Genetic analysis of *Coprinosis cinerea* mutants with defects in fruiting body development

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Few genes have so far been cloned and characterized in fruiting of the heterothallic mushroom *Coprinopsis cinerea*. Fruiting body development normally occurs on the dikaryon. However, the binucleate state of the mycelium hinders easy access of genes. Self-compatible mutants with defects in the mating type pathways can form fruiting bodies without prior fusion to another strain. Uninucleate haploid oidia of such mutants can easily be mutagenized and germinated mycelia tested for defects in fruiting. Mutants can be produced from oidia by classical techniques such as UV treatment or by modern REMI (restriction enzyme-mediated integration) mutagenesis via transformation. Such mutants of self-compatible strains have now been successfully appointed in cloning genes acting in sexual development. Co-isogenic strains of compatible mating types support in genetic characterisation of the mutants.

1. Introduction

1.1. *The wild-type life cycle*

Coprinopsis cinerea (formerly called *Coprinus cinereus*, Redhead et al. 2001) is an excellent model to study fruiting body development in the basidiomycetes. It easily grows in the laboratory and completes its life cycle (Fig. 1, Kües 2000) within two weeks on its natural substrate horse dung as well as

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on artificial substrates on yeast extract – malt extract – glucose basis (Walser et al. 2001).

The life cycle of the heterothallic *C. cinerea* starts with germination of basidiospores that contain one type of haploid nuclei. The resulting primary mycelia are called monokaryons. They have simple septa and one or sometimes two genetic identical haploid nuclei in their hyphal compartments. Monokaryons constitutively produce in high numbers single-celled, uninucleate haploid mitotic spores (oidia) on specialized aerial structures, the oidiophores (Polak et al. 1997, 2001, Kües et al. 2002a, Fischer and Kües 2003). As long as nutrients are available, monokaryons can grow indefinitely. In nature however, dikaryons are prevailing because as soon as they meet, monokaryons of different mating type will fuse to form this secondary mycelia. The dikaryon is characterized by a vigorous mycelium of usually faster growth compared to the parental monokaryons. It has two distinct haploid nuclei in the hyphal compartments (Iwasa et al. 1998) and clamp cells at the hyphal septa (Buller 1933, Badalyan et al. 2004). Under specific environmental conditions, fruiting bodies are formed on the dikaryon (Moore 1998, Kües 2000, Wösten and Wessels 2005). Oidia production on the dikaryon is repressed in the dark. In light, oidia are produced but in much lower numbers than on the monokaryons (Kertesz-Chaloupková et al. 1998, Kües et al. 2002b). The uninucleate haploid oidia are short-lived and serve in distribution of the species to new substrate and as fertilizing agent in

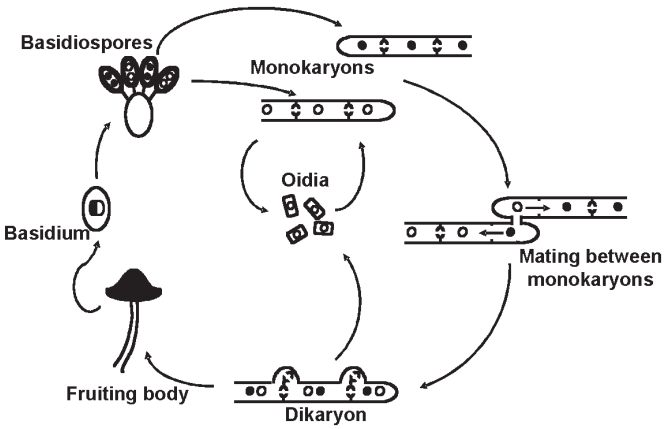

Figure 1. Life cylce of *Coprinopsis cinerea* (modified from Kües 2000). Filled and open circles indicate haploid nuclei of different mating type, a larger half filled-half open circle in the basidium the diploid nucleus obtained by fusion of two haploid nuclei of different mating type. For simplicity of the diagram, oidia production is only shown for one monokaryon.

fusion with monokaryons of different mating type (Brodie 1931, Kemp 1977, Kües 2002, Fischer and Kües 2005).

