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Abstract

Most heterothallic basidiomycetes use small lipopeptide pheromones as part of mate recognition. *Schizophyllum commune* has scores of pheromones that must be specifically recognized by mating receptors. A correlation between a phenylalanine residue near the C-terminus of several pheromones and the ability of those pheromones to activate receptor Bar4 was recognized. We hypothesized that the phenylalanine residue would be critical for Bar4 activation and tested the hypothesis by making site-directed mutant pheromones and testing these pheromone variants in matings. The data support the hypothesis and add to our understanding of which amino acid residues within pheromones are critical for specific recognition by pheromone receptors.

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A carboxy-subterminal aromatic residue in *Schizophyllum commune* mating pheromones controls specific recognition by Bar4 receptor

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Most heterothallic basidiomycetes use small lipopeptide pheromones as part of mate recognition. *Schizophyllum commune* has scores of pheromones that must be specifically recognized by mating receptors. A correlation between a phenylalanine residue near the C-terminus of several pheromones and the ability of those pheromones to activate receptor Bar4 was recognized. We hypothesized that the phenylalanine residue would be critical for Bar4 activation and tested the hypothesis by making site-directed mutant pheromones and testing these pheromone variants in matings. The data support the hypothesis and add to our understanding of which amino acid residues within pheromones are critical for specific recognition by pheromone receptors.

Many of the Agaricomycotina fungi express quite a few mating pheromones and seven-transmembrane-domain pheromone receptors (most recently reviewed by Raudaskoski and Kothe, 2010). A large number of lipopeptide mating pheromones are coded species-wide by *Schizophyllum commune*, but any individual has genes for only a small subset of the species' estimated 80–100 pheromones. More than twenty of the genes that encode these pheromones have been cloned and sequenced (for list and references, see Table 3 in Fowler *et al*., 2004). One attempt at classifying these pheromones placed them into five groups according to similarity of the predicted mature pheromones' amino acid sequences (Fowler *et al*., 2004). The subsets of receptors activated by the pheromones follow a pattern that closely correlates with pheromone groups arranged by sequence similarity. Three pheromone groups (III, IV,V) arranged by similarity activate three completely distinct sets of receptors. The remaining two pheromone groups (I and II) also had corresponding receptors that were distinctly activated only by pheromones within their respective groups, with one exception: pheromone receptor Bar4 is activated by pheromone Bap3(1) from group I and by pheromones Bap3(3) and Bbp2(6) from group II (Table 1). Comparison of the amino acid sequences of group I and II pheromones showed that all five group I pheromones had tryptophan (W) in the carboxy-subterminal position except pheromone Bap3(1), which has phenylalanine (F) in the carboxy-subterminal position and can activate receptor Bar4. Both group II pheromones also have F in the carboxy-subterminal position. For all seven wild-type pheromones of groups I and II that have been characterized, pheromones with F in the carboxy-subterminal position can activate Bar4 and pheromones with W in that position cannot activate Bar4 (Fowler *et al*., 2004). We wondered if that single F residue could be the key to recognition as an activating ligand by Bar4. In other tests for critical amino acid residues within fungal lipopeptide mating pheromones, single amino acids have been crucial. One amino acid in a pheromone can determine activation or failure of activation of a receptor, or produce activity with a different receptor without losing the original pheromone activity (Olesnicky *et al.,* 2000; Fowler *et al.,* 2001). We are interested in the rules and patterns that govern pheromone and pheromone receptor interactions.

Table 1. Wild-type and mutant pheromone sequences and activities

Group	Name	Predicted Pheromone[1]	Receptors Activated[2]
		Wild-type pheromones	
I	Bap3(1)	ERVGTGGTATA**F**C	Bar2, **Bar4**, Bar5
II	Bap3(3)	ERHGSGNMTY**F**C	**Bar4**, Bar7, Bbr8
II	Bbp2(6)	EREGDGNMTY**F**C	**Bar4**, Bar7, Bbr8
I	Bap1(1)r	EREGGSDCTA**W**C	Bar2, Bar3, Bar5, Bar6
		Mutant pheromones	
I	Bap3(1)F54Y	ERVGTGGTATA**Y**C	Bar2, **Bar4**, Bar5
I	Bap3(1)F54W	ERVGTGGTATA**W**C	Bar2, Bar5
I	Bap1(1)rW30F	EREGGSDCTA**F**C	Bar2, Bar3, **Bar4**, Bar5

[1]All pheromones of *S. commune* are cleaved from larger precursors and are predicted to be carboxymethylated and farnesylated on the C-terminal cysteine residue (see review of Raudaskoski and Kothe, 2010).
[2]Receptors Bar1 though Bar9 were each tested individually. Tester strains in order from Bar1 to Bar9 were: V151-20, T26, V160-21, V147-1, V112-17, V123-29, V119-19, V142-3, V118-7. Addition Bar4 tester V131-5 was also used.

