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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.

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

at 7.5 kV cm^{-1} (1.5 kV in a 0.2 cm cuvette) voltage gradient, 25 μF capacitance, and 200 Ω resistance. After electroporation, 1 mL of 1 M sorbitol was added to the cuvette, and next the cuvette was placed on ice for 10 min. Finally, 100 μL of the transformation solution was plated onto regeneration complete medium (RCM) (Pe'er and Chet 1990) plate and cultivated at 28 °C.

PMT of Pleurotus ostreatus

Protoplasts preparation and PEG/$CaCl_2$-mediated transformation of *P. ostreatus* were performed as described previously (Qiu *et al.* 2010; Dong *et al.* 2012). The introduced foreign DNA was the linear DNA (50 μL of 0.5 μg $μL^{-1}$).

Transformants screening and subculture

To screen the *Rhizopus nigricans GFP* transformants, the PDA and RCM plates inoculated the transformation solution were incubated at 28 °C overnight and overlaid with 15 mL molten PDA medium containing 300 μg mL^{-1} geneticin; the plate was incubated at 28 °C for 3 d. Colonies on the plate were randomly picked for fluorescent microscopic observation (DM4000B, Leica, Wetzlar, Germany). The spores of the mycelia colonies with bright and stable fluorescence were selected and inoculated onto fresh PDA plate for subculture.

For the screening of *Pleurotus ostreatus hph* transformants, the PDA and RCM plates were inoculated at 28 °C for 7 d, and then overlaid with 15 mL molten PDA medium containing 80 μg mL^{-1} hygromycin B. After another 7 d of incubation, inoculate mycelial colonies grown on the surface of the upper layer medium onto fresh PDA with hygromycin B for subculture.

Transformant identified by PCR and semi-quantitative RT-PCR

The integration of introduced *GFP* gene in the transformants of *Rhizopus nigricans* was confirmed by PCR. The template DNA was the total DNA extracted from transformant using cetyltrimethylammonium bromide (CTAB) procedure (Zhang *et al.* 1996). The primers were GFP-S (5-ATGGTGAGCAAGGGC GAGGAGC-3) and GFP-X (5-TTATCTAGATCCGGTGGAT CCCGG-3), defining a 798 bp production. The amplification conditions were 94 °C for 30 s, 55 °C for 40 s, and 72 °C for 1 min, and the cycle was repeated 30 times. Similarly, to verify the replacement *PGS* promoter by the homologous recombination fragment in the transformants of *Pleurotus ostreatus*, the PCR primers were hph-1 (5-GGAAGTGCTTGACATTGGGGAG TT-3) and hph-2 (5-TACTTCTACACAGCCATCGGTCCAG-3) for the amplification of the *hph* gene at 94 °C for 1 min, 56 °C for 1 min, and 72 °C for 1 min in 30 cycles. The expected PCR product size was 750 bp. In addition, semi-quantitative RT-PCR was carried out as described elsewhere (Dong *et al.* 2012) with slight modifications. Total RNA was extracted from transformant of *P. ostreatus* using TRIzol (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Primers were PGSF (5-AATTTGGATTCCAACGGGA-3) and PGSR (5-GGGCTATTGATCGCTTCTC-3) for reverse transcription and amplifying glucan synthase gene; and AC-1 (5-GATAGAACCACCAATCCAAAC-3) and AC-2 (5-AAGTCATC ACCATCGGTAACG-3) for reverse transcription and amplifying housekeeping gene β-actin. The expected PCR product sizes were 759 bp and 750 bp. Reverse transcription of the mRNA was carried out at 42 °C for 60 min by using MMLV Reverse Transcriptase 1st-Strand cDNA Synthesis Kit (Epicentre, Madison, WI, USA). The PCR conditions were 94 °C for 40 s, 49 °C for 40 s, and 72 °C for 1 min in 30 cycles.

5 μL of the PCR reaction aliquots was analyzed on 1 % agarose gels stained with ethidium bromide. The electrophoresis bands of RT-PCR reaction were photographed and the density of each band was quantified using image analysis software, UVI band V. 97 software (UVI Tech, Cambridge, UK).

Southern blot analysis of total DNA of the GFP transformants of Rhizopus nigricans

Total DNA isolated from selected transformant of *R. nigricans* was digested with Sac I, and transferred onto positively charged Hybond N^+ membranes. Probes for detection of the *GFP* gene were created using a *GFP* gene PCR amplicon with the same PCR primers and amplification conditions as above. Probes labelling, DNA hybridization and detection were performed as described in the manual of the DIG-high prime DNA labelling and detection Starter kit I from Roche (Indianapolis, IN, USA).

Table 1 – The transformation efficiencies of different transformation methods for *R. nigricans* and *P. ostreatus*.

