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Isolation and Characterization of Dikaryotic Mutants from *Pleurotus ostreatus* by UV Irradiation

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Protoplasts of the wild type strain of *Pleurotus osteatus* were mutagenized with UV light, and 3,000 colonies were examined for abnormal mycelial and fruiting phenotypes. Forty one strains displayed variant phenotypes in mycelia and fruiting processes. The variant phenotypes were classified into 6 groups: (1) auxotrophic strains, which are incapable of growing on minimal media and can only grow when provided with their specific requirements; (2) abnormal vegetative strains, which grow very slowly on minimal and complete media; (3) primordiumless strains, which fail to develop to the formation of primordia; (4) maturationless strains, which form primordia, but do not form mature fruiting bodies; (5) specifically colored strains, which have specific bluish grey or bluish white pileus; (6) poorly spored strains, which fail to produce basidiospore or which produce few spores. These variant strains may be useful in genetic breeding programs and for the studies of fungal development and genetics.

KEYWORDS: Color of pileus, Developmental mutants, *Pleurotus ostreatus*, UV mutagenesis, auxotrophs

Oyster mushroom, *Pleurotus ostreatus* is commercially important in the world mushroom market and it is especially appreciated in East Asia. Besides its importance for food production, *P. ostreatus* has received increasing attention for its use in biobleaching, catalysis of difficult chemical conversions, and the pharmaceutical industry (Vyas *et al.*, 1994). *P. ostreatus* has effects on increasing macrophage and lymphocyte activities (Kurashige *et al.*, 1997), reducing cholesterol levels (Bobek *et al.*, 1998), and increasing antihepatoma and antisarcoma acitvities (Wang *et al.*, 2000). Recently, its ability to degrade toxic substances such as bisphenol A has been noted (Hirano *et al.*, 2000). Its life cycle alternates between monokaryotic (haploid) and dikaryotic mycelial phases (Eugenio and Anderson, 1968). Fruiting occurs under appropriate environmental conditions, and true diploidy occurs only at the basidia (Larraya *et al.*, 2002). Many developmental mutants which are blocked at certain stages of fruiting in the dikaryotic strain (Takemura and Kamada, 1969) and homokaryotic fruiting strain of *Coprinus* (Muraguchi *et al.*, 1999) have already been induced by UV irradiation. A sporeless strain of *P. ostreatus* has been described by Eger (Eger *et al.*, 1976). This strain was obtained by mating two single spore isolates derived from the same spore print obtained from a fruiting body of *P. ostreatus* (Block *et al.*, 1958). However, the fruiting bodies of the sporeless strain are trumpet-shaped with the stipe attached to the centre of the head and is considered poor quality by mushroom producers. Variable auxotrophs of monokaryotic (Yoo *et al.*, 1985) and dikaryotic *Pleurotus* (Yoo, 1993) isolated from spores and protoplasts have been used for strain improvement in Genus *Pleurotus* by protoplast fusion (Yoo and Lee, 1994).

In edible mushrooms, the process of fruiting body formation is scientifically and commercially important. Therefore, we isolated not only abnormal vegetative mutants but also morphological and developmental mutants in the formation of fruiting bodies, which will be used for strain improvement and developmental studies of fungi.

Material and Methods

Strains and culture condition. *P. ostreatus* ASI 2029 stocked at National Institute of Agricultural Science and Technology was used in this experiment. Dikaryotic protoplasts were isolated from mycelium as described by Yoo *et al.* (1985).

Minimal medium (MM) described by Raper and Raper (1972) and complete medium (MCM), which was minimal medium supplemented with 0.2% yeast extracts and 0.2% bacto peptone, were used to culture the mycelium. Protoplasts were regenerated on MCM containing 0.6 M sucrose.

