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**Molecular mechanisms regulating cell fusion and heterokaryon formation in filamentous
fungi**

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ABSTRACT

For the majority of fungal species, the somatic body of an individual is a network of interconnected cells sharing a common cytoplasm and organelles. This syncytial organization contributes to an efficient distribution of resources, energy and biochemical signals. Cell fusion is a fundamental process for fungal development, colony establishment and habitat exploitation and can occur between hyphal cells of an individual colony or between colonies of genetically distinct individuals. One outcome of cell fusion is the establishment of a stable heterokaryon, culminating in benefits for each individual via shared resources or being of critical importance for the sexual or parasexual cycle of many fungal species. However, a second outcome of cell fusion between genetically distinct strains is formation of unstable heterokaryons and the induction of a programmed cell death reaction in the heterokaryotic cells. This reaction of non-self rejection, which is termed heterokaryon (or vegetative) incompatibility, is widespread in the fungal kingdom and acts as a defense mechanism against genome exploitation and mycoparasitism. Here, we review the currently identified molecular players involved in the process of somatic cell fusion and its regulation in filamentous fungi. Thereafter, we summarize the knowledge on the molecular determinants and mechanism of heterokaryon incompatibility, and place this phenomenon in the broader context of biotrophic interactions and immunity.

INTRODUCTION

Cell-cell fusion is an essential biological process that occurs in organisms throughout the tree of life. It is involved in both sexual and asexual developmental processes in most species and has been shown to occur in multicellular and in unicellular organisms. Somatic cell fusion events are widespread in eukaryotic organisms, including animals, where they are important for muscle differentiation, placental development and for formation of multinucleate giant cells in the immune system (1–4).

Somatic cell fusion is a highly regulated event that requires reciprocal recognition as well as coordinated molecular processes in fusion partners. However, indiscriminate cell fusion comes with risks, including fusion with infected, impaired or genetically different fusion partners, which can result in a disadvantaged heterokaryon. Thus, cells have evolved mechanisms to discriminate genetically identical from genetically non-identical (non-self) cells. There are diverse types of genetic recognition mechanisms that organisms use to distinguish self from non-self. In mammalian cells, self/non-self recognition mechanisms mediate pathogen defense, while in basal eukaryotic invertebrates, such as the colonial ascidian *Botryllus schlosseri* and the cnidarian *Hydractinia symbiolongicarpus*, self/non-self recognition functions when individuals fuse to form vascular and hematopoietic chimeras (5). In bacteria, such as *Proteus mirabilis*, swarming colonies can recognize each other as non-self and establish a visible boundary, whereas genetically identical swarms merge (6, 7). In protists such as *Dictyostelium discoideum*, self/non-self recognition limits “self” genotypes to sporulating fruiting bodies (8, 9). In plants, self/non-self recognition is important for both pathogen defense and the prevention of self-fertilization for out-crossing species (10, 11). In filamentous fungi, the syncytial lifestyle combined with cell fusion to form the interconnected hyphal networks that are the growth habit

of these organisms, make them superb models for studies on self/non-self recognition mechanisms. In this chapter, we summarize the current knowledge about somatic cell fusion events in filamentous fungi that govern both self and non-self recognition processes and that act prior to and after cell fusion events.

SOMATIC FUSION IN FILAMENTOUS FUNGI AND ITS CONSEQUENCES: BENEFITS AND DETRIMENTS

In filamentous fungi, somatic cell fusion (*anastomosis*) is essential to develop the hallmark of filamentous fungal growth: an interconnected, multinucleated, syncytial network (Figure 1). Fusion has been reported in more than 73 species of filamentous fungi covering over 20 genera (12). Unlike other organisms, such as plants or animals, fungi lack biological transport networks (vascular systems). Instead, these organisms build a mycelial network to distribute cytoplasm, organelles (including nuclei), nutrients and other resources within the syncytium, facilitating growth and rapid spatial expansion of the fungal colony (13–16). In fact, filamentous fungi form the most extensive biological networks characterized so far (17). Anastomosis in hyphae is also critical for host colonization and virulence of pathogenic and symbiotic fungi (18, 19), and for mycoparasitism (20).

