Isolation and characterization of developmental variants in fruiting using a homokaryotic fruiting strain of *Coprinus cinereus*

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Developmental variants in fruiting of *Coprinus cinereus* were induced by mutagenizing oidia of the homokaryotic fruiting strain CopD5-12 with UV light. Through screening of 2,696 isolates, 1,018 strains exhibited defects in fruiting and were classified into 8 groups: (1) knotless variants, which fail to form hyphal knots, the first visible sign of fruiting; (2) primordiumless variants, which form hyphal knots but fail to develop fruit-body primordia; (3) maturationless variants, which form fruit-body primordia but do not form mature fruit bodies; (4) elongationless variants, which form mature fruit bodies with short stipes; (5) expansionless variants, which form mature fruit bodies with unexpanded pilei; (6) sporeless variants, which fail to produce black basidiospores, resulting in fruit bodies with white pilei after maturation; (7) compound type, which includes variants exhibiting several of the phenotypes described above; (8) others, including variants that produce a "dark stipe" even under in light/dark conditions, which is formed under continuous darkness in the wild-type. Two elongationless variants were characterized histologically.

Key Words——basidiomycete; *Coprinus*; developmental variants; fruit-body morphogenesis; homokaryotic fruiting.

The mushroom *Coprinus cinereus* produces a well-organized multicellular structure, the fruit body, whose development has been studied genetically, histologically, and biochemically (Takemaru and Kamada, 1972; Gooday, 1975; Moore et al., 1979). This fungus is amenable to molecular genetic analysis (Binninger et al., 1987, Seitz et al., 1996; Maida et al., 1997; Muraguchi and Kamada, 1998; Murata et al., 1998). Thus, *C. cinereus* provides an excellent system for molecular analysis of multicellular development in fungi.

Developmental mutants in model organisms, such as *Drosophila melanogaster*, *Caenorhabditis elegans*, and *Arabidopsis thaliana*, have proved to play important roles in dissecting developmental sequences and cloning of genes responsible for the developmental processes (Lewis, 1978; Nüslein-Volhard and Wieschaus, 1980; Wood, 1988; Weigel and Meyerowitz, 1994). In *C. cinereus*, induction of developmental mutants in fruiting has been done extensively using a dikaryon as the parental wild type (Takemaru and Kamada, 1972). However, it is difficult to identify recessive developmental mutations, because recessive mutations are masked by their wild-type alleles in the dikaryon.

Homokaryotic fruiting is known in some basidiomycetous species (Stahl and Esser, 1976; Leslie, 1979; Leslie and Leonard, 1979; Esser et al., 1979; Miyake et al., 1980). Strains exhibiting homokaryotic fruiting should provide a good system to induce and identify developmental mutations, because mutations in such strains directly bring about mutant phenotypes regardless of their dominance. In *C. cinereus*, the *AmutBmut* strain, which has mutations in the *A* and *B* mating-type gene loci, is known to exhibit homokaryotic fruiting (Swamy et al., 1984), and using this strain, some recessive developmental mutations have been identified (Kanda and Ishikawa, 1986; Kanda et al., 1989; Chiu and Moore, 1990).

In the present study, we induced developmental variants by mutagenizing oidia of a homokaryotic fruiting strain of *C. cinereus*, CopD5-12, with UV light. Of the variants induced, two elongationless variants were characterized histologically.

Materials and Methods

Strains and culture conditions 5302 (*A2B2*) is a wild-type homokaryon of *C. cinereus*, which is used as a standard strain in our laboratory. CopD5-12 (*A12B12*) is a basidiospore derivative from a fruit body of *C. cinereus* collected in the field, and has a trait of homokaryotic fruiting. Genetic control for the homokaryotic fruiting of CopD5-12 will be reported elsewhere. Culture conditions, matings, and fruiting conditions were as previously described (Kamada et al., 1984).

Preparation of oidia suspension A quarter of a colony grown for 7 d on a CY-1 plate in a Petri dish 9 cm in diam was picked out together with the agar medium and homogenized in 20 ml of sterilized water with a Waring blender (Ace Homogenizer, Nihonseiki, Japan) at 15,000 rpm for 5 min. One ml of macerated hyphae was spread on CY-1 medium and cultivated at 28°C for various durations. A mycelial lawn was flooded with 5–

10 ml of liquid minimal medium and scratched with a spreader in order to harvest oidia. Oidia were purified by filtration through glass wool. The number of oidia was measured with a Thoma's hemocytometer.