Mutagenesis. Protoplast suspensions at a concentration of 10^7 protoplasts/*ml* were treated with UV light as

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Table 1. List of solutions used for the screening of auxotrophic mutants

	1	2	3	4	5	6	7	8
1	Choline							
2	Cystine	Biotine						
3	Citrulline	Glutamate	Adenine					
4	Cytosine	Histidine	Nicotinic acid	Aneurine				
5[a]	Folic acid	Isoleucine	Methionine	Pyridoxine	Arginine			
6	Guanine	Inositol	Ornithine	Pantothenic acid	Serine	Alanin		
7	Glycine	Leucine	Proline	PABA	Thymine	Tryptophan	Aspartate	
8	Glutamine	Lysine	Phenylalanine	Riboflavin	Tyrosine	Treonine	Valine	Asparagine
9[b]	$(NH_4)_2SO_4$			$Na_2S_2O_3$				

Genotypes of auxotrophic mutants were identified by means of modified Holliday's solution (Holliday, 1956). UV-induced mutants were cultured on minimal media containing many kinds of amino acids (0.5 mg/*ml*), vitamins (0.1 mg/*ml*) and nucleic acids (0.1 mg/*ml*). The solution 5[a] contains folic acid, isoleucine, methionine, pyridoxine, arginine, serine, thymine, tyrosine, and the solution 9[b] contains $(NH_4)_2SO_4$ and $Na_2S_2O_3$.

described by Yoo *et al.* (1985). About 10^6 protoplasts were mutagenized by UV light for an exposure time that resulted in 5~20% survival rates and then plated on MCM containing 0.6M sucrose. UV-treated mutants were tested by mycelial growth on minimal and complete media and subsequently were characterized by the induction of fruiting bodies.

Isolation of auxotrophic mutants. The isolations of auxotrophic mutants were performed as described by Yoo (1993). After 15 days of incubation of protoplasts at 25°C, colonies which were regenerated on hypertonic media were inoculated again on complete media and allowed to develop into macroscopic colonies. Small mycelia plugs were then taken from each colony and were transferred to complete and minimal media. The isolates which grew on complete media but didn't grow on minimal media were cultured by transfer to complete and minimal media for confirming auxotrophic strains. The identification of auxotrophic mutations was achieved by screening their growth on a series of screening media (Table 1). To screen nutritional requirements of auxotrophic mutants, mycelia were transferred to minimal media containing yeast extract (1 mg/*ml*), casamino acid (5 mg/*ml*) and nucleic acid components (50 ug/*ml*), simultaneously. Mycelia were then transferred to complete, minimal, and minimal media supplemented with a combination of amino acid, vitamins, purines or pyrimidines. After testing, genotypes of auxotrophic mutants could be identified. Fruiting characteristics of isolated auxotrophs were tested in a sawdust bottle.

Isolation of fruiting mutants. In order to test for the fruiting phenotype, the resulting colonies were cultivated in 570 g sawdust substrates containing polar tree plus 20% rice bran and 210 *ml* deionized water, which were autoclaved for 1 h at 121°C. Each bottle was inoculated with a single agar plug and was incubated at 27°C for 20~25 days under low intensity light. When mycelia were grown completely on sawdust media, conditions for fructification were promoted by opening the bottle, and placing them in a growth chamber at 20°C and 90% relative humidity. Cultures were illuminated for 14 hours per day. Primordia appeared after a further 4~10 days of growth, and basidiocarps were harvested 5 days later (total time from inoculation to harvesting: 45 days). Variant strains that exhibited abnormal fruiting in the first test were retested for their fruiting phenotypes. Fruiting phenotypes of the variants were scored as the most advanced stage in fruiting. Spore prints from same sized healthy fruiting bodies of UV-treated mutants were collected under aseptic conditions using a pre-sterilized spore print box.

