短　報（Short communication）

Morphological changes in a γ-ray irradiation-induced mutant of the ectomycorrhizal agaricomycete *Tricholoma matsutake* during in vitro spawning on barley-based substrates

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Abstract

Tricholoma matsutake is an ectomycorrhizal agaricomycete that produces the prized mushroom "*matsutake*" when grown in pine forests. At present, *T. matsutake* cannot be artificially cultivated for fruiting. Using barley-based substrate cultivation, a *T. matsutake* mutant, designated G1, which was generated by irradiating *T. matsutake* NBRC 33136 with γ-rays, produced protuberances that were composed of non-aerial hyphal tissues. The protuberances occurred in three consecutive independent experiments but did not grow into fruiting bodies. The issue of whether the protuberances represent the initial stage of fruiting needs to be clarified. However, because of morphological changes in the spawn, the G1 strain may be useful in developing a cultivar of *T. matsutake*.

Key words: Agaricales, fruiting, mushroom cultivation, mutagenesis

1. Introduction

Tricholoma matsutake is an ectomycorrhizal agaricomycete that produces the prized mushroom *"matsutake"* in association with the Pinaceae (Yamada et al. 2014). "*Matsutake*" is hardly ever cultivated artificially; however, Kawai and Ogawa (1976) documented the occurrence of "primordia" on the surface of a growth substrate, which was mainly composed of vermiculite and a Murashige and Skoog-based medium. Kawai and Ogawa (1976) described the primordia as differentiated tissues, but a more detailed analysis on the extent of the differentiation in relation to naturally occurring primordia was not provided. The protocol has never been used for commercially cultivating *T. matsutake* to yield fruiting bodies. In fact, to the best of our knowledge, no further reports of fruiting body production or even morphological changes after cultivating *T. matsutake* as spawn have been documented, indicating that more suitable fungal strains and protocols for the artificial production of the *T. matsutake* fruiting bodies are needed.

Ohta (1994) recorded fruiting body production when cultivating another edible ectomycorrhizal mushroom, *Lyophyllum shimeji* (known as "*honshimeji*"), using spawn substrates composed of barley and saw dust. To achieve the artificial cultivation of "*honshimeji*", Ohta (1994) initially selected, from ~50 *L. shimeji* isolates, four strains that behaved somewhat like saprophytic cultivated mushrooms using spawn substrates composed of rye and saw dust. The *L. shimeji* cultivation system for fruiting is highly reproducible and currently used during its mass production for commercial trading. There are two key elements in the success of "*honshimeji*" production: (i) the isolates can grow as spawn without host plants and easily produce fruiting bodies using a protocol similar to that generally used for cultivated mushrooms; and (ii) the barley-based spawn substrate provides starch derived from grains rather than cellulose derived from hardwood as the carbon source, which some ectomycorrhizal fungi can use (Kusuda 2009).

We previously isolated *T. matsutake* strain G1, after irradiating *T. matsutake* strain NBRC 33136 (= ATCC MYA-915, Y1) with γ-rays, as a mutant that pleiotropically altered the phenotype. Compared with wild-type, it produces a hedgehog-like mycelial colony morphology with fuzzy aerial hyphae rather than a flat morphology, significantly increased amylase and cellulase activities, and parasitic, rather than symbiotic, interactions with *Pinus densiflora* (Murata et al. 2019). Because *T. matsutake* strain G1 appears to have converted from a symbiont to a saprophyte, whether the fungal mutant exhibits some morphological changes relevant to fruiting body production on a barley-based spawn substrate was of interest; *T. matsutake* strain G1 grows faster than *T. matsutake* NBRC 33136 in the spawn substrate (Murata

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et al. 2019). In the present study, we allowed *T. matsutake* strain G1 to produce protuberances that may correspond to the initial stage of fruiting using a modified *L. shimeji* spawn-cultivation protocol during the first 2-year period after mutant isolation.