Mutagenesis and screening Ten ml of oidia suspension in a 9 cm Petri dish was irradiated by a 10 W UV germicidal lamp (Toshiba) set 10 cm from the Petri dish. The oidia suspension was stirred gently during irradiation using a clip as a stirring bar. An aliquot (0.2 ml) of UV-irradiated oidia suspension was spread on a plate of minimal medium supplemented with adenine, arginine, choline, histidine, methionine, and nicotinic acid (10^{-4} M each).

After incubation at 25°C for 5 d in the dark, survivors were picked out under a binocular microscope and inoculated on yeast-maltose-glucose (YMG) medium (Rao and Niederpruem, 1969). The resulting colonies were then subcultured on slant YMG medium for at least 20 d at 28°C in a 12 h light/12 h dark regime in order to test for fruiting phenotypes. The same colonies were also subcultured on minimal medium to test for auxotrophy. Variant strains that exhibited abnormal fruiting in the first test were retested for their fruiting phenotypes on two new YMG slants. Fruiting phenotypes of the variants were scored as the most advanced stage in fruiting.

Results

Preliminary experiments for mutagenesis We first tried to increase the germination percentage of oidia, which was very low (about 10%) in both 5302 and CopD5-12 when oidia were harvested from usual cultures. When oidia are harvested from a colony formed from a one-point inoculum, the age of oidia varies depending on the area in the colony. We assumed that this heterogeneity in age of oidia might be a reason for the low germination rate. To obtain oidia of homogeneous age, macerated mycelia were spread on YMG plates, and oidia were harvested after incubation for various times. The germination percentage of the oidia harvested increased as the incubation proceeded and attained 30% in 6 d cultures (Fig. 1). Thus, we chose to use oidia harvested from 6 d cultures of macerated mycelia for mutagenesis.

To determine the duration of UV-irradiation to give 10% survival, oidia were UV-irradiated for various durations and examined for survival (Fig. 2). UV-irradiation for 25 s gave 10% survival and was used for mutagenesis in later experiments.

Isolation of developmental mutants The parental strain, CopD5-12, exhibits normal fruiting (Fig. 3). Oidia of CopD5-12 were mutagenized with UV light, and 2,696 isolates were examined for fruiting phenotypes. Of these, 1,018 strains displayed variant phenotypes in fruiting, which were classified into 8 groups (Table 1; typical variants are shown in Fig. 4): (1) knotless variants, which fail to form hyphal knots, the first visible sign of fruiting; (2) primordiumless variants, which form hyphal knots but fail to develop fruit-body primordia; (3) maturationless variants, which form fruit-body primordia,
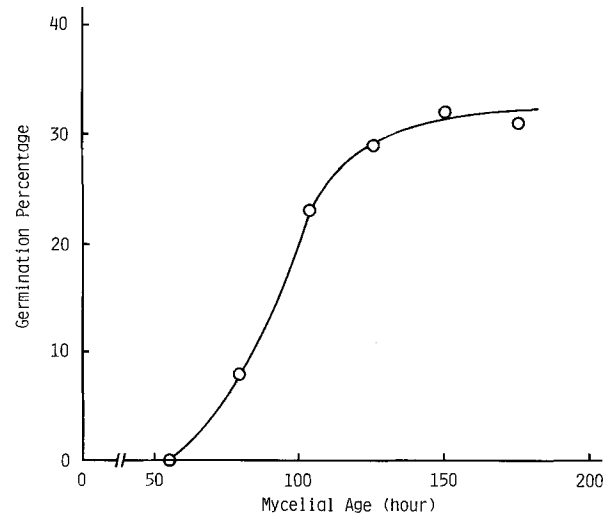

