Interspecific Hybridization of *Ganoderma lucidum* and *G. tsugae* by Protoplast Fusion

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Abstract: Two strains of *Ganoderma lucidum* and one cultivar strain of *G. tsugae* were used as parents for interspecific hybridization. The 2-day old mycelia were protoplasted using 2 % Novozyme in 0.6 M mannitol to yield 2 x 10^5 protoplasts g^{-1} mycelium. Fusion of protoplasts was chemically induced by 30% polyethylene glycol. Then the protoplast mixture was plated onto regeneration medium with 0.6 M mannitol. Screening was carried out with the isolated protoplast regenerants by observing their growth rates at 18 and 37°C. The selected fast-growing hybrids showed unique DNA fingerprints by arbitrarily primed polymerase chain reaction. Also, they were somatically incompatible with the parents. Three hybrids were found fertile producing basidiomes and abundant basidiospores. From the sequences of nuclear interspacers 1 and 2 and mitochondrial small ribosomal DNA, the 3 hybrids were genetically more similar with *G. lucidum*. Thus protoplast fusion can be applied in strain improvement of Lingzhi.

Key words: *Ganoderma,* Lingzhi, protoplasts, hybrid, DNA fingerprints, ribosomal DNA

1 Introduction

Laccate *Ganoderma* spp., commonly called lingzhi, are supreme traditional Chinese medicine now gaining worldwide popularity. Lingzhi refers to a group of fungi including *G. lucidum, G. tsugae* and *G. sinense*.[1-4] These species are sexually incompatible and show differences in nucleotide sequences of nuclear ribosomal DNA and mitochondrial ribosomal DNA genes and other molecular properties[5-9] *G. lucidum* grows at a wider range of temperatures than *G. tsugae*[1] (Figure 1). These medicinal fungi are found to show different medicinal efficacies, and their bioactive components include immunomodulatory protein LZ-8, polysaccharides and terpenes.[4, 10-14] In nature these fungi occupy different biogeographies and/or defend their own territories.[2, 3, 7, 9] The present study expands the growth temperature of a lingzhi cultivar by artificially introducing the wild germplasm of another lingzhi species by protoplast fusion which brings two genomes in a common cytoplasm.[15-18] Hybrids expanding the high and low temperature tolerance will significantly reduce the investment and running cost (by energy saving) of mushroom production. This protoplast fusion biotechnology is advantageous for introducing one or more polygenic trait(s) and traits with unknown genetic mechanisms, and/or to cross the reproduction barrier of two biological species. Although application of protoplast fusion in edible and medicinal mushrooms for artificial breeding has been carried out in laboratory studies, real application has not been reported. This may reflect the problem of using laboratory isolates, e.g. auxotrophic mutants, in experimental design to render commercial exploitation, and/or the decreased quality of the artificial hybrid, e.g. sterility, poor growth and/or fruiting, or the failure in generating hybrids.[15, 16, 18, 19] In particular, no one has attempted to do protoplast fusion of *G. lucidum* and *G. tsugae*, both of which are lingzhi. This study tested the validity of the biotechnological breeding scheme with two different strains of *G. lucidum* to fuse with *G. tsugae.*

