Heterologus Expression of Mating type Genes in Basidiomycetes

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Mating type genes in basidiomycetes encode two types of transcription factors (HD1 and HD2) and pheromones and pheromone receptors. Usually, mating type genes are so dissimilar in DNA sequence (allelic genes and genes from different species) that they do not cross-hybridize. In homobasidiomycetes, directly next to the *A* mating type locus encoding the transcription factors is a highly conserved gene *mip* that allows positional cloning. A candidate gene for positional cloning of the *B* mating type genes encoding the pheromone-pheromone receptor system is *cla4*/*ste20*. With more and more mating type loci cloned from different species, evolution of these loci and their genes can be addressed by sequence analysis and by function by transformation into other species, here *Coprinopsis cinerea*. Transformation of cloned mating type genes into heterologous hosts can lead to activation of mating type controlled development. Heterologous expression of mating type genes is especially interesting for species in which no transformation system exists. Since in *C. cinerea* an *A* null-mutant is available without functional transcription factor genes, self-compatibility of cloned *A* genes from homothallic species can also be tested.

1. Introduction

1.1. *Breeding systems in the basidiomycetes.*

An estimated 85-90% of all basidiomycetous species are heterothallic and need to undergo mating between two compatible monokaryotic strains for sexual development (karyogamy and meiosis) to occur. The remaining 10-

Genetics and Cellular Biology of Basidiomycetes-VI. A.G. Pisabarro and L. Ramírez (eds.)
 ISBN 84-9769-107-5

15% of species are homothallic, i.e. self-compatible. These fertile homokaryotic strains undergo karyogamy and meiosis without mating to another strain. From such truly homothallic species, secondarily homothallic species have to be distinguished. Secondarily homothallic species carry two nuclei of opposite mating type in the basidiospores which germinate into fertile dikaryons (Whitehouse 1949, Quintanilha and Pinto-Lopes 1950).

Sexual development in heterothallic species is controlled by either one or two mating type loci. Species with one mating type locus are called bipolar, because two different mating type specificities segregate in the haploid progeny of a cross. These mating types correspond to the mating types of the parental strains of the cross. Species with two mating type loci are tetrapolar, and four different mating types are found among the progeny of a cross. Two of the four mating types are parental by passing on parental alleles at both mating type loci. The two alternate mating types are newly formed by recombination between the two mating type loci. Approximately 30-40% of heterothallic species are estimated to be bipolar and 60-70% to be tetrapolar (Whitehouse 1949, Quintanilha and Pinto-Lopes 1950).

In most cases, the single mating type locus of bipolar species are called *A*, the two mating type loci of tetrapolar species *A* and *B*. Usually, mating type loci in the basidiomycetes are multi-allelic. Different mating type specificities are indicated by numbers (*A1*, *A2*, *A3*, ...; *B1*, *B2*, *B3*...). In species with two mating type loci, every distinct *A* and *B* combination defines a specific mating type, i.e. *A1B1*, *A1B2*, *A2B1* and *A2B2* strains are all different in mating behaviour. Of these, *A1B1* and *A2B2* strains are compatible as are *A1B2* and *A2B1* strains. For a successful mating, fusing monokaryons need to be different at both mating type loci. In consequence, tetrapolar breeding systems promote outbreeding (Whitehouse 1949, Quintanilha and Pinto-Lopes 1950).

The *A* and the *B* mating type genes in tetrapolar species regulate different steps in sexual development explaining why both loci have to be different between two mates (for details see reviews by Casselton and Olesnicky 1998, Kües 2000, Kües et al. 2002a, 2004, Casselton and Riquelme 2005).

1.2. *The mating type loci in tetrapolar species*

Mating type loci have been cloned and functionally analyzed from the tetrapolar species *Coprinopsis cinerea*, *Schizophyllum commune* and *Ustilago maydis*.