Fig. 1. Germination of CopD5-12 oidia of *C. cinereus*. Oidia were harvested from the mycelial lawn made from macerated mycelia and spread on YMG plate medium. Germination percentages were scored after incubation at 28°C for 2 d.
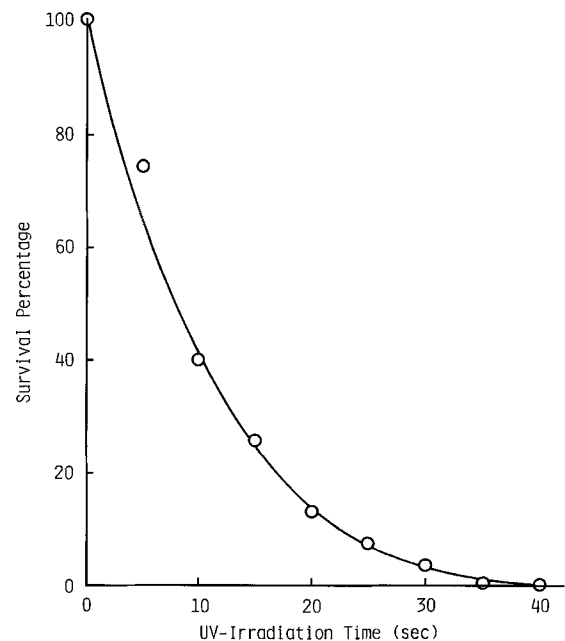

Fig. 2. Survival curve of CopD5-12 oidia. Suspension of oidia was UV-irradiated for various durations, then spread on minimal medium. Survival percentages were scored after incubation at 28°C for 2 d.

but do not form mature fruit bodies; (4) elongationless variants, which form mature fruit-bodies with short stipes; (5) expansionless variants, which form mature fruit-bodies with unexpanded pilei; (6) sporeless variants, which fail to produce black basidiospores, resulting in fruit-bodies with white pilei after maturation; (7) compound type, which includes variants exhibiting several of phenotypes described above; (8) others, including three variants, Uar801, Uad351, and Uad290, which produce
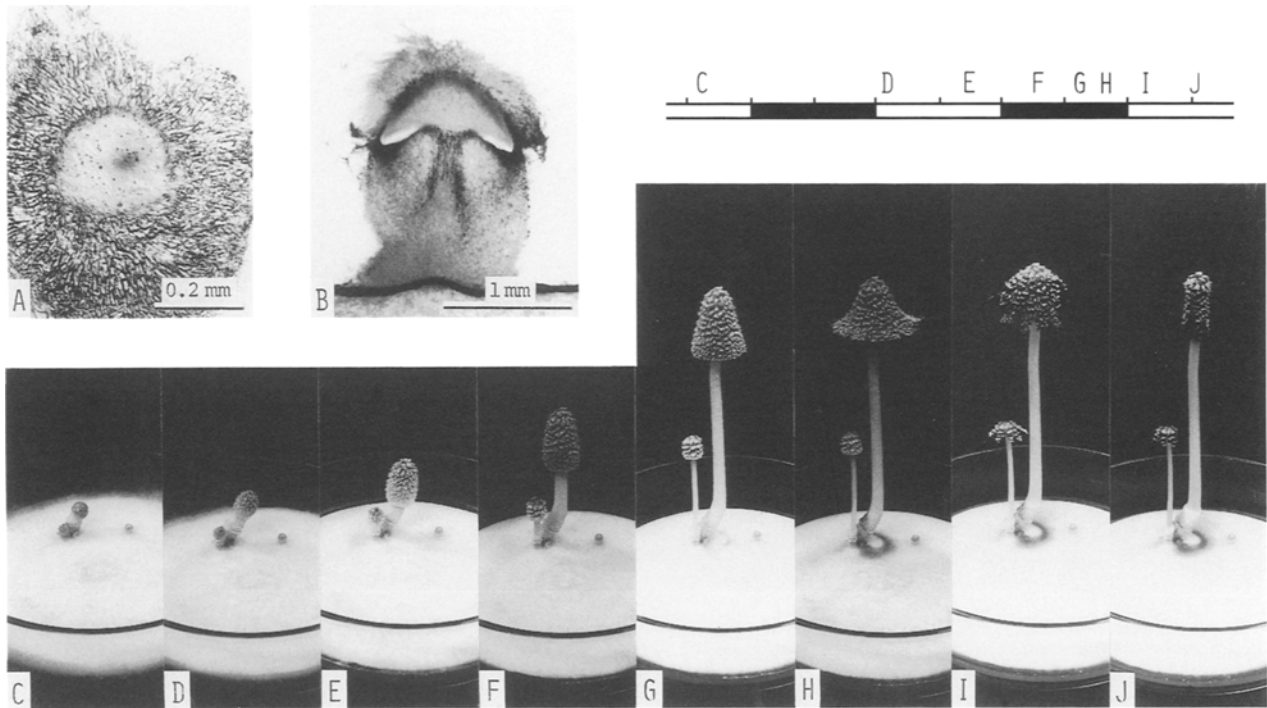
Fig. 3. Homokaryotic fruiting of CopD5-12. A, a median section of a hyphal knot; B, a median section of a primordium; C–J, maturation stages; E–G, stipe elongation; G–H, pileus expansion; D–E, meiosis occurs in basidia.; E–G, sporulation occurs, making the pileus black.; H–J, autolysis. The 12 h light/12 h dark regime of stages C–J is indicated above the photographs.