Results and Discussion

Survival ratio after UV irradiation. Figure 1 showed the effect of UV light on the survival ratio of *P. ostreatus* protoplasts. At a UV light exposure time between 50 and 70 sec, the survival ratio was rapidly decreased. These

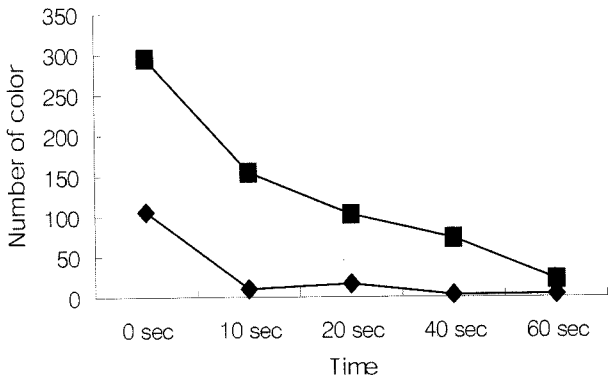

Fig. 1. Effect of UV light on the survival of *Pleurotus ostreatus* protoplasts. Suspensions of 1×10^5 protoplasts (◆) or 1×10^6 protoplasts (■) were treated by UV irradiation for various durations, then spread on MCM containing 0.6 M sucrose. Survival percentages were scored after incubation at 28°C for 5 days.

results were in general agreement with the survival ratio observed in other *Pleurotus* spp. (Yoo *et al.*, 1985). UV irradiation for 60 sec was used for mutagenesis in later experiments.

Characterization of UV-induced mutants. The genetic markers of mutants in fungi are the morphological traits of mycelium, the color of the spore print, and auxotrophic- or drug-resistant mycelial growth. Earlier genetic analysis of abnormal fruiting bodies provided an important suggestion that some developmental steps of fruiting might be controlled by genetic determinants (Takemaru and Kamada, 1972). In *Coprinus*, developmental variants in fruiting of UV-induced mutants were isolated (Muraguchi *et al.*, 1999; Takemura and Kamada, 1969). These studies suggested that the distinct morphological traits of fruiting bodies can be used as genetic markers for selection of mutants in *P. ostreatus*. We selected mutants by testing the characteristics of vegetative mycelium and fruiting bodies. Protoplasts of ASI 2029 were mutagenized with UV light, and 3,000 isolates were examined for abnormal mycelial and fruiting phenotypes that are useful for genetic markers. Various characteristics of selective mutants were compared with those of the wild type. Of these, forty one strains displayed variant phenotypes in mycelia and fruiting. Variant phenotypes were classified into 6 groups (Table 2, typical phenotypes are shown in Fig. 2).

Auxotrophic mutants: Auxotrophic mutants were isolated on selection media by plating of mutagenized protoplasts. About 10^6 protoplasts were mutagenized and then as many as 73 colonies of auxotrophic mutants were selected. The isolation frequency of auxotrophic mutants from protoplasts was 2.4%. To characterize their nutrient requirements, the following 73 colonies were identified by means of modified Holliday's solution (Holliday, 1956). Twenty six of 66 auxotrophic colonies showed one, two or three nutrient requirements for growth. After transfer to minimal medium and minimal medium supplemented with required nutrients, 26 isolates were shown to be true auxotrophs, and each genetic marker was identified (Table 3). Auxotrophic mutants were classified as amino acid requiring mutants (21 colonies), vitamin requiring colony (UVM 1146) and colonies requiring amino acids and vitamins simultaneously (UVM 345, 648, 677, 998). These mutant colonies grew very slowly and sparsely on minimal medium but were completely restored by the addition of required nutrients. Forty of 66 auxotrophic mutants failed to grow on minimal media but grew on several media supplemented with many kinds of nutrients or on complete media. These auxotrophs might require the addition of multiple nutrients for growth and some substances which are present in the complete medium but absent from the minimal media. The requirement of multi-nutrients in the auxotrophs made it difficult to identify genetic markers.

Abnormal vegetative mutants: Abnormal vegetative mutants were selected based on their vegetative characteristics and subsequently were tested by the induction of fruiting bodies (Table 4). These selected colonies exhibited sparse growth both on minimal and complete media. It was suggested that this characteristic resulted from serious mutation(s) (Yoo, 1993). In the fruiting procedure, some of the abnormal vegetative colonies were grown in a sawdust bottle. Formations of primordial and fruiting bodies of these colonies were delayed and the amount of spore was decreased as compared to the normal strain. But there were no strains that displayed abnormal mushroom morphology.

