

# A Large Pheromone and Receptor Gene Complex Determines Multiple *B* Mating Type Specificities in *Coprinus cinereus*

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## ABSTRACT

Pheromone signaling plays an essential role in the mating and sexual development of mushroom fungi. Multiallelic genes encoding the peptide pheromones and their cognate 7-transmembrane helix (7-TM) receptors are sequestered in the *B* mating type locus. Here we describe the isolation of the *B6* mating type locus of *Coprinus cinereus*. DNA sequencing and transformation analysis identified nine genes encoding three 7-TM receptors and six peptide pheromone precursors embedded within 17 kb of mating type-specific sequence. The arrangement of the nine genes suggests that there may be three functionally independent subfamilies of genes each comprising two pheromone genes and one receptor gene. None of the nine *B6* genes showed detectable homology to corresponding *B* gene sequences in the genomic DNA from a *B3* strain, and each of the *B6* genes independently alter *B* mating specificity when introduced into a *B3* host strain. However, only genes in two of the *B6* groups were able to activate *B*-regulated development in a *B42* host. Southern blot analysis showed that these genes failed to cross-hybridize to corresponding genes in the *B42* host, whereas the three genes of the third subfamily, which could not activate development in the *B42* host, did cross-hybridize. We conclude that cross-hybridization identifies the same alleles of a particular subfamily of genes in different *B* loci and that *B6* and *B42* share alleles of one subfamily. There are an estimated 79 *B* mating specificities: we suggest that it is the different allele combinations of gene subfamilies that generate these large numbers.

**M**ATING is an essential step in the life cycle of the mushroom *Coprinus cinereus* and converts an asexual monokaryotic mycelium with uninucleate cells into a fertile binucleate-celled dikaryotic mycelium on which the characteristic mushroom fruit bodies develop (Figure 1). Somatic cell fusion is sufficient for mating. Fusion is followed by an exchange of nuclei between mates and rapid migration of donor nuclei through the established cells of each recipient, a process that necessitates the disruption of the complex septa that normally separate cells (Giesy and Day 1965). When a migrating nucleus reaches a tip cell, growth of the dikaryon is initiated and all subsequent cell divisions result in the formation of a structure known as the clamp connection. The clamp connection ensures that each cell of the vegetative dikaryon contains one nucleus from each mate until nuclear fusion occurs at a late stage in fruit body differentiation (Casselton 1978).

Two sets of mating type genes, *A* and *B*, control mating and do so by regulating the formation and maintenance of the dikaryon (Swiezynski and Day 1960). The initial

migration of donor nuclei is triggered when mates possess different *B* genes (Figure 1B). Different *A* genes promote the formation of a clamp cell and synchronized division of the two tip cell nuclei (Figure 1C), which results in the formation of a binucleate tip cell and uninucleate clamp and subterminal cells. *B* gene compatibility is again required for fusion of the clamp cell to the subterminal cell to complete the clamp connection and permit the clamp cell nucleus to pass into this cell. Remarkably, the mating type genes of both *A* and *B* are multiallelic. For *C. cinereus* there are an estimated 160 versions of the *A* locus and 79 of the *B* locus (Raper 1966) giving a total of more than 12,000 mating specificities. Of particular interest at the molecular level is how such large numbers of *A* and *B* mating specificities are generated and how so many different versions of the mating type gene products can be distinguished yet promote the identical pathway of sexual development.

By cloning five *A* loci of *C. cinereus*, we have shown that multiple *A* specificities are derived from different combinations of three pairs of multiallelic and functionally redundant genes (Pardo *et al.* 1996; Kües *et al.* 1994). Like the  $\alpha 1$  and  $\alpha 2$  mating type proteins of the budding yeast *Saccharomyces cerevisiae* (Herskowitz 1988; Dolan and Fields 1991), these encode two homeodomain-containing subunits of a heterodimeric regulatory protein.

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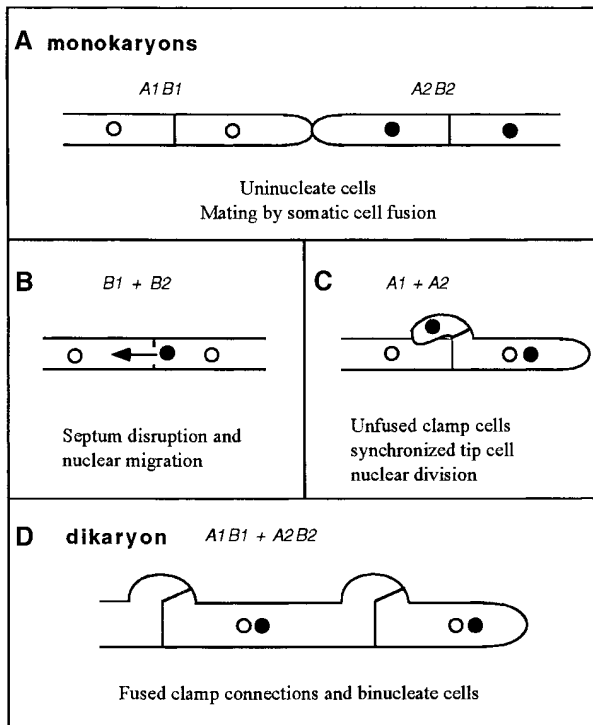


Figure 1.—A and B mating type gene regulation of sexual development. (a) Monokaryons with uninucleate cells mate by hyphal fusion. (b) Different B genes promote nuclear migration and septal breakdown. (c) Different A genes promote synchronized nuclear division and clamp cell development. (d) Different A and B genes act to maintain binucleate cells in the dikaryon by formation of clamp connections; different B genes promote fusion of the clamp cells induced by different A genes.