Table 1. UV-induced and spontaneous variants of homokaryotic fruiting in *Coprinus cinereus*.

Exp.	Depress	Knotless	Primor-diumless	Matura-tionless	Elonga-tionless	Expan-sionless	Sporeless	Compound type	Others	Var./Isol.
I	77	150	39	90	7	5	33	10	9	
II	69	132	39	91	9	3	34	11	8	
III	69	95	11	12	3	0	6	2	4	
Total	215 (8.0)	377 (14.0)	89 (3.3)	193 (7.2)	19 (0.7)	8 (0.3)	73 (2.7)	23 (0.9)	21 (0.8)	1018/2696 (37.8)
Spon.	0 (0.0)	1 (0.4)	4 (1.6)	1 (0.4)	4 (1.6)	0 (0.0)	1 (0.4)	1 (0.4)	0 (0.0)	12/249 (4.8)

Depress phenotype means that aerial hyphae are reduced in colonies and senescent hyphae in the colonies undergo autolysis. Values in parenthesis represent percentage of the variants. Spon., spontaneous variants.

a "dark stipe" even under light/dark conditions (Fig. 5). The dark stipe is an etiolated primordium and is formed under continuous darkness in the wild type (Tsusué, 1969). A total of 3,958 isolates were also screened for auxotrophic mutations, and three auxotrophs were identified: one adenine-requiring, one arginine-requiring and one nicotinic acid-requiring.

Characterization of elongationless mutants Uad435 and Uad605 displayed defects in stipe elongation in the maturation stage of fruiting (Fig. 6). These elongationless variants were analyzed histologically, and their developmental sequences were compared with that of the wild type (Figs. 7, 8). Veil cells and stipe cells in Uad435 are globose compared with those of the wild-type (Fig. 7B). Uad605 exhibits a dumpy pileus in an

early stage of fruiting (Fig. 7C), and subsequently the primordial shaft bulges horizontally (Fig. 8C). Thus, Uad435 and Uad605 not only produce short stipes in the maturation stage of fruiting, but also exhibit defects in early stages of fruit-body development.

