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The efficient genetic transformation of *Cordyceps militaris* by using mononuclear protoplasts

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ABSTRACT

As a highly valued edible mushroom, *Cordyceps militaris* has attracted considerable attention because it contains a variety of bioactive ingredients. However, the unknown number of cell nuclei and lack of optimized protoplast preparation conditions have hindered the studies of protoplast fusion breeding and genetic engineering to increase the content of bioactive constituents. To further promote protoplast fusion and understand the function of genes involved in the synthesis of active ingredients, this study aimed to investigate the number of nuclei in *C. militaris* and increase the yield of protoplasts derived from mononuclear mycelia as well as the regeneration rate. The results observed with a fluorescence microscope showed that the conidia, blastospores, and mycelia of *C. militaris* were all mononuclear. The maximum protoplast yield of 2.25×10^7 protoplasts/g fresh weight (FW) was achieved when 4-day-old mononuclear *C. militaris* mycelia were incubated in an enzymolysis solution (pH 6.5) composed of 1.00% lysing enzyme, 1.00% lywallzyme, and 0.8 M KCl at 32 °C for 3.0 h. The maximum regeneration rate (36.5%) was obtained when protoplasts prepared under the above optimal conditions were spread onto regeneration media (RM) containing 1.0 M sorbitol. Linear DNA fragments containing the *bar* gene were efficiently transformed into protoplasts by polyethylene glycol (PEG)-mediated transformation, and 87 PCR-positive mutants containing the *bar* gene were obtained. These results provide effective methods for the preparation and regeneration of protoplasts derived from mononuclear mycelia, which lays an important foundation for efficient genetic transformation in *C. militaris*.

1. Introduction

As an important edible fungus, *Cordyceps militaris* contains a variety of bioactive ingredients that are similar to those in *Cordyceps sinensis* and is thus widely used as its substitute (Shrestha et al., 2012). Its fruit bodies contain cordycepin (Lin et al., 2018; Yi et al., 2014), cordycepic acid (Deng et al., 2013), polysaccharides (Bi et al., 2018), pentostatin (Xia et al., 2017), and novel carotenoids (Dong et al., 2013). *C. militaris* is found to exert many beneficial effects, as it possesses anti-fatigue (Zhong et al., 2017), anti-inflammatory (Kim et al., 2017), anti-tumor (Liu et al., 2017), and anti-oxidant (Yang et al., 2016) properties. Increasing the content of bioactive components in *C. militaris* by genetic engineering and protoplast fusion breeding is becoming a research hotspot. However, the function of many genes involved in the synthesis of bioactive components remains unknown. Knocking out genes using genetic transformation is a powerful method for studying gene function. Due to its thick cell wall, transforming foreign DNA into *C. militaris* cells is difficult. If the cell wall barrier is removed, transforming exogenous DNA into *C. militaris* cells using polyethylene glycol (PEG)-mediated protoplast transformation is easier. The preparation of high-quality protoplasts is the most critical step for cellular fusion and genetic transformation.

Protoplasts have been widely utilized for fungal transformation, and the most commonly used method for protoplast preparation is the enzymatic digestion of cell walls. Because the cell wall composition of different fungi are clearly different, the enzymolysis conditions of different fungi for preparing protoplasts are also different (Aloulou-Abdelkefi et al., 2017), and optimizing the enzymolysis conditions of *C. militaris* for protoplast isolation is necessary. Mononuclear *C. militaris* materials are indispensable for knocking out target genes and

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protoplast fusion. To our knowledge, there are no reports on observing the number of *C. militaris* nuclei or optimizing the preparation conditions for *C. militaris* protoplasts. In this study, we aimed to observe the number of *C. militaris* nuclei (conidia, blastospores, and mycelia) and optimize the preparation conditions of protoplasts from mononuclear *C. militaris*. The types and concentrations of osmotic stabilizers in the regeneration media (RM) were also studied. The phosphinothricin acetyltransferase (*bar*) gene was transformed into *C. militaris* to verify the viability of the protoplasts.

2. Materials and methods

2.1. Strains, culture conditions, and plasmid

A laboratory and commercial strain of *C. militaris*, CM10 (Ningyang County Haixin Biological Technology Co., Ltd.), was used throughout the study. This strain was cultured on potato dextrose agar (PDA) plates (20.0% potato, 1.0% dextrose, 0.3% KH_2PO_4, 0.1% $MgSO_4$, and 2.0% agar, w/v) at 25 °C for 20 days.

The plasmid pCAMBIA0390-Bar-KO7, containing a *bar* gene under the control of the *Aspergillus nidulans trpC* promoter and *trpC* terminator, was constructed based on pCAMBIA0390 in our laboratory (Supplementary Fig. 1).

C. militaris transformants were cultured on selective PDA containing glufosinate ammonium (300 μg/mL) at 25 °C for 20 days in the dark.

2.2. Collection of conidia, blastospores, and mycelia for nuclear observation

To observe the number of nuclei, three kinds of *C. militaris* materials (conidia, blastospores, and mycelia) were collected. Conidia were harvested from wild-type (WT) *C. militaris* plates using sterile double-distilled water (ddH_2O) and filtered through four-layer lens papers. Conidia were inoculated into 100 mL of potato dextrose broth (PDB, 20.0% potato, 1.0% dextrose, 0.3% KH_2PO_4, and 0.1% $MgSO_4$, w/v) and cultured at 25 °C on a shaker at 150 rpm for 96 h in the dark. The PDB culture was filtered through cotton wool to remove the mycelia. The filtrate was centrifuged at 10,000 *g* for 5 min to obtain blastospores. Coverslips were inserted into the PDA plates with CM10 at a 45-degree angle and cultured at 25 °C until the mycelia grew onto the coverslips. The above three materials were used for fluorescence staining and nuclear observation.

