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ORIGINAL ARTICLE

Protoplasting, regeneration and transformation of medicinal mushroom *Ganoderma multipileum* using succinate dehydrogenase mutation gene as a selection marker

Tzu-Ho Chou[1] · **Shean-Shong Tzean**[1]

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Abstract *Ganoderma multipileum* is a medicinal mushroom possessing potent medicinal secondary metabolites. However, in order to improve metabolic activity via molecular breeding, optimized protoplasting, regeneration and genetic transformation systems are mandatory but remain unexplored in *G. multipileum*. Thus, we sought to fill this gap by testing different parameters for protoplasting, regeneration, and genetic transformation based on the carboxin resistance marker gene. According to our results, the best viable protoplasting conditions were pH 6.0, mycelia age 4 days, enzyme concentration 7.5 mg/mL, 0.6 M sucrose osmoticum and 3 h of enzyme digestion. In a transformation study, a carboxin resistance gene cassette was constructed using PCR mutagenesis and restriction digestion of the native succinate dehydrogenase subunit B gene in *G. multipileum*. A PCR-restriction fragment length polymorphism strategy was also applied by PCR point mutagenesis to create an *Age*I restriction site. The transformation system using carboxin resistant cassette proved amenable. The *Sdh* gene structure showed an intron structure similar to that of other Basidiomycetes and Ascomycetes. Alignment and analysis of the *Sdh* gene sequences in numerous fungi revealed conservation at a carboxin resistant point mutation site that could be applied to a wide range of fungal species. Further *Sdh* gene phylogenetic analysis revealed congruence with species phylogeny but with low bootstrap supporting values.

✉ Shean-Shong Tzean
sst@ntu.edu.tw

Tzu-Ho Chou
cartonchou@gmail.com

[1] Department of Plant Pathology and Microbiology, National Taiwan University, Taipei 10617, Taiwan

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Introduction

Ganoderma lucidum (Mycobank, MB#148413) was originally isolated from a specimen collected from temperate UK (Curtis 1781; Fries 1821). Despite the notable ecological deviation, an isolate isolated from *Eucalyptus* in tropical India in 1973 was identified to be conspecific to *G. lucidum*. This strain was later deposited with the American Type Culture Center (ATCC 32471) in the United States and also in Food Industry Research and Development Institute Bioresource Collection and Research Center (BCRC 36123) in Taiwan. *G. lucidum* BCRC 36123 has been reported to have immunomodulation, antihepatoma and liver protective potential correlated with polysaccharides, and/or triterpenoids etc. (Chyr and Shiao 1991; Su et al. 2013). More recently, Wang et al. (2009) conducted a comprehensive phylogenetic study of the *Ganoderma* genus, including *G. lucidum* BCRC 36123, using the sequences of nuclear rDNA and the internal transcribed spacer (ITS). The results revealed that *G. lucidum* BCRC 36123 was conspecific to *G. multipileum* erected by Hou in 1950 in Taiwan (Hou 1950; Wang et al. 2009). In recognition of its potent medicinal usage, more recently, the genome of *G. multipileum* has been deciphered (unpublished paper). Comparison of the genome of *G. multipileum* with the recently published genome of *G. lucidum* 260125–1 (Chen et al. 2012) revealed only 85 % similarity (data not shown), which further confirmed the distinction between the two species.

Now that the genome of *G. multipileum* has been deciphered, uncovering gene regulation and function is a prelude to exploring the proteomics and metabolomics of this

species in order to improve its metabolic activity via molecular breeding. Therefore, establishing a transformation platform becomes essential. However, our preliminary transformation study disclosed that *G. multipileum* was unable to express a functional hygromycin phospho-transferase gene, which may due to a change in the first two amino acids of this protein in this organism (Kilaru et al. 2009). Even more surprisingly, *G. multipileum* can utilize bialaphos—a chemical herbicide used commonly in selection media—as substrate and showed a linear dosage–growth relationship. In fact, both these unique traits are significant obstacles impeding efficient transformation. These results contrasted greatly to several other studies on transformation in Basidiomycetes, i.e., *Agaricus bisporus*, *Pleurotus ostreatus*, *G. lucidum*, in which using the protoplast–polyethylene glycol (PEG) method, in combination with either GUS, hygromycin or bialaphos as an indicator or selection marker in normal dosage worked as expected (Case et al. 1979; Sonnenberg et al. 1988; Peng et al. 1993; Li and Li 1999; Sun et al. 2001; Kim et al. 2004; Zhou et al. 2008; Xu et al. 2012).