In addition to fusion within a colony, cell fusion can occur between genetically different colonies. Such fusion events of non-clone individuals can be viable and lead to the coexistence of genetically different nuclei in a common cytoplasm (heterokaryon). Heterokaryon formation in filamentous fungi has potential benefits; e.g. it results in functional diploidy and mitotic recombination during the parasexual cycle, which can be especially important for highly clonal species (21, 22). However, heterokaryon formation has risks, as infectious agents can be

transmitted via fusion events (23–26). Within a heterokaryon, allorecognition processes determine the fate of the fused cells: compatible genotypes lead to viable heterokaryons indistinguishable from a homokaryotic colony, while heterokaryotic cells resulting from the fusion of incompatible genotypes are rapidly compartmentalized and undergo a programmed cell death (PCD) reaction, termed Heterokaryon (or Vegetative) Incompatibility (HI or VI) (27, 28). It has been proposed that HI limits genome exploitation and represents a defense mechanism that has important adaptive value in the highly competitive biotopes inhabited by most filamentous fungi (29). HI has been shown to limit the propagation of deleterious agents such as mycoviruses, DNA transposons and senescence plasmids (24, 30–32). However, a recent study by Bastiaans et al. showed that fusion among fungal colonies is mutually beneficial, relative to the absence of fusion upon non-self recognition, suggesting an interplay between beneficial aspects of cell fusion versus the risk factors associated with indiscriminate fusion with other partners (33).

Unlike somatic fusion where genetic similarity between the fusion partners is favorable, in out-crossing species of filamentous fungi, fusion between genetically dissimilar partners is necessary for sexual reproduction. In heterothallic fungi, genetic dissimilarity is determined by the existence of at least two different mating types. Interestingly, in some fungal species, mating type also serves as an HI factor during somatic fusion, preventing the existence of different mating types within a heterokaryotic colony (34, 35). In species capable of outcrossing, individuals that are somatically incompatible undergo sexual reproduction and production of meiotic progeny, indicating that non-self recognition involved in HI processes must be inactive during the sexual cycle.

105 **RECOGNITION, CELL FUSION AND COLONY ESTABLISHMENT**

106 In addition to hyphal fusion within a mature colony, somatic fusion events also contribute to the
107 onset of colony formation. For example, cell fusion between germinated asexual spores
108 (germlings) merges numerous individuals into one functional unit, providing the basis for the
109 developing mycelial colony in a number of fungal species (Figure 1) (36). The ability of
110 germlings to form a network contributes to fitness, as indicated by more rapid colony
111 establishment and subsequent asexual spore formation (36–39). However, fusion can be
112 particularly risky for germlings. For example, a post-fusion incompatibility reaction (see below)
113 would likely result in death of both germlings. Therefore, most HI reactions described so far are
114 suppressed at the germling stage (40).

115 Germlings are especially tractable for analyzing the cellular mechanisms and components
116 involved in the cell fusion process (2). In recent years, more than 60 proteins involved in
117 germling fusion have been identified (41, 42). Most of the cellular components mediating
118 germling fusion processes are also required for hyphal fusion in a mature colony, indicating that
119 both processes share common molecular machinery. Mutual recognition and attraction of the
120 fusion partners employs an intricate signaling network, which comprises conserved eukaryotic
121 factors, such as MAP kinase modules, NADPH oxidases, the striatin-interacting phosphatase and
122 kinase (STRIPAK) complex and calcium-regulated factors, but also fungal specific proteins
123 (Figure 2). A central hub within this network is a MAP kinase (MAPK) cascade homologous to
124 the pheromone response pathway of *Saccharomyces cerevisiae* (43, 44). Analysis of the
125 subcellular dynamics of the MAPK MAK-2 during germling fusion in *N. crassa* revealed an
126 unusual mode of communication associated with localization of MAK-2 to cell tips during
127 chemotropic interactions (45, 46). During the tropic interactions between germlings or hyphae,

