

Crosses with *Amut Bmut*-t homokaryons of *Coprinus cinereus*

Yi Liu, José D. Granado*, Eline Polak, and Ursula Kües – Institut für Mikrobiologie, ETH Zürich, Schmelzbergstrasse 7, CH-8092 Zürich, Switzerland

*Present address: Institut für Pflanzenbiologie, University of Zürich, Zollikerstr. 107, CH-8008 Zürich, Switzerland

In this paper we describe the initial genetic analysis of some developmental REMI and UV mutants of the self-compatible homokaryon AmutBmut. We show that such homokaryons can mate with each other although in fruitbodies we often found spores of only one parent. Crosses with monokaryons of different mating types gave some indications about numbers of mutations and linkage of genetic markers. In most cases, however, we observed an uneven distribution of markers, most likely because of loss of certain progeny. Our results necessitate the construction of monokaryons as closely related to homokaryon AmutBmut as possible but with different mating type loci.

Fusion of two compatible monokaryons of *Coprinus cinereus* leads to the formation of a dikaryon. On the dikaryon, fruitbodies develop with basidia, where karyogamy and meiosis occur. Successful dikaryon formation is governed by the two mating type loci *A* and *B* (Casselton and Olesnick 1998 Microbiol. Mol. Biol. Rev. 62:55-70). Specific mutations in both mating type loci give rise to self-compatible *Amut Bmut* homokaryons, which can form fruitbodies without the need to mate with another strain (Swamy et al. 1984 J. Gen. Microbiol. 130:3219-3224). Because of this unique feature, extensive use of homokaryon AmutBmut (*A43mut*, *B43mut*, *pab1*) has been made in generating mutants in the fruiting pathway (Pukkila 1994 pp. 276-281, in *Mycota I*, Wessels and Meinhardt, Eds., Springer: Berlin). Our group generated mutants by both REMI and UV mutagenesis (Granado et al. 1997 Mol. Gen. Genet. 256:28-36; Kües et al. in prep). For further analysis, it is important to identify those mutants that harbor only a single genetic defect and, if possible, to map the respective genes. The REMI mutants differ from the UV clones by ectopic chromosomal integration of functional copies of the para-aminobenzoic acid (*pab*) synthase gene *pab1*⁺ inserted in the *Escherichia coli* plasmid pTZ18R (Granado et al. 1997 *ibid*). In the case of REMI mutants, it is therefore critical to show linkage of a *pab1*⁺ insertion to a respective mutant phenotype. Due to the insertion of *pab1*⁺, REMI mutants are prototrophic, although typically they should still have the non-functional *pab1* copy present at the natural chromosomal location. A cross with a compatible *pab1* strain offers the possibility to follow the distribution of the inserted *pab1*⁺ gene and a particular developmental defect in sexual progeny.

