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SHORT COMMUNICATION

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Isolation of a homothallic mutant in *Lentinula edodes*

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Abstract To obtain a homothallic mutant in *Lentinula edodes*, basidiospores derived from the common *Bmut* dikaryon (*A1B1mut* × *A2B1mut*) were treated with UV irradiation. Of a total of approximately 5000 monosporous cultures recovered, a single basidiospore isolate was found to produce the hyphae bearing clamp connections without mating. This mutant strain could form fruit bodies, and all its single basidiospore isolates developed into colonies with clamp connections. Such homothallic behaviors were transmitted from the mutant strain to the next generation. During the germination and following hyphal elongation in a single basidiospore of mutant strain, clamp connections were clearly detected in multicellular hyphae, which contained two nuclei in each cell. Their clamp connections were morphologically variable, viz., pseudo, abnormal, and true clamps. Amplified fragment length polymorphism (AFLP) profiles among the basidiospore isolates of mutant strain were identical, indicating that the mutant strain produced isogenic basidiospore progeny.

Key words DNA fingerprinting · Homothallic mutant · *Lentinula edodes* · Mutagenesis

Shiitake, *Lentinula edodes* (Berk.) Pegler, is the major edible mushroom in Asia, particularly in China and Japan. The mating system of this fungus, leading to the formation of a dikaryon that can produce fruit bodies, is known to be bifactorial heterothallism (tetrapolarity) controlled by two unlinked multiallelic incompatibility factors, *A* and *B* (Takemaru 1961). The regulatory role of the *A* and *B* incompatibility factors in sexual morphogenesis is evident from genetic studies on mutation in either or both of the two factors (Raper and Raper 1966; Casselton 1978). Mutants in *A* and *B* factor genes (*Amut* and *Bmut*) have been previously isolated in *Schizophyllum commune* Fr. (Raper et al. 1965) and *Coprinus cinereus* (Schaeff.: Fr.) S.F. Gray (Haylock et al. 1980; Swamy et al. 1984). In edible mushrooms, *Bmut* strains have been recovered in *Pleurotus ostreatus* (Jacq.: Fr.) Kummer (Obatake et al. 2002) and *L. edodes* (Murakami and Hasebe 2000), whereas *Amut* strains have not been isolated so far.

These incompatibility factor mutants are useful for elucidating the construction and function of mating-type genes. Recent molecular studies on the loci of *A* and *B* factors in *S. commune* and *C. cinereus* have revealed that *A* factor genes code for homeodomain proteins functioning as transcription factors and *B* factor genes code pheromone and pheromone receptors (Casselton and Kües 1994; Kothe 1999; Kamada 2002). On the other hand, specific mutations in both mating-type loci give rise to homothallic strains (*Amut Bmut*), which can form dikaryotic hyphae bearing clamp connections and fruit bodies without mating (Swamy et al. 1984). Because of this unique feature, extensive use of *Amut Bmut* strains has been made in generating recessive mutants in fruiting pathway (Kanda and Ishikawa 1986; Kües 2000; Kamada 2002).

In this article, we describe isolation of a homothallic mutant in *L. edodes*. The mutant produces hyphae bearing clamp connections without mating and forms fruit bodies that release isogenic basidiospore progeny.

Strains of *L. edodes* listed in Table 1 were used in this study. The representative mutant strain (24AB1; TMIC-1823) and wild-type dikaryotic strain (TMIC-1358) were deposited at the Tottori Mycological Institute Culture Collection. To obtain the homothallic mutant of *L. edodes*, we attempted to mutate an *A* incompatibility factor in basidiospores from the common *Bmut* dikaryon of *L. edodes*. A common *Bmut* dikaryon was constructed by mating between two *B1mut* homokaryons, (2 × 6) D1-5 of *A1B1mut* and (2 × 6) D1-7 of *A2B1mut*, which were reported by

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Table 1. List of strains of *Lentinula edodes* used in this study

Strain	Description or genotype	Source
TMIC-1358	Wild-type dikaryon	TMIC
(2 × 6) D1-5	*Bmut* homokaryon, *A1B1mut*	Murakami and Hasebe (2000)
(2 × 6) D1-7	*Bmut* homokaryon, *A2B1mut*	Murakami and Hasebe (2000)
Common *Bmut*	Dikaryon cross between (2 × 6) D1-5 and (2 × 6) D1-7	This study
Homothallic mutant strain		
AB	Mutant from basidiospores of common *Bmut*	This study
24AB	Progeny from AB	This study
24AB1 (TMIC-1823)	Progeny from 24AB	This study
24AB1-1	Progeny from 24AB1	This study
24AB1-2	Progeny from 24AB1	This study
24AB1-3	Progeny from 24AB1	This study
24AB1-4	Progeny from 24AB1	This study

