# Non-self recognition and programmed cell death in filamentous fungi

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Non-self recognition resulting in programmed cell death is a ubiquitous phenomenon in filamentous ascomycete fungi and is termed heterokaryon incompatibility (HI). Recent analyses show that genes containing predicted HET domains are often involved in HI; however, the function of the HET domain is unknown. Autophagy is induced as a consequence of HI, whereas the presence of a predicted transcription factor, VIB-1, is required for HI. Morphological features associated with apoptosis in filamentous fungi are induced by various stresses and drugs, and also during HI. Future analyses will reveal whether common or different genetic mechanisms trigger death by non-self recognition and death by various environmental onslaughts.

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## The importance of identity

The ability to distinguish oneself from another is a ubiquitous phenomenon among all living things. As a filamentous fungal colony grows across a substrate in nature, it will encounter many other microorganisms, including bacteria, fungal colonies of the same or of different species, protist species and insects. Interactions between different fungal species, such as *Podospora anserina* and *Coprinopsis cinerea*, can result in the generation of hydrogen peroxide and death of hyphae [1•]. Non-self recognition between genetically different isolates of the same fungal species often results in compartmentalization and death of hyphae that undergo fusion between the two strains, a phenomenon termed 'heterokaryon incompatibility' (HI; Figure 1) [2]. Macroscopically, this interaction often results in a 'barrage', or demarcation zone between incompatible fungal colonies. Although the formation of a barrage is often used to infer genetically different individuals, an association between HI and barrage-formation is not always observed [3].

HI is genetically regulated in filamentous ascomycete species by *het* loci (named after heterokaryon) [2,4,5]. The observed acquisition of novel *het* genes through interspecies gene transfer in *Ophiostoma novo-ulmi* indicated that new *het* specificities can undergo fixation within a population [6••]. DNA polymorphisms associated with *het* allele specificity can be under selection for diversity, a feature common to genes under balancing selection [7••,8,9], an observation that is consistent with their role in mediating non-self recognition. HI also prevents transmission of mycoviruses by hyphal fusion [10]. These observations support the hypothesis that non-self recognition confers a selective advantage and that genetic mechanisms favor *het* gene divergence and subsequent fixation in populations of filamentous ascomycete fungi.

## The genetics of recognition

Two types of genetic system, termed 'allelic' and 'non-allelic', regulate HI in filamentous ascomycete fungi [2,4,5]. In allelic HI, non-self recognition is triggered by alleles of different specificity at the same locus, for example at *het-s* in *P. anserina* (Figure 2) [4]. The *het-s* locus is unusual in that allelic specificity is not stable because the HET-s can fold into two different conformations; one which doesn't trigger HI and one, an infectious prion form, [HET-s], which does [11]. The [HET-s] prion also functions as a meiotic drive element in crosses between *het-S* and *het-s* strains [12,13]. Recent structural studies indicate that the prion-forming domain of [HET-s] is essential for conferring non-self recognition and HI [14•]; the corresponding region in the non-prion form, HET-S, is also required for HI [15].

An example of non-allelic incompatibility between closely linked genes is the *het-c/pin-c* haplotype in *Neurospora crassa*, whereas non-allelic interactions among the unlinked loci, *het-R* and *het-V*, mediate HI in *P. anserina* (Figure 2) [16••,17]. Interestingly, five of the six molecularly characterized *het* interactions involve predicted proteins that share a common ~150 amino acid domain, termed the HET domain (Pfam06985; *N. crassa het-6*, *tol* and *pin-c* and *P. anserina het-D* and *het-E*). Outside of the HET domain, these genes are dissimilar in sequence and domain structure (Figure 3). HET domain genes often interact with another gene (non-allelic HI interactions). For example, in *N. crassa*, *het-c/pin-c* HI requires a plasma membrane protein (HET-C) and a HET domain protein (PIN-C; Figure 3) [16••]. A non-self recognition complex between *un-24* (encoding ribonucleotide reductase) and *het-6* (a HET domain gene) loci is associated with an inversion [7••]; specific alleles at *un-24* and *het-6* are in