2 Materials and Methods

2.1 Artificial hybridisation of *G. tsugae* and *G. lucidium* by protoplast fusion

A cultivar *G. tsugae* Murr. (P2) provided by Peninsular Innovations Limited and two field isolates of *G. lucidum* (W. Curt.: Fr.) P. Karst. (P1 and P3) were used.[7, 9] Their identities were confirmed by sequences of nuclear ribosomal DNA repeat (ITS1, 5.8S and ITS2) and mitochon drial small subunit of ribosomal DNA (Mt-SSU-rDNA).[7, 9] Figure 2 shows the breeding strategy. Both parents in the form of vegetative mycelia were cultivated to a log phase by incubating in a complete medium (CM) broth consisting of (g L^{-1}): $MgSO_4 \cdot 7H_2O$, 0.5; KH_2PO_4, 0.46; K_2HPO_4, 1; peptone, 2; D-glucose, 20; at 28°C in darkness at 120 rpm. Using aseptic techniques, the actively growing mycelia were harvested by filtration through a nickel sieve of 1 mm in diam and rinsed with an osmotic buffer (0.6 M sucrose solution).[7, 16] Then the mycelium was incubated in an osmotic buffer containing 10 mg mL^{-1} Novozyme (Sigma, v2010) in 0.6 M mannitol (osmotic stabilizer) at 10 rpm for 1-3 h to enzymatically remove the fungal cell wall.[7] Yield of protoplasts was quantified using a hemacytometer under phase contrast light microscopy. When sufficient protoplasts were released, protoplasts were harvested by filtrating through a column of glass wool packed in a syringe to hold the undigested mycelia and concentrated by centrifugation at 2,000 x g for 10 min.[7, 16] Protoplasts in the supernatant were harvested and separated from the cell debris. By centrifugation at 6000 x g for 15 min, the pellet of concentrated protoplasts was harvested. The two batches of protoplasts of *G. tsugae* and *G. lucidum* were resuspended in osmotic buffer and mixed together in 30% (w/v) polyethylene glycol (MW 3350, Sigma-Aldrich, 95904) in 0.01 M $CaCl_2$ for 10 min at 28°C in darkness.[16] Then the protoplast mixture was plated onto regeneration medium consisting of (g L^{-1}): D-glucose, 4; yeast extract, 4; malt extract, 10; and agar, 15 in 0.6 M mannitol for regeneration of cell wall.[7, 15, 16] After incubation at 28°C in darkness for 3-5 days, 100 visible colonies were picked and transferred to CM as purified putative isolates. Screening and selection for the desirable hybrids was done by incubating the putative hybrids at two growth temperatures (18 and 37°C) on CM for 3 day using 24 well plates. The two parents were examined in parallel. Five replicates were done for each isolate. The isolates showing faster growth rates were selected.

The fast growing putative hybrids were tested for their fruiting abilities. Fruiting tests were carried out in the mushroom cultivation complex, The Chinese University of Hong Kong (CUHK) for experimental production (30 bags per isolate). The fruiting tests were repeated for three times. Fruiting trial was carried out using autoclavable plastic bags each of which contained 500 g of a formulated fruiting substrate, consisting of (%, w/w): sawdust, 60; milled coconut fibre, 20; wheat bran, 14; sucrose, 5; (agricultural grade) and lime, 1 (modified from[20, 21]). Mycelial running was carried out at 28°C in darkness. When the compost was completely colonized, the colonized compost was transferred to a fruiting environmental chamber in the mushroom cultivation complex, CUHK. Basidiomes were induced by a photoperiod of 12 h light 12 h dark, incubation at 28°C and relative humidity of 85% or above. The putative hybrids were then paired with the parents in CM plate in somatic incompatibility test. After 1 to 2 weeks at 25°C in darkness, the plate was checked whether the two isolates' colonies formed a barrier in between.

2.2 Molecular characterization of the hybrids

Genomic DNA was extracted from harvested mycelium from broth cultures, frozen in liquid nitrogen and purified.[7, 9, 22, 23] The concentration and purity of a DNA sample were measured by the spectrophotometric absorbance and the ratio of OD_{260}:OD_{280}. A sample having a ratio greater than 1.8 was considered suitable for use, and its concentration and purity were further checked by agarose gel electrophoresis using ethidium bromide staining. Arbitrarily-primed polymerase chain reaction was used for putatively identifying the hybrid.[22, 23] The arbitrary primers used in this study are as follows: *Eco*RI ext, TAGGCGTATCACGAGGCCCT; IS5, 5'-