Taking another approach, Keon et al. (1991) first reported the cloning of the housekeeping gene encoding mitochondrial succinate dehydrogenase in the tricarboxylic acid cycle, and introduced a point mutation in the histidine residue at the C terminal end of *Sdh* subunit B. The mutation conferred on the mutated *Sdh* gene resistance to carboxin (2,3 dihydro-5-carboxanilido-6-methyl, 1,4, oxathin, vitavax)—a systemic fungicide targeting and inhibiting the function of *Sdh* (Keon et al. 1991). Since then, carboxin resistance has been applied widely in fungal transformation, particularly in Basidiomycetes (Keon et al. 1991; Honda et al. 2000; Ngari et al. 2009; Shima et al. 2009). Indeed, the point mutation in this endogenous gene offers great advantages over traditional transformation systems, not only in its sensitivity and efficiency but also because it avoids concerns about the use of antibiotic genes in homologous or heterologous expression, which may potentially contaminate the environment.

In this study, we tested several crucial parameters, i.e., mycelial age, pH of lysing enzyme, concentration, osmoticum variety and concentration, and protoplast viability, and optimized the parameters for maximum yield of viable protoplasts based on statistical analysis, Furthermore, we performed PCR-RFLP (restriction fragment length polymorphism) for rapid identification of the transformants. The phylogenetic relatedness assay indicated conservation of carboxin resistance of the *Sdh* gene across a wide range of Ascomycetes and Basidiomycetes, suggesting that the carboxin-transformation platform has great potential for application in a wide range of fungi, including *G. multipileum* for molecular breeding and other genetic studies.

Materials and methods

Strains and cultivation

Ganoderma multipileum strain BCRC 37180 was kept on YMSA (0.4 % yeast extract, 0.4 % peptone, 0.8 % malt extract, 1.2 % glucose, 1.2 % sucrose, 1.5 % agar) and subcultured once per month. Transformants were cultured by hyphal tip excision and subcultured three times to ensure monokaryotic identity.

Protoplasting

Fungal strain BCRC 37180 was grown on cellophane overlaid on YMSA for 5 days. Mycelia were scraped from 40 of 9-cm culture plates and suspended in 90 mL YMSB (0.4 % yeast extract, 0.4 % peptone, 0.8 % malt extract, 1.2 % glucose, 1.2 % sucrose) in a sterile steel cup. The mycelia were homogenized at low speed for 15 s. The resulting homogenate was mixed with 110 mL YMSB then poured onto 15-cm Petri dishes at 10 mL per plate. The suspensions were then cultured in the dark at 30 °C for 5 days. A predefined workable protocol for *G. multipileum* protoplasting is pH 5.8, 5-day-old mycelia, lysing enzyme 7.5 mg/mL, sucrose as osmoticum and shaking 3 h for 50 rpm, then 1 h for 100 rpm for digestion. In order to optimize conditions further, we tested and refined the variable parameters. We centrifuged the collected mycelia in sterile 50-mL falcon tubes and discarded the supernatant. On average, we obtained 80 g wet weight mycelia per batch. The mycelia were then transferred to a 125-mL flask containing lysing enzyme solution (40 mL 0.6 M sucrose in potassium phosphate buffer at pH 5.8, 900 mg lysing enzyme (L1412, Sigma-Aldrich, St. Louis, MO) filtered first through a 0.45 μm filter followed by a 0.22 μm filter). The mixture was incubated for 2 h in a 30 °C water bath in a horizontal reciprocal shaker, initially at 50 rpm followed by a further incubation for 1 h under the same conditions but at 100 rpm. The lysed mycelia were shaken to mix, then filtered through one layer of Kimwipe paper and one layer of cheesecloth. The filtrates were run through 25G2 glass sinter. The resulting protoplasts were centrifuged with a swing bucket centrifuge at 3000 rpm for 10 min, the supernatants centrifuged again and the collected protoplasts were pooled together. The protoplast suspensions were washed twice with 1 M STC buffer (1 M sorbitol, 20 mM $CaCl_2$, 10 mM Tris buffer, pH 7.5). The protoplasts were counted under a light microscope with a hemocytometer. Different parameters were tested, i.e., using 1 g mycelia subjected to lysing enzyme digest (Table 1), two layers of Kimwipe instead of 25G2 glass sinter for filtration.

Table 1 Factors affect *Ganoderma multipileum* protoplasting

Factor	Different parameters
pH of lysing enzyme[a]	5.0, 5.5, 5.8, 6.0
Lysing enzyme concentration (mg/mL)	5.0, 7.5, 10
Osmoticum	KCl, Mannitol, Sucrose, $MgSO_4 \cdot 7H_2O$
Enzyme digestion time (h)	2, 3, 4, 5
Mycelium age (days)	4, 5, 6

[a] pH of the lysing enzyme solution: pH 5 was adjusted with sodium acetate buffer; pH 5.5, 5.8 and 6.0 were prepared with potassium phosphate buffer

Regeneration

Approximately 10,000 protoplasts were first suspended in 3 mL RMB (0.4 % yeast extract, 0.4 % peptone, 0.8 % malt extract, 1.2 % glucose, 0.5 M sucrose), then RMA (RMB with 1 % agarose) preheated to 42 °C was added, mixed by shaking the plate gently, then allowed to solidify. The plates were incubated for 5 days at 30 °C in the dark.