The *A* mating type loci of *C. cinerea* and *S. commune* and the *b* mating type locus of *U. maydis* contain genes encoding two types of homeodomain transcription factors known as HD1 and HD2 proteins. Generally, to induce sexual development, an HD1 protein from one mate has to interact with an HD2 protein of the other mate (Fig. 1). In the simple case in *U. maydis*, there is one pair of divergently transcribed *HD1* and *HD2* genes (*bE* and *bW*) with about 25 different alleles. In a mating, there are therefore two compatible protein interactions: bE1-bW2 and bE2-bW1 (compare Fig. 1). However, one such protein combination is sufficient. In *C. cinerea*, there are at least three independently interacting *HD1-HD2* gene pairs. Each gene pair has a few alleles that are freely recombining with the alleles from the other gene pairs. This recombination generates the estimated number of 160 different *A* mating type specificities. For sexual development, it is, however, enough if mates carry different alleles at any one of the three possible *HD1-HD2* gene pairs. In *S. commune* with a high number of different *A* mating type specificities (>280), the situation is similar. So, far only one complete gene pair (*Aα* locus) has been characterized in this species and one non-allelic *HD2* gene (from the *Aß* locus) been cloned (for details see reviews by Hiscock and Kües 1999, Casselton and Olesnicki 1998, Casselton and Challen 2005).

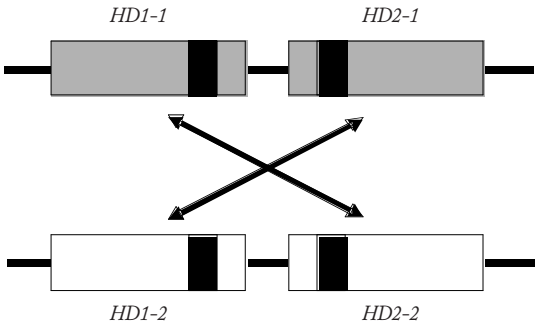

Figure 1. Schematic presentation of a mating type locus encoding HD1 and HD2 genes for homeodomain transcription factors. The simplest case with one pair of divergently transcribed genes are shown. Genes for proteins of the different classes of homeodomain transcription factors (HD1 and HD2) are specified by the first number. The second number defines the allele of a respective gene. DNA dissimilarities between alleles are indicated by boxes of different shading. The more conserved regions encoding the homeodomain DNA-binding motif are given in black boxes. The two possible functional interactions between products from allelic gene pairs are indicated by the arrowheads. These HD1-1/HD2-2 and HD1-2/HD2-1 interactions are redundant in function

The *B* mating type loci of *C. cinerea* and *S. commune* and the *a* locus of *U. maydis* contain genes for pheromones and pheromone receptors (Fig. 2). Pheromones and pheromone receptors of different specificity have to interact for induction of sexual development. In *U. maydis*, there are only two alleles of the *a* locus and each has a pheromone gene and a receptor gene. Three independent groups each comprising one pheromone receptor gene and up to three pheromone genes have been described in *C. cinerea*. Different combinations of the various alleles of these groups are expected to give rise to the 80 *B* specificities estimated to exist in nature. Two groups with each one pheromone receptor gene and up to 8 different pheromone genes are described for the *B* locus in *S. commune*. Similarly for this species, 80 different *B* specificities are expected worldwide to exist (for details see reviews by Casselton and Olesnicky 1998, Kothe 2001, Casselton and Challen 2005, Riquelme et al. 2005).