Monokaryon PG78 in analysis of defects in fruitbody development. A *pab1* auxotrophic monokaryon was available to us with s. n. PG78 (*A6*, *B42*, *pab1*, *trp1*:1.6) (Granado et al. 1997, *ibid*). First, we performed a cross with REMI mutant B-1918 (*A43mut*, *B43mut*, *pab1*, ::*pab1*⁺), which has a single *pab1*⁺ plasmid integration (data not shown). Mutant B-1918 does not form mature fruitbodies. However, the stipes of fruitbody primordia elongate extensively under dark-light fruiting conditions without parallel development of the cap tissue (J. D. Granado, unpublished). Such a phenotype is known as "etiolated" or "dark stipe" phenotype (Elliott 1994 *Reproduction in Fungi. Genetical and Physiological Aspects*, Chapman & Hall: London). Progeny of PG78 x B-1918 were plated on YMG/T complete medium (Granado et al. 1997, *ibid*) and consecutively tested for auxotrophies. The distribution of the auxotrophic markers was unequal with 58% of prototrophic clones, 40% of *pab1* + *trp1* auxotrophic clones and 2% of *pab1* auxotrophic clones (Table 1). This result suggested a close linkage between the *pab1*⁺ insertion and the natural *trp1*⁺ locus ($P > 0.99$). We did not perform the tedious microscopical analysis with this F1 generation to identify clones with fused clamps, which indicates the presence of *A43mut* and *B43mut*. By simple statistics, 11 (25%) of the 43 analyzed clones should have had this genotype offering the possibility to fruit. However, only 2 out of all clones formed tiny fruitbody initials (~1 mm in size) and one, JG1-33 (*A43mut*, *B43mut*, *pab1*, ::*pab1*⁺), had the "etiolated stipe" phenotype. JG1-33 was backcrossed to the parent PG78. In the progeny, we found the same uneven distribution of *trp1* and *pab1* as before in the F1 generation (Table 1). Loss of *trp1* progeny was also observed in the control cross PG78 x AmutBmut (Table 1), suggesting that this phenomenon is intrinsic to the PG78 and AmutBmut backgrounds. In the last two crosses, where we analyzed a high number of clones, *trp1* : *trp1*⁺ clearly segregated in an 1:2 ratio ($P > 0.5$ and $P > 0.25$, respectively), indicating that 25% of the *trp1* progeny was lost.

In the following, we examined clamp cell formation to monitor the inheritance of the *A* mating type locus amongst the PG78 x JG1-33 progeny. Only 39% of all germinated clones were without clamp cells (Table 2), indicating that half of the *A6* progeny was missing ($P > 0.05$). This was also the case in the progeny of a cross between monokaryon PG78 and the prototrophic REMI mutant E-1754 that has a defect in nuclear distribution ($P > 0.75$; Table 2; E. Polak, unpublished) and in the progeny of a cross between monokaryon PG78 and homokaryon AmutBmut ($P > 0.25$; Table 2). Interestingly, within the group of viable *A6* clones *trp1* : *trp1*⁺ distributed 1 : 1 unlike the *A43mut* clones (not shown). However, it is not clear from these data if there is a direct connection between the under representation of *trp1* and that of the *A6* mating type. Loss of certain phenotypes within progeny has been observed before in crosses with other strains. For example, a recessive gene *slg* is known to affect spore germination. Another recessive gene, blocks basidiospore development (Pukkila 1993 pp. 249-264 in *Genetics and Breeding of Edible Mushrooms*, Chang et al., Eus, Gordon and Breach: Y-Parc, Switzerland). Presence of such a mutation in one of the parental strains should lead to a 50% loss of all clones. The loss of 25% of certain phenotypes as found here and also in other studies (Moore 1981 Curr. Genet. 3:145-150) implies

a negative interaction between two unlinked loci in a single haploid nucleus that came together from the different parental strains by karyogamy and meiosis. It is possible that the loss of 25% of progeny connects to translocations which commonly occur in *C. reinkei*, also with the *trp1* chromosome (Pukkila and Casselton 1991 pp.126-150, in *More Gene Manipulations in Fungi*, Bennett and Lasure, Eds., Academic Press: San Diego).