Vector construction

The length of gDNA (3449 bp) of *Sdh* gene subunit B in *G. multipileum* was amplified using primers gm*Sdh*B-Sense-0503/gm*Sdh*B-AS-0503, then cloned into pBluescript KS+ (Agilent, Santa Clara, CA). A point mutation was subsequently introduced using PCR, switching histidine to leucine with one nucleotide change from CAC to CTC. This version also included a point mutation that shifted a non-cutter site into an *Age*I restriction site for further use in PCR-RFLP. We amplified short fragments containing the point mutation with primer gm*Sdh*B-Sense-1490-0503 and gm*Sdh*B-Sense-HIS mutation-0503, then digested the resulting fragment with *Blp*I in order to replace the original sequence with this modified version (Fig. 2a,b). The resulting pBS-Gm*Sdh*Bm plasmid was transformed into *Escherichia coli* strain Top10 to avoid recombination.

PEG-mediated transformation

An aliquot of protoplast suspension (80 μL) with $>10^7$ protoplasts was added to 20 μL 50 % PEG (polyethylene glycol MW 4000, Sigma), and mixed thoroughly before further addition of 5 μg linear plasmid DNA. The samples were spun briefly (5 s) at a few hundred rpm to mix the contents of the tube, then incubated on ice for 30 min, before adding 1 mL ice-cold 50 % PEG and incubating at 25 °C for 30 min then transferring to a Petri dish. RMB (3 mL) was added and mixed before adding 12 mL RMA (RMB containing 1 % agarose) and mixing thoroughly. After growth for 2–3 days, the plates were overlaid with AY agar medium (144 mM acetate–acetic acid buffer at pH 6.5 and 0.5 % yeast extract, 1 % agarose) containing 0.75 ppm carboxin to select transformants. Transformants were selected for 7–10 successive days.

PCR-RFLP analysis

Transformants grown on YMSA with 1 ppm carboxin grew rather more slowly than the wild type. After subculturing three times using the hyphal tip excision isolation technique, we cultured the transformants on YMSA without carboxin for 2 weeks. Mycelia were harvested and gDNA extracted using the CTAB method (Saghai-Maroof et al. 1984), and transformants' DNA was amplified using PCR with primers gm*Sdh*B-Sense 1490–0503 and gm*Sdh*B-AS-0503. The amplicons were examined using 1.5 % TAE gel electrophoresis. From among the multiple bands observed, the band with a size of around 1900 bp was excised, purified and submitted to a second round of PCR with the same primers. The amplified fragments were purified and digested with *Age*I. The mutated *Sdh* subunit B should display two bands, one around 600 bp and the other 1300 bp.

Phylogenetic analysis

A total of 70 species of Ascomycetes and Basidiomycetes, and their *Sdh* subunit B gene fragments, were retrieved from the NCBI GenBank database, compiled into fasta format using Bioedit, and analyzed by MEGA5.0 using neighbor-joining method with JTT model, bootstrap 500 times. To test the model, we used MEGA5.0 (Tamura et al. 2011) to search for the best model. The best model was WAG+G+I with G=0.56, I=0.14. The CIPRES science gateway was implemented to perform the RAxML (Stamatakis 2006) and MrBayes (Huelsenbeck and Ronquist 2001; Ronquist and Huelsenbeck 2003) analyses. The models were set to the best model and others remained default. The resulting trees were visualized with Figtree and saved in PDF format then further modified using Adobe illustrator CS3 and Photoshop (Adobe Systems, San Jose, CA).

Results

Protoplasting and regeneration

To establish and optimize a genetic transformation system for *G. multipileum*, we first tested protoplasting conditions for the best yield of viable protoplasts. Previous studies in

Table 2 *Ganoderma multipileum* protoplast yields under different protoplasting conditions

Factor[a]	Range	Yield of protoplasts ($\times 10^5$/mL)	Regeneration rate (%)	Viable protoplast ($\times 10^5$/mL)
pH of lysing enzyme solution[a]	5.0	0	0	0
	5.5	33.3	0.78	0.2597
	5.8	17	0.96	0.1632
	6.0	49.5	0.55	0.27225
Mycelia age (days)[b]	4	73.8	2.55	1.845
	5	17	0.96	0.1632
	6	115	0.31	0.3565
Lysing enzyme concentration (mg/mL)[c]	5.0	0.6	1.09	0.00654
	7.5	17	0.96	0.1632
	10	7.6	0.95	0.0722
[d]Osmoticum (OM)	KCl	1.3	0.13	0.00169
	Mannitol	0	0	0
	Sucrose	17	0.96	0.1632
	$MgSO_4$ $7H_2O$	6.5	3.29	0.21385
Enzyme digestion time (h)[e]	2	6.1	0.42	0.02562
	3	300	0.41	1.23
	4	17	0.96	0.1632
	5	3.6	1.34	0.04824
All best conditions test 1[f]		3	4.83	0.1449
All best conditions test 2[f]		3.5	3.53	0.12355
All best conditions Test 3[f]		2.5	4.32	0.108
All best conditions Test 4[f]		1	4.425	0.0425
OM test 1[g]	0.6 M $MgSO_4$ $7H_2O$	11.5	1.37	0.15755
OM test 2[g]	0.6 M $MgSO_4$ $7H_2O$	5.5	1.87	0.10285