2.3. Fluorescence staining of C. militaris and nuclear observation

To observe the number of nuclei in each cell, Fluorescent Brightener 28 (FB28) (Sigma-Aldrich, Germany) and 4′,6-diamidino-2-phenylindole (DAPI) (Solarbio, China) were used to stain the cell walls and nuclei of *C. militaris*, respectively. The conidia and blastospores were separately mixed with 0.5 mL of formaldehyde solution (10%, w/v) for 2 h. The coverslips with mycelia were soaked in the formaldehyde solution (10%, w/v) for 2 h. The fixed conidia and blastospores were obtained by centrifugation (5000 *g*, 10 min), and fixed mycelia were obtained by air drying. Next, 100 μL of FB28 (2.5 μg/mL) and 100 μL of DAPI (2.5 μg/mL) were mixed with each material for 30 min. The stained materials were observed under a 1000× fluorescence microscope (Leica, Germany) to observe the number of nuclei in each cell.

2.4. Protoplast isolation

Since *C. militaris* mycelia are mononuclear and easy to collect, they were selected as the material for preparing protoplasts. Briefly, 100 μL of *C. militaris* conidia (1×10^6 conidia/mL) were inoculated into 100 mL of PDB medium and cultured under stationary conditions at 25 °C in the dark. The mycelia were collected by filtering through cotton wool and rinsed with ddH_2O until almost no spores were observed under the microscope.

Table 1
Combinations of lytic enzymes used in the enzymolysis solution.

Treatment	Enzyme
EC 1	1.00% lysing enzyme
EC 2	1.00% Yatalase
EC 3	1.00% lywallzyme
EC 4	0.50% lysing enzyme + 0.50% Yatalase
EC 5	0.50% lysing enzyme + 0.50% lywallzyme
EC 6	0.50% Yatalase + 0.50% lywallzyme
EC 7	0.33% lysing enzyme + 0.33% Yatalase + 0.33% lywallzyme

Lytic enzymes used for cell wall removal are an essential factor and directly affect the release of protoplasts. A variety of lytic enzymes commonly used in filamentous fungi, including lysing enzyme from *Trichoderma harzianum* (Sigma-Aldrich, USA), Yatalase (Takara, Japan), and lywallzyme (Guangdong Institute of Microbiology, China), were tested at different combinations (Table 1) and dissolved in four commonly used osmotic stabilizers (0.8 M KCl, 0.8 M $MgSO_4$, 0.8 M mannitol, 0.8 M glucose).

To optimize the concentration of KCl used in the enzymolysis solution, different concentrations of KCl (0.2, 0.4, 0.6, 0.7, 0.8, 0.9, 1.0, 1.2, 1.5, and 2.0 M) as an osmotic stabilizer were tested. To optimize the concentration of the enzyme mixture (lysing enzyme and lywallzyme at a ratio of 1:1), different concentrations of the enzyme mixture (0.25, 0.50, 0.75, 1.00, 1.25, 1.50, 1.75, 2.00, 2.25, 2.50, 2.75, 3.00%) were tested. To optimize the digestion temperature, different temperatures (18, 20, 22, 24, 26, 28, 30, 32, 34, 36, 38, and 40 °C) were tested. To optimize the pH of the enzymolysis solution, different pH values of the enzymolysis solution (3.0, 4.0, 5.0, 5.5, 6.0, 6.5, 7.0, 8.0, and 9.0) were tested. To optimize the digestion time, different digestion times (1.0, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0, and 5.0 h) were tested. Mycelia were harvested at different ages (2–8 days) to determine the optimal age for the release of protoplasts. Enzymes were dissolved in osmotic stabilizers and sterilized by filtration with 0.22-μm membrane filters (Merck Millipore, USA). Next, 200 mg of wet mycelia were resuspended in 1.0 mL of enzymolysis solution and digested in an incubator under gentle shaking at 90 rpm. After digestion, the mixture was filtered through four-layer lens paper to remove the undigested mycelial debris. The filtrate was centrifuged at 3000 *g* for 10 min, and the collected protoplasts were washed twice with osmotic stabilizer solution and resuspended in STC buffer [18.2% sorbitol, 10.0 mM Tris−HCl (pH 7.5), 25.0 mM $CaCl_2$, w/v] to a final concentration of 1×10^8 protoplasts/mL. Finally, the protoplasts were observed under a 400× microscope and counted with a hemocytometer. The protoplast yield was calculated according to the following formula:

$$Yp = Np/V$$

where Yp is the yield of protoplasts (number of protoplasts per milliliter), Np is the number of protoplasts, and V is the volume of enzymolysis solution. All the experiments were repeated three times.

2.5. Protoplast regeneration

PDA media with different concentrations of osmotic stabilizers (KCl, NaCl, mannitol, sorbitol, glucose, sucrose) were used as the RM. The concentration of protoplasts was diluted to 1×10^3 protoplasts/mL using STC buffer, and 200 μL of diluted protoplast suspension was directly coated onto RM plates. As a control, the concentration of protoplasts was diluted to 1×10^3 protoplasts/mL using ddH_2O. Next, 200 μL of protoplast suspension diluted with ddH_2O was directly coated onto PDA plates without an osmotic stabilizer. All coated plates were cultured at 25 °C for 10 days (Supplementary Fig. 2). The protoplast regeneration rate was calculated based on the number of colonies according to the following formula: