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Review

Liposome-mediated mycelial transformation of filamentous fungi

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ABSTRACT

Liposome-mediated transformation is common for cells with no cell wall, but has very limited usage in cells with walls, such as bacteria, fungi, and plants. In this study, we developed a procedure to introduce DNA into mycelium of filamentous fungi, *Rhizopus nigricans* LH 21 and *Pleurotus ostreatus* TD 300, by liposome-mediation but with no protoplast preparation. The DNA was transformed into *R. nigricans* via plasmid pEGFP-C1 and into *P. ostreatus* via 7.2 kb linear DNA. The mycelia were ground in 0.6 M mannitol without any grinding aids or glass powder for 15 min to make mycelial fragments suspension; the suspension was mixed with a mixture of the DNA and Lipofectamine 2000, and placed on ice for 30 min; 100 μL of the transformation solution was plated on potato dextrose agar (PDA) plate and cultivated at 28 °C for transformant screening. The plasmid and the linear DNA were confirmed to be integrated into the host chromosome, proving the success of transformation. The transformation efficiencies were similar to those of electroporation-mediated protoplast transformation (EMPT) of *R. nigricans* or PEG/$CaCl_2$-mediated protoplast transformation (PMT) of *P. ostreatus*, respectively. The results showed that our procedure was effective, fast, and simple transformation method for filamentous fungi.
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Introduction

Many filamentous fungi are genetically engineered and industrially important eukaryotes. They secrete a variety of enzymes to decompose macromolecules in nature, and contribute to approximately 60 % of the industrial enzymes (Østergaard & Olsen 2010). Their edible fruiting bodies, organic acids, polysaccharides, and secondary metabolites are valuable sources of food and medicine components (Magnuson & Lasure 2004; Shen *et al.* 2007; Petrič *et al.* 2010; Wang *et al.* 2011). Furthermore, filamentous fungi are being increasingly used as hosts to produce homologous or heterologous proteins (Punt *et al.* 2002; Ward 2012). Therefore, the development of an effective DNA transformation approach is very important for filamentous fungi to identify the gene function and improve its genetic characters.

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Since the first report of transformation in *Neurospora crassa* by using PEG/$CaCl_2$-mediated protoplast in 1979 (Case et al. 1979), a number of methods have been developed to deliver DNA into about 100 species in all major groups of filamentous fungi, including oomycetes, zygomycetes, ascomycetes, and basidiomycetes (Fincham 1989; Verdoes et al. 1995; Meyer 2008; Ward 2012). Besides PEG/$CaCl_2$-mediated protoplast transformation (PMT), other common methods are protoplast electroporation (Kothe & Free 1996), liposome-mediated protoplast transformation (Radford et al. 1981), lithium acetate-mediated mycelial transformation (Dhawale et al. 1984), biolistic transformation (Klein et al. 1987), and *Agrobacterium*-mediated transformation (Beijersbergen et al. 2001). However, those methods have a few shortcomings, such as complicated procedures, expensive instruments, and low transformation rate. For example, protoplast-mediated transformation relies on laborious protoplast preparation and regeneration; biolistic transformation, although does not involve protoplast manipulation, requires special equipment and suffers from high cost; lithium acetate-mediated transformation can only apply to limited number of species in filamentous fungi; finally *Agrobacterium*-mediated transformation is time-consuming and prone to low transformation rate due to various factors, such as fungal species specificity, *Agrobacterium* strains specificity, initial amount of fungal material and bacterial cells, the *vir*-gene inducer acetosyringone concentration, cocultivation time and temperature (Meyer 2008; Michielse et al. 2008).

Liposome-mediated transformation has high DNA packaging capacity, low immunogenicity and large-scale production, so is broadly used for gene delivery in gene therapy (Joshi & Muller 2009). Liposomes contain hydrophilic head groups and hydrophobic tails, and attain positive charges. They form bimolecular lipid vesicles in an aqueous environment. In this process, negatively charged DNAs are attracted and engulfed in the vesicles, which protects the DNAs from degradation by nucleases. DNAs enter cells via endocytosis of the liposomes which adhere and fuse with the negatively charged cell membrane (Fraley et al. 1979; Barampuram & Zhang 2011). Therefore, the vesicles carrying DNAs must attach directly to cell membrane for successful transformation. For cells with walls, transformation usually combines with other techniques (Radford et al. 1981; Allshire 1990). Using liposome to deliver genes into fungal mycelium has not been reported.