[a] Other parameters: 5-day-old mycelia digested at 30 °C for 4 h in 0.6 M sucrose osmotic stabilizer with lysing enzyme 7.5 mg/mL

[b] Other parameters: mycelia digested with lysing enzyme 7.5 mg/mL pH 5.8 at 30 °C for 4 h containing 0.6 M sucrose osmotic stabilizer

[c] Other parameters: 5-day-old mycelia digested at 30 °C for 4 h in 0.6 M sucrose as osmotic stabilizer pH 5.8

[d] Other parameters: 5-day-old mycelia at 30 °C for 4 h, pH 5.8, lysing enzyme 7.5 mg/mL

[e] Other parameters: 5-day-old mycelia digested at 30 °C in 0.6 M sucrose osmotic stabilizer, pH 5.8, lysing enzyme 7.5 mg/mL

[f] Parameters: 4-day-old mycelia, digested at 30 °C for 3 h in 0.3 M $MgSO_4$ $7H_2O$ osmotic stabilizer, lysing enzyme 7.5 mg/mL in pH 6.0

[g] Parameters: 4-day-old mycelia digested at 30 °C for 3 h in 0.6 M $MgSO_4$ $7H_2O$ osmotic stabilizer, lysing enzyme 7.5 mg/mL in pH 6.0

Ganoderma lucidum utilized sucrose and mannitol as osmotica, so we tested mainly these osmotica.

Table 2 displays the calculated protoplast yields under the conditions listed in Table 1. Of the enzyme in osmotica adjusted to different pH, pH 6.0 yielded 4.95×10^6 protoplasts/mL. The best conditions were mycelia 6 days old; lysing enzyme concentration 7.5 mg/mL; sucrose as osmoticum and 3 h of enzyme digestion.

As we did not know whether the protoplasts yielded were viable or not, we calculated the regeneration rate as shown in Table 2. The best conditions for protoplast regeneration were pH 5.8; mycelia age 4 days; lysing enzyme 5.0 mg/mL; $MgSO_4$ $7H_2O$ as osmoticum and 5 h of enzyme digestion.

Concomitantly, we calculated viable protoplasts by calculating the protoplasts yields and regeneration rates listed in Table 2, i.e., viable protoplasts per reaction. From Table 2, the best conditions were pH 6.0; mycelia age 4 days; enzyme concentration 7.5 mg/mL; osmoticum $MgSO_4$ $7H_2O$ and 3 h of enzyme digestion.

Next, we tried all "best" conditions four times with two replicates each to determine the best molar concentration using 0.6 M $MgSO_4$ $7H_2O$ as osmoticum. The results showed that the regeneration rates were elevated; however, protoplast yields were low. The best conditions when combined together did not generate the best results in our surveys (Table 2).

The protoplasts yielded using optimal conditions are shown in Fig. 1a and protoplasts after different regeneration times on YMSA plates in Fig. 1b–e. Figure 1b,c are 16 h after regeneration and Fig. 1d,e 27 h after