128 MAK-2 assembles with its two upstream kinases MEK-2 and NRC-1, the scaffold HAM-5 and
129 the adaptor protein STE50 in an oscillating manner at the plasma membrane of cell tips (45, 47,
130 48). Within one cell, formation of the MAK-2 complexes at the cell tips alternates with the
131 membrane recruitment of another protein, called SOFT (SO), a factor that is only conserved in
132 filamentous ascomycete fungi and is essential for germling/hyphal fusion (45, 49). The
133 membrane recruitment of the MAK-2 complex or SO alternates between the two fusion partners
134 in a coordinated manner with one phase lasting between 8 and 12 minutes. These observations
135 indicate that the two fusion cells switch between two physiological states in a highly coordinated
136 manner. The current working model suggests that the cells take turns in signal sending and signal
137 receiving. Mathematical modeling revealed that this unique mode of communication would
138 allow signaling via a single receptor/ligand pair employed by both partner cells (50). The
139 spatiotemporal coordination of signal sending versus signal receiving would enable genetically
140 and developmentally identical cells to achieve mutual attraction and fusion, while avoiding self-
141 stimulation. While the nature of the predicted signal and receptor activating the MAPK module
142 remains unknown, the linkage of the MAK-2 module and the SO protein within the fusion
143 signaling network is beginning to unfold. In *Sordaria macrospora*, a homolog to SO, called
144 PRO40, functions as a scaffold for a second MAPK module, the cell wall integrity pathway (51).
145 Similar to the MAK-2 pathway, this MAPK module is also essential for somatic fusion in several
146 filamentous fungi (43, 51–53). In *N. crassa*, nuclear entry of the MAPK of the cell wall integrity
147 pathway, MAK-1, is controlled by the STRIPAK complex in a MAK-2-dependent manner (54).
148 STRIPAK complexes are multi-protein assemblies, which mediate a plethora of biological
149 functions in eukaryotic organisms. Their presence in filamentous fungi was first shown in *S.*
150 *macrospora*, and their essential role for somatic fusion has been established for several species

(54–59). Besides the STRIPAK complex, an additional target of MAK-2 is the transcription factor PP-1, which in turn controls the expression of several fusion-mediating factors, including genes encoding the STRIPAK complex, *so*, *mak-1*, *mek-1* and NADPH oxidase (NOX) encoding genes (39). NOX complexes are essential for somatic fusion in several filamentous fungi and their potential interaction with the MAPK signaling modules as targets or activators is currently a major research topic in the cell fusion field (2, 60–62). In general, placing the numerous identified fusion factors into the described signaling network will be one of the main future challenges in this research field. Given, however, the experimental tractability of fungal model systems, hyphal and germling fusion may well advance as paradigms for studying eukaryotic signaling networks and their subcellular dynamics.

Non-self recognition events acting pre-fusion

While the molecular basis of chemotropic interactions between genetically identical germlings has been studied extensively, there are only a few studies focusing on processes involved in chemotropic interactions between genetically non-identical germlings. For example, pre-recognition of incompatible strains has been reported in *Tuber borchii* and *Glomus mosseae*, in which incompatible hyphae avoid fusion with each other (63, 64). Although the genes and mechanisms behind these findings remain to be revealed, these observations suggest a link between fusion signaling and non-self recognition. This hypothesis was supported recently in a study by Heller et al. showing that the polymorphic “greenbeard” genes *doc-1*, *doc-2* and *doc-3* mediate kind discrimination in *N. crassa* populations and which act at a distance during chemotropic interactions. Kind discrimination divides *N. crassa* populations into separate communication groups; only individuals with identical sets of *doc* genes (kind individuals) show

chemotropic growth and cell fusion. Germlings from different communication groups grow past each other, even when in close proximity, to find a partner of their own communication group. If non-kind germlings are in close proximity, MAK-2 oscillation reinforcement is repressed resulting in an inability of cells to establish chemotropic interactions (65). These findings show that cellular communication in fungi is even more complex than initially anticipated: Due to non-self recognition mechanisms acting at a distance, cells must not only avoid self-stimulation during chemotropic interactions, but also stimulation by non-kind individuals.