When Uad435 was mated with a compatible wild-type strain, 5302, the resultant dikaryons produced fruit-bodies with longer stipes, suggesting that Uad435 carries a recessive mutation. When Uad605 was mated with 5302, the resultant dikaryons produced dumpy fruit-body primordia, resulting in mature fruit bodies with short stipes. This result suggested that Uad605 harbors a dominant mutation.
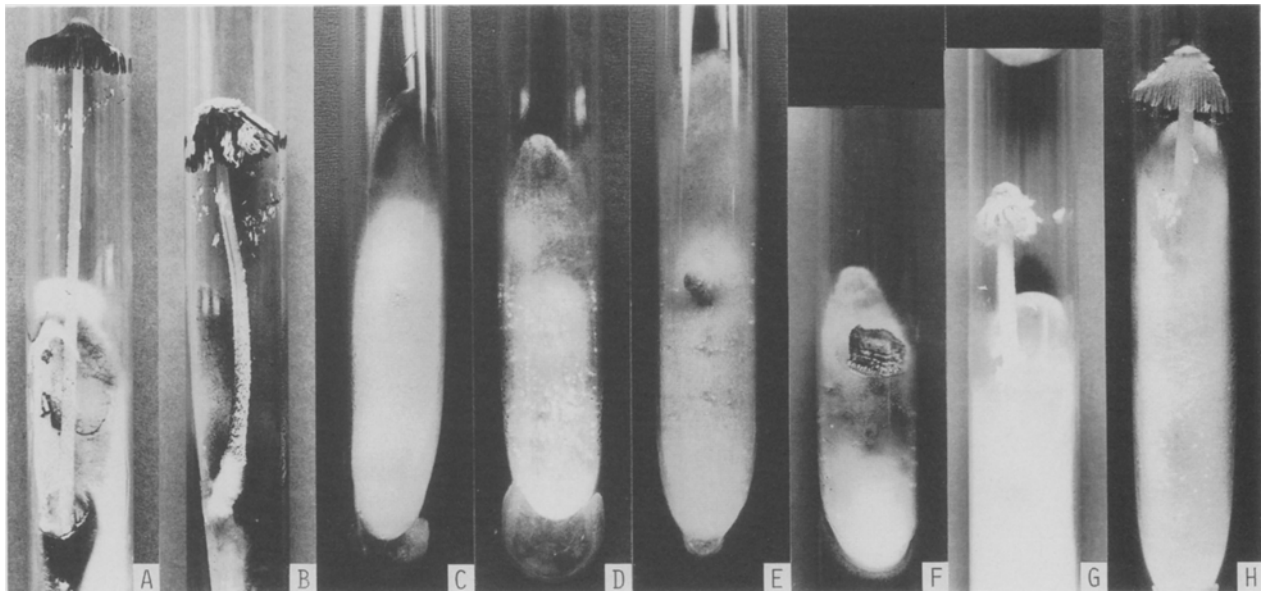
Fig. 4. Typical variants in fruiting. A, a dikaryotic wild-type fruit-body; B, a homokaryotic wild-type fruit-body; C, knotless; D, primordiumless; E, maturationless; F, elongationless; G, expansionless; H, sporeless.
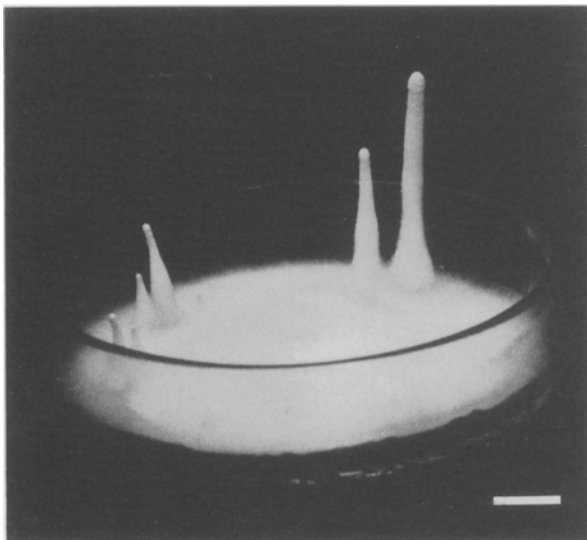

Fig. 5. Dark stipe variant Uar801. The scale bar represents 1.0 cm

Discussion

Developmental variants in fruiting were induced by ir-radiating oidia of a homokaryotic fruiting strain, CopD5-12, with UV light. The percentage of the induction of developmental variants is surprisingly high (1,018/2,696, 37,8%) compared with that of auxotrophic muta-tions (3/3,958, 0.08%). A high frequency of induction in screening for fruiting variants using a dikaryon has also been reported in this fungus (Takemaru and Kamada, 1972). These high frequencies suggest that develop-ment is affected by defects in various housekeeping processes in addition to fruiting-specific processes. In addition, frequencies of spontaneous variations suggest that these steps might be instable in the homokaryotic fruiting (Table 1).

Elongationless mutants Stipes in both Uad435 and Uad605 fail to elongate in the maturation stage of fruit-ing. However, their phenotypes are easily distinguisha-ble from each other (Fig. 6). Moreover, dominance tests suggested that Uad435 carries a recessive mutation, whereas Uad605 carries a dominant mutation. These results suggest that Uad435 and Uad605 have mutations in different loci.