Primordiumless and maturationless mutants: The developmental process of normal, dikaryotic fruiting in *P. ostreatus* may be divided into only two stages, the primordia and fruiting body stages. After a few days incubation under fruiting conditions, primordia are developed. Primordia usually develop into the adult fruiting body within in a few days.

All of the 3,000 colonies mutagenized with UV light were examined for abnormal fruiting phenotypes. In order to characterize the developmental mutants, the resulting colonies were cultivated under fruiting conditions and were checked periodically for fruiting body formation (Table 5). Simultaneously, the mycelial growing rates of

Table 2. Classification of UV-induced mutants in *Pleurotus ostreatus*

Phenotypes	Mutants
(1) Auxotrophic strains	UVM 10, 251, 293, 345, 534, 566, 592, 632, 647, 648, 677, 732, 733,755, 913, 998, 1040, 1084, 1146, 1298,1239, 1310, 1319, 1794, 1806, 1816
(2) Abnormal vegetative strains	UVM 491, 1032, 1623, 1807, 2101, 2298, 2683
(3) Primordiumless strains	UVM 831
(4) Maturationless strains	UVM 534, 790
(5) Specific colored strains	UVM 676, 1068
(6) Poorly spored strains	UVM 676, 1284, 1573, 1799, 2697

Protoplasts of the normal strain, ASI 2029, were mutagenized with UV light, and 3,000 isolates were selected by subsequently testing the vegetative mycelial characteristics and the fruiting characteristics. Various characteristics of selective mutants were compared with those of the wild type. Of these, forty one strains displayed variant in mycelia and fruiting phenotypes and were classified into 6 groups.

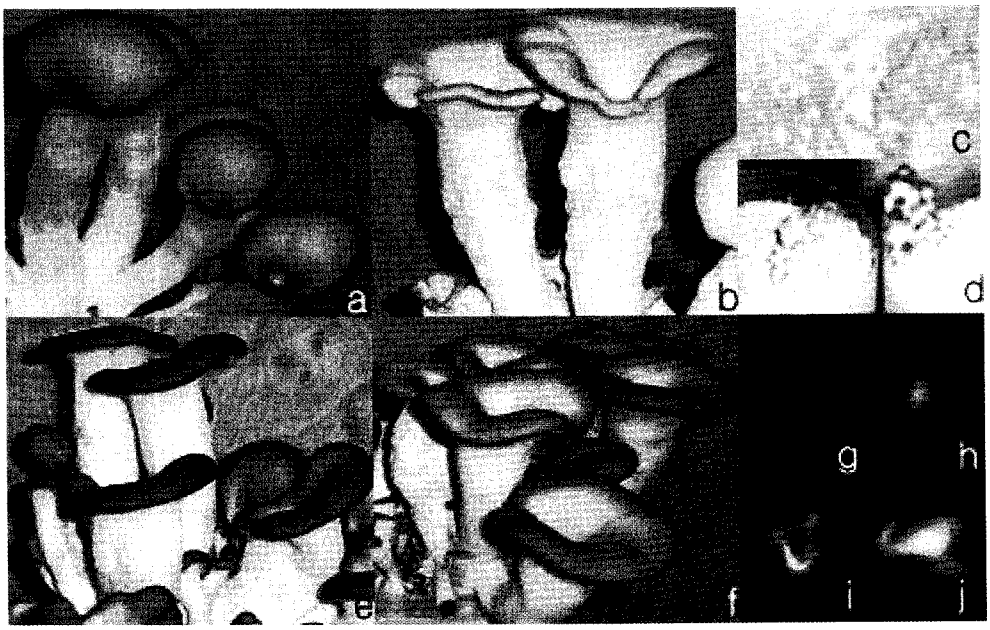

Fig. 2. Basidiocarps of UV-induced mutants. 3,000 isolates of UV-induced mutants were examined for abnormal phenotypes by induction of fruiting. Fruiting characteristics of mutants were compared with those of the wild type. Mutant strains reproducibly displayed variant fruiting phenotypes. a: Wild type strain, ASI 2029, b: UVM 1068, specific colored strain, c: primordium of UVM 1068, d: young fruiting body of UVM 1068 (left) and ASI 2029 (right), e: UVM 1032, abnormal vegetative strain, f: UVM 1799, poorly spored strain, g: spore print of UVM 1799 (level 0), h: spore print of UVM 1068 (level 1), i: spore print of UVM 2683 (level 3), j: spore print of ASI 2029 (level 5).