**Figure 1**
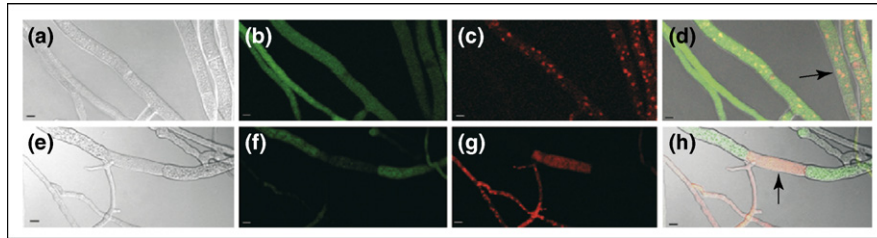


Confocal micrographs showing hyphal fusion and heterokaryon formation between two colonies that are all isogenic at all *het* loci **(a–d)** (one strain [KD02-10] was transformed with cytoplasmic GFP vector and the second strain [RM01-01] was transformed with a histone H1 construct tagged with dsRed) or between strains that differ in *het-c* haplotype **(e–h)** (one strain [KD06-15] was transformed with the cytoplasmic GFP vector and the second strain is the same as used in [a–d]). Panels **(a)** and **(e)**, differential interference contrast (DIC) micrographs. Panels **(b)** and **(f)**, fluorescent micrographs showing cytoplasmic GFP fluorescence. Panels **(c)** and **(g)**, fluorescent micrographs showing nuclear dsRed fluorescence. Panels **(d)** and **(h)** are merged images. The arrow in panel (d) shows a healthy heterokaryotic hypha, whereas the arrow in panel (h) shows compartmentalized fusion cell. Note that the nuclear H1-dsRed signal is diffuse in the incompatible fusion cell (g,h), presumably because of nuclear degradation. Scale bar=10 μm.

severe linkage disequilibrium. HI that is associated with the *het-6* haplotype requires genetic differences at both *un-24* and *het-6*. In *P. anserina*, non-self recognition mediated by *Pahet-C/het-D* or *Pahet-C/het-E* requires a glycolipid transfer protein (PaHET-C) [18] and a HET domain protein (HET-E or HET-D) [19]. Interestingly, in the plant species *Arabidopsis thaliana*, an ortholog of *Pahet-C*, ACD11, has been implicated in programmed cell death (PCD) [20].

Predicted HET domain genes are specific to and common in filamentous ascomycete genomes. The *N. crassa* genome contains ~50 HET domain genes, the human pathogen *Aspergillus fumigatus* has seven, whereas the plant pathogen *Stagonospora nodorum* has ~150. Genetic analysis and modeling analysis indicate that the number of *het* loci required for non-self recognition in fungal populations is between 7 and 12 [2,21], suggesting that not all predicted HET domain genes are involved in HI. Phylogenetic analysis of HET domain genes in the genomes of different *Aspergillus* species (the numbers of genes range between 7–38) indicate multiple gene duplication events, rapid diversification and gene loss [22••]. HET domain genes involved in HI are highly polymorphic within fungal populations. For example, alleles of different specificity at *N. crassa pin-c* or *het-6* show only ~47–68% identity at the amino acid level (depending on the allele comparison) [7••,16••]. In *P. anserina het-E* and *het-D*, sequence variation is associated with allelic specificity [19]. Although polymorphisms associated with specificity have been identified