NON-SELF RECOGNITION MECHANISMS ACTING POST-FUSION

In spite of non-self recognition mechanisms that act pre-fusion, fusion between genetically different strains occurs frequently. Anastomosis between compatible genotypes results in formation of a viable heterokaryon, while anastomosis between incompatible genotypes triggers HI, resulting in a PCD reaction leading to death of the fusion cells (Figure 3). This outcome is widespread in fungi and represents the most probable event after heterokaryon formation. Heterokaryon (or Vegetative) incompatibility can be observed by the appearance of a demarcation line, which separates the genetically incompatible strains, termed ‘barrage’. One of the first accounts of this type of rejection of non-self in filamentous fungi was made in the early 20th century by the pioneering mycologist Dorothy M. Cayley on the plant pathogenic ascomycete *Diaporthe perniciosa* (66). After confronting mycelia of monosporic cultures that exhibited the barrage phenotype Caley noted that ‘*like must meet like and unlike shows aversion to unlike*’. HI reactions in nature, mostly on dead tree trunks, or on an agar plate under laboratory conditions, can be detected by assessing whether barrage lines form at interaction zones between genetically distinct individuals (Figure 4) (67). Microscopically, incompatible heterokaryotic

fusion cells are rapidly isolated from the rest of the mycelia by septal plugging (68, 69). Septal plugging in response to stress can be mediated by specialized peroxisome-derived organelles called Woronin bodies (70–73), although the role of the Woronin body in HI is unclear. However, a family of septal pore-associated (SPA) proteins and the fusion protein SO have been associated with septal plugging during HI in *N. crassa* (74, 75). The HI reaction leads to vacuolization of the fusion cells accompanied by cell wall thickening, lipid droplet accumulation, reactive oxygen species (ROS) production and intracellular *de novo* formation of septa (Figure 5) (70, 76). Furthermore, nuclear DNA degradation during cellular dismantling occurs, drawing parallels between fungal PCD and *apoptosis* in metazoans (77). The complete cellular lysis of an incompatible hyphal compartment can vary in duration, ranging from 20 minutes to more than 6 hours, depending on the genetic determinants triggering the reaction. Autophagy also plays a role in HI, although the autophagosomal process is not required for the execution of cell death (78, 79). It has been proposed that autophagy has a protective role during HI by limiting the spread of the PCD signals, similar to what has been reported in plant immune responses (80).

Genetic control and molecular characterization of heterokaryon incompatibility

Early efforts to understand the HI reaction led to genetic mapping of loci involved in its control for a small number of ascomycete species, so called *het* (heterokaryon) or *vic* (vegetative incompatibility) loci (81, 82). Genetic analyses revealed that the number of *het/vic* loci ranges from seven to twelve for different species in sampled populations. Non-self recognition in HI systems may be mediated by alternate alleles at a single *het* locus (allelic HI systems) or by alternate alleles at more than one *het* locus (non-allelic HI systems). Within the three species (*N. crassa*, *Podospora anserina* and *Cryphonectria parasitica*), which have served as model

organisms for studying HI, non-allelic HI systems predominate (Figure 6). Independent of the type of HI system, particular hallmarks of molecular evolution are shared between *het* loci. Foremost, alleles at a particular *het* locus are highly polymorphic in populations and are often found in hypervariable genomic regions (83). Second, alleles conferring alternate specificity are maintained in wild populations in nearly equal allelic frequencies, which is an indication of non-neutral balancing selection operating on these loci (83, 84). The observed signatures of balancing selection can be explained by the associated benefits of preserving incompatible alleles and are in accordance with the proposed adaptive value of HI. The underlying evolutionary process is an example of negative frequency-dependent selection in which the rare genotype is advantageous – strains carrying a less frequent *het* allele would potentially form a higher number of incompatible heterokaryons and therefore possess a fitness advantage by diminishing conspecific parasitism (85, 86). In some cases, the evolutionary pressure on HI determinants surpasses speciation events resulting in trans-species polymorphism in which closely related species share ancestral *het* alleles. Balancing selection and trans-species polymorphisms are described for other self/non-self defining loci such as the major histocompatibility complex (MHC) in vertebrates and the ‘S’ locus in flowering plants (87, 88).