the mutants were tested on minimal and complete media. Developmentally aberrant strains, which reproducibly display abnormal fruiting results, were selected. There were three strains that dominantly displayed abnormal characteristics in their mushroom developmental stages. Primordia in the normal strain appeared after a further 3~4 days of growth and basidiocarps were harvested 4~5 days later. Mutants UVM 534 and 790 formed primordia but did not form mature fruiting bodies during the 90 days of observation. Mutant UVM 831 failed to develop primordia. The periods of time for the formation of maturationless strains to take place were increased as compared to that of the normal strain. These primordiumless or maturationless strains that exhibited abnormal fruiting in the first test were retested for their fruiting phenotypes under other conditions and in other places. Also, mycelial growing rates of three stains were abnormal both on minimal and complete media. The rate of mycelial growth in the primordiumless UVM 831 strain was decreased as compared to that of maturationless UVM 534 and 790 strains. The frequency of UV-treated variants in this study was very low (1.3%) as compared with that (18.4%) observed in *Coprinus* (Takemura and Kamada, 1969). The frequency observed in *Coprinus* was suggested to result from defects in various housekeeping processes. The very low frequency of UV-treated variants in *P. ostreatus* suggests that recessive mutants might turn out to have normal characteristics.

Specific colored strains: In the fruiting of *P. ostreatus*, morphological phenotypes of fruiting bodies are affected by environmental conditions. Therefore, it is difficult to select organisms having morphological mutations in their fruiting bodies. During the fruiting test, we tried to select strains having morphological fruiting mutations, but there was no strain that reproducibly displayed abnormal fruiting morphology except in the specific coloration of the pileus. The pileus in the normal strain exhibited a dark grey color in the young stage and a brownish grey color in the mature stage. Specific colored strains were selected by visual comparison with the normal strain.

Of the 3,000 colonies that were mutagenized by UV light, only two specific colored strains were selected. To characterize specific colored strains, the resulting colonies were checked for the rates of mycelial growth, the formation of primordia and the quantities of basidiospores (Table 5). Especially, the color of the pileus was compared to that of the wild type. Mutant UVM 676 that grew slowly both on minimal and complete media exhibited a bluish grey pileus of the fruiting body and subsequently exhibited defects in spore formation. Thus, this result suggested that UVM 676 resulted in the bluish grey coloration of the pileus by UV irradiation and that the coloration of the pileus may be partially related to spore production, as in *Coprinus*, where sporeless strains produce a white coloration of the pileus (Muraguchi *et al.*, 1999). Mutant UVM 1068 that grew quickly on minimal and

Table 3. The characterization of auxotrophic strains in *Pleurotus ostreatus*

Strains	Mycelial growth[a]					Genetic marker[b]
	MCM	MM	MM + YE	MM + CAS	MM + NA	
ASI 2029	L	L	L	L	L	–
UVM 10	L	S	M	M	S	Arg
UVM 251	M	S	L	S	S	Lys
UVM 293	M	S	L	L	S	Lys
UVM 345	M	S	M	M	S	Lys, Ino
UVM 534	M	S	L	M	S	Ile, Lys
UVM 566	L	S	M	S	S	Tyr
UVM 592	L	S	M	S	S	Ile, Lys
UVM 632	L	S	M	S	S	Tyr, Val
UVM 647	M	S	L	S	S	Lys
UVM 648	L	S	L	L	S	Ino, Ile, Lys
UVM 677	M	S	M	M	S	Biotine, Ile
UVM 732	L	S	M	S	S	Phe
UVM 733	M	S	M	S	S	Arg
UVM 755	S	S	M	M	S	Lys
UVM 913	L	S	L	L	S	Lys, Tyr
UVM 998	L	S	L	S	S	Gln, Folic acid
UVM 1040	L	S	L	M	S	Gln, Lys, Asn
UVM 1084	M	S	M	M	S	Arg
UVM 1146	M	S	L	L	S	Folic acid
UVM 1298	M	S	M	S	S	Ile
UVM 1239	L	S	M	L	S	Tyr
UVM 1310	L	S	M	M	S	Asn
UVM 1319	L	S	M	S	S	Arg
UVM 1794	L	S	L	M	S	Lys
UVM 1806	M	S	M	S	S	Arg, Met
UVM 1816	L	S	L	L	S	Ile, Lys