AAGTAAAAGTCGTAACAA-3'; and TGF, 5'-AGGCTCCGATAG-3'.[22-25] A 10 µl reaction mixture contained 1X Reaction Buffer, 3.5 mM $MgCl_2$, 0.2 mM dNTPs, 16 pM primers, 1.5 U Taq DNA polymerase (Thermoprime Plus), about 300 ng DNA and ultra pure water.[22, 23, 24, 25] The thermal program was: 2 low-stringency cycles of 94°C for 2 min, 35°C for 1 min and 72°C for 2 min, followed by 35 high-stringency cycles of 94°C for 2 min, 55°C for 1 min and 72°C for 2 min with the last extension time lengthened to 10 min by a thermal cycler PTC-100™ (MJ Research, Inc.). DNA fingerprints were resolved by agarose gel electrophoresis, and images were captured using a gel documentation system (BIO-RAD Gel Doc 1000).

The fungal-specific primers its1 and its2 were used to amplify the region (ITS1, 5.8S and ITS2) of the nuclear ribosomal DNA, and another pair of primers (Ms1 and Ms2) were used to amplify the mitochondrial ribosomal DNA.[7, 9, 26] The nuclear primer sequences were as follows: 5'-TCCTCCGCTTATTGATATGC-3' (its1) and 5'-GGAAGTAAAAGTCGTAACAAGG-3' (its2), respectively. The mitochondrial primer sequences were as follows: 5'CAGCAGTCAAGAATATTAGTCAATG-3' (Ms1) and 5'-GCGGATTATCGAATTAAATAAC-3' (Ms2). A total volume of 10 µL of reaction mixture consisted: 1X Reaction Buffer VI, 2.5 mM $MgCl_2$, 10 mM dNTPs, 10 pM of each primers, 2U Taq DNA polymerase and about 100 ng genomic DNA. Specific PCR programme comprised of: 95°C for 1 min; 60°C for 1 min and 70°C for 1 min for 39 cycles with the last extension time lengthened to 10 min.[7, 9, 23] PCR-amplified fragments were further purified by using GENECLEAN® II KIT. Three volumes of sodium iodide stock solution were added to PCR mixture before adding 1 µL GLASSMILK® purification kit (Biogene 101). The purified DNA was collected for cycling sequencing using DNA sequencing kit (PE Applied Biosystems). A mixture consisting of purified DNA template, 2 µL; forward primer of the target gene, 2 µL; dRhodamine Terminator, 4 µL (PE Applied Biosystems) and ultrapure water to a final volume of 10µL. The following thermal profile was used: 36 cycles of DNA denaturation at 95°C for 30 s, annealing at 50°C for 30 s and extension at 70°C for 1 min.

The products were then purified by ethanol precipitation with 70% ethanol. The pellet was then dried under vacuum. Twelve µL Template Suppresser Reagent (PE Biosystems) were added to resuspend the dried pellet, and the sample was recorded by ABI Prism 310 Genetic Analyser (PE Biosystems). The obtained sequence was processed by software Sequencing Analysis version 3.0 (PE Biosystems). Phylogenetic trees were constructed by UPGMA (unweighted pair-group method using arithmetic averages) provided by PAUP (Phylogenetic Analysis Using Parsimony, Version 4.0 beta8 [27]).

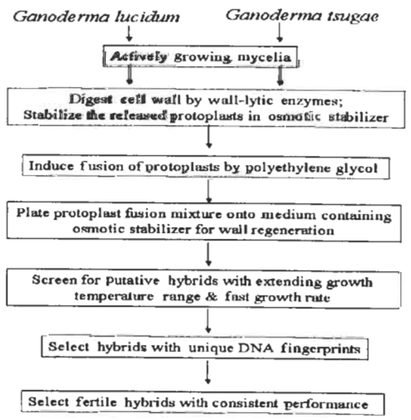

Figure 1. A schematic diagram showing the breeding program to improve cultivar *G. tsugae* by introducing the wild germplasm of *G. lucidum* by protoplast fusion