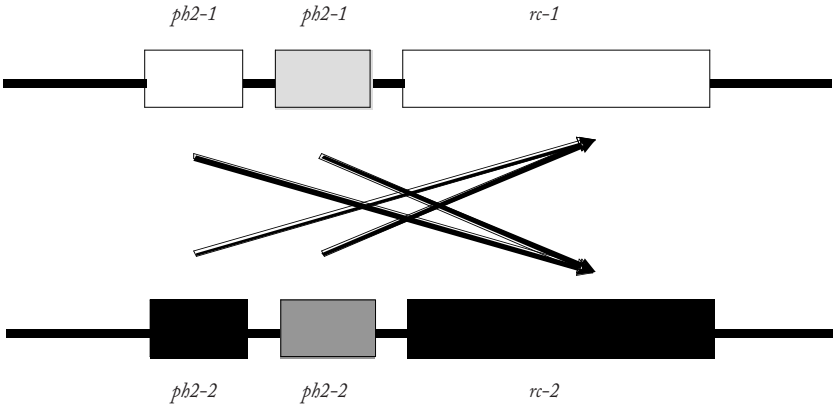

Figure 2. A hypothetical mating type locus encoding two different pheromones (Ph1, Ph2; second number = allele) and one pheromone receptor (Rc; second number = allele) is shown – in reality, there might be less or more pheromone genes and also genes unlinked to mating function that have been integrated into the locus by coincidence. Interactions of pheromones with the respective receptor from an allelic mating type locus are indicated by arrows

1.3. *Cloning mating type loci*

Alleles of mating type loci are very dissimilar in sequence so that they do not cross-hybridize. This feature has been used in cloning the *B* mating type

genes from *C. cinerea* by a genomic subtraction technique selecting unique DNA sequences for incorporation into a cloning vector (O'Shea et al. 1998). In other cases, as in cloning the *B* genes from *S. commune*, mating type genes were identified upon transformation through activation of developmental programs being under control of the mating type genes (Specht 1995). Chromosome walking from closely linked and easily to identify metabolic genes was applied in cloning *A* genes from both *C. cinerea* and *S. commune* (Giasson et al. 1989, Mutasa et al. 1989). Despite the reported homothallic behaviour of strains in *Phanerochaete chrysosporium* (Alic et al. 1987), mating type genes were identified in the genome sequence of this fungus (Martínez et al. 2004). All these approaches to obtain mating type genes are very laborious and often also difficult.

To understand the evolution of mating type loci and the different breeding systems in the basidiomycetes, genes from more species need to be cloned and analyzed. In higher basidiomycetes, a highly conserved gene for a mitochondrial intermediate peptidase (*mip*) is found directly next (< 1 kb) to the *A* mating type genes (James et al. 2004a). This gene has successfully been employed in positional cloning of *A* mating type genes from *Coprinopsis scobicola* (=*Coprinus bilanatus*; Kües et al. 2001), *Pleurotus djamor* (James et al. 2004b), and *Coprinellus disseminatus* (=*Coprinus disseminatus*; James 2003).

Fragments with *B* mating type gene sequences from *P. djamor* were initially identified through PCR using two pairs of degenerate primers, showing that such a PCR approach can be successful (James et al. 2004b). Another approach for isolating *B* mating type genes makes use of positional cloning. Previous studies indicated that in the ascomycete *Pneumocystis carini* (Smulian et al. 2001) and in the basidiomycetes *Cryptococcus neoformans* (Lengeler et al. 2002) and *P. chrysosporium* (James 2003) the p21-activated kinase gene *cla4*/*ste20* is closely linked to pheromone and pheromone receptor genes. The *cla4*/*ste20* gene was cloned from *P. djamor* and shown to reside at a distance of about 28 kb from to a pheromone gene and a pheromone receptor gene (James et al. 2004b). Close linkage between a *cla4*/*ste20* gene and the *a* mating type locus of *U. maydis* has now also been reported (Leveleki et al. 2004), and this syntenic arrangement is also conserved in *C. cinerea* as observed in the genome sequence (http://www.broad.mit.edu/annotation/fungi/coprinus_cinereus/). In conclusion, positional cloning through *cla4*/*ste20* appears to be a most promising strategy to obtain *B* mating type genes from many species.