In the next section we summarize the molecular details for the currently characterized *het* genes and HI systems of each of the three above-mentioned fungal models.

Neurospora crassa. *N. crassa* is a heterothallic ascomycete with a haploid cell cycle, initially used as a model organism to establish the bases of molecular biology and decipher metabolic pathways (89, 90). Classical genetics assays (complementation or dominance/recessiveness) with haploid monokaryotic strains carrying auxotrophic mutations revealed that heterokaryosis between strains of opposite mating types results in growth inhibition

(91). Incompatibility based on differences at the mating type locus is not exclusive to *N. crassa* and is found in several other unrelated species (92, 93). The mating type locus in *N. crassa* is composed of genes that confer mating identity (*mat A* or *mat a*) and are evolutionarily unrelated (termed idiomorphs) (94). The *mat-A1* and *mat-a1* genes of the *mat A* or *mat a* idiomorphs, respectively, encode for transcriptional regulators which trigger HI when co-expressed in heterokaryotic cells (34, 95). The role of mating of *N. crassa* *mat* locus is uncoupled with its HI function as demonstrated by strains carrying mutations in *mat-A1* or *mat-a1* that abolish HI, but that do not impact mating competency (96, 97). Additional genetic analysis for suppressors of mating type incompatibility led to the identification of a recessive mutation called *tol* (tolerant) (98). Strains carrying the *tol* mutation mate normally but do not induce the HI phenotype. TOL actively regulates mating type dependent HI, as shown by introgression experiments between *N. crassa* and the closely related pseudohomothallic species *Neurospora tetrasperma* (99, 100). The *tol* gene encodes for a putative 1011-amino-acid polypeptide with a HET domain and coiled-coil and leucine-rich repeat regions. Transcriptional repression of *tol* during the sexual reproductive phase has been postulated to allow opposite mating type nuclei to co-exist in the dikaryotic ascogenous hyphae (93).

Further investigations on heterokaryosis with nearly isogenic *N. crassa* strains or with the elegant use of strains containing partial chromosomal duplications revealed ten other *het* loci in *N. crassa* (81, 101, 102). Three of these *het* genes have been cloned and their products examined at molecular level. Two of the *het* genes, *het-c* and *het-6*, control HI in association with closely linked (adjacent) genes termed *pin-c* (partner in incompatibility of *het-c*) and *un-24*, respectively (81, 103, 104, 105). Non-allelic interactions between *het-6* and *un-24*, the latter encoding the large subunit of ribonucleotide reductase, are considered crucial for the HI phenotype in the *het-*

266 *6/un-24* system (106). Haplotypes of *het-c/pin-c* and *het-6/un-24* are under balancing selection,
267 show severe linkage disequilibrium – haplotypes are inherited as blocks – and exhibit trans-
268 species polymorphism (84, 107). The product of *het-c* is a glycine-rich cell wall protein and the
269 HI specificity of the allelic variants is determined by indels in a variable region (108, 109). The
270 *pin-c* gene encodes a protein that shares a region of similarity with the products of *het-6* and *tol*,
271 termed the HET domain (~150 aa). Genes encoding predicted HET domain proteins are
272 ubiquitous in ascomycete genomes (83). A HET domain protein is also encoded by the recently
273 characterized *het-e* locus (83), and co-expression of different *het-e* alleles triggers the HI
274 reaction. Proteins encoding a HET domain are a common thread in all molecularly characterized
275 HI systems in *Neurospora*, suggesting downstream similarities in PCD pathways. A combined
276 population genomics and evolutionary approach was used to identify fifteen additional candidate
277 *het* loci, all encoding proteins with a HET domain and which displayed at least two long-
278 diverged haplogroups (83). Additionally, deletion of a transcription factor, *vib-1*, suppresses the
279 HI reaction mediated by genetic differences at all molecularly characterized *het* loci in *N. crassa*
280 and was shown to be required for expression of some *het* genes encoding HET domain proteins
281 (110–112).