TMIC, Tottori Mycological Institute Culture Collection

Murakami and Hasebe (2000). The resulting dikaryon was grown and maintained on malt extract agar (MA) medium (20 g malt extract, 20 g agar, 1 l tap water) at about 23°C. Fruit bodies of the common *Bmut* dikaryon were produced on sawdust (SD) medium prepared with 400 g air-dried hardwood sawdust and 80 g rice bran wetted with 1 l tap water (Murakami et al. 1987). Basidiospores were harvested from the fruit bodies and then suspended in sterilized water. The spore suspension was evenly spread throughout the surface of MA medium in Petri dishes. Immediately after being spread, the spores were irradiated with a Toshiba 10 W germicidal lamp for 30–40 s at a distance of 10 cm and then incubated at 25°C. The UV treatment and the incubation were performed in the dark. After a week of incubation, the germlings of the surviving spores were isolated individually with method described by Miles et al. (1966) and cultured on MA medium. After 10 days incubation at 25°C, colony peripheries were examined microscopically for the occurrence of hyphae bearing clamp connections as a sign of homothallic characteristics, because clamp connection was recognized in homothallic strains in *C. cinereus* (Haylock et al. 1980; Swamy et al. 1984) and *S. commune* (Raper et al. 1965). Of a total of approximately 5000 monosporous cultures recovered, a single isolate was found to produce dikaryotic hyphae bearing clamp connections without mating. The mutant strain could form fruit bodies on SD medium, and all its single basidiospore isolates developed into colonies with clamp connections. Such homothallic behaviors were transmitted from the mutant strain to the next generation.

To examine morphological changes in germlings, slide culturing was performed. Basidiospores from fruit bodies were spread on a thin film of MA medium on a sterilized glass slide. The glass slide was placed in a sterilized Petri dish containing wet filter paper. The culture was incubated at 25°C in the dark. After the incubation, approximately 1000 germlings of each strain were observed under a phase-contrast light microscope. When basidiospores of mutant strain (24AB1-1) were released on a thin film of MA medium, about half the basidiospores showed no sign of germination and the other half were able to germinate. About 25% of germlings survived and produced colonies: the other portion was abnormally swelled and failed to produce colonies. No clamp connection was observed at the first septum in a two-cell hypha incubated for 48 h at 25°C (Fig. 1C). The hypha forming the hock cell was found after 72 h incubation (Fig. 1D). After 96 h incubation, clamp connections were apparently detected in multicellular hyphae, which contained two nuclei in each cell (Fig. 1E), although no clamp connection was detected in the wild-type strain at the same stage (Fig. 1F). These observations strongly suggest that the germlings of the mutant basidiospore developed into dikaryotic hyphae without mating. The clamp connections of the mutant strain were morphologically variable, viz., pseudo, abnormal, and true clamps (Fig. 2). Irregularities in clamp structure were also observed in the *Amut Bmut* strain of *C. cinereus*, in which several pseudo clamps were intermingled with true clamps, and the ratio of pseudo clamp to true clamp varied (Swamy et al. 1984). The irregularities suggest that these mutants exhibit several dikaryotic characteristics including true clamp connections, binucleate cells, and normal fruiting, whereas they retain a few features of incomplete fertilization such as pseudo clamp formation.

To examine genetic relatedness among basidiospore isolates derived from the fruit bodies of the mutant, amplified fragment length polymorphism (AFLP) analysis was carried out according to the procedure of Terashima et al. (2002). Cultured mycelia from each strain were harvested, freeze-dried, and used for DNA extraction as described previously (Matsumoto et al. 2003). Genomic DNA (1 µl from elution fluid in DNA extraction procedure, approximately 20 ng or less) was digested with endonucleases (*Eco*RI and *Mse*I). PCR reactions were carried out in a Takara PCR Thermal Cycler MP (Takara, Shiga, Japan) with Takara *Ex Taq* DNA polymerase (Takara). AFLP primer pairs were E + 0 / M + 0 for preselective amplification, and E + AC / M + CA or E + AC / M + CC for selective amplification. Electrophoresis and detection of amplified fragments were carried out using an ABI Prism 310 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). By AFLP analysis with two primer pair combinations (E + AC / M + CA and E + AC / M + CC), 55 and 37 DNA fragments were detected in the range of 100 to 500 bases, respectively.