Uad435 exhibits a defect in cell shape not only in the stipe tissue but also in the veil. The veil and the stipe in Uad435 are composed of large globose cells compared with cylindrical large cells in the wild-type. This defect appears to culminate in the failure of stipe elongation in the maturation stage of fruiting. The defect in cell shape suggests that there is a common mechanism by which veil cells and stipe cells are formed and elongate cylindri-cally. In fact, stipe and veil cells are similar to each other with respect to helical chitin fibrils in the cell wall (Kama-da and Tsuru, 1993). NG0398, which was induced in a dikaryon with nitrosoguanidine and carries a semi-dominant mutation, $eln1-1$, exhibits a cellular defect simi-lar to Uad435 (Takemaru and Kamada, 1972; Kamada and Takemaru, 1977a, b). This suggests that a reces-sive mutation in Uad435 might be allelic to $eln1-1$. Fur-ther studies are necessary to confirm this.

Uad605 produces dumpy fruit-body primordia, sug-gesting defects in balance between the pileus and the primordial shaft, the lower part of the stipe. The defects are manifested in an early stage of development. We could not attribute the phenotype to defects in a certain tissue. Genetic analysis indicated this phenotype is
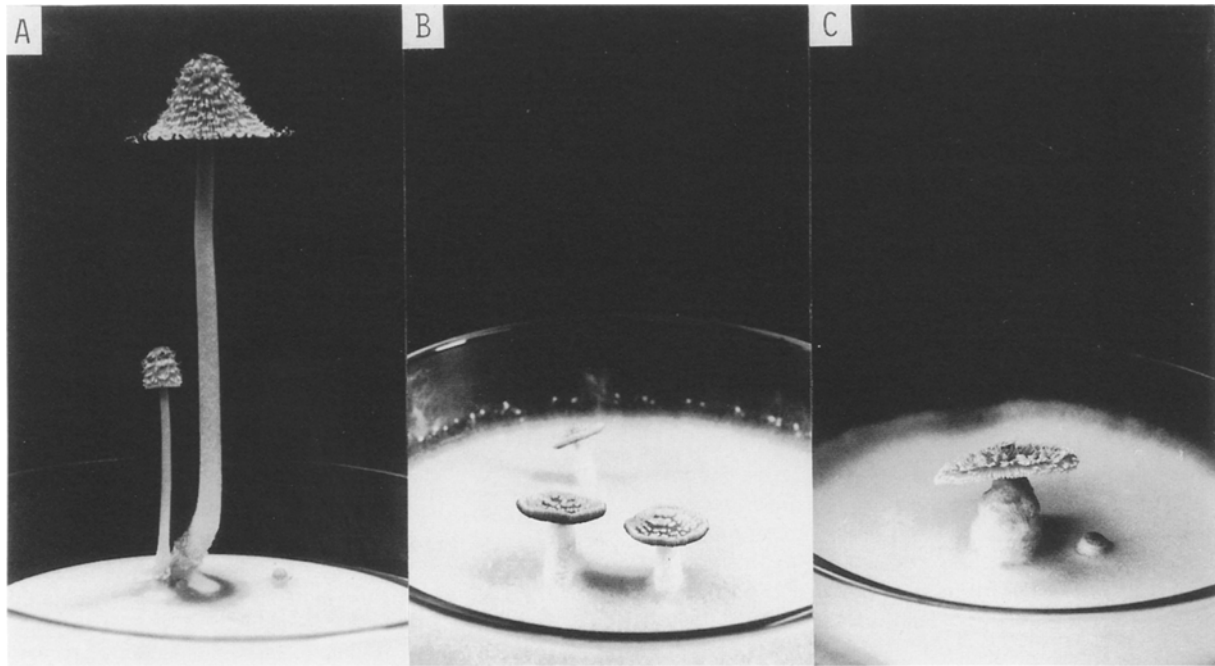
Fig. 6. Mature fruit bodies of the wild-type strain, CopD5-12 (A), and the elongationless variants Uad435 (B) and Uad605 (C).
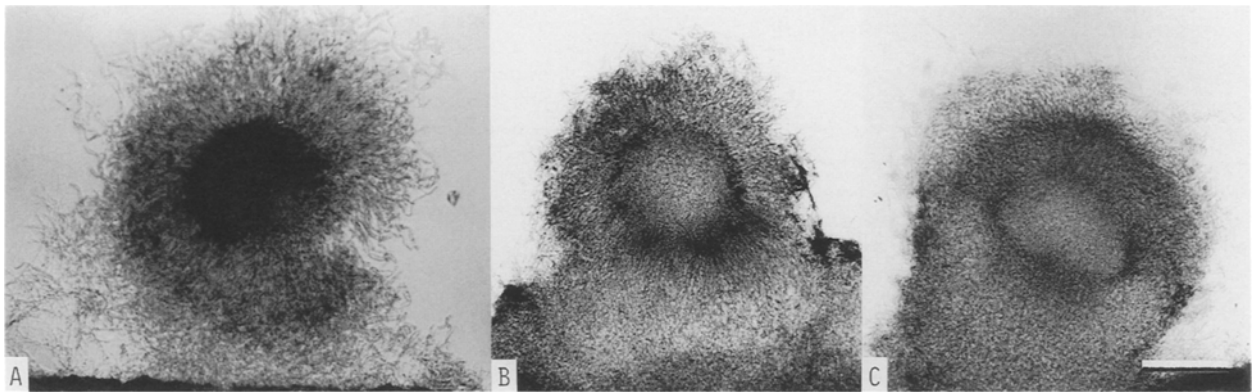