282 ***Podospora anserina*.** *P. anserina* is an ascomycete in the same order (Sordariales) as
283 *Neurospora* (68). Eight HI systems have been genetically defined in *P. anserina* and four have
284 been molecularly investigated (70, 82). To date, none of the characterized *het* loci in *P. anserina*
285 are orthologous to those described in *N. crassa*. As in *N. crassa*, three non-allelic HI systems
286 (*het-d/het-c*, *het-e/het-c* and *het-r/het-v*) depend on a gene encoding a HET domain protein (*het-*
287 *d*, *het-e* and *het-r*) (113, 114). The HET domain itself has been shown to play an essential role in
288 the induction of PCD in *P. anserina* (115). The HET domain is situated at the N-terminus of

these proteins next to a central nucleotide-binding NACHT domain and a C-terminal domain composed of WD40 repeats (116). The NACHT domain is associated with regulation of apoptosis and innate immunity in metazoans and mediates the oligomerization of proteins forming multimeric cell death platforms like the apoptosome (117, 118). The WD40 repeats form a doughnut-shaped beta-propeller structure where individual repeats of 40-42 amino acids, in beta-sheet conformation, revolve around a central axis and which can function in molecular sensing as protein scaffolds (119). APAF1, the human apoptotic factor assembling the apoptosome, has also a WD40 domain which binds to cytochrome *c*, thus regulating its apoptosis-inducing activity (120). The NACHT and WD40 domains have been shown to be functionally important in HET-E to trigger HI (121).

The co-expression of allelic variants of HET-D or HET-E with antagonistic variants of HET-C – a protein with a GLTP (glycolipid transfer protein) fold – induces a PCD reaction (122, 123). In this non-allelic incompatibility system, the *het-c* gene is not linked to *het-e* or to *het-d* (124). Eleven highly polymorphic *het-c* alleles are present in a population of 110 *P. anserina* strains, falling in seven distinct functional classes with polymorphic positions under positive diversifying selection (high ratio of non-synonymous over synonymous substitutions per codon) overlapping with positions defining allelic specificity (125). The allelic specificity of HET-D, HET-E and HET-R is defined by the variability of the WD40 domain (113). Exchange between the WD40 repeats between alleles from the same *het* locus and/or between different paralogous WD40 genes are hypothesized to aid in concerted evolution of alleles (126, 127). The current data supports a molecular model for *het-d/het-c* and *het-e/het-c* HI systems in which direct binding of antagonistic HET-C allelic variants by the WD40 domain of HET-D/HET-E triggers the PCD reaction (128).

One HI system in *P. anserina* that has been extensively studied involves the *het-s* locus, which encodes two functional alleles, *het-s* and *het-S* (129). Importantly, the *het-s* allele encodes a prion protein (the HET-s protein) that can exist as a soluble monomer, in a state termed [Het-s*], or as infectious aggregates (a prion state), termed [Het-s] (130, 131). The appearance of the [Het-s] prion state occurs sporadically at a low rate and converts the [Het-s*] state to the [Het-s] prion, so that strains of *het-s* genotype are exclusively prion-infected or prion-free. Only prion-infected [Het-s] strains trigger HI with [Het-S] strains (132). The HET-S protein is a pore-forming toxin that is activated upon interaction with the HET-s prion protein, resulting in death of the cell (133, 134). The two alleles *het-s* and *het-S* are under balancing selection and in a wild population of *P. anserina*, with 92% of the strains of *het-s* genotype being prion-infected, thus having the HI competent [Het-s] state (24). These observations underline the adaptive value of the allorecognition system in the wild – a conclusion supported in the same study by the correlated distribution of a deleterious plasmid with the frequency of the *het-s* and *het-S* genotypes in populations (24).