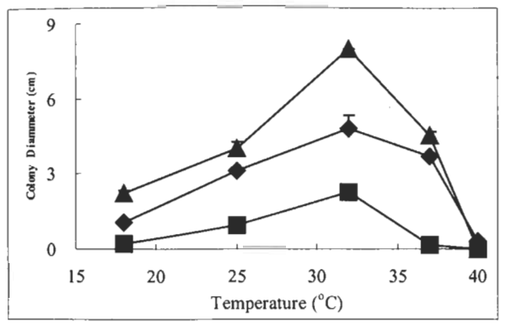

Figure 2. The growth rates and growth temperature ranges of parents *G. tsugae* (P2) and *G. lucidum* (P1 and P3) for protoplast fusion

Symbols: ◆, *G. lucidum* (P1); ■, *G. tsugae* (P2); ▲, *G. lucidum* (P3).

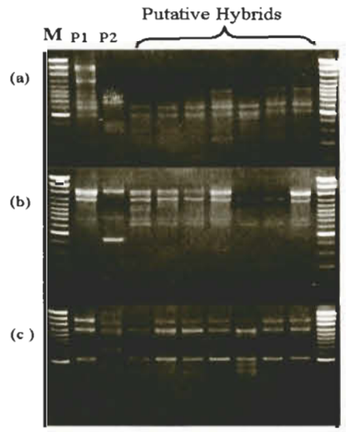

Figure 3. DNA fingerprints of the putative hybrids and parents *G. lucidum* (P1) and *G. tsugae* (P2) by arbitrarily primed polymerase chain reaction

Arbitrary primers: (a) *Eco*RI ext, (b) IS5 and (c) TGF. Lane M, GeneRuler™ 100 bp DNA Ladder Plus, ready-to-use (MBI Fermentas).

3 Results

Figure 1 shows the breeding scheme used in interspecific hybridisation of *G. lucidum* and *G. tsugae* by protoplast fusion and the screening and selection strategy used.

Protoplasts were released from *G. lucidum* (P1, P3) and *G. tsugae* (P2) at yields of 178 - 228 x 10^3 protoplasts g^{-1}. The protoplast regeneration efficiency was 4.0 ± 0.2 %. Both large and small regenerated mycelial colonies were observed. Putative hybrids of fast growth rates at both 18 and 37°C were screened out and selected. Cultivar *G. tsugae* (P2) did not show obvious visible growth at 18 and 37°C (Figure 2). Identity of the hybrids was confirmed by examining the DNA fingerprints and comparing to those of the parents P1 and P2 (Figure 3). Similar results were obtained with the pair of parents P2 and P3 and their hybrids. The DNA fingerprints of the hybrid were not synergistic from those of the parents suggesting the occurrence of somatic recombination in the formation of the hybrid.

The hybrids were dikaryons (diploid-equivalent condition in lingzhi), not haploids generated by protoplasting process, and bore clamp connections. When such a hybrid was paired with its parents, somatic incompatibility

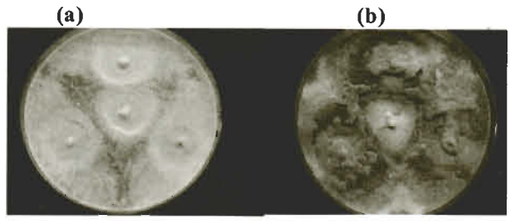

Figure 4. Somatic incompatibility test among 3 hybrids (peripheral colonies) with the parent (a) *G. tsugae* (P2) or (b) *G. lucidum* (P1) (central colony)

in terms of colony delimitation or barrier reaction was observed (Figure 4). All 3 hybrids selected from two pairs of parents (P1 and P2; P2 and P3) were fertile producing basidiomes and basidiospores.