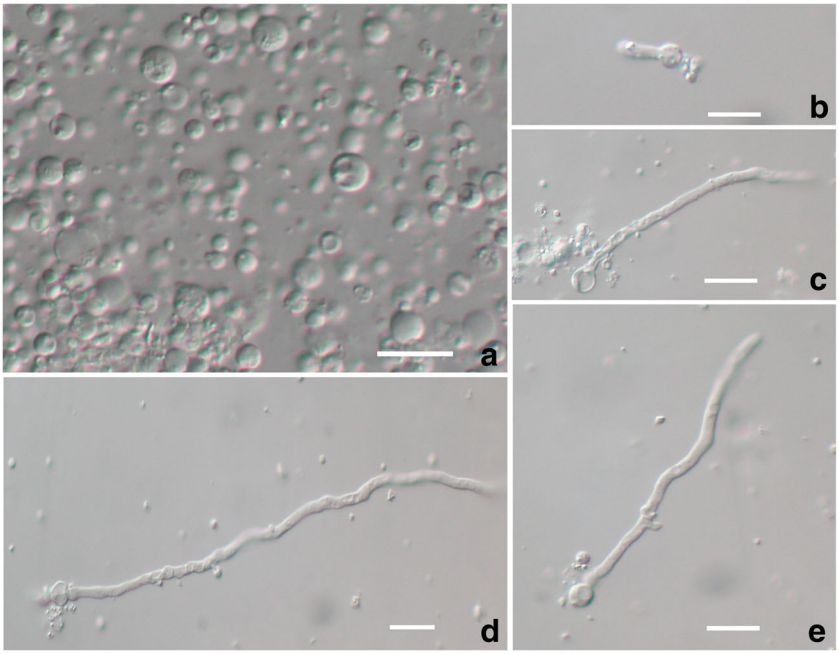


Fig. 1a–e Protoplasts of *Ganoderma multipileum* and regeneration on regeneration medium broth (RMB). **a** Light microscopy of generated protoplasts. **b**, **c** Regeneration of protoplasts forms short (**b**) or long (**c**) hyphae after incubation for 16 h under constant light illumination. **d**, **e** Except for small size or anucleated protoplasts, regenerated protoplasts grow and form longer and wider hyphae, after incubation for 27 h under constant illumination. *Bars* 5 μm

regeneration. The regeneration pattern was not uniform, some grew faster, others slower.

Transformation, PCR-RFLP

The constructed transformation vector is shown in Fig. 2a; the point mutations exchange histidine for leucine and create an *Age*I site. The gene structure defined using Augustus gene prediction software (http://bioinf.uni-greifswald.de/augustus/) is shown in Fig. 2b. The fragment cloned contained the 1000 bp promoter region and 1188 bp terminator region to ensure good expression. An alignment of the *Sdh* subunit B sequence surrounding the point mutated residue is shown in Fig. 3. The phylogenetic tree shown in Fig. 4, which includes sequences retrieved from NCBI GenBank using the neighbor-joining method, depicts the most rational relatedness among taxa. In transformation experiments, the initial transformation rate averaged 0.1 transformants/μg plasmid DNA. The transformants emerged after about 10 days of regeneration and selection. Many false-positive transformants also emerged later. Our tests showed that, after 10 days, a ratio of about two false positive transformants appeared for every three positive transformants. PCR amplification of the *Sdh* subunit B using gm*Sdh*B-Sense 1490–0503 and gm*Sdh*B-AS-0503 primers resulted in multiple bands, as depicted in Fig. 5a. After subjecting the ~1900 bp fragments of transformants, wild type and false positive transformants, to digestion with *Age*I restriction

