Genetic engineering of *Ganoderma lucidum* for the efficient production of ganoderic acids

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*Ganoderma lucidum* is a well-known traditional medicinal mushroom that produces ganoderic acids with numerous interesting bioactivities. Genetic engineering is an efficient approach to improve ganoderic acid biosynthesis. However, reliable genetic transformation methods and appropriate genetic manipulation strategies remain underdeveloped and thus should be enhanced. We previously established a homologous genetic transformation method for *G. lucidum*; we also applied the established method to perform the deregulated overexpression of a homologous 3-hydroxy-3-methyl-glutaryl coenzyme A reductase gene in *G. lucidum*. Engineered strains accumulated more ganoderic acids than wild-type strains. In this report, the genetic transformation systems of *G. lucidum* are described; current trends are also presented to improve ganoderic acid production through the genetic manipulation of *G. lucidum*.

**Ganoderma lucidum and Ganoderic Acids**

Medicinal mushrooms have been used to treat various diseases especially in Asia. *Ganoderma lucidum* is a medicinal mushrooms. This species is known as Ling-zhi in China, Yeongji in Korea and Reishi in Japan. *G. lucidum* is widely used in traditional Chinese medicine and food products to promote longevity and increase vitality.1 *G. lucidum* contains ganoderic acids, which are highly oxygenated C30 lanostane-type triterpenoids; these substances play a major role in the biological effects of this species. Ganoderic acids also possess antitumor, anti-HIV, anti-metastasis, antihistamine, anti-hypertensive, antihepatotoxic, hypocholesterolemic, anti-inflammatory, anti-oxidative, and anti-aging activities.2

Ganoderic acids in *G. lucidum* are biosynthesized from acetyl coenzyme A, and this mechanism is termed as mevalonate pathway. Laonsterol is the common cyclic intermediate of triterpene, and this intermediate is biosynthesized by lanosterol synthase in *G. lucidum*. The biosynthetic pathway upstream of the lanosterol formation includes 11 enzymes in *G. lucidum*.3 However, the final steps following cyclization, including a series of oxidation, reduction, and acylation reactions, remain unclear.2-4

**Genetic Transformation Systems for *G. lucidum***

A suitable selection marker gene is prerequisite for the development of a stable genetic transformation system. Resistance marker genes in *G. lucidum* include hygromycin B phosphotransferase gene (*hph*), geneticin resistance gene (*kan*), phosphinothricin resistance gene (*bar*), and carboxin resistance gene (*cbxR*). *Hph, kan* and *bar* are derived from *Actinomycetes* or from bacteria. Although the genetic transformation of *G. lucidum* through these heterologous resistance genes as selection marker has been reported,5-7 these transformation events are inefficient or difficult to reproduce in other laboratories. We initially attempted to transform the protoplasts of *G. lucidum* using *hph* and *bar* as resistance gene but failed to obtain stable transformants. The poor
expression of selection marker genes, instability and higher false positive ratio are possibly accounted for the difficult in transformation with heterologous resistance genes. We previously developed a selection marker gene cbsK on the basis of a host-derived gene rather than heterologous genes; we then used cbsK to establish a stable genetic transformation system for *G. lucidum*. Molecular hybridization analysis revealed that cbsK is stably integrated in the genome of the transformants. Moreover, the established homologous transformation system has been successfully used to overexpress 3-hydroxy-3-methylglutaryl coenzyme A reductase gene, squalene synthase gene, α-phosphoglucomutase gene, and UDP glucose pyrophosphorylase gene in *G. lucidum*. Our results showed that *G. lucidum* protoplasts were more efficiently transformed to confer carboxin resistance than hygromycin and phosphinothricin resistance; this finding indicates that homologous marker genes may be more efficiently used to establish genetic transformation systems than other genes. *G. lucidum* is also more sensitive to carboxin than to the 3 other antibiotics. Wild-type strains are unable to grow in the presence of 2 mg/L carboxin. *G. lucidum* also fails to grow in the presence of 250, 200, and 100 mg/L hygromycin, phosphinothricin, and gentamicin, respectively. Hence, carboxin used to select *G. lucidum* transformants is more cost effective than other antibiotics.