ASI 2029 is the wild type strain and the UVM series are the UV-induced mutants. UV-induced mutants were examined for auxotrophic phenotypes.

[a]Mycelia were cultured on mushroom complete medium (MCM), minimal medium (MM) and minimal media containing yeast extracts (YE, 1 mg/*ml*), casamino acid (CAS, 5 mg/*ml*) and nucleic acid components (NA, 50 ug/*ml*). The rates of mycelial growth were evaluated by the diameters of mycelial colonies. L: largely (over 40 mm in diameter) and densely growing colony, M: medium colony (from 25 to 40 mm in colony diameter), S: small colony (below 25 mm in colony diameter).

[b]Genotypes of auxotrophic mutants were identified by means of modified Holliday's solution (Holliday, 1956). Mutants symbols: Arg (Arginine), Lys (Lysine), Ino (Inositol), Ile (Isoleucine), Tyr (Tyrosine), Val (Valine), Phe (Phenylalanine), Gln(Glutamine), Asn (Asparagine), Met (Methionine).

Table 4. The characterization of abnormal vegetative strains in *Pleurotus ostreatus*

Strains	Mycelial growth[a]		Days to primordia	Color of Pileus		Formation of spore[b]
	MCM	MM		Young	Mature	
ASI 2029	L	L	3.5	Dark grey	brownish grey	5.0
UVM 491	S	S	6.7	Dark grey	brownish grey	2.5
UVM 1032	S	S	6.0	Dark grey	dark grey	1.8
UVM 1623	S	S	7.0	Dark grey	brownish grey	2.5
UVM 1807	S	S	5.7	brownish grey	light brown	4.3
UVM 2101	M	S	6.0	brownish grey	light brown	2.3
UVM 2298	M	S	4.3	Dark grey	dark bluish grey	1.7
UVM 2683	S	M	5.5	Dark grey	bluish grey	2.7

ASI 2029 is the wild type strain and the UVM series are the UV-induced mutants. UV-induced mutants were examined for abnormal characteristics.

[a]Mycelia were cultured on mushroom complete medium (MCM) or on minimal medium (MM). The rates of mycelial growth were evaluated by the diameters of mycelial colonies. L: largely (over 40 mm in diameter) and densely growing colony, M: medium colony (from 25 to 40 mm in colony diameter), S: small colony (below 25 mm in colony diameter).

[b]Levels of sporulation were visually scored from 0 (no spore) to 5 (normal sporulation).

Table 5. The characterization of primordiumless, maturationless, and specific color of pileus strains in *Pleurotus ostreatus*

Strains	Mycelial growth[a]		Days to primordia	Color of Pileus		Formation of spore[b]
	MCM	MM		Young	Mature	
ASI 2029	L	L	3.5	dark grey	brownish grey	5.0
Primordiumless variants						
UVM 831	S	S	–	–	–	–
Maturationless variants						
UVM 534	M	S	7.0	–	–	–
UVM 790	M	M	6.8	–	–	–
Specific color of pileus						
UVM 676	S	S	3.3	Grey	bluish grey	0
UVM 1068	L	L	4.7	bluish white	bluish white	1.7

ASI 2029 is the wild type strain and the UVM series are the UV-induced mutants. UV-induced mutants were examined for abnormal characteristics.