2. Materials and Methods

Two *T. matsutake* strains were subjects in the experiment: NBRC 33136, which is the wild-type strain, also known as Y1 (Yamada et al. 1999, 2014), and G1, which is a γ-ray irradiation-induced mutant of NBRC 33136 (Murata et al. 2019). The *T. matsutake* strains were cultured and maintained on MMN+V8 agar plates (Murata et al. 1999). To cultivate as spawn using a barley-based substrate, *T. matsutake* mycelia were cultured in the MMN+V8 liquid medium at 23°C for 28 d, washed once in water, and inoculated into the substrate. The inoculated substrates were incubated at 23°C for three months, and then transferred into a fruiting room and further incubated at 16°C with 90% relative humidity until morphological changes occurred; the whole process was performed without light. Unless stated otherwise, the substrate was prepared by blending barley, soil (either 100% vermiculite or 75%:25% vermiculite:pumiceous soil mixture), and Ohta liquid medium at a ratio of 1:1.5:1 on a volumetric basis (Ohta 1994). Prior to the blending, the dried barley was submerged in the liquid medium for at least 1 h. The substrate was then packed into 2/3 of 450-mL jars with polycarbonate screw caps having 8-mm (ø) air holes sealed with Milliseal membrane (Merck Millipore, Darmstadt, Germany) and autoclaved for 60 min. The experiment was performed three times independently under the same culture conditions, except 100% vermiculite was used as soil in the first experiment and the 75%:25% vermiculite:pumiceous soil mixture was used in the second and third experiments.

3. Results and discussion

In the first experiment, *T. matsutake* G1 developed several tiny (~ 5–7-mm ø) protuberances mainly on the sides of the spawn during a 7-month incubation period in the fruiting room (Fig. 1A–C). The protuberances were brown and lightly covered with aerial hyphae. Cross-sections revealed that the protuberances were not simple aggregates of aerial hyphae but were tissue-like, although some uncertainty remains as to whether the latter is related to fruiting bodies, such as a remnant of the inner veil around the pileal margin (Fig. 1D). In the second experiment, the fungus also developed many protuberances of the same size (~5–7-mm ø) around the spawn after a 6-month incubation period in the fruiting room (Fig. 2 A–C). The internal portions exhibited the same tissue-like texture as observed in the first experiment (Fig. 2D). In the third experiment, protuberances occurred at the top of the spawn within 2.5 months after being incubated in the fruiting room (Fig. 3). Here, the tissue-like texture was

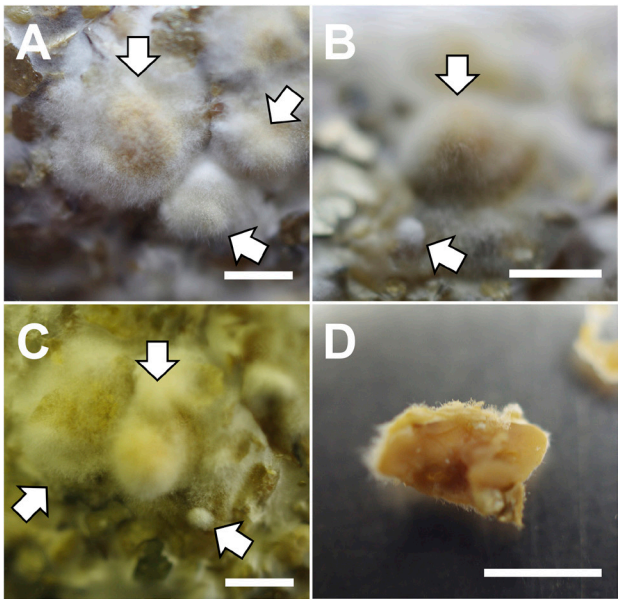

Fig. 1. The morphological changes in *Tricholoma matsutake* G1 in spawn, the 1st experiment.
(A–D) Primordium-like protuberances. (A–C) Protuberances protruding from the spawn are indicated by arrows. (D) Cross-section of the protuberance indicates tissue differentiation. *Scale bars* 5 mm.

