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Construction of novel cold-tolerant strains of *Volvariella volvacea* through protoplast fusion between *Volvariella volvacea* and *Pleurotus eryngii*

Bao-Lin He[a,b,1], Li-Rong You[a,b,1], Zhi-Wei Ye[a,b], Li-Qiong Guo[a,b,*], Jun-Fang Lin[a,b,*], Tao Wei[a,b], Qian-Wang Zheng[a,b]

[a] College of Food Science & Institute of Food Biotechnology, South China Agriculture University, Guangzhou 510640, China
[b] Research Center for Micro-Ecological Agent Engineering and Technology of Guangdong Province, Guangzhou 510640, China

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ABSTRACT

Volvariella volvacea, an edible mushroom that grows at high temperature and humidity, has attracted growing attention because of its high nutrient value, short cropping duration, unique aroma and texture. However, it is difficult to store fresh because it is highly perishable in low-temperature. To expend its cultivation region and season, this study aims to improve the low-temperature tolerance ability and the biological efficiency of this macrofungus. For this purpose, interfamily hybridization between *V. volvacea* and *Pleurotus eryngii* was studied through PEG-mediated protoplast fusion technique. Two low-temperature resistant strains, namely, VP1 and VP2 were successfully obtained through protoplast fusion with biological characteristics screening and molecular determination. The hybrid strain VP1 showed a higher biological efficiency (31.53%), compared with the 9.38% in the parental strain. Fruiting bodies of both VP1 and VP2 had a longer storage life than that of control at 16 °C. The size and amplified fragments length polymorphism (AFLP) pattern of fusants were similar, but differed from the parental V138 species, which implied that fusants are novel strains. This research has achieved interfamily protoplast fusion, which provides an effective way and the theoretical basis for the distant hybridization breeding of edible fungi.

1. Introduction

Volvariella volvacea, the typical edible straw mushroom with high-temperature tolerance, is one of the most extensively cultivated mushrooms in many tropical and subtropical regions of Southeast Asia (Chiu, 1993; Bao et al., 2013). It is widely appreciated for its unique aroma and texture, high nutrition (Chang and Buswell, 1996) and shorter cropping duration compared with other cultivated mushrooms (Cheung et al., 2003). However, *V. volvacea* is sensitive to temperature changes, especially to the low temperature (Guo et al., 2016). When stored at 4 °C, the mycelia of *V. volvacea* will autolyze quickly, followed by the spoilage of the fruiting bodies. This causes great difficulties in product storage and transportation (Ahlawat et al., 2008). Moreover, the biological efficiency (conversion of growth substrate into mushroom fruiting bodies) of *V. volvacea* is only 15% on straw-based substrates and is 30–40% on cotton-waste 'composts', which is considerably low compared with other major cultivated species such as *Pleurotus eryngii*. As one of the exportable products of tropical and subtropical countries, *V. volvacea* industry is one of the critical components of agricultural income. However, short shelf life at low temperature becomes an obstacle for its export (Furlan et al., 1997; Zhao, 2011). Hence, breeding cold-tolerant and high-yield strains of *V. volvacea* is a potential way to address the above-mentioned challenges.

Therefore, breeding a new strain of *V. volvacea* with low-temperature tolerance has drawn the attention of both academic and industry communities. Several techniques have been applied for breeding including single basidiospore breeding (Ahlawat et al., 2005; Xiong et al., 2014), spore (Chiu and Moore, 1999) and protoplast mutation (Mukherjee and Sengupta, 1986). However, *V. volvacea* is a homothallic fungus without clamp connections among mycelium, and the lack of genetic marker makes cross breeding infeasible (Ahlawat et al., 2008; Fu et al., 2010). Besides, spore and protoplast mutation has several drawbacks, such as indefinite stimulation requirement, low mutation rate, and difficult germination. Biotechnological approaches such as protoplast fusion can circumvent these difficulties mentioned above, and help enhance breeding efficiency and expedite *V. volvacea* genetic improvement process.