Because of the partial loss of certain progeny, care has to be taken in mapping the locations of genes within chromosomes. Nevertheless, we concluded from the 12% recombinant phenotypes (*pab1⁺ trp1* and *pab1 trp1⁺*) within the PG78 x JG1-33 progeny (Table 1) that the chromosomal distance between the ectopic *pab1⁺* insertion in JG1-33 and *trp1⁺* is about 0.12 map units. In order to determine whether this *pab1⁺* insertion links to the "etiolated stipe" defect, we submitted selected groups of the PG78 x JG1-33 progeny (Table 3) to standard fruiting conditions (Granado et al. 1997). Within these groups we did not discriminate between the presence of *B43mut* or *B6*. Since *B43mut* and *B6* distribute equally within the progeny (data not shown), half of the analyzed clones (*B43mut* clones) would be expected to initiate fruiting. However, none of the cases came even close to the expectations (Table 3). Thus, we analyzed fruiting within the *A43mut* (*B43mut* + *B6*) progeny of a control cross PG78 x *AmutBmut*. Twenty-one percent of the clones were able to initiate fruiting (Table 3), corresponding to half of the *B43mut* clones ($P > 0.25$). Twenty nine out of the 31 fruiting clones were *trp1⁺*, which suggests either a negative effect of the *trp1* auxotrophy on fruiting and/or, on the PG78 chromosome, a linkage between *trp1* and a non-functional gene that is necessary for fruitbody initiation. From other strains, we recently learned that a *trp1* auxotrophy interferes with fruitbody initiation (U. Kües and M.J. Klaus, unpublished observations). Co-transformation of a compatible *A* gene with a *trp1⁺* selection marker did not induce the fruiting pathway in strain PG78 unlike in other *trp1* monokaryons (Kües et al. 1998 Mol. Gen. Genet. 260:81-91). Together, these observations lead to the conclusion that monokaryon PG78 is not well suited in the genetic analysis of fruiting mutants of homokaryon *AmutBmut*. Nevertheless, we still might conclude from data obtained from the PG78 x JG1-33 progeny that the locus responsible for the "etiolated stipe" phenotype in mutant JG1-33 links to the *pab1⁺* insertion. Clones forming etiolated stipes predominate amongst the few clones that were able to induce fruiting (Table 3). Initials of the 3% exceptional cases (Table 3) did not develop far enough to determine whether primordia are of wild-type or of the "etiolated stipe" phenotype. 80 out of the 81 fruiting inducing clones were *pab* prototrophs. Sometimes, we specifically selected for *trp1⁺* progeny and thus preferentially for a *pab* prototrophic progeny as well because of the close linkage of *trp1⁺* to the *pab1⁺* insertion (Table 3). With the distance of 0.12 map units, we would expect at least 9-10 *pab1* clones amongst those initiating fruitbody development, if the "etiolated stipe" mutation is unlinked to the *pab1⁺* insertion.

Monokaryon PG78 in analysis of defects in hyphal development. Despite the limited use of monokaryon PG78 in analyzing fruiting defects, the strain still has some value for analyzing defects in vegetative development. This is indicated from studies on the prototrophic REMI mutants E-1281 and E-2095, both of which lost the ability to form clamp cells at the hyphal septa (E. Polak, unpublished data). As expected from a single mutation unlinked to *A43mut*, the relative numbers of clones with and without clamp cells reversed in the progeny of cross PG78 x E-1281 with 24% : 75% (Table 4), compared to the 70% : 30% in cross PG78 x *AmutBmut* (Table 2). *pab1⁺* : *pab1* distributes 1 : 2 ($P > 0.25$) within progeny of PG78 x E-1281 (Table 4), indicating 25% loss of *pab1⁺* clones, but we currently do not know whether this relates to the 25% loss of *A6* mating type. In the progeny of cross PG78 x E-2095, only 8% of all clones formed clamp cells (Table 4). Therefore, the loss of clamp cell formation in E-2095 could be caused by a mutation linked to *A43mut*. 8 of the 20 identified *A43mut* clones were *pab* prototrophs suggesting that the insertion of *pab1⁺* segregates independently of the mutation ($P > 0.5$; Table 4). In accordance, 10 out of 22 randomly chosen clampless *pab1⁺* strains formed clamp cells in backcrosses with PG78 and thus should have the *A43mut* genotype. However, only 19% of all clones of the PG78 x E-2095 progeny were *pab* prototrophs (Table 4). One possible explanation for this observation is a loss of the ectopic *pab1⁺* insertion during meiosis (Kües and Stahl 1990 Prog. Bot. 52: 201-225).