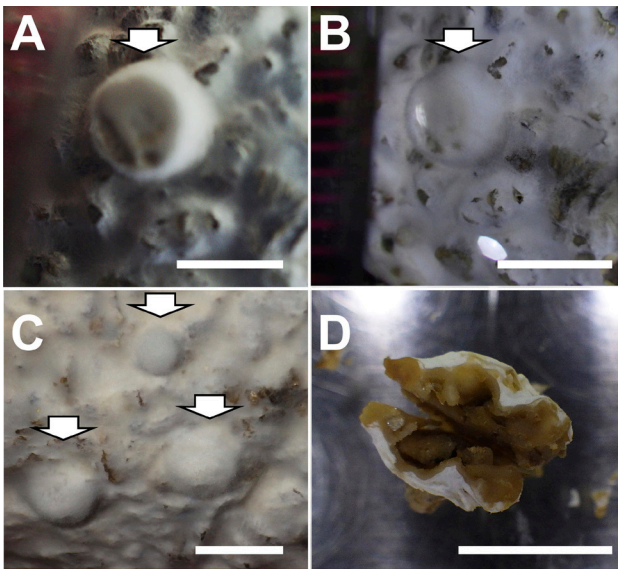

Fig. 2. The morphological changes in *Tricholoma matsutake* G1 in spawn, the 2nd experiment.
(A–D) Primordium-like protuberances. (A–C) Protuberances protruding from the spawn are indicated by arrows. (D) Cross-section of the protuberance indicates tissue differentiation. *Scale bars* 5 mm.

noted inside the protuberances (Fig. 3C). In this series of the *T. matsutake* substrate cultivations, NBRC 33136 never exhibited any morphological changes. In a separate experiment, neither NBRC 33136 nor G1 showed any morphological changes when subjected to the protocols of Kawai and Ogawa (1976).

The protuberances we observed from *T. matsutake* G1 in the present study occurred on the apparently matured spawn, in

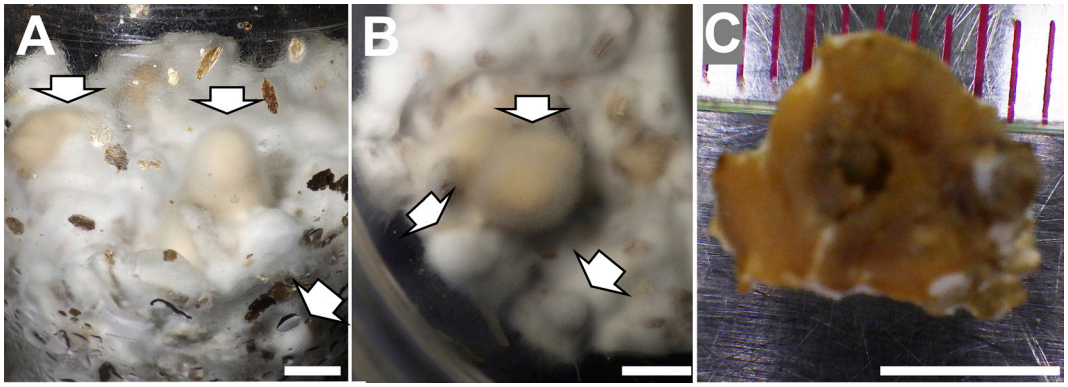

Fig. 3. The morphological changes in *Tricholoma matsutake* G1 in spawn, the 3rd experiment.
(A–B) Primordium-like protuberances. (C) Cross-section of the protuberance indicates tissue differentiation. Protuberances protruding from the spawn are indicated by arrows. *Scale bars* 5 mm.