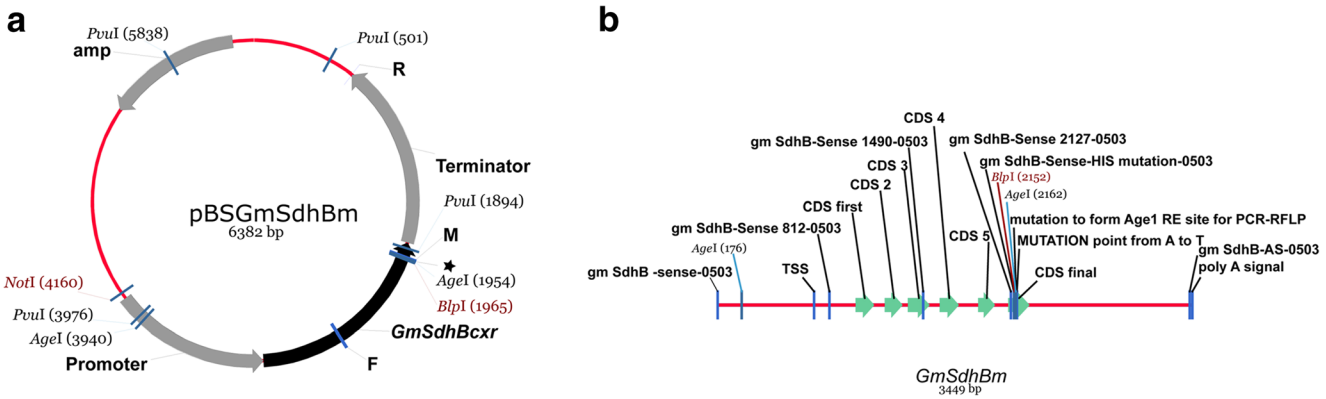
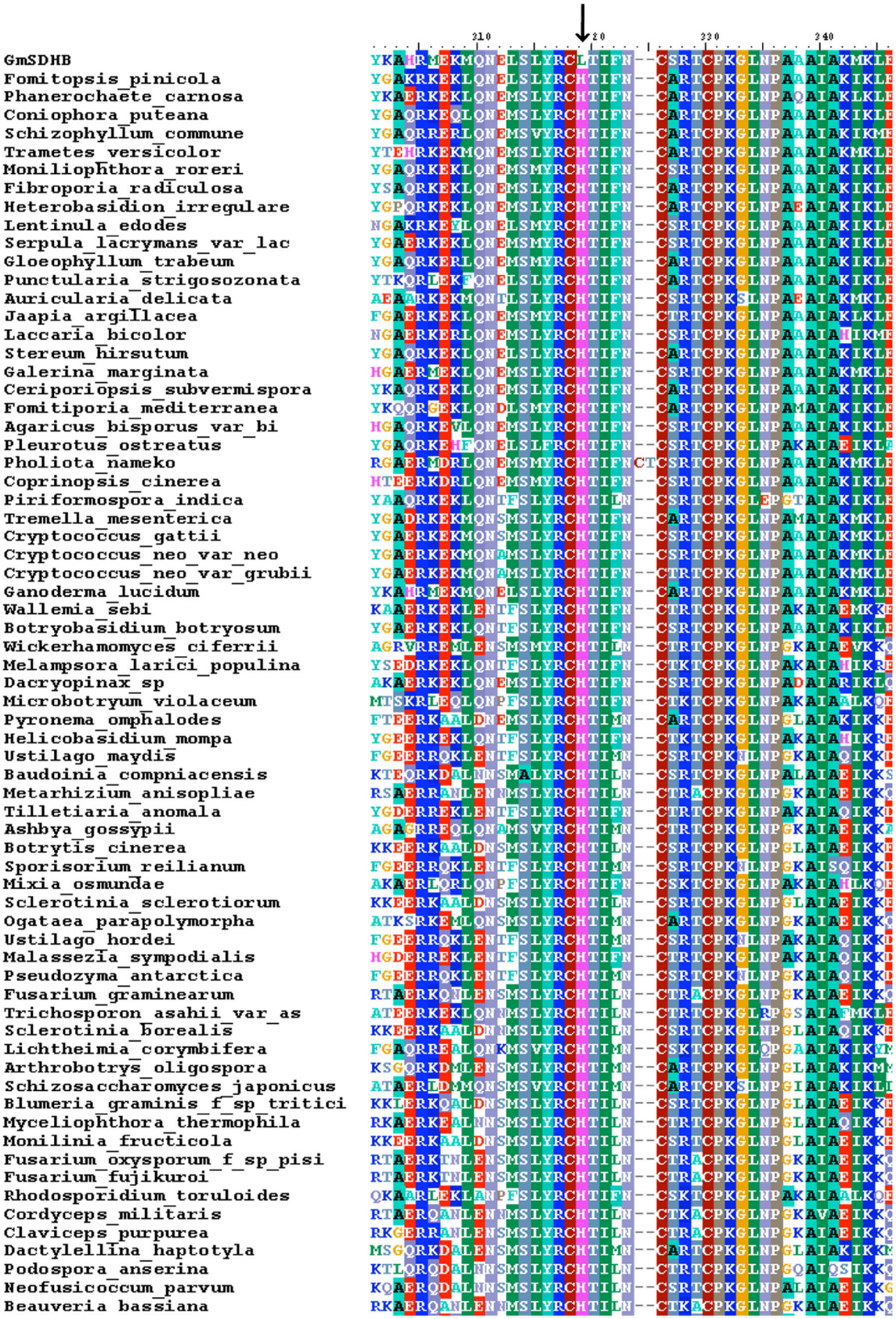


Fig. 2a,b Gm*Sdh*Bm gene structure and construction of the carboxin resistance vector. **a** pBSGm*Sdh*Bm vector. *Amp* Ampicillin resistance gene used for selection of plasmids in *Escherichia coli*; *M* point mutation changing a histidine to a leucine in the coding region; * point mutation to create a restriction site for PCR-RFLP; *F* and *R* represent forward and reverse primers used in PCR-RFLP. **b** Gm*Sdh*Bm gene structure predicted by Augustus gene prediction software (http://bioinf.uni-greifswald.de/augustus/). *Arrows* Exons


210
220
230
240
GmSDHB
Fomitopsis_pinicola
Phanerochaete_carnosa
Coniophora_puteana
Schizophyllum_commune
Trametes_versicolor
Moniliophthora_roreri
Fibroporia_radiculosa
Heterobasidion_irregulare
Lentinula_edodes
Serpula_lacrymans_var_lac
Gloeophyllum_trabeum
Punctularia_strigosozonata
Auricularia_delicata
Jaapia_argillacea
Laccaria_bicolor
Stereum_hirsutum
Galerina_marginata
Ceriporiopsis_subvermispora
Fomitiporia_mediterranea
Agaricus_bisporus_var_bi
Pleurotus_ostreatus
Pholiota_nameko
Coprinopsis_cinerea
Piriformospora_indica
Tremella_mesenterica
Cryptococcus_gattii
Cryptococcus_neo_var_neo
Cryptococcus_neo_var_grubii
Ganoderma_lucidum
Wallemia_sebi
Botryobasidium_botryosum
Wickerhamomyces_ciferrii
Melampsora_larici_populina
Dacryopinax_sp
Microbotryum_violaceum
Pyronema_omphalodes
Helicobasidium_mompa
Ustilago_maydis
Baudoinia_compniacensis
Metarhizium_anisopliae
Tilletiaria_anomala
Ashbya_gossypii
Botrytis_cinerea
Sporisorium_reilianum
Mixia_osmundae
Sclerotinia_sclerotiorum
Ogataea_parapolymorpha
Ustilago_hordei
Malassezia_sympodialis
Pseudozyma_antarctica
Fusarium_graminearum
Trichosporon_asahii_var_as
Sclerotinia_borealis
Lichtheimia_corymbifera
Arthrobotrys_oligospora
Schizosaccharomyces_japonicus
Blumeria_graminis_f_sp_tritici
Myceliophthora_thermophila
Monilinia_fructicola
Fusarium_oxysporum_f_sp_pisi
Fusarium_fujikuroi
Rhodosporidium_toruloides
Cordyceps_militaris
Claviceps_purpurea
Dactylellina_haptotyla
Podospora_anserina
Neofusicoccum_parvum
Beauveria_bassiana

◀ **Fig. 3** Alignment of amino acid residues of succinate dehydrogenase gene (*Sdh*) among Basidiomycetes and Ascomycetes retrieved from GenBank, NCBI. *Arrow* Conserved histidine residue sensitive to the antibiotic carboxin; in Gm*Sdh*B, a point mutation replaces this histidine with leucine to confer resistance to carboxin. This figure was produced by Bioedit (http://www.mbio.ncsu.edu/bioedit/bioedit.html)

enzyme, only the wild type showed a single copy of the *Sdh* subunit B gene. However, one to several copies of *Sdh* subunit B was present in the transformants due to ectopic insertions (Fig. 5b). A search of the whole deciphered genome database of *G. multipileum* BCRC 37180 further verified that only one copy of *Sdh* is present in the genome. Primers used for vector construction, point mutation and creation of restriction sites are listed in Table 3.

Discussion

To investigate the function of genes involved in host colonization capacity, mating and fruiting control mechanisms in the medicinal mushroom *G. multipileum*, establishing amenable transformation systems is essential. In recent advancements, either a dominant auxotrophic marker, i.e., acetamidase-encoding genes (Hynes et al. 1983), or genes resistant to selection antibiotics, fungicides or herbicides such as benomyl, bleomycin, phleomycin, hygromycin B, carboxin, bialaphos (Bar), have been applied successfully to *Saccharomyces cerevisiae* and many filamentous fungi in genetic manipulation studies (Orbach et al. 1986; Punt et al. 1987; Avalos et al. 1989; Austin et al. 1990; Drocourt et al. 1990; Kamper et al. 1995; Kwon-Chung et al. 1998). We previously attempted to

Fig. 4 Phylogenetic relatedness of *G. multipileum* with other fungi based on the succinate dehydrogenase gene using the neighbor-joining method with 500 bootstrap replicates. *Thick bars* Bootstrap supporting value over 70. Tree constructed by MEGA 5.0 with JTT model (http://www.megasoftware.net). *Scale bar* 10 changes

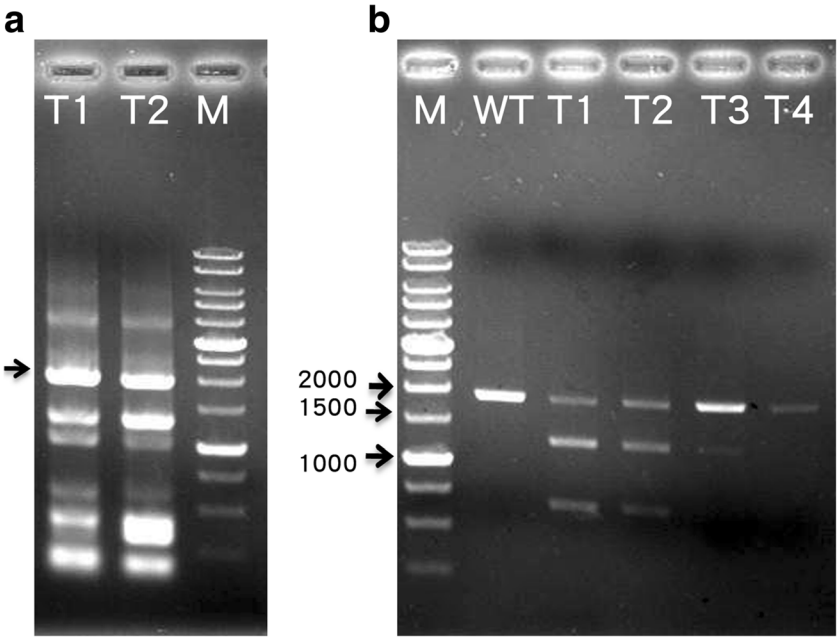


Fig. 5 **a**,**b** Electrophoresis of PCR-amplified *Sdh* subunit B gene fragments from *Sdh* gene point mutated transformants of *G. multipileum* BCRC 37180 without (**a**) or with (**b**) *Age*I restriction. **a** Multiple *Sdh* gene fragments amplified using gm*Sdh*B-Sense 1490–0503 and gm*Sdh*B-AS-0503 primers. The anticipated *Sdh* gene fragment length of *Sdh* subunit B gene is 1900 bp (*arrow*). **b** The 1900 bp amplicon restriction fragment length polymorphism (RFLP) of wild type (WT) and transformants (T1, T2, T3, T4) restricted by *Age*I. Bands at 600 bp and 1300 bp are *Age*I restriction products of transformed carboxin resistance cassette (*arrowheads*). T4 is a false positive transformant. *M* 1 kb marker, length size at 2000, 1500 and 1000 bp denoted (*arrows*)