The B mating type genes of the mushroom fungi, as first shown for *Schizophyllum commune*, encode pheromone precursors and 7-transmembrane helix (7-TM) receptors (Wendland *et al.* 1995; Vaillancourt *et al.* 1997). Signaling by means of peptide pheromones also plays an essential role in mating of the ascomycetous yeasts (Kurjan 1993) and hemibasidiomycetes such as the plant pathogenic *Ustilago spp.* (Bölker *et al.* 1992; Bakkeren and Kronstad 1994; Bölker and Kahmann 1993). These fungi have just two versions of the pheromones and receptors produced by cells of different mating types, and the pheromones are secreted to act as chemoattractants. Pheromone binds to receptors on cells of the opposite mating type and activates a signal transduction cascade that induces several cellular changes that bring about mating competence including the formation of mating projections, which are essential for cell fusion (Kurjan 1993). The mushroom fungi are remarkable in that the pheromone response is initiated only after mating cells have fused and also in having several functionally redundant and multiallelic genes encoding the pheromone and receptor molecules.

In this report, we describe for the first time the organization of a B mating type locus of *C. cinereus*. The locus

contains a large complex of genes encoding six pheromone precursors and three receptors. Our analysis permits us to suggest that the estimated 79 versions of this locus are derived from three sets of multiallelic and functionally redundant genes.

## MATERIALS AND METHODS

**Fungal strains and growth conditions:** *C. cinereus* strains used in this study were: A6B6: H9 wild type, LT2 *trp-1.1.1.6*; A5B6: BM5 *ade-5*, H5 wild type, FA2222 *trp-1.1.1.6*; A6B3: PR94226 *ade-5 cho-1*; A2B3: LCO12 *trp-3*; A6B42: PG78 *pab-1 trp-1.1.1.6*; A42B42: LN118 *trp-1.1.1.6 ade-2*. Media and methods for culturing *C. cinereus* were described by Lewis (1961), with modifications summarized by Mutasa *et al.* (1990). Hosts for transformation were LCO12 and LN118. B6 genes were introduced by cotransformation with plasmids pCc1001 or pDB1 containing the *C. cinereus trp-1* (Binniger *et al.* 1987) or *trp-3* gene (Burrows 1991), respectively. Transformation was performed as described by Casselton and De La Fuente Herce (1989). Routinely, 50 transformants were tested for the expression of an introduced B gene. Frequency of cotransformation was variable, but a positive mating reaction was always detected in 12–50% of transformants.

**Genomic subtraction:** This was based on the method of Kunkel *et al.* (1985) as modified by Moore and Edman (1993). 100 µg high molecular weight DNA from strain 94226 (A6B3) was sonicated in an MSE Soniprep 150 (Sanyo Gallencamp, Leicester, UK) to give fragments of 1.0 kb in size. DNA (5 µg) from strain H9 (A6B6) was digested to completion with *Mbo*I and *Sau*3A1. EDTA (6 µl, 0.5 M) and 3 µl 10 mg/ml proteinase K (Sigma, St. Louis) were added to 60 µl digestion mix followed by incubation at 65° for 30 min. DNA was precipitated and resuspended in 15 µl water to final concentration of 50 ng/µl. Sonicated DNA (25 µg) was mixed with 0.12 µg digested DNA, heated to 100° for 5 min, cooled on ice and added to a final reaction containing 7% phenol equilibrated to pH 7.7 with 0.1 M Tris/HCl, 1.25 M sodium perchlorate and 120 mM sodium phosphate, pH 6.8 made up to 250 µl with water. The reassociation mixture was vortexed using an IKA VXR (Camlab, Cambridge, UK) set at 1800 oscillations per min for 36 hr. The mixture was extracted twice with chloroform, dialyzed for 6 hr against TE (10 mM Tris/HCl pH 7.5, 1 mM EDTA), precipitated and resuspended in 10 µl TE. Reassociated DNA [250 ng (2 µl)] was mixed with 25 ng *Bam*HI cut and dephosphorylated pUC18 (Pharmacia, Piscataway, NJ) and 2 units of T4 DNA ligase (Gibco/BRL, Life Technologies, Paisley, Scotland) and incubated at room temperature for 30 min followed by 2 days at 15°. Ligation mix (2 µl) was electroporated into 20 µl high efficiency electroporation competent *Escherichia coli* DH5α cells.