***Cryphonectria parasitica*.** Six *vic* loci control the incompatibility reaction in *C. parasitica*, an ascomycete causing the chestnut blight disease (135, 136). As in *Podospora*, HI in *Cryphonectria* is observed by the appearance of a barrage between incompatible strains. Moreover, a systematic exploration of the transmission of virulence-attenuating mycoviruses during heterokaryosis showed that genetic differences at five of the six *vic* loci reduced virus transmission between strains, suggesting fitness benefits associated with HI (31, 137). This approach makes *Cryphonectria* an exciting model to study the relationship between HI and its function as a barrier against mycovirus transmission. The six incompatibility systems in *Cryphonectria* are composed of closely linked polymorphic genes or idiomorphs and their

functional characterization indicates mostly non-allelic interactions (31). Three of the HI systems rely on proteins with HET domains and one other involves a protein with NACHT-WD40 architecture. As above, the molecularly characterized *vic* genes are not orthologous to *Podospora* or *Neurospora* molecularly characterized *het* loci. Recently, as a consequence of the molecular characterization of the *vic* genes, a super mycovirus donor strain has been engineered carrying multiple deactivated *vic* genes, that could potentially serve as a more potent biocontrol agent against larger sets of wild isolates that are capable of causing the chestnut blight disease (138).

HI determinants - at the molecular crossroad of allo- and xenorecognition

Experimental evidence supports the hypothesis that HI, an allorecognition process, has a beneficial role in nature, preventing conspecific parasitism and limiting harmful replicon propagation. However, similarities of some fungal HI-inducing determinants with plant and metazoan innate immunity factors, the latter mediating xenorecognition (non-self discrimination between different species), have prompted the idea that fungal allorecognition (HI) may originate from molecular networks with a primary role in heterospecific non-self recognition (139, 140). For example, HET-E (HET-D and HET-R), controlling HI in *P. anserina*, have a similar domain organization to APAF-1 and NLRC4 (NOD-like receptor; NLR), which are intracellular molecular sensors operating in plant and metazoan innate immunity (Figure 7) (141, 142). An extensive analysis of fungal NLR-like proteins – a protein superfamily that includes HET-E, HET-D and HET-R from *Podospora* and VIC4-2 from *Cryphonectria*, suggests a similar role in xenorecognition (143). Furthermore, fungal NLRs not directly involved in HI have been shown to regulate pore-forming toxins that function as *bona fide* HI-factors, like HET-S in *Podospora* (128, 144, 145). These data support the hypothesis regarding the relationship between allo- and

xenorecognition systems in fungi and put the HI phenomenon in a broader context of fungal biotic interactions.

CONCLUSIONS AND FUTURE OUTLOOK

Cell fusion is of capital importance for the fungal life style. It is not only related to mating but it is also important during vegetative growth, facilitating colony establishment, network formation and heterokaryon formation. Non-self recognition mechanisms that function pre- and post-fusion restrict the development of heterokaryons to cells that are more genetically similar. Genetic analyses has identified a large number of genes required for cell fusion, but many of these have not been placed in a cellular pathway, nor is it understood how the different cellular pathways are integrated to accomplish cell fusion events. Another major outstanding question is the relationship between the molecular mechanisms regulating cell fusion events versus those that regulate non-self recognition, both pre- and post-fusion. Unlike alleles at loci required for cell fusion, alleles at loci that function in non-self recognition often display features of balancing selection. The availability of complete fungal genome sequences on the population level will facilitate the identification and characterization of loci that display balancing selection and thus are presumptively involved in either xeno- or allorecognition.

As discussed above, the induction of PCD as a result of non-self recognition and HI seems to be conserved among species of filamentous fungi, even though the proteins used for non-self recognition can vary. However, with the exception of the *het-s/het-S* system of *P. anserina*, the link between the non-self recognition and the death reaction is still unclear for other HI systems. This raises the question as to how death is induced on a molecular level.

Identifying the signaling networks and exact mechanisms that control the PCD pathway will be in the center of interest for further research on fungal HI.

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Figure Legends

Fig. 1: Germling and hyphal fusion in *Neurospora crassa*.

(A - C) Germinating spores undergo mutual attraction and fusion (A: 0 min; B: 40 min; C: 80 min). (D) Consecutive fusion events result in network formation. (E, F) Hyphal branches fuse and form cross connections. (E: DIC; F: cell walls stained with calcofluor white). Asterisks in all images indicate fusion points. Adapted from (38).

Fig. 2: Working model of the molecular events governing germling and hyphal fusion.