Light is also needed for induction of fruiting on the dikaryon (Tsusué 1969, Morimoto and Oda 1973, Lu 1974). However, fruiting occurs only under high humidity when nutrients are low and temperatures are in the range of 25-28°C (Madelin 1956, Walser 1997). Tissue formation within the primordium and fruiting body maturation including karyogamy, meisois and basidiospore production is adapted to the daily dark/light rhythm (Lu 1974, Moore et al. 1979). For the process to correctly proceed, further to initiation, light signals as well as distinct dark phases are required at specific points of development (Lu 1974, 2002, Kamada et al. 1978). Once all tissues in the primordium are established, light induced karyogamy parallels induction of fruiting body maturation. Meiosis directly follows karyogamy in the basidia. Stipes elongate and caps open with proceeding meiosis and basidiospore production and maturation. The mature fruiting body appears black by the colour of the melanized cell walls of the ripe basidiospores. The fruiting body is short-lived. Within a few hours, it quickly undergoes autolysis for spore liberation (Moore 1998, Kües 2000, Kamada 2002).

1.2. *Mutants in fruiting body development on the dikaryon*

Fruiting bodies normally develop on the dikaryon, which hinders genetic analysis of the process. Upon mutagenesis of the dikaryon, one would expect to only detect dominant mutations and a very low total number of mutants in screenings for defects in fruiting. For detection of recessive genes, principally two different nuclei would have to be mutagenised and this in the same cell and in the same gene. Such double mutants should be hard to find, particularly when using for mutagenesis mycelium with many dikaryotic cells in which many nuclei will be left non-mutagenised.

Takemaru and Kamada used macerated mycelium of a *C. cinerea* dikaryon in UV mutagenesis and chemical mutagenesis with NG (*N*-methly-*N*'-nitro-*N*-nitroso-guanidine). Surprisingly, they found abnormalities in fruiting body development in frequencies of over 10% of tested clones, in total 1,594 developmental variants amongst 10,641 tested isolates (Takemaru and Kamada 1969, 1970, 1972). Takemaru and Kamada (1972) suggested three causes for

the high amount of variants in their studies: i. influence of factors other than genes, ii. mutations in dominant genes and iii. easy access of fruiting genes in mutagenesis. Later on, Moore (1981) pointed out, that there are already many recessive genetic defects in fruiting present within the natural genetic pool of *C. cinerea*. For example, he observed in his wild-type strain collection defective alleles in four different genes acting in fruiting body initiation. In addition, we found in strain Okayama 7 (http://www.broad.mit.edu/annotation/fungi/coprinus_cinereus/) a natural defect in an essential fruiting initiation gene due to insertion of a transposon (unpublished). In support of Moore's view, other natural defects in sexual development have been described in tissue formation of the primordium (Muraguchi and Kamada 1998), in formation of basidiospores (Pukkila 1993, Kües et al. 2002b), in activation of fruiting in homokaryons (Uno and Ishikawa 1971, Murata et al. 1998a,b, Muraguchi et al. 1999) and in the process of nuclear exchange during mating (May and Taylor 1988). Furthermore, during crosses, new defects spontaneously arise (see below).

Few genes in fruiting have been cloned and analysed since Takemaru and Kamada did their mutagenesis study on the dikaryon. A respectable reason for the low number of cloned genes in fruiting is certainly the normally required dikaryotic state that requests an enormous work load and clever combinations of classical and molecular approaches when wanting to identify a gene (Muraguchi and Kamada 1998, 2000).