which the fungal hyphae had fully colonized the substrate (Figs. 1–3). This was unlike those reported by Kawai and Ogawa (1976) that developed with apparently little hyphal growth on the major portion of the substrate other than the top surface. In general, agaricomycetes require decent mycelial growth for fruiting, although the extent varies depending upon taxa, strains, growth media, and spatial distribution (Staplers 1978). For example, those commercially cultivated for trading require fully grown spawn to fruit while those used in laboratories do not (Chang and Hays 1978, Stametes 1993, Kamata et al. 2010, Muraguchi 2015). Determining whether the protuberances of the *T. matsutake* G1 spawn in the present study and those reported by Kawai and Ogawa (1976) are the same will require a comparative anatomical analysis of both specimens, which is very difficult. At this point, the fruiting system of the model agaricomycete *Coprinopsis cinerea* is interesting (Muraguchi et al. 2015). *Coprinopsis cinerea* initiates fruiting by forming a "knot," the 1st stage that is solely composed of aerial hyphae, which leads to the 2nd "tissue differentiation" stage, followed by the 3rd "fruiting body primordia" stage. These three stages occur much earlier than karyogamy and meiosis, or even premeiotic replication, required for reaching the 4th "fruiting" stage having sexual spores (Muraguchi et al. 2015). In comparison with the *C. cinerea*-fruiting scheme, the protuberances we observed in the present study of *T. matsutake* G1 could correspond to the 2nd stage.

The morphological changes in strain G1 consistently occurred during cultivation as spawn on a barley-based substrate when the inocula were grown from mycelia transferred from the original slant culture. However, morphological changes did not occur progressively but rather occurred sporadically when inocula were from mycelia that had been stored at 10°C for ~1.5 years. Since this experiment, the mycelia of *T. matsutake* G1 have been transferred multiple times to slants and agar plates for further culture storage. Such trait instability in terms of morphological changes, including primordium formation and fruiting, as observed in the occurrence of protuberances of *T. matsutake* G1, occurs rather ubiquitously even in cultivated mushrooms for unknown reasons.

The protuberances occurred consecutively in the three earlier independent experiments and then the morphological changes occurred sporadically for nearly 2 years, while other traits of *T. matsutake* G1 that characterize this mutant, including colony morphology on agar plates, increased amylase and cellulase activities, and harmful effects on plants, have been maintained to date (Murata et al. 2019). Because *T. matsutake* G1 sporadically produces protuberances, the morphological changes, especially those linked to sexual reproduction, it requires an environment suitable for such important lifecycle events, including the substrate composition, and temperature and moisture fluctuations. In *T. matsutake*'s natural habitat, such moisture and temperature changes occur in the soil as well as in the atmosphere, even within a single day.

To address this problem, we are currently inducing mutations in *T. matsutake* G1, as well as NBRC 33136, by irradiating with heavy-ion beams, featuring dense ionizing radiation with a high-linear energy transfer, represented by those of neutrons and α-particles (Abe et al. 2015). Heavy-ion beams have greater biological effects than γ-rays, which have low-linear energy transfer, and can cause gaps ranging in size from a few bases to over one kilobase in both strands of the genomic DNA, rather than one base in a single strand of DNA, as generally induced by γ-ray irradiation. Such double-strand DNA breaks are repaired by non-homologous end joining, which is highly error-prone (Goodhead 1999, Ichida et al. 2008). This line of irradiation breeding could eventually produce mutants that preferentially express the sexual reproductive stage over the vegetative growth stage.

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γ線で誘発したマツタケ突然変異体の菌床栽培での形態変化

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要旨

マツタケ（*Tricholoma matsutake*）は、マツ林で高級きのこ「まつたけ」をつくる外生菌根性の真正担子菌である。今日までマツタケは人工栽培できない。本研究において、γ線照射により作出したマツタケ変異体G1株が、押し麦を含んだ人工培養基上で組織化した菌糸塊を形成した。この菌糸塊は、G1株作出後3回の反復実験で連続して再現したが、子実体には発達しなかった。この菌糸塊は、子実体原基であるかは不明であるが、G1株におけるこの特性はマツタケ栽培品種開発の手がかりとなる可能性がある。

キーワード：ハラタケ目、子実体形成、きのこ栽培、変異体誘導

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