1.4. *Functional analysis of cloned mating type genes*

Transformation systems that allow functional analysis of cloned potential mating type genes exist for only very few basidiomycetes. In the higher basidiomycetes, functions of cloned *A* and *B* genes from *C. cinerea* and *S. commune* have been tested in strains of the same species (for details see the reviews cited above). The first heterologous expression of *C. cinerea A* mating type genes was achieved in the closely related heterothallic fungus C. *scobicola* (Challen et al. 1993). Subsequently, function of *A* mating type genes from *C. scobicola* in *C. cinerea* was also demonstrated. *C. scobicola A* mating type genes are active with resident genes from various *C. cinerea* monokaryons (Kües et al. 2001; Fig. 3). Transformation experiments with heterologous

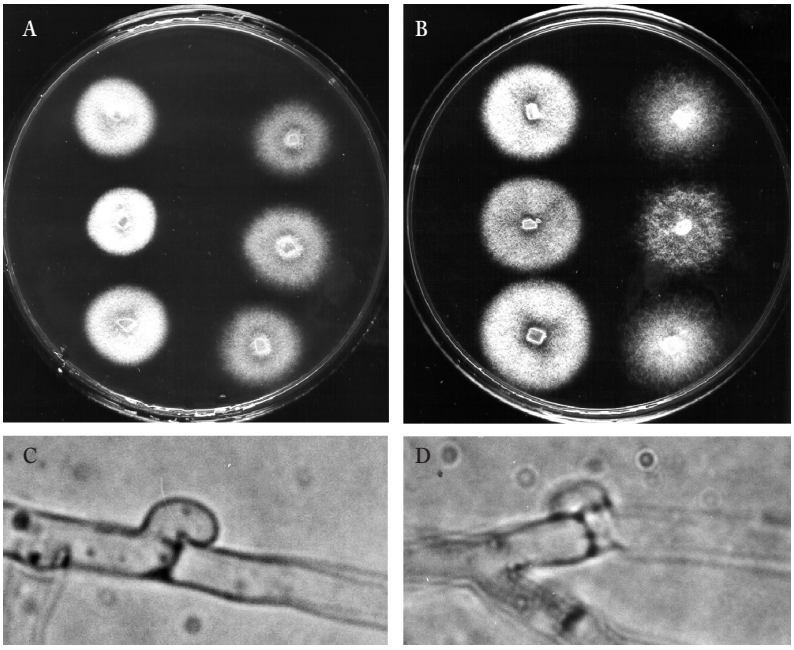

Figure 3. Expression of A mating type genes from a cosmid of *C. scobicola* in *C. cinerea* monokaryon LN118 (*A42, B42, ade-2, trp-1.1,1.6*) and FA2222 (*A5, B6, acu-1, trp1.1,1.6*). *A* and *B* show typical colony morphologies of densely grown monokaryotic clones (left) transformed with the *trp1*$^{+}$ plasmid pCc1001 (Binninger et al. 1987) and of less dense, more fluffy clones co-transformed with pCc1001 and a cosmid with A mating type genes from C. scobicola (right). *C* and *D* show unfused clamp cells found at hyphal septa in the A-activated transformants of LN118 and FA2222, respectively

genes from other species suggest that *C. cinerea* monokaryon 218 (*A3*, *B1*, *trp-1.1,1.6*) reacts best in tests with *A* mating type genes from foreign species (Srivilai, unpublished observation; James et al., in preparation). Different scenarios can be tested in strains that have endogenous mating type genes: the general function of an entire cloned *A* mating type locus within the heterologous species (Kües et al. 2001; Fig. 3) or the behavior of individual *A* genes and whether they interact with foreign *A* genes resident in the new host (Challen et al. 1993).