Mating between strains of related mating type specificities. The complications in the crosses with PG78 described above demand another strategy for analyzing defects in fruiting. The mutations in the mating type loci in homokaryon *AmutBmut* overcome the natural incompatibility between the endogenous mating type products (Hiscock and Kües 1999 Int. Rev. Cytol. in press). Therefore, one might expect that *AmutBmut* homokaryons are also compatible with monokaryons carrying the wild-type mating type loci. We mated homokaryon *AmutBmut* on several YMG/T plates (~20) with its progenitor strain 5026 (*A43*, *B43*; kindly provided by T. Kamada) and with the distantly related monokaryon AT8 (*A43*, *B43*, *trp3*, *ade8*; Kües et al. 1992 Genes Dev. 4:568-577). We readily observed fruitbody formation on the *AmutBmut* side of the crosses. In contrast, we never observed any fruitbodies on the outer edge of an AT8 colony and only once a fruitbody on the outer edge of a 5026 colony. Basidiospores of this exceptional fruitbody and of fruitbodies from the *AmutBmut* sides of the crosses were always *pab1* which suggests that there was no interaction between the nuclei of the two different mating partners at least during later stages of basidiome formation. To test the situation between *A43mut* *B43mut* homokaryons we first crossed the clamp cell defective REMI mutants E-1281 x E-2095 and found clamp cells at hyphae grown in the intermediary growth zone of the two strains (not shown). Therefore, the two different mycelia must have fused and their nuclei complement each other in their defects in clamp cell formation. To study complementation of defects in fruitbody formation, we crossed mutants that are defective in initiation of fruiting, taking advantage of the *pab* auxotrophy of an UV mutant (6-031) and the *pab* prototrophy of REMI mutants (B-436, B-2798 and E-1593). Fruitbodies were formed in the intermediary zones of the crosses

suggesting that the defects in fruitbody initiation are recessive. However, when basidiospores of six independent fruitbodies of 6-031 x B-2798 were analyzed, all were *pab1*⁻. This result suggests that only one type of nucleus migrated into cells of the fruitbodies, although the defects in fruitbody initiation could be complemented either by the presence of the two different nuclei within the same hyphae or by feeding effects of diffusible substances. In contrast, *pab1* : *pab1*⁻ distributed 1 : 1 in the progeny of the one tested fruitbody from cross 6-031 x E-1593 ($P > 0.75$) and 1 : 3 in one out of three tested fruitbodies from cross 6-031 x B-436 ($P > 0.5$); basidiospores of the other two fruitbodies were 100% *pab1*⁻. Obviously, it depends very much on the crossing partners whether both or only one type of nuclei migrate into the basidia of the fruitbodies.

Monokaryon 5401 in analysis of defects in fruitbody development. Since crosses between strains of related mating type specificities not always lead to karyogamy of different parental nuclei, we performed crosses with a more closely related monokaryon of a different mating type, in the hope not to introduce any unwanted and unknown genetical traits into the progeny. Prof. T. Kamada kindly supplied wild-type monokaryon 5401 [*A1*(m), *B1*(m); note that the nomenclature of these mating types relates to strains originally described as *C. macrorhizus*, Maida et al. 1998 Curr. Genet. 32:231-236]. This strain was derived from the same genetic background as monokaryon 5026 and homokaryon AmutBmut. Strain 5401 had been used before to identify gene loci in primordiumless and sporeless AmutBmut mutants (Kanda et al. 1989 Bot. Mag. Tokyo 102:561-564 and Mol. Gen. Genet. 216:526-529). We crossed monokaryon 5401 with UV mutant 6-031 and with homokaryon AmutBmut and found in both progeny an equal distribution of the *pab1* and *pab1*⁻ marker ($P > 0.75$ and $P > 0.05$, respectively; Table 5). In contrast, the *A43mut* mating type was slightly over represented (Table 5). The *A* locus and the *pab1* gene are closely linked on chromosome I (0.5 map units; Lukens et al. 1996 Genetics 144:1471-1477). Thus, for unknown reason, the 3-7% recombination frequency between *pab1* and the *A* locus (Table 5) was somewhat higher than expected from the literature.