Fungal protoplast fusion has been established as means to transfer genetic material and has provided an effective method for genetic manipulation and strain improvement (Chakraborty and Sikdar, 2008;

* Corresponding authors at: College of Food Science & Institute of Food Biotechnology, South China Agriculture University, Guangzhou 510640, China.
[1] Both authors contributed equally to this work.

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Sirisha et al., 2010). Hybrid strains with high biological efficiency have been obtained by certain researchers (Mallick and Sikdar, 2015; Xu et al., 2012). Successful protoplast fusion of *V. volvacea* and *Volvariella bombycina* has been well demonstrated (Zhao and Chang, 1997). Although auxotrophic marker, drug resistance marker, fluorescent label, inactivated protoplast and isozyme analysis have been used to screen fusants from protoplast fusion, there were still many issues to be solved (Ahlawat et al., 2008). Protoplast fusion has been proven to be very feasible for interspecific and intergeneric hybridization for strain improvement in edible mushrooms (Chakraborty and Sikdar, 2010; Patil et al., 2014; Selvakumar et al., 2015). However, there is few research related to interfamily protoplast fusion. In this study, the interfamily protoplast fusion between *V. volvacea* and *P. eryngii* was carried out to improve the cold-tolerant ability of parental *V. volvacea*.

P. eryngii, commonly known as *King Trumpet Mushroom*, is an edible mushroom native to Mediterranean regions of Europe, the Middle East, and North Africa, and is also grown in many parts of Asia (Ohga and Royse, 2004). *P. eryngii* is also valued for its nutritional value (Siwulski et al., 2017) and has more trade-friendly traits than other mushroom species, including sustainable yield, long shelf life, and high biological efficiency of up to 99.34% (Estrada et al., 2009), possibly because of the low water content and firm flesh of its fruiting body (Manzi et al., 2004).

Considering the beneficial characters of two parents, the present study carried out the protoplast fusion between *V. volvacea* and *P. eryngii*, whose fusant would either show low temperature tolerance as *P. eryngii* and the colour and texture as that of *V. volvacea* or augment biological efficiency and shelf life of *V. volvacea*. It is speculated the fusant would process several industrial benefit properties, such as higher biological efficiency and increased low-temperature tolerance.

2. Materials and methods

2.1. Strains and culture media

V. volvacea strain V138 was obtained from Guangzhou Agricultural Science Test Center (Guangzhou, China), and *P. eryngii* strain Pe811 was purchased from the Sanming Mycological Institute of Fujian Province (Sanming, China). Vegetative cultures of both fungi were maintained on Potato-Dextrose-Agar (PDA) medium containing 2% of glucose, 0.3% of KH_2PO_4, 0.15% of $MgSO_4{\cdot}7H_2O$, 2% of agar and 1 L of potato juice which was prepared by boiling 200 g of potato for 30 min and filtering through eightfold gauze under stationary conditions (Chakraborty and Sikdar, 2008). Mycelium were cultured in malt-yeast extract-glucose (MYG) liquid medium containing 2% of glucose, 0.2% of yeast extract and 1 L of potato juice which was prepared by boiling potato as described above. Protoplast were cultured on regeneration medium containing 10 g/L of malt extract, 4 g/L of glucose and 4 g/L of yeast extract, 1 g/L of KH_2PO_4, 1 g/L of $MgSO_4{\cdot}7H_20$, 1 g/L of vitamin B1, 0.6 M osmotic stabilizer.