**DNA procedures:** High molecular weight *C. cinereus* DNA was isolated as described by Mellon *et al.* (1987). For Southern analysis the small scale method of Zolan and Pukkila (1986) was used. Southern blot analysis was carried out according to the method of Mellon *et al.* (1987). For DNA sequencing large overlapping fragments covering the entire unique sequence together with 7 kb of flanking sequence to the left and 3 kb to the right were used. To generate clones for sequencing, 3 µg DNA was digested with DNase I (BRL, Life Technologies) diluted to 0.1 units/µl for 90 to 105 sec. The entire digestion was electrophoresed on a 0.8% gel at 45 V/hr. Fragments (0.5–1.0 kb) were band eluted and ligated into *Sma*I cut and dephosphorylated pUC18 (Pharmacia). DNA sequence was obtained using the PRISM™ Ready Reaction DyeDeoxy™ Terminator Cycle Sequencing Kit (Perkins Elmer, Norwalk, CT)

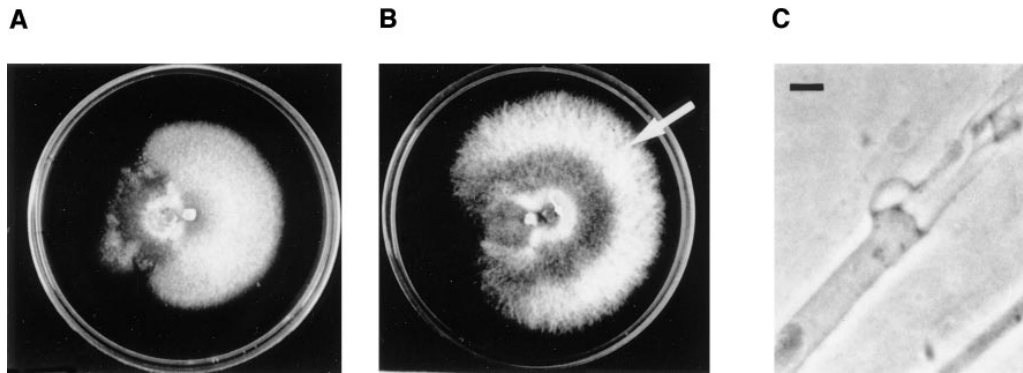


Figure 2.—Change in mating specificity in hosts transformed with *B* mating type genes. (a) Incompatible cross between an untransformed *A2B3* host and *A6B3* tester strain (at right) and an *A6B3* tester strain (at left) in which both strains remain monokaryotic. (b) Compatible cross between an *A2B3* strain transformed with either a *B6* receptor gene or a *B6* pheromone gene, showing dikaryotization (arrowed at right) and an *A6B3* tester strain, remaining monokaryotic (at left). (c) Fused clamp connection present on the dikaryon. Scale bar represents 5  $\mu$ m.

for analysis on an ABI 373 (Perkins Elmer, Norwalk, CT) automated sequencer. Sequence analysis was performed using the Gene Jockey II Sequence Processor, (© P. L. Taylor, 1996) Biosoft, Cambridge, UK. Prediction of the positions of the transmembrane helices in the receptors was based on homology to the two pheromone receptors from *U. maydis* (Bölker *et al.* 1992) and based on predictions from the three programs: Secondary structure prediction of membrane proteins (<http://www.tuat.ac.jp/~mitaku/sosui/>); TMPred ([http://ulrec3.unil.ch/software/TMPRED\\_form.html](http://ulrec3.unil.ch/software/TMPRED_form.html)); DAS - Transmembrane Prediction server (<http://www.biokemi.su.se/~server/DAS/>).

**Plasmids and PCR strategies:** The genomic library from which *B6* was recovered was constructed in the *C. cinereus* cosmid vector pLLC5200 (Pukkila and Casselton 1991) using DNA from strain BM5 (Pardo *et al.* 1996). Routine cloning was in pBluescript (Stratagene, La Jolla, CA) or pUC18 (Promega, Madison, WI) and plasmid amplification was in *E. coli* strain XL-1 Blue or DH5 $\alpha$ . Plasmids containing *B6* genes used for transformation are detailed in Table 2. Five of the genes, those for the three receptors and *phb1.2* and *phb2.1*, were amplified by the polymerase chain reaction (PCR). All three *B6* receptor genes were amplified from genomic DNAs of wild-type *B6* strains and either used directly for transformation or subcloned into pGEM-T (Promega). Primer 1 to receptor *rch2* had a *Hind*III site and primer 1 to *rch3* had a *Bam*HI site introduced at the 5' end for subcloning purposes.

Primers were as follows: *rch1*, primer 1: 5'-CCCCGACGGCC TTGACTGTAGC-3', primer 2: 5'-CTCGCTCTGCTCCCGG ACC-3'; *rch2*, primer 1: 5'-AAGCTTGGGGCGGACGATGCG-3', primer 2: 5'-AAGCTTAGTAAGAGGACATGAGTCCC-3'; *rch3*, primer 1: 5'-GGATCCTTGGACGGGAAGAGGACGG-3', primer 2: 5'-CCCGCGTTTCTTGGAGCCG-3'; *phb2.1*, primer 1: 5'-GGCCAAGGAGAATCAGCGACG-3' primer 2: 5'-GGCCAAGGAGAATCAGCGACG-3'; *phb1.2*, primer 1: 5'-CGT TCTGCCATGGTGCAG-3', primer 2: 5'-CGGCGATGGCGC ACAGC-3'. cDNAs for the receptor genes were obtained by RT-PCR using the Access RT-PCR Kit (Promega) and the following primers designed to the 5' and 3' ends of the genes: *rch3*, primer 1: 5'-AAGCTCAGTTTTCGACGACCTTC-3', primer 2: 5'-GCTGGGATTGCGTGTGAGTTTTTCAG-3'; *rch2*, primer 1: 5'-GCTCCCTCTCAGGACCAACG-3', primer 2: 5'-GGGAAAGCGTTCGGTCTGTTGA-3'; *rch1*, primer 1: 5'-AAGTACCAGCTCTCCCGGTGTTG-3', primer 2: 5'-GACA GCTACTTAACGGAAGCTGAGT-3'. RNA for RT-PCR was extracted from an LT2 strain transformed with a cosmid clone

