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An evolutionarily conserved mechanism underlies interspecies cell–cell signalling in fungi

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When a conidium (vegetative spore) or ascospore (sexually produced spore) of the filamentous fungus *Neurospora crassa* germinates, it produces a long narrow filamentous multinucleate cell called a hypha. Hyphae grow by elongation, they can branch, and the tips of branches can also rejoin by fusion. Growth, branching, and fusion create an interconnected web, called a mycelium, within which cytoplasmic continuity is maintained. Some researchers have focused their studies on hyphal elongation and branching, others on formation of conidia and ascospores, and still others, prominently Andre Fleißner, Nick Read, Louise Glass, and colleagues, on tip fusion. Each of these fundamental processes contributes to the development of species-characteristic mycelial morphology. Using the fluorescently tagged *Neurospora* proteins MAK-2 and SO, they made the startling discovery that when tips of freshly germinated and genetically identical conidia (germlings) came within 15 μm of each other, each tip took turns to send and receive a molecular signal in an oscillatory back-and-forth manner. When one tip accumulated dsRED-SO (or mCherry-SO) in its cortical membrane, the other accumulated MAK-2-GFP. After 3–5 minutes they switched, and the first accumulated MAK-2-GFP while the second accumulated dsRED-SO. Another 3–5 minutes later they switched again. Up to six switches were seen as the tips coordinated their hyphae to grow towards each other, made contact, and fused (Fleißner *et al.* 2009). With each switch the MAK-2 and SO proteins became more and more localized to the site of future tip contact and cell fusion. The nature of the diffusible signal and its receptor is still unknown. Mutation in the *mak-2* and *so* genes abolished this interaction and hence did not show cell fusion.

An engineered variant of MAK-2, called Q100G, can be inhibited by the ATP analogue 1NM-PP1. The inhibitor did not affect wild-type cells. Germlings bearing the variant allele (*mak-2*^{Q100G}) showed a mutant *mak-2* phenotype in the presence of inhibitor. In *mak-2*^{Q100G} and *mak-2-gfp* germling pairs, the *mak-2-gfp* cells showed normal oscillation of MAK-2-GFP in the absence of inhibitor, but this was abolished upon addition of inhibitor and there was no further inter-cell interaction. This implied that MAK-2 activity in one cell was required for MAK-2 recruitment to its partner's tip. Likewise, *mak-2Q100G* and *so-gfp* germling pairs revealed that MAK-2 activity was required for dispersion of SO from the partner's tip.

The following model for the chemical dialogue between the fungal tips was proposed. After SO accumulates in the signal-sending cell tip, a pulse of signal is released which reaches the partner cell, where it recruits and activates MAK-2 in its tip. The hyphal tip cytoplasm is enriched in secretory vesicles which are arranged in an aggregation called the Spitzenkörper or 'apical body'. The Spitzenkörper might specify that only the tip cells engage in the chemical dialogue. Active MAK-2 activates downstream targets for directed growth, and also delocalizes SO from the signal-receiving cell cortex. Possibly, SO delocalization enables the next signal pulse to be loaded. Inactivation of MAK-2 by phosphatases then delocalizes it from the signal-receiving cell's cortex, and it concomitantly accumulates SO. Thus, the signal-receiving cell now becomes a signal-sending cell and releases its signal pulse, which reaches the first cell and causes it to recruit and activate MAK-2. Such oscillation between

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signal sending and receiving directs the two tips to grow towards each other. In the absence of MAK-2, both cells remain in signal sending mode and chemotropic interaction is disrupted.

More recently, Fleißner, Reinhard Fischer, and colleagues fluorescently tagged the MAK-2 and SO homologues in the plant pathogenic fungus *Botrytis cinerea* (respectively, BMP-1 and BcPro40) and showed that in this species also similar oscillatory behaviour occurs in chemotropically interacting germlings – the protein is present, then it disappears, and still later it reappears (Hammadeh *et al.* 2022). Moreover, Δ*bcpro40* and Δ*bmp1* mutant *B. cinerea* strains did not show cell–cell fusion. These findings indicate evolutionary conservation of the inter-cell dialogue machinery. The *N. crassa*/*B. cinerea* evolutionary distance is comparable to that between humans and salmon. Tantalizingly, inter-species *Neurospora*/*Botrytis* cell pairs also engaged in the MAK-2/SO dialogue. However, contact between the tips was not followed by cell fusion, indicating that cross-species cell merger is prevented by post-contact checkpoints.

Hammadeh *et al.* (2022) further showed that the nematode-trapping fungus *Arthrobotrys flagrans* uses the same molecular dialogue to make a ring-like hyphal trap. For this, the tip of the leading hypha fuses with the tip of a trailing peg-like branch. The two tips originate from the same hypha with a distance of typically four hyphal compartments. In most filamentous fungi, including *A. flagrans*, the growing hyphae maintain cytoplasmic continuity. But the hyphae also contain septal pores which can be occluded by peroxisome-derived organelles called Woronin bodies (Riquelme and Sanchez-Leon 2014). Using fluorescently tagged Arthrobotrys MAK-2 and SO homologues (encoded by the *makB* and *sofT* genes, respectively), they showed that the molecular dialogue led to tip fusion. Gene knockout makB and sofT mutants did not make a proper trap. Septal pores must be closed by Woronin bodies to enable physiological switches between closely neighbouring hyphal compartments during trap formation. To test this, they created a *ΔhexA* mutant that lacked a major Woronin body protein and found that in the mutant, as in the *ΔsofT* and *ΔmakB* mutants, the ring failed to close. Thus, the cell dialogue mechanism for mycelial formation was repurposed to form the *A. flagrans* nematode trap.

Fleißner and colleagues reported that *Neurospora* germlings stained with filipin show that tip membranes are enriched for ergosterol, which prompted them to look at the cell dialogue and germling tip fusion in mutants defective in ergosterol biosynthesis (Weichert *et al.* 2016, 2020). The *N. crassa ergosterol* (*erg*) genes encode enzymes of the ergosterol biosynthesis pathway: *erg-1* encodes C-8 isomerase, *erg-2* encodes sterol C-24(28) reductase, *erg-3* encodes sterol C-14 reductase, and *erg-10a* and *erg-10b* encode individually redundant isomers of sterol C-5 desaturase. The *Δerg-1*, *Δerg-2*, and *Δerg-10a/Δerg-10b* mutant germlings showed a ∼30–90% drop in cell–cell fusion (i.e., failure to exchange cytoplasm). The *Δerg-2* mutant accumulated a precursor sterol with a conjugated double bond in its aliphatic side chain, whereas the precursor sterols accumulated in the *Δerg-10a/Δerg-10b* and *Δerg-1* mutants included some that lacked the C-5 desaturation. These studies were of interest to me because several years back Saswati Sengupta in my laboratory made the serendipitous discovery that *erg-1* and *erg-3* mutant ascospores made colonies with a distinctly different morphology on medium supplemented with sorbose compared with the wild type. The colony morphology difference enabled us to develop an assay for repeat-induced point mutation (RIP), a sexual-stage-specific mutational process of fungi (Noubissi *et al.* 2000). However, no one had studied a possible basis for the colony morphology difference until Weichert *et al.* (2016, 2020). How might the presence of sorbose affect the impact of these sterol changes?

Fleißner and colleagues, and other groups, will undoubtedly continue to try and understand how sterol alterations impact mycelial morphology. Moreover, the fact that evolutionarily distant fungi can respond to each other via the MAK-2/SO molecular dialogue will prompt researchers to explore new possibilities in cross-species fungal communication.

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