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Breeding of new *Ganoderma lucidum* strains simultaneously rich in polysaccharides and triterpenes by mating basidiospore-derived monokaryons of two commercial cultivars

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ABSTRACT

The aim of this study was to breed *G. lucidum* strains simultaneously rich in polysaccharides and triterpenes by mating of basidiospore monokaryons of two commercial cultivars, *G. lucidum* JC and AL. By incubation of freshly collected basidiospores, 31 monokaryons were isolated (19 from strain JC, and 12 from strain AC), and showed significant differences in mycelial growth and contents of polysaccharide and triterpene. Mating between the two strains yielded 83 instances of sexual compatibility. Of the hybrids isolated, sixteen ones that showed moderate and fast substrate colonization were evaluated in terms of fruiting. All the tested hybrids produced fruiting bodies but varied considerably in time to first harvest, productivity, yield distribution, and pileus shape and color. Significant differences in polysaccharide content (from 6.79 to 18.21 mg/g dry weight) and triterpene content (from 5.90 to 10.87 mg/g dry weight) were also observed among the hybrids. Notably, high concentrations of polysaccharides (16.63 mg/g dry weight) and triterpenes (10.50 mg/g dry weight) were both present in hybrid H-23, with satisfactory biological efficiency (19.41%). An antagonism test and random amplified polymorphic DNA (RAPD) analysis indicated that H-23 is a new hybrid strain. This hybrid may be suitable for use in commercial production of *G. lucidum* for manufacture of more effective health-promoting and medicinal products. To our knowledge, the present study is the first report on the development of new *G. lucidum* strains via mating by means of basidiospore-derived monokaryons.

1. Introduction

Ganoderma lucidum (Fr.) Karst belonging to family *Polyporaceae* (or *Ganodermaceae*) is the most famous medicinal mushroom. It has been widely used in traditional Chinese medicine in oriental countries like China, Japan, and Korea for more than 2000 years to treat many human diseases, such as gastric ulcer, hepatitis, nephritis, hypertension, and arthritis (Jong and Birmingham, 1992; Mizuno et al., 1995). Pharmaceutically active components of *G. lucidum* include triterpenoids, polysaccharides, ergosterols, proteins, fatty acids, vitamins, and trace elements (Boh et al., 2007; Shiao, 2003; Wasser and Weiss, 1999). Among them, triterpenes and polysaccharides are the two most important functional components with various activities such as antioxidant, antitumor, and immunomodulating (Wagner et al., 2004; Xu et al., 2010).

G. lucidum is distributed in forests in most regions of the world, but sufficient amounts for commercial use cannot be collected. Artificial cultivation of *G. lucidum* was successfully developed on solid substrates in China in the 1970s (Wasser, 2005). In this cultivation process, a large amount of currently available lignocellulosic waste (especially sawdust) was commonly used, but supplementation with small amounts of organic nitrogen sources such as wheat bran is required for a better mushroom yield. Currently, *G. lucidum* is commercially produced on a large scale in China, Korea, and Japan. Although *G. lucidum* has a relatively long cultivation history (more than 40 years), this activity still mainly depends on a limited number of wild-type strains. Because continuous vegetative propagation for commercial production commonly leads to degeneration in the quality of *G. lucidum* strains (Qi et al., 2003), sustained development of the *G. lucidum* industry faces a huge risk.

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To develop new *G. lucidum* strains, various mushroom breeding methods like protoplast fusion, mutagenesis, genetically engineered transformation, and mycelial mating have been applied to this fungus. Successful protoplast fusion of *G. lucidum* with a variety of other mushroom species especially those belonging to different genera or orders of Basidiomycetes has been carried out (Bok et al., 1994; Chiu et al., 2005; Park et al., 1991), and various fusants were developed. Qi et al. (2003) reported that a new *G. lucidum* strain with a distinct mutation was generated after space flight. Genetic transformation of *G. lucidum* has also been achieved to express an exogenous β-glucuronidase gene (Sun et al., 2002). In our previous study, protoplast-derived monokaryons were used in mycelial mating, and a variety of hybrids were bred (Wu et al., 2009). Nonetheless, a practical commercial application of these new strains has not been reported to date.

Given that *G. lucidum* is a typical tetrapolar heterothallic fungus that has a double factor incompatibility system consisting of incompatibility factors A and B (Chen et al., 2012), it forms numerous haploid basidiospores of four incompatibility types in basidia by meiosis during fruiting. In the present study, two commercial *G. lucidum* cultivars were employed as parental strains because of the existence of complementary effect in terms of the number and individual weight of fruit bodies produced between them. Our objective was to isolate monokaryotic cultures by germinating basidiospores of the two strains, and isolated monokaryons were mated between the two strains to obtain dikaryotic hybrids. Using polysaccharide and triterpene contents as major indicators and paying attention to the biological efficiency, we selected well-suitable candidates (among various hybrids) for commercial production of *G. lucidum*.