In *C. cinerea*, mutations in the *A* mating type locus are known that lead to self-compatibility. These mutations originate from large deletions of mating type DNA leading to in frame-fusions of an *HD2* and an *HD1* gene whose products normally do not interact. However, the product of the *HD2-HD1* fusion gene acts in the same manner as normal heterodimers of HD2 and HD1 proteins from different mating types. Activity but not self-compatibility of such fusion genes can be tested by clamp cell production in backgrounds of hosts with different mating type specificity. Self-compatibility can be shown by transformation into a wild-type self-background, i.e. by transformation into a monokaryon that carries the original unfused genes (Kües et al. 1994, Pardo et al. 1996). More elegant is the use of a *A*-null strain such as NA2 (Pardo 1995), where no resident *A* genes can interfere with action of the fusion gene (Fig. 4). The bipolar species *C. disseminatus* is found to have unlinked loci for homeodomain transcription factors and for pheromone genes and pheromone receptors, respectively, suggesting that one of these loci is either inactive or self-compatible (James et al., in preparation). Transformation into *C. cinerea* NA2 can help to clarify whether for example the locus for homeodomain transcription factors confers self-compatibility. Likewise, in strain NA2 one might test self-compatibility of genes for homeodomain transcription factors cloned from homothallic species. More-

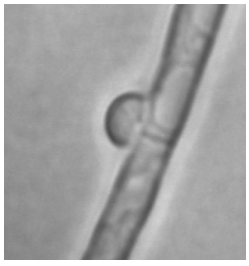

Figure 4. Clamp cell produced in *A* null mutant strain NA2 upon transformation with the self-compatible fusion gene *a2-1/d1-1* generated by deletion from an *A6* wildtype locus (Kües et al. 1994); photo by E. Polak.

over, co-transformation with *A* genes from two different mating types are possible in this strain enabling analysis of the interaction of two genes at a time (Kües et al. 1999).

Functionality of *B* mating type genes can be tested in *C. cinerea* by transformation into a monokaryon and subsequent mating to another monokaryon carrying the same resident *B* mating type locus but a different *A* mating type locus (O'Shea et al. 1998). In monokaryon 218, functional expression of compatible *B* mating type genes is also possible to recognize by a specific colony morphology on complete medium. Positive transformants show retarded colony growth, produce little aerial mycelium and irregular hyphal morphology, the so-called "flat" phenotype (Kües et al. 2002b). When simultaneously transformed with an *A* mating type gene of a specificity compatible to that already present in the monokaryotic strain, clamp cell production apical to a septum is induced by action of the *A* mating type genes and *B*-induced clamp cell fusion to the subapical hyphal cell can be observed. Usually in the mycelium of such transformants, more often than formation of fused clamp cells, *B*-regulated formation of subapical pegs is detectable on septa with non-fused clamp cells (Kües et al. 2002b, Badalyan et al. 2004; Fig. 5). Similar observations in monokaryon 218 can be made when transforming heterologous *B* genes into the strain (Srivilai, unpublished). A *B* null mutant from *C. cinerea* so far is unfortunately not available to test self-compatibility of cloned homologous and heterologous *B* mating type genes.

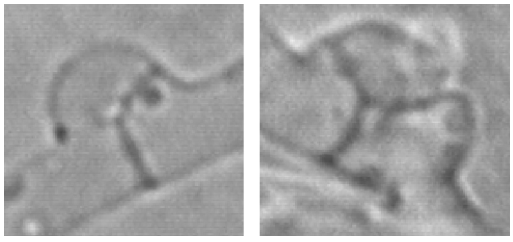

Figure 5. Fused clamp cell (left) and unfused clamp cell accompanied by a subapical peg (right) produced in monokaryon 218 upon co-transformation with compatible *A* and compatible *B* mating type genes.

2. Conclusions

Mating type genes have now been cloned from several different basidiomycete species. Cloning of mating type loci from further species is now

possible by positional cloning. The function of these mating type genes from other species can be addressed by transformation into *C. cinerea*. Our work showed that at least in some cases, foreign mating type genes are functionally expressed. Foreign gene products appear to interact with those from resident genes. Self-compatible *A* mating type genes and specific combinations of pairs of *A* mating type genes can be analyzed in an *A* null background of *C. cinerea*. For the first time, foreign *B* mating type genes are also seen to function in *C. cinerea*.

3. Acknowledgements

PS and WC hold PhD scholarships of the Mahasarakham University (Thailand) and the Rajamangala University (Thailand), respectively. The Göttingen laboratory is supported by the Deutsche Bundesstiftung Umwelt (DBU).

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