As a first gene, the pileus-specific *ich1* gene (for *ichijiku*, the Japanese word for fig) was cloned by first identifying the chromosome it locates on and then complementing the spontaneous recessive *ich1* mutation through transformation of a chromosome-specific library into an *ich1* defective monokaryon followed by crosses of transformants to another compatible *ich1* strain. *ich1* mutants fail to differentiate pileus tissue at the apex of the primordial stipe. Lack of pileus tissue causes a dent in the normally egg-shaped primordia giving the structure a fig-like shape. The abnormal *ich1* primordia rupture during stipe maturation and basidiospores are not formed, unless the defect is complemented by transformation with the wild-type gene. Ich1 is a large protein of 1353 amino acids that contains a potential nuclear targeting signal and has therefore been suggested to act within the nucleus (Muraguchi and Kamada 1998). Moreover, the protein has in its N-terminal half a potential S-adenosyl-methionine (SAM) binding domain similar to known O-methyltransferases (Kües 2000).

Dominant genes might be obtained in analogous strategies but by transformation into a wild-type monokaryon prior to mating of transformants to a compatible wildtype strain. *eln2* (*elongationless 2*) is a constitutively expressed gene that encodes a novel type of microsomal cytochrome P450 enzyme termed CYP502. A dominant *eln2* mutation (originally identified in a self-compatible background, see below) affects stipe tissue formation in the primordia and results in dumpy fruiting bodies with short stipes. The mutant gene was found by altered phenotype on the dikaryon after transforming a wild-type monokaryon and crossing to another strain (Muraguchi and Kamada 2000).

1.3. *Self-compatible mutants in studying fruiting body development*

Fruiting body development has been shown to be controlled by the mating type genes (Tymon et al. 1992, Kües et al. 1998, 2002b). The genes at the *A* mating locus, encoding heomeodomain transcription factors (Hiscock and Kües 1999, Casselton and Challen 2005) control light-induced initiation of fruiting. However, development arrests after tissue formation in the primordia and before karyogamy occurs in the basidia (Tymon et al. 1992, Kües et al. 1998). The genes at the *B* mating type locus, encoding pheromones and pheromone receptors, respectively (Kothe 2001, Casselton and Challen 2005), support the *A* mating type genes in their function in initiating fruiting body development. Primordia are formed in higher numbers and at an earlier time when both pathways are active. Moreover, after completion of tissue formation in the primordia, development continues leading to mature fruiting bodies. This suggests that the *B* mating type genes are required for induction of karyogamy (Kües et al. 2002b).

Consistent with the above results from monokaryons transformed with heterologous mating type genes, mutants with defects in the two mating type loci are self-compatible and have a simplified life-cycle (Fig. 2). Such homokaryotic *Amut Bmut* strains form fruiting bodies with basidiospores without mating to another strain (Swamy et al. 1984, Boulianne et al. 2000; Fig. 3 and Fig. 4). These basidiospores germinate into a self-compatible vigorous mycelia of dikaryon-like appearance (Fig. 3, left photo). The mycelium of *Amut Bmut* homokaryons has clamp cells at most septa (Fig. 4, photo at the left), but only one type of haploid nuclei in its hyphal cells. In submerged

medium, there are mostly two nuclei per hyphal cell and in there aerial mycelium, there is often only one nucleus in a hyphal cell (Swamy et al. 1984, Polak et al. 1997). *Amut Bmut* homokaryons produce uninucleate haploid oidia that again grow into self-compatible mycelia (Swamy et al. 1984; Fig. 3, 2nd photo from left). However, oidia are not constitutively produced in the aerial mycelium as in monokaryons, but asexual spore formation needs illumination as in dikaryons (Polak et al. 1997, Kertesz-Chaloupková et al. 1998). Also like in dikaryons, light induces fruiting on the established mycelium when nutrients are exhausted (Walser et al. 2003, Kües et al. 2004). Upon primordia formation, karyogamy of genetic identical nuclei occurs in the basidia. Meiosis follows and, during fruiting body maturation, the production of four identical basidiospores (Swamy et al. 1984, Kanda et al. 1989a; Fig. 3 photos to the right, Fig. 4). Nowadays, *Amut Bmut* homokaryons [either the original homokaryon AmutBmut from Swamy et al. (1984) or *A43mut*, *B43mut* homokaryon 326 created by Pukkila (1993, 1996) from homokaryon AmutBmut through repeated backcrosses to monokaryon 218] are in most instances used in mutant production (Kanda and Ishikawa 1986, Kanda et al. 1989a,b Chiu and Moore 1990, Pukkila 1994, Granado et al. 1997, Cummings et al. 1999, Inada et al. 2001, Arima, et al. 2004, Kües et al. unpublished;see below). Several genes have been cloned from UV and REMI mutants of such self-compatible homokaryons (Celerin et al. 2000, Inada et al. 2001, Arima et al. 2004, Liu et al. submitted). Amongst cloned functions acting in mushroom formation is a gene *cfs1* for a cyclopropane fatty acid synthase (Liu 2001, Liu et al. submitted) and a gene *eln3* for putative membrane protein with a general glycosyltransferase domain (Arima et al. 2004).