containing the *B* genes isolated from a *B42* strain (J. R. Hallsall and L. A. Casselton, unpublished data). The strain was grown in rotary shake culture in liquid medium for 36 hr, harvested by filtering and frozen in liquid nitrogen. Total RNA was isolated using the hot-phenol procedure and precipitated with lithium chloride (Schuren *et al.* 1993). PCR was used to mutate pheromone genes. The cysteine to arginine change in the CaaX sequence was affected by a T to C substitution in both *phb2.1* and *phb2.2*. These changes were affected by inverse PCR using the primers 5'-GGGGGCTTCCGCGTTA TCG-3' and 5'-AGGAGTGTCGCCAGGTCC-3' (*phb2.1*), and 5'-TACGCCTGGCGGTTATCT-3' and 5'-AGCTTTGTTCCG ATTGTAGCG-3' (*phb2.2*).

## RESULTS

**Isolation of the *B6* locus:** The *B6* locus of *C. cinereus* was isolated by the genomic subtraction technique described by Moore and Edman (1993). Based on our previous analysis of the multiallelic *A* genes, which differ significantly in DNA sequence (Pardo *et al.* 1996), we predicted that different *B* alleles would fail to hybridize to one another. Our strategy, therefore, was designed to isolate small clonable sequences unique to a *B6* strain, H9 (*A6B6*), using reassociation with excess sheared DNA from a *B3* strain PR94226 (*A6B3*) to subtract sequences common to both genomes. Following the subtraction and cloning of residual fragments, 23 plasmids were recovered in *E. coli* that contained 200–500-bp inserts of genomic DNA derived from the *B6* strain. Southern hybridization analyses showed that only two of these sequences were unique to the *B6* genome.

One of the fragments was chosen to probe a cosmid genomic library constructed with DNA from a *B6* strain. Five overlapping clones were isolated initially and each tested for *B* gene activity by transformation into an *A2B3* host strain (LCO12). Transforming DNA predominantly integrates ectopically in *C. cinereus* (Binniger *et al.* 1987) so introduction of a second compatible *B* mating type gene would alter rather than replace the

**TABLE 1**  
Genetic analysis to demonstrate that a unique DNA sequence cosegregates with *B6*

	<i>B6</i>		<i>B3</i>		% recombination
	+	-	+	-	
<i>ade-5</i>	20	3	3	21	12.7
<i>cho-1</i>	18	5	7	17	25.5
DNA marker	47	0	0	47	0

Cross: *A6B3ade-5 cho-1* (PR94226) × *A5B6ade-5<sup>+</sup> cho-1<sup>+</sup>* (H5). The 47 sexual spore progeny were scored for genotype and for the presence of a 2.8-kb *EcoRI* fragment present in PR94226 but not H5 genomic DNA. + = *ade-5<sup>+</sup>/cho-1<sup>+</sup>*/presence of 2.8-kb fragment. - = *ade-5/cho-1*/absence of 2.8-kb fragment.

endogenous mating specificity of the transformed strain. Heterozygosity for the *B* locus does not confer a morphologically recognizable phenotype in *C. cinereus*, so to test for *B* gene activity it was necessary to demonstrate a change in mating behavior in a mating test assay.

Transformants were crossed to a tester strain having different *A* genes but the same *B3* specificity (PR94226, *A6B3*). The common *B3* genes would normally not permit dikaryon formation, but introduction of a *B6* gene should provide the necessary compatible *B* gene interaction to permit dikaryosis. Four of the five clones tested caused a change in *B* mating specificity in the *B3* host (Figure 2). Matings were initiated by placing inocula of each strain side by side on an agar plate. The untransformed host was unable to form a dikaryon with the *A6B3* tester strain and the two monokaryons grew alongside each other but remained discrete (Figure 2A). In transformants containing *B6* sequences, nuclei from the tester strain migrated through the cells of the transformant strain to convert it to a fluffy dikaryotic mycelium (Figure 2B, arrowed). Thus, *B*-regulated nuclear migration occurred, albeit in one direction only (unilaterally). Microscopic observation of the dikaryon confirmed that the second *B*-regulated function, clamp cell fusion, had also occurred (Figure 2C).

The five clones initially isolated covered a 17-kb unique sequence present in the *B6* genomic DNA together with some 30 kb of homologous flanking sequence at one border and 2 kb of homologous sequence at the other border. A sixth clone was isolated which provided a further 20 kb of homologous flanking sequence at the other border. The overlapping regions of three clones, cSO1, cSO2 and cSO3, used for subsequent analysis are shown above the physical map presented in Figure 4.