[a]Mycelia were cultured on mushroom complete medium (MCM) or on minimal medium (MM). The rates of mycelial growth were evaluated by the diameters of mycelial colonies. L: largely (over 40 mm in diameter) and densely growing colony, M: medium colony (from 25 to 40 mm in colony diameter), S: small colony (below 25 mm in colony diameter).

[b]Levels of sporulation were visually scored from 0 (no spore) to 5 (normal sporulation).

complete media displayed a specific bluish white color during primordia and fruiting body development (Fig. 2a, 2b, and 2c). In general, the specific color of the mushroom is affected by the temperature of the culture and the pH of the media. But UVM 1068 exhibited the same color of pileus from young to mature stages in ten repeat samples that were tested in two other places and with using other materials in a sawdust bottle. Thus, we could suggest that UVM 1068 is a specific colored variant caused by UV irradiation.

Poorly spored mutants: A sporeless strain of *P. ostreatus* has been described by Eger *et al.* (1976). However, this sporeless strain has been considered as having a "poor" quality by mushroom producers and consumers. In this study, we tried to select the sporeless mutants by evaluating the quantity of dispersed spores.

Spore production of all 3,000 colonies mutagenized with UV light was evaluated by macroscopic observations. When the diameter of the pileus reached 30 mm, the fruiting bodies were collected and placed on pre-sterilized supports. Spore prints from healthy fruiting bodies were visually scored from 0 (no spores) to 5 (normal sporulation) (Fig. 2f to 2j). Firstly, ten poorly spored colonies that scored below level 1 in the first testing were selected. These putative colonies were retested for their sporulation and finally, five strains reproducibly observed to be poorly spored in five independent assays. To characterize poorly spored strains, the resulting colonies were checked for rates of mycelial growth, the formation of primordia and the morphologies of fruiting bodies (Table 6). Except for UVM 676, which is a specific colored strain, the morphologies of mycelia and fruiting bodies of poorly spored strains were almost normal and the coloration of the pileus had a tendency to be lighter than that of the normal strain. Since we only selected by macroscopic testing, the selected sporeless strains (UVM 676 and 1799), in which no spores were observed on the supports, will be confirmed by microscopic or spectrophotometric testing. Also, the quality of poorly spored strains was not as good as in previous studies (Block *et al.*, 1958; Eger *et al.*, 1976). To improve the quality of these strains, further studies will be performed by mating two single spore iso-

Table 6. The characterization of poorly spored strains in *Pleurotus ostreatus*

Strains	Mycelial growth[a]		Days to primordia	Color of Pileus		Formation of spore[b]
	MCM	MM		Young	Mature	
ASI 2029	L	L	3.5	dark grey	brownish grey	5.0
UVM 676	S	S	3.3	grey	bluish grey	0
UVM 1284	L	L	4.3	dark grey	grey	0.3
UVM 1573	L	L	4.7	dark brown	light brown	0.3
UVM 1799	L	L	3.7	brownish grey	light grey	0
UVM 2697	M	L	4.3	dark grey	brownish grey	0.2

ASI 2029 is the wild type strain and UVM series are the UV-induced mutants. UV-induced mutants were examined for abnormal characteristics.

[a]Mycelia were cultured on mushroom complete medium (MCM) or on minimal medium (MM). The rates of mycelial growth were evaluated by the diameters of mycelial colonies. L: largely (over 40 mm in diameter) and densely growing colony, M: medium colony (from 25 to 40 mm in colony diameter), S: small colony (below 25 mm in colony diameter).

[b]Levels of sporulation were visually scored from 0 (no spore) to 5 (normal sporulation).

lates derived from these strains.

The selections of auxotrophs and developmental variants were achieved by determining vegetative and fruiting characteristics. Forty one UV-induced mutants were isolated and were classified into 6 groups. Of these mutants, we isolated twenty six auxotrophs, two specific colored and five poorly spored variants. These mutant strains that exhibited selectable, defective or abnormal phenotype, will be very useful materials for use in genetic breeding programs and for the studies of fungal development and genetics.

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