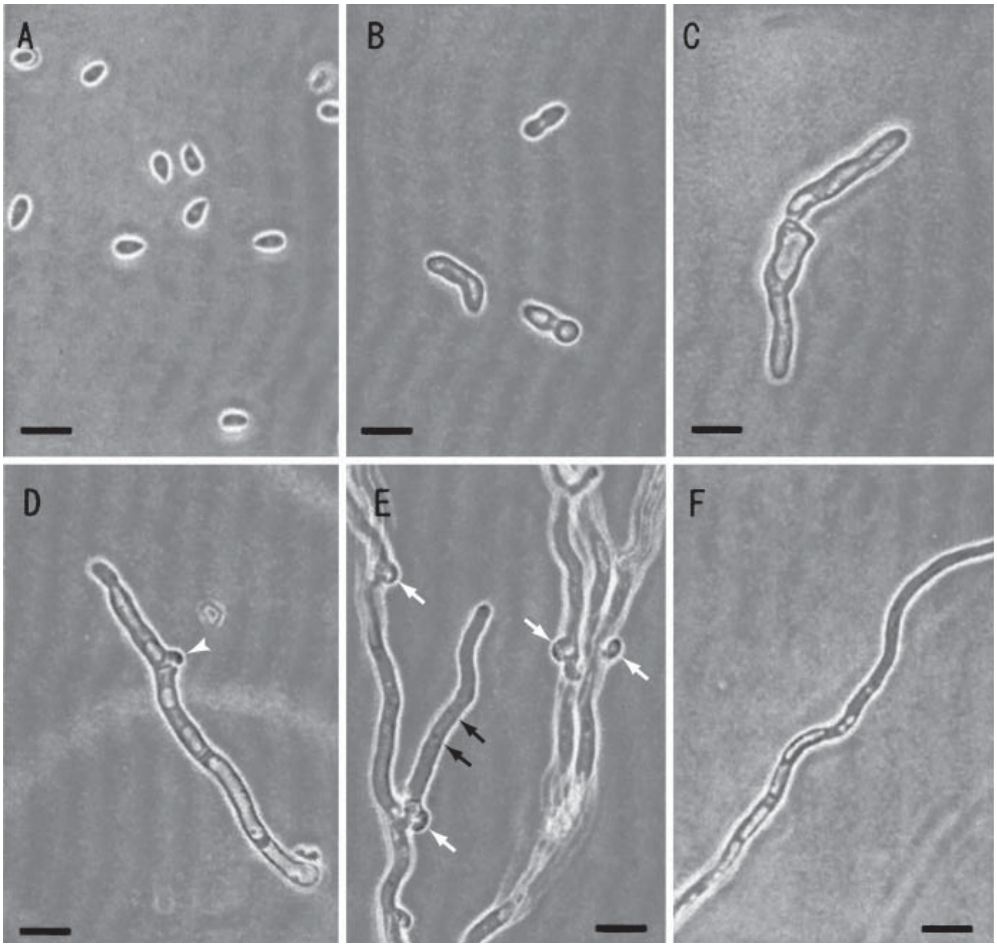

Fig. 1. Germination and subsequent hyphal elongation of basidiospores from a mutant strain 24AB1-1 (**A**, **B**, **C**, **D, E**) and a wild-type strain TMIC-1358 (**F**) of *Lentinula edodes*. Basidiospores were incubated on a thin film of malt extract agar (MA) medium at 25°C for 0 h (**A**), 24 h (**B**), 48 h (**C**), 72 h (**D**), and 96 h (**E**, **F**), and observed under a phase-contrast light microscope. *White arrowhead* (**D**) indicates a hock cell at septum in germling; *white arrows* (**E**) indicate clamp connections at septa in multicellular hyphae; *black arrows* (**E**) indicate nuclei. *Bars* 10 μm

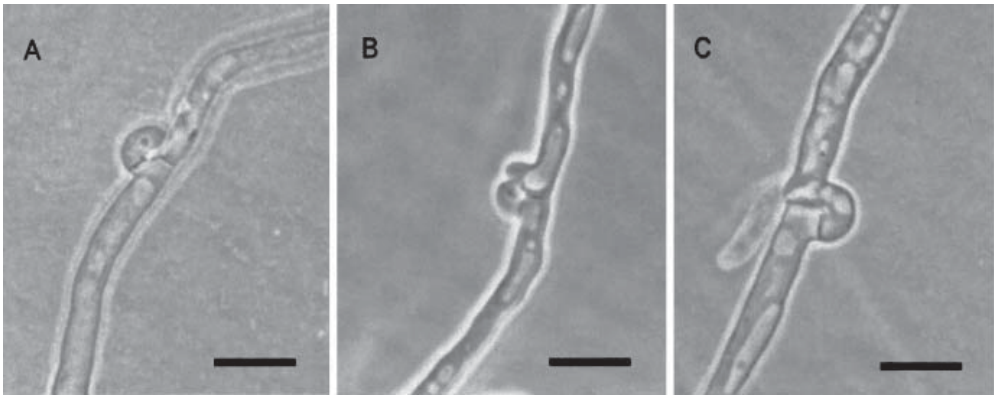

Fig. 2. Structures of clamp connections in the mycelium of mutant strain 24AB1-1 of *Lentinula edodes*. **A** Pseudo clamp connection. **B** Abnormal clamp connection. **C** True clamp connection. The mutant strain 24AB1-1 was cultured in a thin film of MA medium at 25°C for 7 days, and the colony periphery was observed under a phase-contrast light microscope. *Bars* 10 μm