Additional evidence that the unique sequence isolated was from the *B* locus was obtained from progeny analysis of a genetic cross between a *B3* (PR94226) and

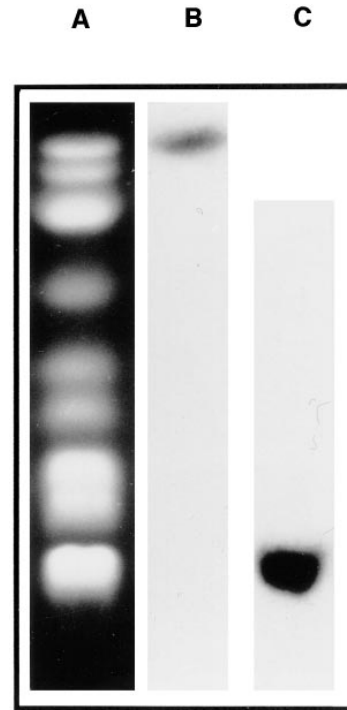


Figure 3.—Assignment of *A* and *B* genes to pulse-field gel separated chromosomes of *C. cinereus*. Gel shows chromosome separation of *C. cinereus* strain FA2222 (A). The *A5* genes were shown to hybridize to the largest 5.1-Mb chromosome (B), and the *B6*-linked sequence to one of the two smallest chromosomes of approximately 2.2 Mb (C).

a *B6* (H5) strain. The *B* locus is known to map to linkage group II and is 15.75 and 28.0 map units, respectively, from the metabolic markers *ade-5* and *cho-1* (Day and Anderson 1961). A 2.8-kb *EcoRI* fragment from cSO2 that did not hybridize to genomic DNA of the *B3* parent strain was shown to cosegregate with *B6* and to have the predicted linkage to *ade-5* and *cho-1* (Table 1).

The Southern blots were used to check the other unique sequence recovered from the genomic subtraction. This did not segregate with mating type and was not studied further.

*C. cinereus* has 13 chromosomes that can be separated by pulse field gel electrophoresis (Zolán *et al.* 1992), although the size of the chromosomes differs slightly between strains. Figure 3 (lane A) permits us to distinguish most of the chromosomes of strain FA2222. The *A* mating type genes map to genetic linkage group I, and this has previously been shown by hybridization to be present on a large 5.1-Mb chromosome (May *et al.* 1991). This is illustrated in lane B which shows the result of probing the filter with a gene from the *A5* complex. The *B* locus maps to genetic linkage group II. The unique sequence containing *B6* gene activity identified this as being present on one of the two smallest chromosomes of ~2.2 Mb (lane C). Interestingly, the *A* and *B* genes of *S. commune* are also present in the largest and



ses to be homologous in genomic DNAs from nine strains, all of which have different mating specificities. Cosmid clones covering some 20–30 kb of sequence either side of the unique *B6* region contained no other unique *B*-specific sequences.

The six small open reading frames found within *B6* encode polypeptides of 53–72 amino acids in length and each contains the C-terminal CaaX motif CVIA or CVIS. The predicted protein sequences are given in Figure 5. There is little sequence similarity between the six proteins other than the CaaX box, five residues 14–20 amino acids from the C terminus and a further five at the N terminus. Maturation of a fungal pheromone involves the addition of a farnesyl group to the cysteine residue of the CaaX box, cleavage of the terminal three amino acids and truncation of the N-terminal sequences to generate a short active peptide of 9–15 residues (Caldwell *et al.* 1995). In the *C. cinereus* proteins, there are two highly conserved amino acids 14–16 residues from the C terminus (ER/DR indicated by † in Figure 5). If this represents a proteolytic cleavage site in pheromone maturation, peptides of 11–13 amino acids would be generated, a size in accordance with those from other fungal species (Bölker and Kahmann 1993; Caldwell *et al.* 1995).

We identified the three genes encoding 7-TM receptors using a BLAST search. The N-terminal sequence of the proteins in each case showed significant homology to the Ste3p  $\alpha$ -pheromone receptor of *S. cerevisiae* (Nakayama *et al.* 1985; Hagen *et al.* 1986), receptors encoded by the *pra1* and *pra2* mating type genes of *U. maydis* (Bölker *et al.* 1992), and the receptors encoded by the *bar1* and *bbr1* genes of *S. commune* (Wendl and *et al.* 1995; Vaillancourt *et al.* 1997), all of which recognize CaaX-modified pheromones. The three *C. cinereus* genes contain either four or five introns and the coding regions were derived by comparing genomic with cDNA sequences. An alignment of the three receptor sequences with putative positions of the seven transmembrane domains is presented in Figure 6. The three proteins vary in length; *rcb1* is 558, *rcb2* is 518 and *rcb3* is 423 amino acids. The major conservation in sequence occurs throughout the 300 amino acids of the transmembrane domains. The C-terminal domains are highly variable in length and sequence, retaining only 16 conserved residues between all three proteins.

**Transformation studies demonstrate that all nine *B6* genes can activate *B*-regulated development:** All nine genes identified by sequence analysis were tested for *B6* function in the *B3* host strain (Table 2). When no suitable restriction sites were available, we used PCR to amplify just a single gene including 5' and 3' flanking sequences sufficient for ectopic expression. We introduced each gene individually into a *B3* host and determined its ability to change the mating specificity of the host, using the test illustrated in Figure 2. All nine genes permitted the *B3* host to mate with a *B3* tester strain

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M-----KY PALPVFAILG ALLVLIPLPW HWRARNVATL SIIAWLFVMN
M-APSHDPTY PLFPILFSLG FILSAIPFWS HLQAWNSGTC AFMIWTGLAC
MKLSFDPPSY PAFPIFAFIG FILVLIPLPW HLQAWNSGTC LFMIIWTAIGC
* c c* * * c*c*ccc* c* **cc* * c*c* c* cc * c
-----I-----