Method[a]	Grinding aid	*R. nigricans*		*P. ostreatus*		
		Colony on PDA[b]	TE[c] (cfu $μg^{-1}$ DNA)	Colony on B− PDA[d]	Colony on B+ PDA[d]	TE[c] (cfu $μg^{-1}$ DNA)
LMMT	No	+	8.2 ± 1.9	+	−	0
	Glass powder	−	0	+	+	20.0 ± 4.2
	Quartz sand	−	0	−	−	0
EMPT		+	6.0 ± 2.2			
PMT				+	+	22.2 ± 3.3

a Control for each method was the same except that no foreign DNA was added.
b Presence (+) or absence (−) of colony on PDA plate inoculated with the transformation solution.
c Data are means and standard error (SE) of four independent experiments.
d Presence (+) or absence (−) of colony on PDA plate in lower layer medium containing no hygromycin B (B− PDA) or in upper layer medium containing hygromycin B (B+ PDA) after the transformation solution was inoculated.

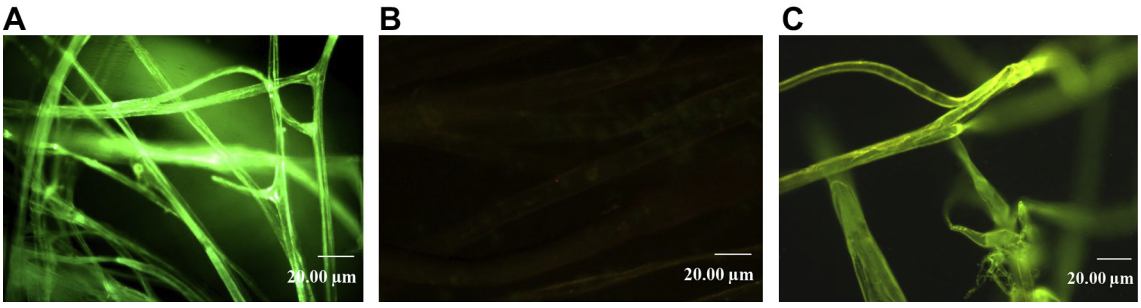

Fig 1 – Fluorescence micrographs of the *GFP* transformant of *R. nigricans* (200×). (A) Transformant obtained by LMMT. (B) Wild type. (C) Transformant obtained by EMPT.

Statistical analysis

Statistical analysis was performed using standard analysis of variance (ANOVA) techniques. Comparisons between the means of band density in the semi-quantitative RT-PCR products were made using the least significant difference (LSD) test.

Results

Comparison of transformation efficiency (TE) among different treatments

In this study, we conducted two different LMMT of *Rhizopus nigricans* and *Pleurotus ostreatus*. The foreign DNA for *R. nigricans* is plasmid pEGFP-C1 containing *GFP* gene and that for *P. ostreatus* is linear DNA-containing hygromycin B resistance gene. And we applied three grinding manners on the mycelia: no-grinding aid, grinding using glass powder, and grinding using quartz sand, particles harder than glass powder. To compare those with the traditional methods, we also did EMPT of *R. nigricans* and PMT of *P. ostreatus*. Table 1 lists the results of those different methods.

After mycelia of *R. nigricans* was ground with glass powder or quartz sand, liposome-mediated transformation did not produce any colony on the PDA plates inoculated with the transformation solution. However, no-grinding aid led to transformants emitting bright and stable fluorescence (Fig 1A), proving the success of transformation because the control, where no plasmid was added, produced colonies but with no fluorescence (Fig 1B); the TE was 8.2 ± 1.9 cfu μg^{-1} plasmid DNA. Similar fluorescence intensity was observed from the protoplast transformants of *R. nigricans* with a TE of 6.0 ± 2.2 cfu μg^{-1} plasmid DNA (Fig 1C; Table 1).

Interestingly, the effect of grinding on liposome-mediated transformation of *P. ostreatus* was different from that of *R. nigricans*. In the treatment of no-grinding aid, colonies were observed only in the medium with no hygromycin B. In quartz sand grinding, no colonies were seen in any of the media containing hygromycin B or no hygromycin B. While colonies grew well in medium containing hygromycin B after grinding with glass powder, the control, which had no hygromycin B resistance gene, could only grow in medium with no hygromycin B. It demonstrated that grinding with glass powder resulted in successful transformation; its efficiency, 20 ± 4.2 cfu μg^{-1} DNA, is comparable to the 22 ± 3.3 cfu μg^{-1} DNA in the protoplast transformation of *P. ostreatus* (Table 1).

Verification on the integration of foreign GFP gene into the chromosome of Rhizopus nigricans *transformant*

One *GFP* transformant of *R. nigricans* from each of LMMT and EMPT was randomly selected and repeatedly sub-cultured three times. The total DNA samples of the transformants gave a single band matching the genomic DNA of *R. nigricans*. The expected PCR amplifications were obtained from all DNA samples of the transformants using the primer pairs for the *GFP* gene (Fig 2). This strongly indicated that the *GFP* gene was integrated into the host chromosome.

To further examine the copy number of the introduced *GFP* gene integrated into the chromosome of the liposome-mediated transformant, southern blot analysis on the DNA from the F1–F6 spore progenies of one randomly selected transformant were performed. The result shows one copy of the *GFP* gene stably present in the genome of the transformant offspring (Fig 3). As a control, *GFP* gene was not detected in the untransformed mycelium.

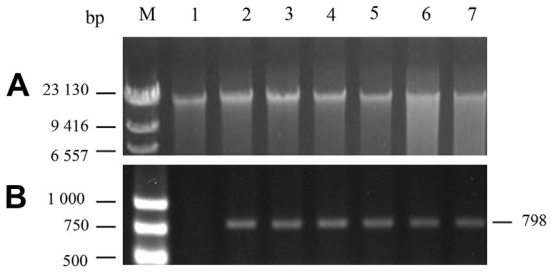

Fig 2 – PCR for identifying the recombinant *GFP* sequence in the transformants of *R. nigricans*. (A) Total DNA isolated from the transformants and its spore progeny; (B) PCR amplification was carried out on total DNA using primers (GFP-S and GFP-X) defining a 798 bp product spanning the *GFP* gene. Lane 1: Wild type (Untransformed original strain); Lane 2–4: Spore progeny F1–F3 of the transformant obtained by LMMT; Lane 5–7: Spore progeny F1–F3 of the transformant obtained by EMPT; M: DNA molecular size markers.

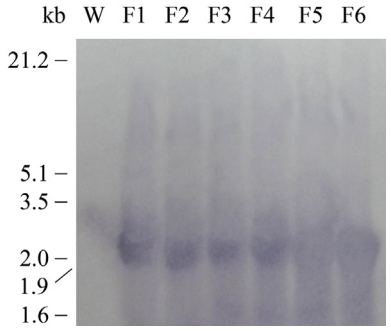

Fig 3 – Southern blot analysis of the spore progenies F1–F6 of one randomly selected *GFP* transformant of *R. nigricans* obtained by LMMT after digesting chromosomal DNA with Soc I and hybridization with a DNA probe directed against a 798 bp fragment of *GFP* gene. W: Wild type (Untransformed original strain); F1–F6: Spore progenies F1–F6 of one *GFP* transformant.

*Verification on the integration of foreign hygromycin B resistance gene (*hph*) into the chromosome of* Pleurotus ostreatus *transformant*

Three subculture F3 generations of the *P. ostreatus* transformants from liposome and protoplast transformation were randomly selected respectively. The isolated total DNA and PCR results all indicated that the *hph* gene was integrated into the host chromosome (Fig 4).

In addition to *hph* gene, the foreign DNA segment also contains *gpd* promoter of *Aspergillus nidulans* followed by *P. ostreatus PGS* gene. To further examine the stability and functionality of foreign DNA in *P. ostreatus* transformants, the mRNA expression levels of *PGS* gene in the above transformants were measured by semi-quantitative RT-PCR, and they were 2–4 times higher in liposome transformants than in wild type (Fig 5).

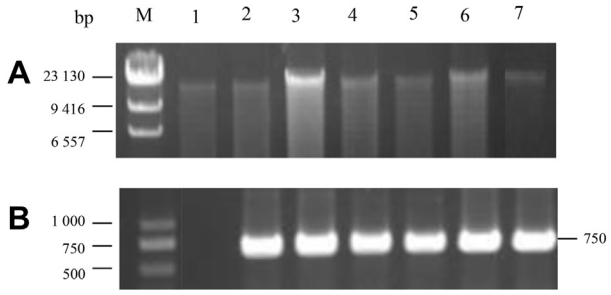

Fig 4 – PCR for the identification of the recombinant *hph* sequence in the transformants of *P. ostreatus*. (A) Total DNA isolated from the subculture F2 generation of the selected transformants; (B) PCR amplification was carried out on total DNA using primers (hph-1 and hph-2) defining a 750 bp product spanning the *hph* gene. Lane 1: Wild type; Lane 2–4: Transformants obtained by LMMT; Lane 5–7: Transformants obtained by PMT; M: DNA molecular size markers.