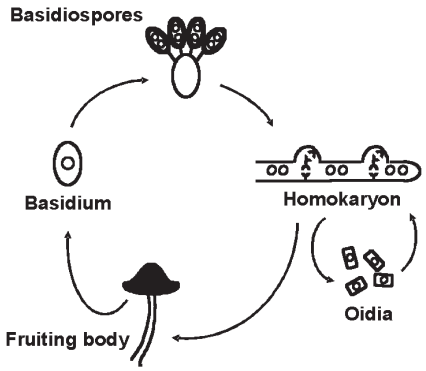

Figure 2. Life cycle of the self-compatible *Coprinopsis cinerea* homokaryon AmutBmut that is defective at both mating type loci (Swamy et al. 1984). Small open circles indicate haploid nuclei, a large open circle the homozygotous diploid nucleus in the basidium

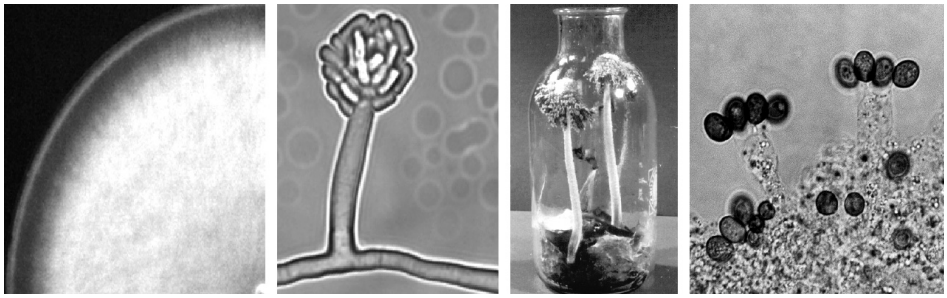

Figure 3. *Coprinopsis cinerea* strain AmutBmut (*A43mut, B43mut, pab1-1*), a homokaryon originally isolated by Swamy et al. (1984). From left to right: vegetative mycelium, an oidiophore produced upon light induction, mushrooms on horse dung and basidia with each four basidiospores. Photos are of courtesy of Yi Liu, Eline Polak, Markus Aebi and Jose Granado

Alternatively to *Amut Bmut* homokaryons, self-compatible strains with defects in the mating type pathways downstream of the mating type genes might be used for mutant production and gene recovery, e.g. the Cop5D mutant being defective in a gene *pcc1* for an HMG box transcription factor acting in the *A* mating type pathway (Murata et al. 1998a,b, Muraguchi et al. 1999, Muraguchi and Kamada 2000). The dominant *eln2* gene is an example of a gene detected through mutagenesis of homokaryon Cop5D (Muraguchi and Kamada 2000, see above).

2. Material And Methods

C. cinerea homokaryon AmutBmut (*A43mut*, *B43mut*, *pab1-1*; Swamy et al. 1984, May et al. 1991) was used in mutant production of UV mutant 6-031 (*A43mut*, *B43mut*, *pab1-1*, *skn1*, *mat*, *bad*) and REMI mutant B-1918 (*A43mut*, *B43mut*, *pab1-1*, *dst3*) (Liu et al. 1999 and submitted, Chaisaena et al. unpublished). Monokaryons JV6 (*A42*, *B42*), 218 (*A3*, *B1*, *trp-1.1,1.6*, *bad*) and PS001-1 (*A42*, *B42*; co-isogenic to homokaryon AmutBmut) were used in crosses (Kertesz-Chaloupková et al. 1998, Srivilai et al. in preparation). R1428 (*A8*, *B7*, *dst1-2*) was kindly supplied by T. Kamada. Growth conditions and all genetic methods are given in Walser et al. (2001).

3. Results And Discussion

3.1. *Mutant production with self-compatible* Coprinopsis cinerea *homokaryons*

The *pab1-1*-auxotrophic homokaryon AmutBmut (Fig. 3, Fig. 4) carrying the mating type alleles *A43mut* and *B43mut* (Swamy et al. 1984, May et al. 1991) has repeatedly been used in the past to create mutants in fruiting body development including meiosis and basidiospore production (Kanda and Ishikawa 1986, Kanda et al. 1989a,b Chiu and Moore 1990, Pukkila 1994, Granado et al. 1997, Kües et al., unpublished). Mutagenesis is easy since the haploid oidia can be used both for classical UV mutagenesis (Kanda et al. 1989 a,b) as well as for transformation in modern REMI (restriction enzyme-mediated integration) mutagenesis (Granado et al. 1997). Upon light illumination, oidia production on *A43mut*, *B43mut* homokaryons is abundant with numbers of up to 10^9 spores per plate (Kertesz-Chaloupková et al. 1998). Established UV and REMI mutagenesis protocols of oidia from homokaryon are given by Walser et al. (2001).

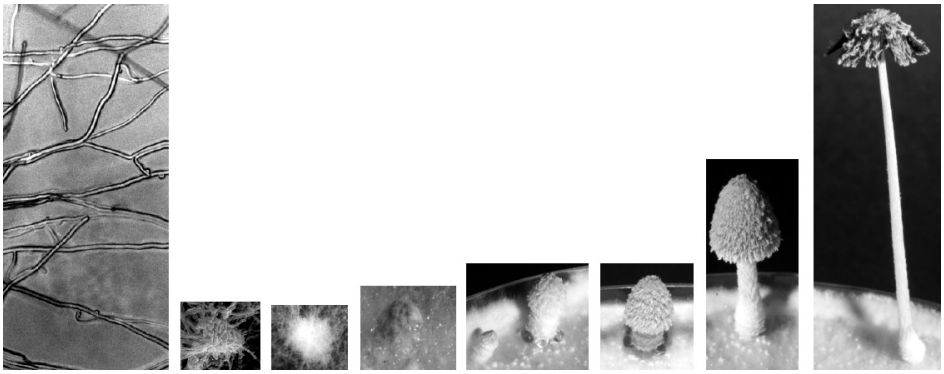

Figure 4. Stages in fruiting body development of *Coprinopsis cinerea* homokaryon AmutBmut (not to scale; adapted with alterations from Boulianne et al. 2000). Within the established mycelium (note the clamp cells at some of the septa in the left photo), loose aggregates (primary hyphal knots) form in the dark by localized intense formation of short hyphal branches with restricted tip growth. Upon reception of a light signal, hyphae aggregate into the compact secondary hyphal knots, in which tissue formation occurs. Correct tissue formation in the primordia needs changing day-night rhythms. Karyogamy in the basidia is induced by a light signal and directly followed by meiosis and basidiospore formation. Parallel to meiosis and basidiospore formation, the stipe elongates and the cap opens. Since it is a highly synchronized process, the developmental stages within the basidia can be predicted by the outer appearance of stages in mushroom development (further details can be found in Lu 1974, 2002, Moore et al. 1979, Kües 2000, Liu 2001, Walser et al. 2003, Kües et al. 2004).

Both UV and REMI mutagenesis has been performed by our group with homokaryon AmutBmut. About 10,000 mutants were screened for behaviour in fruiting. More than 1,200 mutants were detected that were affected in mycelial growth, asexual sporulation and/or fruiting body development (Granado et al. 1997, Kües et al. unpublished, Polak 1999). Using the scheme in Fig. 4, mutants were classified into three major groups with each several hundreds of mutants. The first group comprises defects in fruiting body initiation (block in primary hyphal knot formation and block in secondary hyphal knot formation), the second defects in primordia development up to the stage of karyogamy and the third defects in fruiting body maturation including defects in meiosis and basidiospore formation, respectively (Kües et al. unpublished). The frequencies of mutant production in homokaryon AmutBmut is thus as high as in the original mutant screens with dikaryons performed by Takemaru and Kamada (1972) and as high as in screens with other self-compatible homokaryons of *C. cinerea* (Cummings et al. 1999, Muraguchi et al. 1999).

3.2. *Gene cloning with mutants of self-compatible* Coprinopsis cinerea *homokaryons*

A gene in secondary hyphal knot formation (*skn1*) has recently been cloned by direct complementation of the AmutBmut UV mutant 6-031 (Liu et al., submitted). For transformations, an AmutBmut genomic library was used present in a cosmid carrying the wild-type *C. cinerea pab1* gene (Bottoli et al. 1999). *pab1* complements the *pab1-1* auxotrophy of homokaryon AmutBmut and encodes a para-aminobenzoic acid synthase (James et al. 2002).

Whilst the defect in the early step of fruiting was complemented in the original UV mutant, complete fruiting body development was not achieved (Liu et al. submitted). Crosses of mutant 6-031 with unrelated monokaryons suggested further mutations in later stages of fruiting to be present in the mutant. However, the results of crosses were difficult to interpret because of large progeny fractions were unable to initiate fruiting or development arrested at different stages in development. Large quantities of progeny from parallel crosses between homokaryon AmutBmut and the same monokaryons also failed to initiate fruiting whilst others initiated but did not complete

fruiting. Therefore, failure of initiation and completion of fruiting body development in the progenies of crosses of mutant 6-031 and monokaryons were in many instances likely not due to the *skn1* defect (Liu et al. 1999, Liu 2001, Srivilai et al. in preparation). Sequencing of the DNA fragment complementing the defect in fruiting body initiation in the *skn1* mutant identified the wild-type *cfs1* gene for a potential cyclopropane fatty acid synthase (Liu et al. submitted).

Handling REMI mutants might also not be as easy as originally thought. REMI mutants can carry more than one insertion (Granado et al. 1997, Liu et al. 1999), requesting separation by crosses prior to cloning the interesting insertion by plasmid rescue or PCR-mediated approaches (for techniques of inserted DNA recovery see Cummings et al. 1999 and Walser et al. 2001). However, genetic analysis of progeny of AmutBmut REMI mutants with unrelated monokaryons can be as difficult as with the UV mutants (Liu et al. 1999). As another hindrance found by other researchers (Inada et al. 2001), REMI insertions in some instances are unlinked to the mutant phenotype.

In conclusion, careful genetic analysis is advisable for both UV and REMI mutants before starting cloning genes. So far, this was difficult to perform for mutants of homokaryon AmutBmut.

3.3. *Creating monokaryons with different mating type specificities that are co-isogenic to* Coprinopsis cinerea *homokaryon AmutBmut*

In the past, few attempts have been made to create co-isogenic monokaryons in *C. cinerea* that distinguish just by mating types (Pukkila 1993). Therefore, we crossed *A43mut*, *B43mut* homokaryon AmutBmut to monokaryon JV6 with an *A42*, *B42* mating type and to monokaryon 218 with an *A3*, *B1* mating type. In the first generations, the fruiting abilities of *A43mut*, *B43mut* strains were very poor. In contrast, in higher filial generations of backcrosses to homokaryon AmutBmut, the mycelial appearance of clones in the progenies resembled that of homokaryon AmutBmut and the fruiting abilities raised above 90% of all *A43mut*, *B43mut* clones (Srivilai et al. in preparation). As a positive side effect from the first filial generation of the cross with monokaryon 218, we isolated a spontaneous *A43mut*, *B43mut* mutant with dumpy mushrooms (UFO1, see Fig. 5).