VIYAVNTIYW AGSIRDVAPV YCDIATKLIH GASHALPLAT LCICKHLEMV
LNGFINSLWV RGNSENPAPV WCDISSKLIH GVSIGIPAAT LCISRRLLAL
LNLFINSDIW HCNADWAPI WCDISTRILV GISVAIPAA S LCINRRLYKI
c c*ccc* *c c **c ***ccc**c * c*c** *c *** cc* c
-----III-----

SSSRVTSYDV SDKRRRMIFE GVMCFVLPMI FMAHYIVQGG HRYDIIQIEFG
TSVSTVSVTR ADKRRMVIWD LCISFGIPAI IMVLYHYVQGG HRFDILEDVGG
ASCQTATISR AQKRRAVMID LAIGLGFPL QMLQFIVQGG HRYDIFQDVG
c* * c cc*c* cc c c c c* c * *ccc*** **c**ccc *
-----IV-----

CQPTIYISIP AIFIVWFPP LFAVISFILA AMALHFFVRR RLVLAAHLQN
CYPVYNTL AYFLYIAWPV ILGIFSPVFS CLTLRSEFWR RAQF-SQLVT
CYPTIVNTTP AYPLVFFWPP IICLVSAVYC ILLTRAFMKR RAQF-GQLIS
* * c * c * * cc c * cc cc*c* * * * c cc*
-----V-----

ANSALTPNRY IRLIAMALTI MIWNTSLTAF NLYNNVFPFG- LRPWINWADV
SNSSMSMSRY LRLMILALVD IVCTVPLSAY QLYNLGTHGVP LHPWWSWEET
SNNSLTVHRY FRMLSLATLE LVLHLPIHTTY GLYLCTFSRSP IYPTWSWSDI
c*cccc ** c**c c* c ccccc c* * c ** c* c
-----VI-----

HSNFSRVDLF PTVFLPD--Y FIRAMMLFWW AMPVSSLIFF LFFGFGEEAM
HYGFSRVDRI PALFWRSSST YVVGVELARW LRVLCAPLFF ALFGFASEAQ
HHNWIYIDTF PRVLWGHYDV QVVTELELRW ALVFCAPLFF GFFGFAQEAR
* * c * cc c cc * c* ccc** c**c **
-----VII-----

KEYRKVGAWI SRVILRRKTN EKGELLGSTG NSRRRLHLVD LKNKNASMIS
KQYKRAYWAI VKPF----- --GIEPHSAK
KQYRKVFLPI AKPF----- --GYNPSSN
*c*ccc * c c cc
-----VIII-----

KPIPGSFCKT SQSPSATVAG SPPPYKKSFD VVSPSTSTNC STVVNANSPL
QPIKVALPSW VKSANLDSTL SPKPHMRQHK QHVSLASTAN ASVDIASTVV
EK-KNTFVHV GKPPSI-PTF TPRP----- LH--LAST-- PTLNDKSTSF
c *c cc cccc c c* * c c cc cc cc
-----IX-----

KKANEIDLEA GDDFSYQAQS NASTATQLAP FAMVERSSST RSSGPIYSEF
----- GSDLEKSACR SSFSSIRSAP PTYSPSPVPHH VALDVLDIQR
----- TRTSLSSNS SGGTKVACSP P----- AFRLDDIKV
c c cc c c* *c c c c
-----X-----

IPSPTLSYCS ILSPSTAPST SAFSPSSFPE SVGPDLSNRA SEMDLVESYY
SLFPSSPLSS DSTVFCPEP REKLPEPYTY SLPKSOPTRT SF-----
KLSSSPGTVS DFP----- SPTSMTTRF DD-----
c * c * * cc * *
-----XI-----

EMQRDIRREE TRRTHLRGPA SPAYHRFPSP TLYPVALPHP GSALPPVNGI
-----ERS NPSSPLSPSN HFGFHRFPSP PTIYPTTIAH PSAVAVDVI
-----FDS NAHSKV-----
c c c *cc***** c c ** c* *
-----XII-----

LVTVHRQASV DELPSTHS-- --- 558 B6 rcb1
RATIRPLSQA SDNLMFQQR TMV 518 B6 rcb2
----- 423 B6 rcb3
c*cc c c c

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Figure 6.—Alignment of the three *B6* receptor sequences. Wild-type amino acid sequences of receptor proteins. The predicted seven transmembrane domains (labeled I–VII) are shown by lines under the sequence. \* indicates identical amino acids, c indicates conserved amino acid substitutions. The percent similarity/identity in sequence for *rcb1* and *rcb2* is 53/32, for *rcb1* and *rcb3* is 59/35 and for *rcb2* and *rcb3* is 65/47.

and were thus active in conferring mating functions. Thus a single pheromone or receptor gene was sufficient to trigger the change in mating response in a compatible host.