transform *G. multipileum* with the glufosinate ammonium resistant gene; however, surprisingly, this herbicide can be utilized as a substrate for growth, which was proportional to the concentration over 1000 ppm compared with wild type. Also, no inhibition was shown toward a wide range of antibiotics, such as Geneticin (G418), Kanamycin, Neomycin, Phleomycin and the fungicide Benomyl (data not shown). Generally, *G. multipileum* exhibited high resistance to hygromycin, even at 400 ppm, and was recalcitrant to transformation; only false positive transformants were selected by the hygromycin shuttle vector constructed previously. Several reasons have been suggested to account for this deficit. For example, in *Schizophyllum commune*, an AT-rich region may correlate with the failure to obtain hygromycin resistance gene (*hph*) transformants (Scholtmeijer et al. 2001). In the case of *Coprinopsis cinerea*, two amino acids residues in the N terminal region may be involved in the repression of *hph* gene expression (Kilaru et al. 2009).

In this study, we used carboxin as an alternative selection marker. We circumvented the difficulties exerted by hygromycin, and succeeded in obtaining positive transformants. Using the succinate dehydrogenase gene (*Sdh*) to construct the point mutated transformation cassette had several advantages. For instance, point mutated *Sdh* was analogous to the native endogenous *Sdh*, thus transformants do not cause environmental pollution concerns, and there is no need to get rid of the marker. In addition, the wild type *G. multipileum* was highly sensitive to carboxin, at concentrations even as low as 0.75 ppm, thus leading to effective selection. Besides, carboxin costs much less than hygromycin.

Table 3 Primers used in this *G. multipileum* study

Primer name	Primer sequence 5′→3′	Purposes of primers
gm*Sdh*B-Sense-0503	ACCTCCATTACCCAACGGAAACCAC	Construct
gm*Sdh*B-Sense 812–0503	CTGGTCTGGTGAGGCTTGGATGAGG	Sequencing
gm*Sdh*B-Sense 1490–0503	TTGACAGGAACGCTGGCAAGGAT	PCR-RFLP
gm*Sdh*B-Sense 2127–0503	CGGATGGAGAAGATGCAGAATGAGC	Sequencing
gm*Sdh*B-AS-0503	TTTATTGCACGTCGCCGGTGAGAAC	Construct, PCR-RFLP
gm*Sdh*B-Sense-HIS mutation-0503	GAGCTCAGCCTGTACCGGTGCCTCAC	Point mutation switch His to Leu and creation of an *Age*I restriction site

To overcome the low transformation rate, we varied several key factors systematically, i.e., pH, concentration and digestion time of lysing enzyme solution, mycelia age and osmoticum variety, all of which may result in lower efficiency. Eventually we optimized the conditions and came up with the following formulation: pH 6.0, 7.5 mg/mL lysing enzyme, 3 h of digestion time, 4-day-old mycelia, 0.6 M sucrose as osmoticum. These conditions were optimal for protoplast yield, viability and regeneration, in turn improving the transformation rate to five transformants/μg DNA.

Comprehensive analysis of the sequence of the *Sdh* gene among an array of Basidiomycetes and Ascomycetes retrieved from GenBank, NCBI, respresenting 70 taxa, revealed a conserved histidine amino acid residue. Rationally, a point mutation switching histidine to leucine will confer resistance to carboxin selection on the mutant (Fig. 3). Consequently, the carboxin selection marker can be applied to an immense number of fungi, including yeasts or filamentous forms as shown by the phylogenetic analysis (Fig. 4). Obviously, these results support many previous reports (Keon et al. 1991; Honda et al. 2000; Kilaru et al. 2009; Ngari et al. 2009; Shima et al. 2009).

In conclusion, the carboxin transformation system has been applied successfully to the transformation of *G. multipileum* regarding a homeodomain protein and pheromone receptor in combination with dual mating of semicompatible monokaryotic strains (data not shown). However, the transformation efficiency by no means is high or efficient. In future perspectives, protoplasting, intact propagules lithium acetate treatment, electroporation, biolistic transformation, CRISPR/*CAS9* gene knockout and choice of selectable markers should be tested and refined (Ruiz-Diez 2002; Lin et al. 2011; Shen et al. 2014) to facilitate the molecular study of *G. multipileum*, especially in monokaryotic fruiting, define gene function in fruiting and molecular farming of active metabolites.

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