Reliable genetic transformation methods should be established for the genetic engineering of *G. lucidum*. For instances, Agrobacterium tumefaciens-mediated transformation (ATMT), polyethylene glycol-mediated transformation (PMT), restriction enzyme-mediated DNA integration (REMI), and electroporation transformation have been developed for the genetic engineering of *G. lucidum*. The transformation efficiency through ATMT is 10–15 transformants per 10^7 protoplasts of *G. lucidum*, and this finding is comparable to that through REMI, PMT and electroporation methods, which yield a transformation efficiency of 4–20 transformants per μg DNA per 10^7 protoplasts. Mitotic stability analysis revealed that the introduced DNA is stably integrated in the genome of transformants obtained through ATMT and PMT methods. The reproducibility of the ATMT and PMT methods have also been confirmed through the overexpression of different *G. lucidum* homologous genes. PMT, REMI and electroporation methods usually result in the ectopic integration of DNA into the genome of *G. lucidum* with one or more copy numbers. By contrast, single-copy integration events are mainly detected in *A. tumefaciens*-mediated transformants. Therefore, ATMT may be applicable to perform target gene disruption in *G. lucidum*. PMT, REMI and electroporation are the optimum choice when multiple copies of target genes are expected to integrate in the genome. However, the genetic transformation methods of *G. lucidum* remain underdeveloped and inefficient compared with those available for bacteria and yeast. The transformation efficiency and transformant stability should be improved.

The promoters used to facilitate the expression of target genes in *G. lucidum* include glyceraldehyde-3-phosphate dehydrogenase (GPD) gene promoter from *Lentinula edodes*, GPD gene promoter from *L. edodes*, and cauliflower mosaic virus 35S promoter. Homologous promoters are widely applied to genetically manipulate *G. lucidum* because these promoters are more efficient than heterologous promoters. Although these constitutive promoters provide simplicity and relatively constant expression level of target genes, these promoters are unsuitable for the expression of deleterious gene products and metabolite production, in a specific stage, as well as under unique conditions. Controllable promoters should be developed to facilitate the conditional expression of target genes in *G. lucidum*. However, the expression of heterologous genes in *G. lucidum* is prevented by several factors. Shi et al. observed a weak fluorescence signal when the enhanced green fluorescent protein (EGFP) is transformed into *G. lucidum*, this finding may be attributed to the low translational efficiency of EGFP. We also found that the expression of hph and bar is not as efficient as that of the homologous cbsK. Similar observations have been detected in other basidiomycetes; in particular, methylation and AT-rich sequences hamper the expression of the bacterial hph and kan. To efficiently express heterologous genes in *G. lucidum*, codon optimization should be applied in future studies. This process has resulted in an increased steady-state mRNA level and an improved translational efficiency of heterologous genes in filamentous fungi.

### Overproduction of Ganoderic Acids by Genetically Modified *G. lucidum*

Our recent work showed that the overexpression of the ganoderic acid biosynthetic gene 3-hydroxy-3-methylglutaryl coenzyme A reductase gene increases the production of total crude ganoderic acid. However, the production of individual ganoderic acids is not increased in the transgenic *G. lucidum*. The result indicates the presence of downstream flux control points in the ganoderic acid biosynthetic pathway. When the squalene synthase gene is overexpressed, the accumulations of total and individual ganoderic acids is enhanced; this finding indicates that the squalene synthase is another key enzyme in ganoderic acid biosynthesis in *G. lucidum*. Our previous work revealed more than one disadvantages in the ganoderic acid biosynthetic pathway. Therefore, the overexpression of a single pathway gene may be insufficient to maximize ganoderic acid accumulation in *G. lucidum*. Csernetic et al. reported that the amount of carotenoid produced by *Mucor circinelloides* transformed with 3 isoprenoid biosynthetic genes is greater than that of carotenoid produced by strains bearing only one isoprenoid biosynthetic gene. In the production of penicillin by *Aspergillus nidulans*, 3 biosynthetic genes are simultaneously expressed; and a greater amount of penicillin accumulates in *A. nidulans* with the 3 biosynthetic genes than in the strains overexpressing only one biosynthetic gene. Thus, the co-expression of numerous biosynthetic genes in *G. lucidum* can enhance the production of ganoderic acids.