In a second series of transformation experiments we introduced all nine *B6* genes into a *B42* host (LN118). In this case six of the genes, members of groups 2 and

TABLE 2  
Functional analysis of *B6* mating type genes by transformation and mating tests

Plasmids	Gene	Fragment	Altered <i>B</i> specificity in host strain		
			<i>B6</i>	<i>B3</i>	<i>B42</i>
Pheromones					
pSB6.35	<i>phb3.2</i>	1.27-kb PCR	⊥	⊥	⊥
pSB6.33	<i>phb3.1</i>	2.0-kb <i>Bam</i> HI/ <i>Xho</i> I	⊥	⊥	⊥
pSB6.34	<i>phb2.2</i>	1.8-kb <i>Eco</i> RI	⊥	⊥	⊥
pSB6.30	<i>phb2.1</i>	3.0-kb PCR	⊥	⊥	⊥
pSB6.9	<i>phb1.2</i>	4.8-kb <i>Hind</i> III	⊥	⊥	⊥
pSB6.39	<i>phb1.1</i>	1.6-kb <i>Bam</i> HI/ <i>Eco</i> RI	⊥	⊥	⊥
Receptors					
pSB6.31	<i>rcb3</i>	2.6-kb PCR	⊥	⊥	⊥
pAB6.25	<i>rcb2</i>	3.1-kb PCR	⊥	⊥	⊥
pSB6.18	<i>rcb1</i>	3.3-kb <i>Bam</i> HI	⊥	⊥	⊥

Genes were introduced into three host strains: LT2, *A6B6 trp-1* LCO12, *A2B3 trp-3* and LN118, *A42B42 trp-1* by cotransformation. Transformants were mated to tester strains with the same *B* mating type specificity as the host. ⊥ indicates that a proportion of transformants (generally 12–50%) had been cotransformed with a gene that changed *B* mating specificity and permitted dikaryon formation with the tester strain. ⊥ indicates that none of the transformants had a changed *B* mating specificity. Restriction fragments were cloned into either pBluescript (Stratagene) KS+ or KS–; PCR products were cloned into pGEM-T (Promega).

3, were able to alter the mating specificity of the host but the two pheromone genes and receptor gene comprising group 1 had no effect. Significantly, when we tested for DNA sequence homology, we found that the region containing the three group 1 genes hybridized to *B42* genomic DNA. The region of DNA homology is indicated by the gray box in Figure 4. A correlation between DNA sequence homology and ability to activate *B*-regulated development leads us to suggest that *B6* and *B3* contain different alleles of all nine genes, whereas *B6* and *B42* share alleles of the three genes which constitute group 1.

**The CaaX sequence is essential for efficient pheromone gene function:** The CaaX sequence is a signal for isoprenylation of pheromones and the fact that X is alanine or serine in the *C. cinereus* proteins indicates that the isoprene will be a farnesyl group, a characteristic of all fungal lipopeptide pheromones characterized to date (Caldwell *et al.* 1995). Prenylation facilitates membrane localization and increases hydrophobicity and may also play a role in interaction with other proteins (Glomset *et al.* 1990; Marshall 1993). It has been shown for both *S. cerevisiae*  $\alpha$ -pheromone (Marcus *et al.* 1991) and *U. maydis* *mfa1* pheromone (Spellig *et al.* 1994) that absence of the CaaX modification results in a thousandfold or more loss in normal activity. To determine whether C-terminal modification was essential for efficient function of the *C. cinereus* peptides we replaced the cysteine of the CaaX box of both *phb2.1* and *phb2.2* with an arginine residue.

Both of the modified pheromones were still active in changing the mating specificity of the transformed host but the development of dikaryotic hyphae was delayed

(3 days compared with 1–2 days) and only very few dikaryotic hyphae were detected in the overall monokaryotic growth of the mating partners. Dikaryotic growth was maintained on subculture but growth was far less vigorous than when the wild type pheromone gene was expressed. We conclude that, as in yeast and *U. maydis*, the unmodified pheromone has some activity but isoprenylation leads to much greater efficiency. In a similar type of experiment, Vaillancourt *et al.* (1997) made C-terminal truncations of two pheromone precursor genes of *S. commune* by replacing the cysteine codon of the CaaX motif with a stop codon. Their results indicate that a truncated pheromone is inactive.

## DISCUSSION

We have described the organization of a mating type locus of the mushroom *Coprinus cinereus*, a gene complex that determines compatibility in mating by regulating part of a developmental sequence that permits cells to proceed to sexual reproduction. We identified nine genes in the *B6* complex, which encode six pheromone precursors and three 7-TM receptors (Figures 4, 5, and 6). The genes lie within 17 kb of DNA sequence, which is unique to *B6* when compared with genomic DNA of a strain containing a *B3* mating specificity. Any one of the nine genes is sufficient to change *B* mating specificity when introduced by transformation into a *B3* host.

In the basidiomycete fungi, unlike the ascomycetous yeasts, the genes encoding the mating pheromones and receptors are sequestered into a dedicated mating type locus. At the simplest level, in the yeast-like *Ustilago maydis*, there are just two “alleles” of this locus, *a1* and

*a2*, each containing the genes encoding a mating type-specific pheromone and 7-TM receptor (*mfa1* and *pra1* in *a1* and *mfa2* and *pra2* in the *a2* locus). The DNA sequences of the *a1* and *a2* loci are very dissimilar, thus ensuring that the two genes are inherited as an inseparable unit and loci containing a cross-compatible combination of pheromone and receptor genes cannot be generated by recombination. In the mushroom fungi, *S. commune* and *C. cinereus*, we find a similar lack of sequence similarity in different "alleles" of the loci; indeed, we exploited this nonhomology to isolate a *B6*-specific sequence by genomic subtraction.