Analyzing the fruiting behavior of the *A43mut* [*B43mut* + *B1*(m)] progeny, we found 50% of all clones (66 out of 129 clones) from the cross 5401 x AmutBmut and 27% of all clones (34 out of 124 clones) from the cross 5401 x 6-031 to initiate fruitbody formation. This reduction by half correlates well with a defect in fruitbody initiation in a single gene (*fbil* for fruitbody initiation gene 1) that is unlinked to *A43mut* ($P > 0.5$). However, another 16% of the clones (20 out of 124 clones) formed fruitbodies in crosses with both parental strains, indicating that the *fbil*⁻ wild-type gene and *B43mut* was present within these clones. Furthermore, 17% of the *A43mut* progeny (21 out of 124) developed fruitbody initials only when crossed to 6-031 suggesting that these contained the active gene *fbil*⁻ and probably the *B1*(m) mating type genes. 22% of the *A43mut* progeny (27 out of 124) initiated fruitbody development only in crosses with monokaryon 5401 showing that they contain *B43mut* and likely the mutated gene for fruitbody initiation. The remaining 18% of the clones (22 out of 124) did not form fruiting bodies with either parental strain. Adding up these data, 60% of the clones of the *A43mut* progeny (34 + 20 + 21 clones) should carry the wild-type gene and a maximum of 40% the mutated gene. Moreover, at least 65% of the *A43mut* clones (34 + 20 + 27 clones) should contain *B43mut*. This over representation of *B43mut* within the *A43mut* progeny agrees with values we obtained within the limitations of certainty of the microscopical test with which we analyzed all the *A43mut* mycelia for the presence of fused clamp cells. According to this visual test, 78% of all *A43mut* clones (97 out of 124) of cross 5401 x 6-031 and 70% of all *A43mut* clones (90 out of 129) of cross 5401 x AmutBmut had fused clamp cells. These results show that markers also did not segregate perfectly in the progeny of crosses with monokaryon 5401 although the higher frequency of fruitbody formation within progeny of crosses makes it a better strain for such analysis than monokaryon PG78.

General conclusions. We discovered a number of possible problems (loss of progeny, instabilities of ectopic DNA insertions, migration of only one nucleus into fruitbody cells) to follow genetical traits within progeny of crosses involving REMI and UV mutants of homokaryon AmutBmut. In consequence, it can be difficult to decide whether a mutant contains one or more mutations and whether a *pab1*⁻ insertion in REMI mutants links to the mutation of interest. It appears that it is better to maintain the same genetic background. Truly isogenic strains distinguished only in their mating type specificity so far do not exist in *C. cinereus*. We will thus have to develop an optimal partner for crosses, e.g. by repetitive backcrossing to the AmutBmut background (see Pukkila 1993 for further discussion). For analyzing REMI mutants we will have to introduce a *pab1* auxotrophy into a generated suitable tester strain by mutagenesis.

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Table 1. Inheritance of auxotrophic markers in progeny of crosses between monokaryon PG78 and *Amut Bmut* homokaryons

Cross	Phenotype of progeny, number and percentage of clones*				Total isolates
	trp ⁺ pab	trp ⁺ pab ⁻	trp ⁻ pab	trp ⁻ pab ⁻	
PG78 x B-1918	17 (40%)	-	1 (2%)	25 (58%)	43 (100%)
PG78 x JG1-33	38 (30%)	8 (6%)	7 (6%)	74 (58%)	127 (100%)
PG78 x <i>AmutBmut</i>	76 (36%)	n. a.	134 (64%)	n. a.	210 (100%)

*Basidiospores from each one fruitbody were plated on YMG/T and auxotrophies determined on minimal medium MM (Granado et al. 1997) supplemented with 100 mg/L trp and 5 mg/L pab where appropriate; n.a.: not applicable.