2. Materials and methods

2.1. Fungal strains

Commercial *G. lucidum* strains JC and AL, stored at the Mycological Research Centre, Fujian Agriculture and Forestry University, were used in this study. They were maintained in potato dextrose agar (PDA) slants at 4 °C, and subcultured every month at 25 °C for 7 d. In commercial cultivation on short logs, strain JC produced fruit bodies with characteristics of short stipe (less than the pileus radius), thick pileus, varnished pileus surface, and heavy individual weight, while strain AL yielded a large number of fruit bodies but with less individual weight and gave high productivity.

2.2. Collection of basidiospores

To obtain basidiospores of *G. lucidum* strains JC and AL, the two strains were cultivated in polypropylene bags (17 × 33 cm) each containing 1800 g of a substrate composed of 80% of mixed sawdust, 15% of wheat bran, 4% of corn powder, and 1% of gypsum with 65% moisture content. During fruiting, once the fruiting bodies matured (i.e., the typical white color of the growing zone disappeared), those with heavy individual weight (more than 50 g), fine pileus shape and varnished appearance were selected without harvesting from the substrate, and pieces of sulfate paper (12 × 12 cm) were placed under them for 12 h to collect only dispersing basidiospores. Such collected basidiospores (referred to as *spore print*) were ready to use immediately.

2.3. Germination and isolation of monokaryotic strains

Sterilized distilled water (5 μL) was dotted on a spore print under sterile conditions, scraped with an inoculation loop, and then the loop was directly immersed into 5 mL of sterilized distilled water in a 10-mL plastic tube to resuspend the spores. The concentration was adjusted to approximately 1×10^6 spores/mL after proper dilution or by resuspending more spores. The resultant spore suspension (0.1 mL) was pipetted into 0.9 mL of sterilized malt broth (20%, w/v) in 1.5-mL Eppendorf tubes and incubated at 28 °C.

Aliquots (10 μL) of the broth were withdrawn at intervals of 12 h during incubation, dotted onto glass slides, and covered with coverslips. They were examined under a microscope (DM750, Leica, Germany) at 40× magnification. Once germinated spores were noticed, 0.1 mL of the broth was spread on PDA plates (90-mm diameter Petri dishes). After further 4-h incubation mainly intended for absorption of excess broth on the surface by the solid medium, the germinating spores were picked up cautiously with an inoculation needle under a microscope, transferred into slants, and incubated at 25 °C for 7 d. Monokaryotic strains were confirmed microscopically by the absence of clamp connections on the mycelium. The monokaryons derived from strain JC were designated as J strains, and those from AL were designated as A strains.

2.4. Determination of the mycelial growth, and polysaccharide and triterpene production of monokaryons

For determination of mycelial growth of monokaryons, a mycelial agar plug (5 mm in diameter) cut from the periphery of young, actively growing 10-day-old colonies was inoculated into the center of a 90-mm Petri dish containing the PDA medium. The inoculated dishes were incubated at 25 °C. The diameter of mycelial colonies after 7-day incubation was measured along two perpendicular directions and averaged. The mycelial radial growth rate was calculated by dividing the measured diameter of a colony by the incubation time (d) and was expressed as the radial extension length per day (mm/day).

To measure the polysaccharide and triterpene production of the monokaryons, submerged cultures were performed to obtain mycelia. For the seed culture, an entire slant was squashed into approximately soybean-sized agar blocks with a sterile inoculated rake, and a half of the agar blocks was transferred into a 250 mL flask containing 80 mL medium, which consisted of (per liter) 35 g glucose, 5 g peptone, 4 g yeast extract, 1.0 g KH_2PO_4, and 0.5 g $MgSO_4$. The inoculated flasks were cultivated at 150 rpm and 30 °C for 4 d, and then homogenized in a polytron. The homogenate (5 mL) was transferred into a 250 mL flask containing 75 mL medium as previously described, and cultured in the same manner as the seed culture for 6 d. Cultured mycelia were separated from the broth by centrifuging at 8000 × *g* for 10 min and washing twice with distilled water. The mycelia pellet obtained was dried at 60 °C until a constant weight was reached, and utilized for the analysis of polysaccharide and triterpene contents.

2.5. Mono-mono mating and hybrid isolation

Each of the JC-derived 19 monokaryotic mycelia was paired with 12 monokaryons of AL, yielding a total of 228 crosses. For mating, a mycelial block (of soybean like size) of monokaryotic cultures was placed opposite of another monokaryotic mycelium block at a 1-cm distance on PDA in tubes (15 × 150 mm). After incubation at 25 °C for 7 d or more, successful mating (compatibility) was microscopically confirmed by examining clamp connections on the mycelium within the contact zone and on the obverse sides of donor inoculum blocks. For each detected mating, a mycelial plug was isolated from the junction between two monokaryotic mycelia, transferred to slants, cultured at 25 °C, and maintained as hybrids. Hybrids that showed poor growth were discarded without further investigation.