To test whether the subterminal F is a key residue for Bar4 activation by pheromones from groups I and II, site-directed changes in codons for the subterminal residues, codons 30 and 54, were made in two pheromone genes, *bap1(1)r* and *bap3(1)*, respectively, using the Quik-change oligonucleotide-based site-directed mutagenesis kit (Stratagene, La Jolla, CA; Kothe, 1999; Fowler *et al.,* 2004). Oligonucleotide primers used in this study are shown in Table 2. The plasmid templates for mutagenesis, containing a genomic copy

of a wild-type pheromone gene, were *pTF9045-12* [*bap3(1)*] and *pTF9073-2* [*bap1(1)r*] (Fowler *et al.,* 2004). Mutagenesis of the genes was confirmed by DNA sequencing across the mutagenized sites (University of Illinois Urbana-Champaign Core Sequencing Facility). Several new pheromones were produced from these altered genes. Each mutant pheromone has a single amino acid difference at the carboxy-subterminal amino acid position compared to its wild-type progenitor. A tryptophan auxotrophic strain of *S. commune* (V201-106, *trp1*$^{-}$*, ΔB-MAT*; FGSC#9350), which has no endogenous mating pheromone or receptor activity due to a large deletion in the *B-MAT* locus (Raper and Raper, 1973; Fowler *et al.*, 1998), was co-transformed with two plasmids, one containing the pheromone gene to be tested and the second containing wild-type *trp1* (*pRHV1*, Horton and Raper, 1995). Protoplasts were generated for transformation with Novozyme 234 and transformed by a PEG-mediated method (Specht *et al*.; 1988 Horton and Raper, 1991), using 20 ug of the pheromone gene plasmid and 10 ug of *pRHV1*. Co-transformants were identified through selection on CYM agar lacking tryptophan (Raper and Hoffman, 1974) followed by test matings with *S. commune* strains V160-21 (Bar3) and T26 (Bar2) to identify pheromone activation by the transformants. Those tryptophan prototrophic colonies that could convert either test mate to a dikaryon, indicating they were producing a pheromone, were saved and used in further matings with additional tester strains that represented each of the nine Bars (***B***-*MATα* **r**eceptors), including Bar4 (Fowler *et al.*, 2004). Table 1 reports the sequences and activities of the wild-type and mutant pheromones. A minimum of three independent transformants of each pheromone gene was tested.

The data in Table 1 support the hypothesis that F plays a critical positive role for activation of Bar4. The mutant pheromone Bap3(1)F54Y also extends the range of possible residues in the subterminal position by showing that Bar4 responds if tyrosine (Y) is in the carboxy-subterminal position. However, like four previously characterized wild-type group I pheromones including Bap1(1)r (Fowler *et al*., 2004), if a mutant Bap3(1) has a subterminal tryptophan [Bap3(1)F54W], then Bar4 is not activated. In a second test of the effectiveness of pheromones with subterminal F to initiate signaling through Bar4, Bap1(1)r was altered from a wild-type pheromone that has W in the subterminal position and does not stimulate Bar4 to the mutant Bap1(1)rW30F that can activate Bar4. Additionally, we note that Bap1(1)r is affected by the mutational change with regard to Bar6, but in an opposite manner to Bar4. Bar6 activation appears dependent on W in the carboxy-subterminal position of Bap1(1)r and does not tolerate F in that position [Bap1(1)rW30F]. In no case did altering a pheromone to a different aromatic residue at the carboxy-subterminal position result in a completely inactive pheromone.

Extrapolating from the activity range of characterized wild-type pheromones and the interfertility of *S. commune*, each version of *B-MAT* must produce a suite of pheromones that collectively can activate all non-self mating receptors, including some pheromones with partially redundant activities. This arrangement maintains a high potential for outbreeding in the species. In this mutagenesis study, we have identified the functional importance of aromatic residues in the carboxy-subterminal position of pheromones in groups I and II. The gene for receptor Bar4 has not yet been isolated. It will be interesting to determine how similar Bar4 is to the pheromone receptors that recognize either group I or group II pheromones, but not both.

Table 2. Oligonucleotides used for site-directed mutagenesis

Name	Oligonucleotide sequence[1]	Mutant pheromone produced
060905-1	GGTACTGCGACCGCC**TGG**TGCGTTGTCGCATGAG	Bap3(1)F54W
060905-2	CTCATGCGACAACGCACCAGGCGGTCGCAGTACC	Bap3(1)F54W
060905-3	GGTACTGCGACCGCC**TAC**TGCGTTGTCGCATGAG	Bap3(1)F54Y
060912-1	CTCATGCGACAACGCAGTAGGCGGTCGCAGTACC	Bap3(1)F54Y
060905-5	CTCTGACTGCACGGCG**TTT**TGTGTGGTGGCGTAG	Bap1(1)rW30F
060905-6	CTACGCCACCACACAAAACGCCGTGCAGTCAGAG	Bap1(1)rW30F

[1]Codon change is shown in bold. Primers are used in consecutive pairs.

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