The genetic manipulation of transcription factors is an alternative approach to...
increase the production of secondary metabolites efficiently. In contrast to genetic engineering approaches that target a single gene, transcription factor approaches usually affect numerous genes involved in the biosynthetic pathway, as a result, the integrated upregulation or downregulation of the related biosynthetic pathway occurs.\textsuperscript{22,23} Transcription factors have been manipulated to improve the production of desired products in various organisms. Liu et al.\textsuperscript{24} reported that the ectopic expression of a BRZ1-1D transcription factor in tomato enhances carotenoid accumulation. In \textit{E. coli}, the overexpression of the regulatory transcription factor FadR enhances the accumulation of fatty acid.\textsuperscript{22} Davuluri et al.\textsuperscript{25} reported that the RNAi-mediated suppression of an endogenous photomorphogenesis regulatory gene named DET1 enhances flavonoid content in tomatoes. The genome and transcriptome of \textit{G. lucidum} have been sequenced.\textsuperscript{4,26} Yu et al.\textsuperscript{27} performed the proteomic and biochemical analyses of \textit{G. lucidum}. Omics analysis helps elucidate the regulatory mechanisms of ganoderic acid biosynthesis; omics analysis also facilitates the identification of the related transcription factors. Likewise, transcription factor engineering can be applied to manipulate the ganoderic acid biosynthetic pathway for the hyperproduction of ganoderic acids.

The remarkable heterogeneity of ganoderic acids is attributed to the differential modification of a common lanostane ring skeleton. The recovery of individual ganoderic acids from \textit{G. lucidum} is particularly difficult because of the similar physicochemical characteristics of various analogs and low content. Different individual ganoderic acids exhibit different bioactivities.\textsuperscript{2} Consequently, the enhancement of the production of individual ganoderic acids is an important issue. In the biosynthetic pathway, the lanostane skeleton undergoes various modification processes, such as oxidation, reduction, and acylation, mediated by cytochrome P450 and acyltransferases, to form different individual ganoderic acids. Some candidate cytochrome P450 and acyltransferase genes have been identified in the \textit{G. lucidum} genome.\textsuperscript{6} After the genes encoding the modification enzymes are identified and characterized, genetic engineering is an efficient method to perform the directed production of desired individual ganoderic acids.

**Summary and Perspectives**

In conclusion, genetic transformation systems for \textit{G. lucidum} have been remarkably developed. The homologous genetic transformation system has been considered as a practical method to genetically improve \textit{G. lucidum}. The genetically modified \textit{G. lucidum} strains can accumulate high amounts of ganoderic acids.

Gene knockout and genome editing are important mechanisms involved in genetic engineering of \textit{G. lucidum}; these mechanisms have shown great potential for the gene deletion and modification of target genes in vivo.\textsuperscript{28} These tools have also been successfully applied in other filamentous fungi; indeed, these tools provide insights into the genetic engineering of \textit{G. lucidum}. Gene deletion systems and genome editing approaches haven’t been reported in \textit{G. lucidum}, these techniques should be further investigated.

The omics analysis of \textit{G. lucidum} has obtained valuable information to help understand the regulatory mechanisms of ganoderic acid biosynthesis. With the advancements in ganoderic acid biosynthesis and genetic manipulation tools for \textit{G. lucidum}, genetic engineering can be applied to improve ganoderic acid production.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

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