td>
</tr>
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<td>idi-3</td>
<td>Induced by het-c/e and r/v incompatibility, signal peptide</td>
<td>ND</td>
<td>11</td>
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<tr>
<td>mod-A</td>
<td>Modifier of het-c/e, c/d, and r/v incompatibility, SH3-binding motif</td>
<td>NCU07121.1 (e-135)</td>
<td>VIL</td>
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<tr>
<td>mod-D</td>
<td>Modifier of het-c/e incompatibility, G protein (\alpha) subunit</td>
<td>NCU05206.1 (e-176)</td>
<td>IRV</td>
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<tr>
<td>mod-E</td>
<td>Modifier of het-r/v, HSP90</td>
<td>NCU04142.1 (0.0)</td>
<td>VR</td>
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<tr>
<td>pspA</td>
<td>Induced by het-c/e and r/v incompatibility, subtilisin-like serine protease</td>
<td>NCU00673.1 (0.0)</td>
<td>IR</td>
</tr>
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<td>S. cerevisiae apoptosis-like PCD genes</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>ATP4</td>
<td>F(<em>{1})F(</em>{0})-ATPase</td>
<td>NCU00502.1 (7e-54)</td>
<td>IR</td>
</tr>
<tr>
<td>CDC48</td>
<td>Cell division cycle, AAA ATPase, fusion of ER-derived vesicles</td>
<td>NCU00018.1 (0.0)</td>
<td>Unknown</td>
</tr>
<tr>
<td>HEL10</td>
<td>Unknown</td>
<td>ND</td>
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</tr>
<tr>
<td>HEL13</td>
<td>Unknown</td>
<td>ND</td>
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<tr>
<td>NSR1</td>
<td>Unknown</td>
<td>ND</td>
<td>42</td>
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<tr>
<td>PPA1</td>
<td>Vacuolar H(^+)-ATPase</td>
<td>NCU00794.1 (3e-41)</td>
<td>IR</td>
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<td>SAR1</td>
<td>ER to Golgi transport</td>
<td>NCU00381.1 (7e-77)</td>
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<tr>
<td>STMI</td>
<td>Suppressors of pop2 and tom2</td>
<td>NCU00252.1 (1e-13)</td>
<td>Unknown</td>
</tr>
<tr>
<td>YCA1</td>
<td>Metacaspase</td>
<td>NCU09982.1 (2e-90)</td>
<td>Unknown</td>
</tr>
<tr>
<td>Ygl129c</td>
<td>Mitochondrial small ribosomal subunit</td>
<td>NCU08120.1 (3e-18)</td>
<td>IL</td>
</tr>
</tbody>
</table>

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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 (<1e-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, PPSA, CDC48, NSR1, SAR1, and YCA1 detected 10, 27, 66, 67, 3, 4, 21, 11, 7, and 2 proteins with e values of <1e-10, respectively. ND, not detected.

c ER, endoplasmic reticulum.
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 aspartil 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-1 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-rihet-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-rihet-v. The presence of autophagic bodies in vacuoles, concomitant with relocalization of ID1-7, 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, Bel-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-XL (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 ATP hydrox 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 NACH-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 ascomycetous 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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