Table 2. Inheritance of the *A* mating type locus in progeny of crosses between monokaryon PG78 and *Amut Bmut* homokaryons

Cross	Phenotype of progeny, number and percentage of clones*		Total isolates
	Clamprps (<i>A43mut</i>)	No clamprps (<i>A6</i>)	
PG78 x JG1-33	145 (61%)	91 (39%)	236 (100%)
PG78 x E-1754	66 (65%)	36 (35%)	102 (100%)
PG78 x <i>AmutBmut</i>	147	63	210

*Basidiospores from each one fruitbody were plated on YMG/T. Presence of the respective mating type in germinated clones was microscopically determined by presence (*A43mut*) or absence of clamp cells (*A6*) at the hyphal septa.

Table 3. Fruiting behaviour in progeny of crosses between monokaryon PG78 and *Amut Bmut* homokaryons

Cross	Analyzed Genotypes	Phenotype of progeny, number and percentages of clones*			Total isolates
		Wild-type primordia	"Enolated stipes"	Tiny initials	
PG78 x JG1-33 ^{a,c}	<i>A43mut trp1</i> ⁺ + <i>A43mut trp1</i>	-	10 ^d (7%)	2 ^e (1%)	145 (100%)
PG78 x JG1-33 ^{b,c}	<i>A43mut trp1</i> ⁺	-	45 ^e (8%)	1 ^e (0.2%)	534 (100%)
PG78 x JG1-33 ^b	<i>A43mut trp1</i> ⁺ + <i>A6 trp1</i> ⁻	-	23 ^e (8%)	-	300 (100%)
PG78x <i>AmutBmut</i> ^{a,c}	<i>A43mut trp1</i> ⁺ + <i>A43mut trp1</i>	31 (21%)	-	-	147 (100%)

* Fruiting was tested as described by Granado et al. (1997).

^a Basidiospores of each one fruitbody were germinated on YMG/T and consequently clones were either trp or trp⁺ and either pab or pab⁻.

^b Basidiospores of each one fruitbody were germinated on MM/pab and consequently clones were either pab or pab⁻ but always trp⁻.

^c The *A6* progeny was eliminated by microscopy for clamp cells at hyphal septa.

^d One clone was trp⁺ pab, all others trp⁺ pab⁻.

^e All clones were trp⁻ pab⁻.

Table 4. Clamp cell production in the progeny of crosses between monokaryon PG78 and clampless mutants of homokaryon AmutBmut

Cross	Phenotype of progeny, number and percentages of clones*				Total isolates
	Clamps		No clamps		
	pab ⁻	pab	pab ⁺	pab	
PG78 x E-1281	-	42 (24%)	53 (30%)	78 (45%)	173 (100%)
PG78 x E-2095	8 (3%)	12 (5%)	41 (16%)	188 (75%)	249 (100%)

* Basidiospores of each one fruitbody were plated on YMG/T and auxotrophies determined on minimal medium MM supplemented with 100 mg/L trp and 5 mg/L pab where appropriate. Presence of clamps at hyphal septa was determined by microscopical inspection.

Table 5. Inheritance of the *A* mating type locus in progeny of crosses between monokaryon 5401 and *Amut Bmut* homokaryons

Cross	Phenotype of progeny, number and percentages of clones*				Total isolates
	pab ⁻		pab ⁺		
	Clamps (A43mut)	No clamps [A1(ro)]	Clamps (A43mut)	No clamps [A1(m)]	
5401 x 6-031	108 (49%)	-	16 (7%)	95 (43%)	219 (100%)
5401 x AmutBmut	123 (56%)	-	6 (3%)	90 (41%)	219 (100%)

*Basidiospores of each one fruitbody were plated on YMG/T and auxotrophies determined on minimal medium MM (Granado et al. 1997) supplemented with 5 mg/L pab where appropriate. Presence of the respective mating type in germinated clones was microscopically determined by presence (*A43mut*) or absence of clamp cells [*A1(m)*] at the hyphal septa.