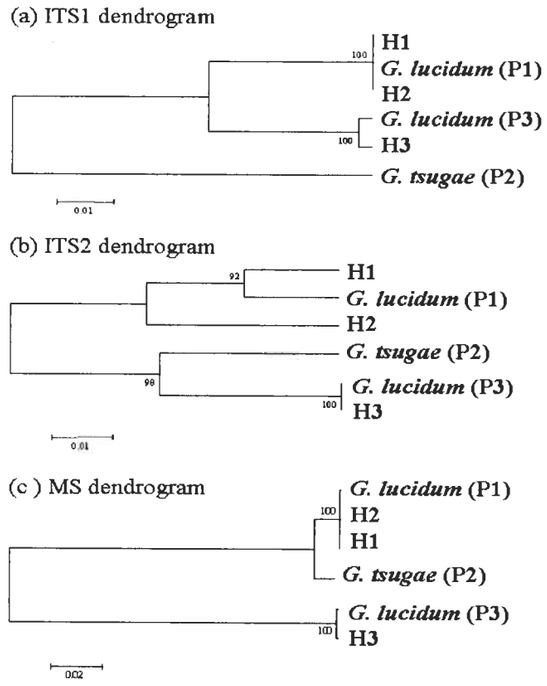

Figure 5. UPGMA dendrograms of parents *G. lucidum* and *G. tsugae* and their 3 hybrids based on the sequences of (a) nuclear ribosomal spacer ITS1, (b) nuclear ribosomal spacer ITS2 and (c) mitochondrial ribosomal DNA (mt-SSU rDNA) region
G. lucidum (P1) was protoplast fused with *G. tsugae* (P2) to generate hybrids H1 and H2.
G. lucidum (P3) was protoplast fused with *G. tsugae* (P2) to generate hybrid H3.

The nuclear and mitochondrial ribosomal DNA sequences of 3 hybrids of two pairs of parents for protoplast fusion were aligned (Figure 5). All the hybrids showed higher similarity to the *G. lucidum* parent (P1 or P3) than *G. tsugae* parent (P2). Hybrids H1 and H2 differed from *G. lucidum* parent (P1) in ITS2 sequences, and H3 differed from *G. lucidum* parent (P3) in ITS1 and MS sequences besides their unique DNA fingerprints.

4 Discussion

There are two reports on interspecific hybridisation using protoplast fusion involving *G. lucidum*.

They were on *G. lucidum* and *Pleurotus ostreatus* and *G. lucidum* and *Grifola umbelleta*.[18, 19] In both cases, no fertile basidiomes were produced in the claimed hybrids. Only the hybrid of *P. florida* and *G. lucidum* was detected by random amplified polymorphic DNA markers.[18] This study provides a method for producing fertile lingzhi hybrids of two *Ganoderma* species discriminated by somatic incompatibility, growth rate, growth temperature range, nuclear and mitochondrial ribosomal DNA sequences besides the basidiome morphology. This study provides a solution to produce stable fertile mushroom hybrid, which is derived from more than one species. In mushroom breeding, multiple spores may be mixed to give production spawn (seed culture for mushroom production). Mushroom farmers who do not have facility and technique of keeping cultures practise this 'multispore' spawn. They collect the mushrooms as sources of the 'multispores' for inoculation. However, this limits to intraspecific hybridization. When *G. tsugae* was confronted with *G. lucidum* in agar media, somatic incompatibility occurred by forming barrier between the two mycelial colonies. Thus mix spawn/multiple individuals could not be used in lingzhi production, as two or more fungal individuals would 'waste' the energy and resources in somatic incompatibility to define their territories, and this accounts for the observation of decreasing yield in later flushes in outdoor cultivation of mushroom *Lentinula edodes*.[28] In cultivation, 'multispore' spawn of heterogeneous genetic make-up does not provide a consistent performance in production. A study of the phylogenetic relationship among the genus *Ganoderma* subgenus *Ganoderma* is important, and the success of protoplast fusion may be correlated with the evolutionary relationship among these species. In this study, the genetically close relationship of the 3 hybrids to their corresponding parent *G. lucidum* may actually be a result of the selection criteria for expanding the growth temperature ranges and fast growth rates. A diversity of putative hybrids was generated during protoplast fusion, and several subcultures were needed to 'stabilize' the hybrids selected. This study shows a scheme which can be modified with the desirable traits as the selection criteria in screening and selection of the hybrids. Protoplast fusion provides a resource for selection.

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