2.2. Preparation and regeneration of protoplast

Protoplast isolation was conducted according to methods described by Zhao et al. (2010), with slight modifications. In order to obtain protoplast, 5 mL mycelial masses of each fungus were inoculated into potato dextrose broth and statically incubated for 3 days at 32 °C for V138, and for 5–6 days at 25 °C for Pe811. Mycelium was rinsed with sterile distilled water and osmotic stabilizer (0.6 M D-mannitol), blotted dry using sterilized filter paper, and suspended in 2 mL of filter-sterilized lywallzyme (Guangdong Institute of Microbiology, Guangzhou, China) solution (15 mg/mL in 0.6 M) for cell wall digestion. The mycelia of V138 and Pe811 were obtained by centrifugation and washed three times with osmotic stabilizer, then absorbed excess water with sterile filter paper. A 250 mg (wet weight) equivalent of mycelia was immediately treated at 30 °C for 3 h with 1 mL of lywallzyme solution. During this period, protoplast generation was followed by observation under the optical microscope. The protoplasts were separated from the undigested and fragmented mycelia by being filtered through 4-layer filter paper and obtained by centrifugation. The supernatant was discarded and the sedimented protoplasts were suspended immediately in osmotic stabilizer solution. Protoplast yield (cells/mL^{-1}) was determined by using a Neubauer hemocytometer. Protoplast regeneration rate (%) = (A–B)/C × 100%, where A is the total number of colonies counted on PDA medium containing 0.6 M osmotic stabilizer, B is the number of colonies counted on PDA medium without osmotic stabilizer, and C is the number of protoplasts (Zhu et al., 2016).

2.3. Lethal time of V138 normal mycelium and protoplast regeneration mycelium at 0 °C

Lethal time of V138 mycelium at 0 °C was investigated according to the method established by Liu et al. (2011) with slight modifications. Colonies of normal mycelium and protoplast regeneration mycelium incubated on RM regeneration medium (PDA medium containing 0.6 M D-mannitol as the osmotic stabilizer) were transferred to fresh medium and exposed at 0 °C for 12 h (to 156 h with a 12-h interval). The chilled treated plates were incubated at 30 °C for one week and observed for detecting mycelial growth. The mycelium was regarded as dead if it did not regrow and record the lethal time.

2.4. Inactivation of protoplast of P. eryngii

For heat inactivation, the isolated protoplast of *P. eryngii* was taken at a density of 10^6 protoplasts/mL, and then was treated at 50, 55 and 60 °C for 30, 60 and 90 min, respectively. After serial dilution, the inactivated protoplast suspensions were regenerated by regeneration PDA medium. The plates were then incubated at 25 °C for 6 days. The inactivated protoplasts were then used for fusion (Zhao et al., 2011).

2.5. Protoplast fusion between V138 and pe811

Both the protoplasts of normal viable V138 and heat-inactivated protoplasts of Pe811 were taken at a density of 10^6-10^7 protoplasts/mL for fusion. Fusion was carried out according to Chakraborty and Sikdar (2008) with slighted modifications. An equal volume of both protoplasts was mixed in a tube and gently centrifuged at 900*g* for 10 min, the supernatant was rinsed off and added 1 mL fusion solution (40% of polypropynoglycol 4000 in a 0.05 mol L^{-1} solution of $CaCl_2$), then maintained at 30 °C for 30 min. The suspensions were then washed with 0.6 M of mannitol solution and centrifuged at 900*g* for 10 min. Later the protoplast suspension was coated on regeneration medium. The plates were incubated at 30 °C, the protoplast regeneration and development of colonies were observed.

2.6. Screening of the putative fusants at low temperature (0 °C)

Colonies and mycelium were regrown in the plate after 5-7 days, later covered with a layer of PDSA solid medium (1.0% agar), treated at 0 °C for lethal time of V138 protoplast regeneration mycelium. The dishes were kept at 30 °C for 3–5 days and developed colonies were transferred to new PDSA plate dishes. These developed colonies were regarded as the putative fusants. The screening process is repeated three times at 0 °C. The surviving strains are considered to be fusants and then are used for further identification.

2.7. Identification of the putative fusants

2.7.1. Antagonistic test

The putative fusants and parents, from Section 2.6, were active by subculturing 3–5 times. The putative fusants and parents were co-cultured in 20 mL of PDA in 90 mm petri dishes at 25 °C for several days.