**Figure 2**
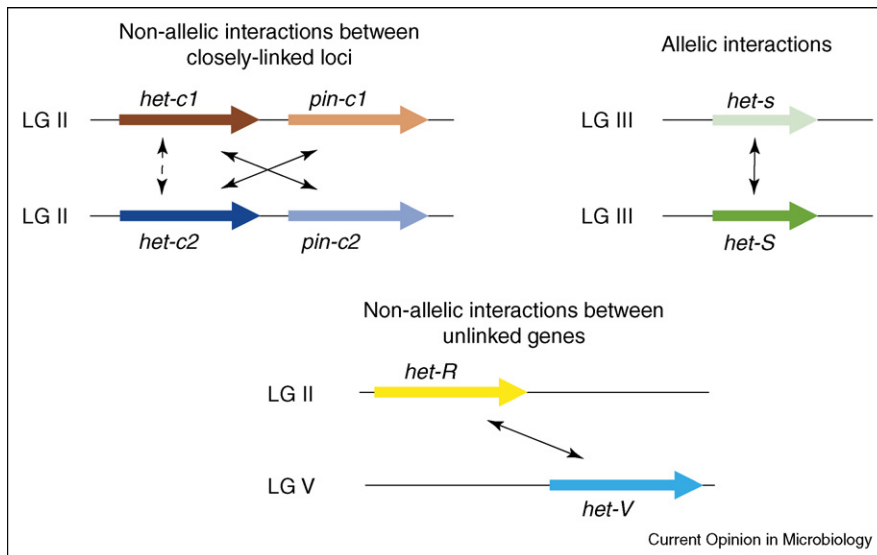


Non-self recognition resulting in HI through non-allelic interactions of closely-linked *het* genes (*N. crassa het-c/pin-c*), non-allelic interactions between unlinked *het* genes (*P. anserina het-V/het-R*) and allelic interactions (*P. anserina het-s/het-S*).

**Figure 3**



Interactions between predicted HET domain proteins (*pin-c*, *tol*, *het-6*, *het-E and het-D*) and their partners (*het-c*, *mat*, *un-24* and *Pahet-c*). Interactions between *un-24* and *het-6* are inferred on the basis of population analyses and a decrease in fitness in meiotic progeny; specific alleles at *het-6* and *un-24* show severe linkage disequilibrium [7,9].

in the non-allelic or gene complex partners of HET domain genes (such as *N. crassa het-c*, *un-24* and *Pahet-C*; [2]), these genes do not show similar levels of duplication, divergence and loss among filamentous ascomycete species [22••].

Little is understood about how molecular interactions between alternate *het* genes mediate non-self recognition and trigger HI. Loss-of-function mutations at *het-c*, *pin-c*, *tol*, *mat*, *Pahet-c*, *het-e* and *het-s* do not affect the vegetative growth phenotype of mutants. However, such mutants fail to distinguish self from non-self and will form vigorous heterokaryons with strains with which they were formerly incompatible. Thus, incompatibility cannot be a result of disruption of an essential cellular function encoded by *het* genes. Models depict physical interaction between proteins encoded by *het* loci as a pre-requisite for non-self recognition. In support of this hypothesis, a HET-C heterocomplex composed of alternative HET-C proteins is associated with HI in *N. crassa*, suggesting that a HET-C/PIN-C heterocomplex might play a role in non-self recognition [16••,23].

## Signaling death
Compartmentalization by septal plugging, increased septation, vacuolization of plugged compartments and accumulation of lipid bodies are common microscopic features associated with HI. Two new approaches to identify components of the death pathway have been used recently: the characterization of genes induced during HI [24–26]; and the identification of mutations which suppress HI [16••,27].

The *P. anserina het-R/het-V* system shows temperature sensitivity; strains containing incompatible *het-R* and *het-V* alleles grow like the wild type at 32 °C, but undergo massive cell death when shifted to 26 °C [24]. Genes induced during *het-R/het-V* incompatibility (*idi* family) include genes for predicted cell wall proteins (*idi-1*, *idi-2* and *idi-3*), a predicted bZIP transcription factor (*idi-4*) and orthologs of autophagy genes in *Saccharomyces cerevisiae* (*idi-6* and *idi-7*) [17,26,28••]. During autophagy, organelles and cytoplasm are engulfed in specialized vesicles termed autophagosomes and targeted to the vacuole for degradation and recycling. The *idi* genes are induced under nitrogen starvation and treatment with rapamycin, a specific inhibitor of the TOR (for 'target of rapamycin') kinase; treatment of the cells with rapamycin mimicked the effects of HI [24]. Autophagy, first described as a cellular response to nutrient starvation, has been associated with PCD in metazoans [29]. However, *P. anserina* mutants in the *S. cerevisiae* orthologs of *ATG1*, a protein kinase involved in the induction of autophagy and *ATG8*, a gene required for autophagosome formation, showed accelerated cell death associated with HI [28••]. Although the bZIP DNA-binding domain of IDI-4 binds to the *idi-7* promoter, inactivation of *idi-4* does not affect HI [25,30]. These observations indicate that the induction of autophagy during HI is a cell survival response in conflict with death signaling pathways.

In *N. crassa*, mutations in a putative transcription factor gene, *vib-1*, (for vegetative incompatibility blocked) suppress both *mat* and *het-c/pin-c* incompatibility [27,31,32].