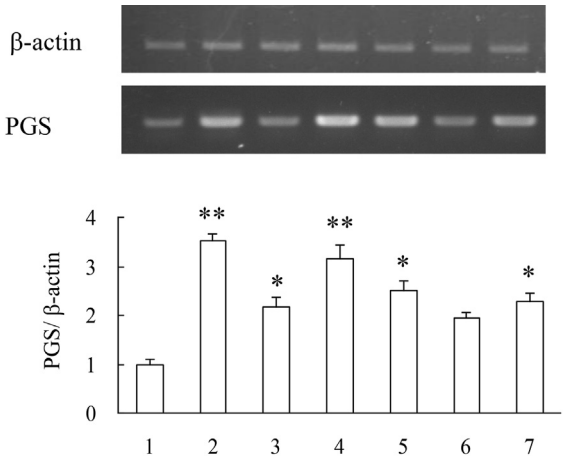

Fig 5 – Semi-quantitative RT-PCR analysis of *PGS* mRNA in the transformants derived from Fig 4. The amount of *PGS* mRNA, expressed as the ratio of densitometric measurement of the sample to the corresponding internal standard (β-actin), is shown in the upper panels. Data are shown as mean ± SEM for three independent experiments. $^{*}p < 0.05$ comparing to wild type; $^{**}p < 0.01$ comparing to wild type. 1: Wild type; 2–4: Transformants obtained by LMMT; 5–7: Transformants obtained by PMT; M: DNA molecular size markers.

Discussion

Since the first liposome-mediated transformation on *Escherichia coli* (Fraley *et al.* 1979), several type of cells have been genetically modified by this method, including Gram negative bacteria (Kawata *et al.* 2004), Gram positives bacteria (Makins & Holt 1981; Van der Vossen *et al.* 1988; Yu *et al.* 2001; Kawata *et al.* 2004), archaea (Metcalf *et al.* 1997), and mammalian (Aune & Aachmann 2010). Nevertheless, this method is not very effective on bacteria with cell wall which blocks the passage of large molecules or vesicles, and has to be combined with other techniques such as competent cell/$CaCl_2$, protoplast preparation, and electroporation or PEG/$CaCl_2$-mediation. The same situation arises in fungi and plants due to their cell walls, and protoplasts are required for liposome-mediated transformation (Radford *et al.* 1981; Deshayes *et al.* 1985; Allshire 1990; Judelson *et al.* 1991; Zhu *et al.* 1993; Wordragen *et al.* 1997). However, protoplast preparation is laborious and of low efficiency; in addition, protoplasts are not always physiologically identical to the original cells. Therefore, liposome-mediated transformation is far from routine in plants and fungi (Ruiz-Diez 2002; Rao *et al.* 2009).

In plants, liposome-mediated transformation without protoplast is only carried out on callus of tobacco with low TE (Rosenberg *et al.* 1990). As far as we know, there has been no such report in fungi. In the present study, such transformation was successfully completed on not only lower fungi zygomycetes *Rhizopus nigricans* but also higher fungi basidiomycetes *Pleurotus ostreatus* with efficiencies similar to those of conventional protoplast transformation. In the lower fungi, vegetative mycelium does not have septate except those

found in reproductive structures (de la Providencia et al. 2005). However, in arbuscular mycorrhizas *Scutellospora reticulata* and *Glomus clarum*, a septum forms 9–18 min after the cut of mycelium to prevent the loss of cytoplasm (de la Providencia et al. 2007). The mycelium of higher fungi has septum with a central pore of about 70–500 nm in diameter (Deacon 2005). In many basidiomycetes and ascomycete, septal pores are rapidly plugged by Woronin body or septal pore cap (SPC), organelles surrounded by closely associated unit membrane, in the event of mycelial wounding (Markham 1994; Jedd 2011). So the mechanism of LMMT of filamentous fungi probably was the following. In lower fungi, the DNA-containing liposome entered the mycelial cell when aseptate mycelium was injured and was kept inside the cell by the subsequently formed septum; in higher fungi, the liposome entered the mycelial cell by fusing with the membranes of Woronin body and SPC. However, if the wounding of mycelium was too severe to be repaired, the cell could not survive; that explains why no colony was observed in the treatment of grinding with glass powder on the soft mycelium of *R. nigricans*, and in the treatment of grinding with quartz sand on the relatively hard mycelium of *P. ostreatus*.

PMT could integrate multiple copies of genes into host chromosome and lead to high exogenous protein production in filamentous fungi (Meyer 2008). There has not been report whether liposome-mediated transformation is able to achieve the same result. Our study clearly demonstrated that only one copy of the plasmid containing *GFP* gene was inserted into *R. nigricans* chromosome when delivered to the mycelium by liposome.

By our knowledge, this is the first study to introduce the long linear DNA (7.05 kb) into filamentous fungi using liposome-mediated transformation method, and the first simple, fast, and effective transformation procedure for filamentous fungi.

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