The signal emitting cell releases the ligand in a pulse-like manner, probably by exocytosis. Binding of the signal molecules to their cognate receptors results in assembly and activation of the MAK-2 module at the plasma membrane. MAK-2 phosphorylates MOB-3 of the STRIPAK complex, thereby promoting nuclear entry of MAK-1. In the nucleus MAK-2 activates the transcription factor PP-1, which controls cell fusion factor encoding genes. Activation of MAK-2 involves ROS production by the NOX complex either upstream or downstream of the MAP kinase cascade. Adapted from (38).

Fig. 3: Heterokaryosis and its possible outcomes.

Genetically distinct individuals can undergo hyphal anastomosis. If there are no allelic specificity differences at *het* loci, a viable heterokaryon is established and nuclei (blue and brown dots) are exchanged. If allelic specificity is different between the two strains for any of the *het* loci, septal plugging isolates the heterokaryotic compartments and cell death occurs.

Fig. 4: Macroscopic visualization of vegetative incompatibility.

The heterokaryon (vegetative) incompatibility reaction is visualized by the occurrence of a demarcation line called ‘barrage’ that separates the incompatible strains. **A.** Evidence of barrage on wood (spalted wood) occurring in the wild. **B.** Barrage reaction (black arrows) between genetically incompatible *Podospora anserina* strains. Identical individuals fuse without inducing allorecognition PCD and do not form the barrage (white arrows).

Fig. 5: Microscopic visualization of programmed cell death during vegetative incompatibility.

A time course of compatible and incompatible hyphal fusion in *Neurospora crassa*. The programmed cell death reaction is followed by the fluorescent vital dye (membrane staining) FM4-64. **A.** Fusion between two *N. crassa* strains that have identical specificities at all *het* loci. Arrow shows fusion pore (p). Nuclei or large vacuoles (v) are transported through the pore with the cytoplasmic flow. **B.** Fusion between two *N. crassa* strains that differ in *het* specificity. Heterokaryotic cells are compartmentalized by septal plugs (solid arrow and insert). Permeabilization of the plasma membrane leads to increased cytoplasmic staining and vacuolization; vacuoles burst as cell death proceeds (*). Bar= 10 μ M. Adapted from (146).

Fig. 6: Incompatibility systems and genetically identified *het* (*vic*) loci in model filamentous ascomycete species.

Round-headed arrows connecting the *het/vic* genes indicate non-allelic HI systems and squared-headed arrows indicate allelic HI systems. Blue arrows indicate that the incompatibility reaction influences the distribution of mycoviruses that result in hypo-virulence in *C. parasitica*. Genes in red encode for proteins with a HET domain and boxed genes (loci) are still not identified molecularly.

Fig. 7: Domain organization of fungal and metazoan NLRs (NOD-like receptors) and NLR-like proteins.

The heterokaryon determinants HET-E (also HET-D and HET-R as paralogues of HET-E) and VIC4 present a typical NLR-like domain organization. NLRs have a tripartite domain organization with a central nucleotide-binding and oligomerization (NOD) domain, an N-terminal effector domain and a C-terminal sensor domain. The sensor domain can be composed of various repeated motifs (LRR or WD40, in the examples presented here) that trigger the activation of the receptors upon recognition of defined molecular cues. The recognition of the signal activates the formation by the receptors of multimeric protein platforms. The oligomerization of the receptors is mediated by the NOD domain (NACHT or NB-ARC type) in characterized cases, such as APAF1 (the human apoptosis-controlling factor) and NLRC4 (an innate immunity receptor). *Abbreviations:* CARD (Caspase Recruitment Domain); LRR (Leucine Rich Repeats).

Septal plugging/Vacuolization/Shrinkage of plasma membrane/Organelle degradation

Cell Death

mat-A
mat-a

het-e1

het-c

un-24

het-5

het-10

het-7

het-d

het-8

het-i

het-9

N. crassa

tol

het-e2

pin-c

het-6

het-s

het-c

het-v

het-b

het-S

het-e

het-d

het-r

het-q

het-z

P. anserina

vic1a

vic2

vic3a

vic4-1

vic6

vic7

vic1b

vic2a

vic3b

vic4-2

pix6

vic7a

C. parasitica