Rhizopus nigricans, the most common species of *Rhizopus*, has extensive application in brewing, the 11α-hydroxylation of 16α, 17-epoxyprogesterone (Wu et al. 2011), and is also used as a mycoremediation to tolerate and remove heavy metal and toxic compounds (Tomasini et al. 2001; Bai & Abraham 2002). Therefore, it is very valuable to develop a routine DNA transformation method in *R. nigricans* (Horiuchi et al. 1995). *Pleurotus ostreatus*, popularly named oyster mushroom, is a widely cultivated edible and medicinal mushroom in China and East Asia. It has short growth time, high adaptability and productivity, high lignocellulose degrading enzyme activities, and high bioactive β-glucans yield (Castanera et al. 2012; Chai et al. 2013). In this study, we developed a new transformation procedure for the above two filamentous fungi using liposome-mediated mycelial transformation (LMMT) independent of any other compound and techniques; our results showed that the new method was efficient, simple, and fast.

Materials and methods

Strains, DNAs, and liposome

The filamentous fungi *Rhizopus nigricans* strain LH 21 (Henan Lihua Pharmaceutical, Anyang, Henan, China) was cultivated on potato dextrose agar (PDA) medium plate at 28 °C for 3 d as described previously (Du et al. 2006). *Pleurotus ostreatus* TD 300, often used as a commercial cultivation strain in China, was cultivated on PDA medium at 28 °C for 6 d as described elsewhere (Wang et al. 2011).

Plasmid pEGFP-C1 (Clontech, Mountain View, CA) contains the variant green fluorescent protein (*GFP*) gene under the control of the cytomegalovirus (CMV) promoter (Cormack et al. 1996). The linear DNA (7.2 kb) is a homologous recombination fragment replacing glucan synthase gene promoter in *P. ostreatus* TD 300; it comprises of four DNA segments: DNA sequence preceding the promoter of glucan synthase gene of *P. ostreatus* (*PGS*) (1.015 kp), hygromycin B resistance gene (*hph*) of *Escherichia coli* expression cassette (2.822 kp) from plasmid PAN7-1 (Punt et al. 1987), the promoter of glyceraldehyde-3-phosphate dehydrogenase (*gpd*) gene of *Aspergillus nidulans* (2.206 kp) also from plasmid PAN7-1, and the 5′ end part sequence of *PGS* (1.008 kp) (Chai et al. 2012).

Lipofectamine 2000 (1 mg mL^{-1}, Invitrogen, Carlsbad, CA) is a cationic liposome formulation, and contains lipid subunits that can form liposomes in an aqueous environment.

Liposome-mediated mycelial transformation (LMMT)

50 μL of Lipofectamine 2000 and 50 μL of 2 μg μL^{-1} pEGFP-C1 were mixed and placed on ice for 30 min. 0.5 g mycelia of *Rhizopus nigricans* was collected from PDA medium plate and added in sterile mortar, and then ground with 5 mL 0.6 M mannitol for 15 min to mycelial fragments suspension. To compare the grinding results, 1 g sterile glass powder (10 μm in diameter, hardness 5 on the Mohs' scale. Tatsumori, Tokyo, Japan) or quartz sand (5–10 μm, hardness 7 on the Mohs' scale. Dongfeng, Guoyi, China) were added into the mortar, respectively. 1 mL of the mycelial fragments suspension was added into the mixture of the pEGFP-C1 and Lipofectamine 2000, and then mixed well by pipetting and placed on ice for 30 min. 100 μL of the transformation solution was plated on PDA plate and cultivated at 28 °C.

Pleurotus ostreatus is applied to the same procedure to carry out the transformation, except the foreign DNA was 0.5 μg μl^{-1} of the linear DNA.

Electroporation-mediated protoplast transformation (EMPT) of Rhizopus nigricans

0.5 g mycelia of *R. nigricans*, collected from PDA medium plate, was ground with 1 mL 0.6 M mannitol for 15 min in sterile mortar to make mycelial fragments suspension; then the suspension was treated by lytic enzyme to form protoplast (Qiu et al. 2010). 80 μL of protoplast suspension (10^6 mL^{-1}) and 20 μL of pEGFP-C1 plasmid (2 μg μL^{-1}) were transferred to an ice-cold 0.2 cm electroporation cuvette (Gene Pluser, 2 mm gap cuvette. BIO-RAD, Hercules, CA, USA) and electroporated

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