Fig. 7. Longitudinal sections of the hyphal knots of the wild-type strain, CopD5-12 (A), and the elongationless variants, Uad435 (B) and Uad605 (C). The bar represents 0.2 mm.
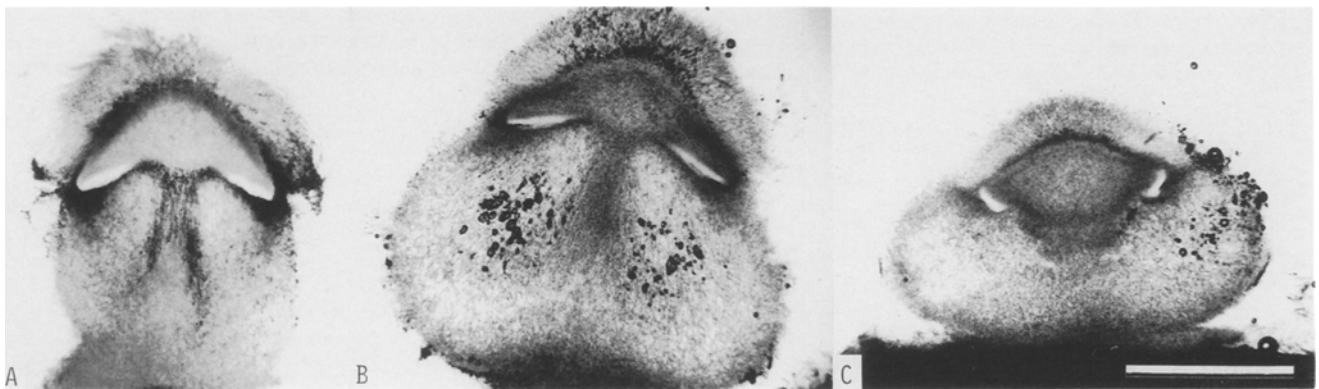

Fig. 8. Longitudinal sections of the primordia of the wild-type strain, CopD5-12 (A), and the elongationless variants Uad435 (B) and Uad605 (C). The bar represents 1.0 mm.

dominant. If the mutant gene in Uad605 was introduced into the wild-type strain, the transformants would display the dominant phenotype in fruiting. This transformation would allow us to clone the mutant gene. Experiments to clone the gene are in progress.

Dark stipe mutants Maturation of fruit-bodies in *C. cinereus* requires appropriate light conditions (Kamada et al., 1978). Through the screening for developmental mutants, three variants were isolated that produce a "dark stipe" even under a 12 h light/12 h dark regime. Since the dark stipe is formed by the wild-type dikaryon cultured under continuous darkness (Tsusué, 1969), these variants are suggested to have defects in light reception or light signal transduction pathways.

Action spectrum analysis indicates that blue or near-UV light is effective for fruit-body maturation in basidiomycetes (Morimoto and Oda, 1973; Eger-Hummel, 1980; Durand and Jacques, 1982). To date, genes involved in blue light reception and blue-light signal transduction have been identified and cloned in *Arabidopsis thaliana* and *Neurospora crassa* (Ahmad and Cashmore, 1993; Carattoli et al., 1995; Ballario et al., 1996; Ballario and Macino 1997; Linden and Macino, 1997; Linden et al., 1997). However, many of the components in the blue-light reception system remain to be elucidated. Molecular analysis of the dark stipe variants identified in *C. cinereus* may provide further insight into the mechanisms underlying the blue-light reception system.

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