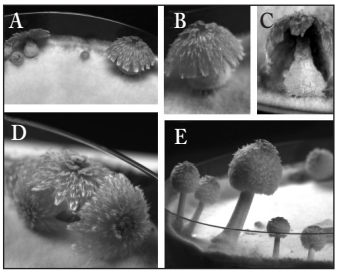

Figure 5. A.-D. Mutant UFO1 (*A43mut*, *B43mut*, *pab1-1*) forms dumpy mushrooms due to a semi-dominant defect in stipe elongation (*eln*). Moreover, it has no basidiospores due to a *bad* defect in basidiospore formation obtained from monokaryon 218 (Pukkila 1993, Kües et al. 2002). **E.** The UFO1 x 218 dikaryon forms medium-sized mushrooms suggesting that the *eln* defect in mutant UFO1 is semi-dominant. Mushrooms have white caps by lack of basidiospore production due to the homozygotous *bad* situation in the dikaryon.

3.4. *Co-isogenic, mating compatible monokaryons in crosses with the secondary hyphal knot UV mutant 6-031 of* Coprinospis cinerea *homokaryon AmutBmut*

When crossing the *skn1* mutant 6-031 with the compatible co-isogenic monokaryons, it was easy to separate the *skn1* mutation from a *mat* mutation (primordia maturation) and a *bad* mutation (basidiospore formation) that were also present in the mutant. Patterns of inheritance of such crosses were clear (Liu et al. submitted, Srivilai et al. in preparation, Fig. 6).

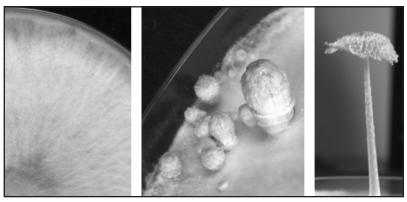

Figure 6. Phenotypes of *A43mut, B43mut* progeny of UV mutant 6-031 crossed with the compatible co-isogenic monokaryon PS001-1. From left to right: a *skn1* clone unable to initiate fruiting, a *mat* clone unable to produce mature fruiting bodies and a *bad* clone forming white mushrooms without spores.

3.5. *AmutBmut REMI mutant B-1918*

B-1918 is a REMI mutant of homokaryon AmutBmut that forms in light "etiolated stipes", also called "dark stipes" (Liu et al. 1999; Fig. 7). In the wild-type, etiolated stipes appear when a strain did not receive enough light (Lu 1974). T. Kamada kindly supplied monokaryon R1428 (*A8*, *B7*, *dst1-2*) that carries a recessive defect in a light receptor and causes in dikaryons etiolated stipe formation in light when present in both type of haploid nuclei (Yuki et al. 2003). A B-1918 x R1428 dikaryon forms mature fruiting bod-

ies (Fig. 7), indicating that the two mutants do not carry the same *dst* defect. In the homokaryotic *A43mut*, *B43mut* situation, the *dst1-2* gene gives in light rise to etiolated stipes (Fig. 7).

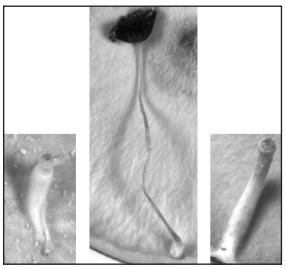

Figure 7. Mutant B-1918 forms etiolated stipes in the dark (left). In crosses with R1428, mature mushrooms arise on the dikaryon. *A43mut, B43mut, dst1-2* clones from the progeny AmutBmut x R1428 form etiolated stipes in the light and do not give rise to mature fruiting bodies.

4. Conclusions

The self-compatible *C. cinerea* homokaryon AmutBmut has been used for producing mutants in fruiting body development. In the past, genetic analysis of these mutants was difficult to perform by lack of co-isogenic compatible monokaryons. We now have co-isogenic strains that allow fast genetic access of mutants and clear-cut interpretations of inheritance of mutant genes in progenies of crosses with AmutBmut mutants.

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