The *mat* locus encodes transcription factors required for entry into sexual reproduction; fusion between opposite mating types during vegetative growth results in HI (Figure 3). Recent results suggest that *vib-1* is required for the expression of HET domain genes, *pin-c*, *tol* and *het-6* [33]. Recently, a strain containing mutations in an ortholog of *vib-1* in *Aspergillus nidulans* was shown to be defective in protease production in response to nutrient limitation [34]. These data suggest that mutations in *vib-1* might suppress HI both because HET domain genes are not expressed and because downstream effectors of HI might be lacking.

## More than one way to die?

PCD mechanisms are ubiquitous in both prokaryotic and eukaryotic species. Filamentous fungal genomes contain the complement of genes involved in PCD in *S. cerevisiae*, and also have homologs of genes, involved in PCD in metazoans, that are not present in *S. cerevisiae* or *Saccharomyces pombe* [2,22••,35,36]. Several biochemical assays have been used to assess cellular changes associated with apoptosis in filamentous fungi, including deoxynucleoti-dyltransferase-mediated dUTP nick end labeling (TUNEL) for DNA degradation, Annexin-FITC (fluorescein) binding for the presence of phosphatidylserine on the outer leaflet of the plasma membrane and 2,7′-dichlorodihydrofluorescein diacetate for reactive oxygen species (ROS) production. In addition, the exclusion of propidium iodide and the blockage of cell death by treatment with the translation inhibitor cycloheximide are used to differentiate apoptosis from necrosis. The results of these assays show that apoptosis in filamentous fungi occurs in response to treatment with hydrogen peroxide, phytosphingosine, antifungal proteins (PAFs), amphotericin B and farnesol [37••,38–40,41•]. Farnesol is involved in quorum sensing in *Candida albicans* [42], but causes apoptosis in *A. nidulans*, implying that this signaling molecule might be used in interspecies antagonistic interactions [37••]. Apoptotic characters such as DNA degradation are also associated with HI in *N. crassa* [43], entry into stationary phase in *A. fumigatus* [44] and asexual sporulation in *A. nidulans* [45].

In *C. albicans*, mutations that block Ras signaling — including those in *ras1*, *cdc35* (encoding adenylate cyclase), *tpk1* and *tpk2*, which encode regulatory subunits of PKA — suppress or delay the apoptotic response upon exposure to low levels of acetic acid, $H_2O_2$ and amphotericin B [46,47•]. By contrast, mutations that result in constitutive activation of the RAS pathway, such as $RAS1^{val13}$ (DARas), accelerate entry into the apoptotic pathway in *C. albicans* and *Colletotrichum trifolii* [41•,47•], although Ras activation by itself is not sufficient to kill cells. Treatment of *C. trifolii* with proline suppressed apoptosis associated with DARas as well as apoptosis associated with a variety of stresses [41•]. In *A. nidulans*, resistance to PAFs and farnesol was associated with a dominant-interfering mutations in the α–subunit of G protein, $fadA^{G203R}$ [39] or mutations in the Gβ subunit (Δ*sfaD*), respectively [37••], whereas mutations that hyperactivated G protein signaling (Δ*flbA*) resulted in increased sensitivity to farnesol. A predicted poly(ADP-ribose) polymerase (PARP) ortholog in *A. nidulans* was also shown to be required for farnesol-induced nuclear condensation [48]. PARP is a highly conserved enzyme that is implicated in the stress response and in apoptosis in metazoans [49]. Crosstalk between cAMP and G protein signaling occurs in several fungi [50], although the exact mechanisms vary among fungal species. These observations suggest that alterations of nutrient signaling pathways might mediate the commitment of hyphae to enter the apoptotic and HI pathways.

## Conclusion: life after death?

The process of PCD in filamentous ascomycete fungi occurs as a result of non-self recognition, treatment with various drugs and during developmental processes. Work in *P. anserina* and *N. crassa* suggest that protein interaction and conformation alterations might be a molecular mechanism for non-self recognition. Genetic analyses of HI and of apoptosis induction by drug treatment suggest that signaling pathways involved in nutrient sensing might be recruited to trigger death. Analyses in *P. anserina* indicate that the induction of cell survival mechanisms, such as autophagy, also occur in concert with death signaling, thus complicating analysis of signal transduction mechanisms associated with death. Future work will determine the commonalities and differences in PCD or apoptosis induced by HI between a variety of environmental triggers. Specifically, do all of these triggers mediate death by a common signaling and dismantling pathway, or are there many ways to die, depending on which cellular pathway is perturbed?

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