**Origin of multiple *B* mating specificities:** The mushroom species such as *S. commune* and *C. cinereus* are unique amongst the fungi in having several functionally redundant and multiallelic genes encoding signaling elements. In *S. commune* the functionally redundant genes map to two discrete loci termed *B $\alpha$*  and *B $\beta$* . Each locus has 9 different "alleles," which in different combinations could generate a predicted 81 unique *B* mating specificities. Sequencing of an  $\alpha$  and a  $\beta$  locus has identified a single receptor gene and three pheromone precursor genes in each. Southern blot analyses have established that the genes within each locus are embedded in up to 8.5 kb of locus-specific DNA sequence, which would act to keep each set of genes together as a functional unit, as it does in the *U. maydis* loci (Wendland *et al.* 1995; Vaillancourt *et al.* 1997). The classic alleles of *B $\alpha$*  and *B $\beta$*  thus derive their different specificities from three genes. In *C. cinereus*, genetic evidence suggests that there is only a single *B* locus (Haylock *et al.* 1980). The locus we have described contains many more genes than either the *B $\alpha$*  or *B $\beta$*  loci of *S. commune* and we suggest that multiple *B* mating specificities in *C. cinereus* can be generated by functionally redundant genes at this single locus.

We have previously shown that the large numbers of *A* mating specificities in *C. cinereus* are generated by three sets of multiallelic and functionally redundant genes at the single *A* locus. Different alleles of the three sets of genes can be combined in all possible combinations and just a few alleles of each set is sufficient to generate the predicted 160 different *A* mating specificities. The 79 predicted *B* mating specificities could also be generated by three groups of multiallelic genes. In this instance each group is composed of two pheromone genes and one receptor gene, each equivalent to the three genes that make up the separate *B $\alpha$*  and *B $\beta$*  alleles in *S. commune* and similarly kept together as a single functional unit by DNA nonhomology. Our hypothesis predicts that *B* loci will share one or more groups of genes and need to differ in the alleles of only a single group to acquire a unique *B* mating specificity. Introduction of a gene into a host strain that already has the same allele of a gene subfamily will not activate the pheromone response and Southern blot analysis should show that the transformed gene is homologous to a

sequence in the host strain. This is what we observed for the three *B6* genes that comprise group 1 (Figure 4) when tested for DNA homology and function in a *B42* host. We concluded that *B6* and *B42* share this group of genes but have different alleles of genes in the two groups we designated 2 and 3. Preliminary sequence data confirm that the group 1 genes are shared by *B6* and *B42* (J. R. Halsall and L. A. Casselton, unpublished data).

The three receptor proteins encoded by the *C. cinereus* *B6* genes have only a moderate level of sequence identity (32–35%). In *S. commune*, different alleles of the *B $\alpha$*  *bar* gene encode proteins that have as much as 90% sequence identity, but these proteins show only 15% sequence identity with the functionally redundant *B $\beta$*  *Bbr1* protein (Wendland *et al.* 1995; Vaillancourt *et al.* 1997). The low level of sequence identity between the *C. cinereus* *B6* receptor proteins would be consistent with them being members of different subfamilies.

A remarkable degree of specificity is demanded of the different pheromones and receptors encoded by the *B* genes. A single receptor can be triggered by several different pheromones but not those encoded within the same *B* locus or from different subfamilies. Similarly a single pheromone can trigger several different receptors but not those encoded within the same locus or different subfamilies. In both *S. commune* and *C. cinereus* it is surprising to find so many pheromone genes. The level of functional redundancy in both species is increased by having so many pheromone genes. At present, we have no knowledge as to how the specificity in receptor-ligand recognition is achieved. In both species, the sequences of the pheromone precursors are surprisingly different even where two or more are known to activate the same receptor.

**Mating behavior of transformants:** In a wild-type mating between compatible monokaryons, there is generally a reciprocal exchange of nuclei that leads to the dikaryotization of both mating partners. In our experiments, the transformed hosts nearly always behaved as unilateral nuclear acceptors in crosses to tester strains (Figure 2B) but on occasion, failed to accept nuclei, and dikaryon only appeared at the junction of the mated strains. In *S. commune*, the mating behavior of strains transformed with receptor or pheromone genes is different. Strains transformed with a pheromone gene are characteristically unilateral donors of nuclei to the untransformed mating partner, whereas strains transformed with a receptor gene can neither donate nor receive nuclei (Wendland *et al.* 1995; Vaillancourt *et al.* 1997). This has led to a plausible model that may explain how pheromones and pheromone receptors regulate the normal bilateral migration of nuclei during mating (Vaillancourt *et al.* 1997). It is suggested that the pheromones can diffuse and thus activate the *B*-regulated response in the mating partner whereas the receptor cannot diffuse and only activates the response



within cells of the transformant. The mating behavior of the *C. cinereus* transformants appears to be at variance with this model, but it should be remembered that there are many subtle differences in the biology of these two species, in particular in the expression of the *B* genes (Haylock *et al.* 1980). Unlike *S. commune*, heterozygosity for *B* genes does not lead to continuous nuclear migration or abnormal cell and colony morphology or, as shown in this study, to the inability to accept nuclei in mating.

In animals, rhodopsin-like receptors constitute large families that have evolved different specificities to respond to a variety of ligands (Troemel *et al.* 1995; Dulac and Axel 1995). In fungi such as *C. cinereus* and *S. commune*, we see a similar evolutionary process that has created a family of functionally redundant receptors and corresponding ligands. The large family of genes we now describe will afford us a unique opportunity to answer questions of universal biological interest.

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