AFLP profiles among basidiospore isolates (24AB1-1, 24AB1-2, 24AB1-3, and 24AB1-4) from mutant strain (24AB1) were identical (Fig. 3). Identical AFLP profiles were also obtained from four mutant strains (AB, 24AB, 24AB1 and 24AB1-1) that belong to different generation progenies from each other. This result indicates that the mutant is a homokaryon containing two genetically identical nuclei pair in each cell, which then potentially produces isogenic basidiospore progeny.

From the results that the single basidiospore isolates of the mutant developed into dikaryotic colonies and gave identical AFLP profiles, we assume that the mutant recovered in this study is mutated in both *A* and *B* incompatibility factors. However, it has not been determined whether the mutation responsible for homothallic phenotype occurred in the incompatibility factors or in another gene such as *pcc1* associated with fruit body formation in a homokaryon without mating in *C. cinereus* (Murata et al. 1998). There-

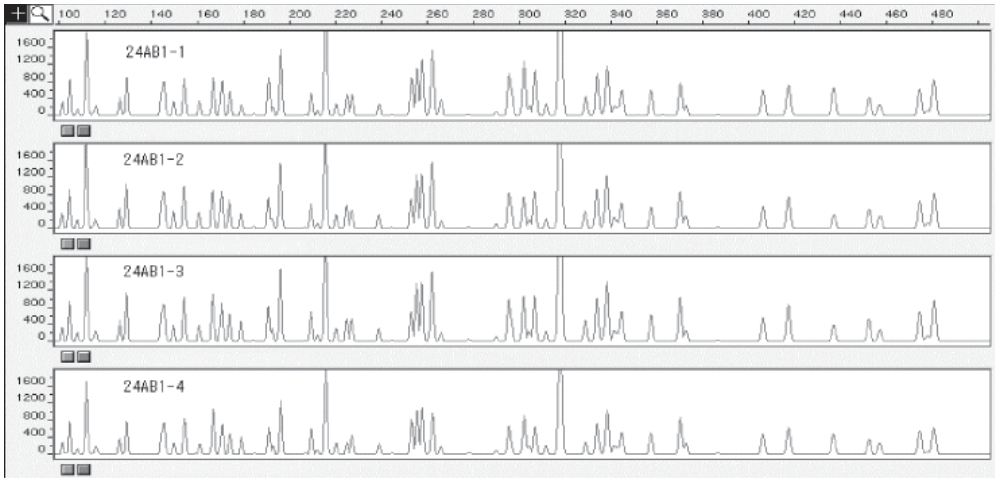

Fig. 3. Representative electrophoresis image of amplified fragment length polymorphism (AFLP) analysis in single basidiospore isolates derived from mutant strain 24AB1 of *Lentinula edodes*. These DNA fragments were produced with the E + AC / M + CC primer combination

fore, genetic identification and characterization of this mutation remain to be investigated.

Fruit body formation is one of the most typical and attractive morphological differentiation in mushrooms. Developmental mutants are very useful to understand the molecular mechanisms responsible for fruit body formation. Initially, developmental mutants in *C. cinereus* were induced by mutagenizing the macerated dikaryotic mycelium (Takemaru and Kamada 1972). However, it was difficult to identify recessive mutations in the dikaryon because they were masked by their wild-type alleles. The use of basidiospores from the *Amut Bmut* strains in isolating developmental mutants has a number of advantages over the use of those from the wild-type dikaryons. First, it is clear that all genes essential for fruiting are present in an active form. Second, because of the presence of a single haploid genome, all possible mutations will be detected, regardless of whether they are dominant or recessive. Because of these advantages, it is common to use *Amut Bmut* strains and other homokaryotic fruiting strains in UV and in restriction enzyme-mediated integration (REMI) mutagenesis (Kanda and Ishikawa 1986; Granado et al. 1997; Muraguchi et al. 1999; Cummings et al. 1999).

Each of the several developmental mutants previously reported in *L. edodes* has a heterokaryotic background (Komatsu and Kimura 1964; Murakami et al. 1987; Hasebe et al. 1991). Instead, the homothallic mutant recovered in this study facilitates generating extensive and various developmental mutants that enable us to elucidate the mode of gene expressions during fruit body formation under the specific homokaryotic background.

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