Phytochemistry xxx (2016) 1–8

Contents lists available at ScienceDirect

Phytochemistry

journal homepage: www.elsevier.com/locate/phytochem

Overexpression of the homologous lanosterol synthase gene in ganoderic acid biosynthesis in *Ganoderma lingzhi*

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ARTICLE INFO

Article history:
Received 25 July 2016
Received in revised form
10 November 2016
Accepted 16 November 2016
Available online xxx

Keywords:
Medicinal mushroom
Ganoderma lucidum
Fermentation
Genetic engineering

ABSTRACT

Ganoderic acids (GAs) in *Ganoderma lingzhi* exhibit anticancer and antimetastatic activities. GA yields can be potentially improved by manipulating *G. lingzhi* through genetic engineering. In this study, a putative lanosterol synthase (LS) gene was cloned and overexpressed in *G. lingzhi*. Results showed that its overexpression (OE) increased the ganoderic acid (GA) content and the accumulation of lanosterol and ergosterol in a submerged *G. lingzhi* culture. The maximum contents of GA-O, GA-Mk, GA-T, GA-S, GA-Mf, and GA-Me in transgenic strains were 46.6 ± 4.8, 24.3 ± 3.5, 69.8 ± 8.2, 28.9 ± 1.4, 15.4 ± 1.2, and 26.7 ± 3.1 μg/100 mg dry weight, respectively, these values being 6.1-, 2.2-, 3.2-, 4.8-, 2.0-, and 1.9-times higher than those in wild-type strains. In addition, accumulated amounts of lanosterol and ergosterol in transgenic strains were 2.3 and 1.4-fold higher than those in the control strains, respectively. The transcription level of LS was also increased by more than five times in the presence of the *G. lingzhi* glyceraldehyde-3-phosphate dehydrogenase gene promoter, whereas transcription levels of 3-hydroxy-3-methylglutaryl coenzyme A enzyme and squalene synthase did not change significantly in transgenic strains. This study demonstrated that OE of the homologous LS gene can enhance lanosterol accumulation. A large precursor supply promotes GA biosynthesis.

1. Introduction

Mushrooms are an important source of bioactive compounds with medicinal value. For instance, *Ganoderma lucidum*, a well-known traditional Chinese medicinal mushroom, has been widely used in Asia to treat diseases (Bisho et al., 2015; Paterson, 2006). Recently, the updated nomenclature of *Ganoderma* species has been reported based on morphology and multilocus phylogeny (Zhou et al., 2015). *G. lucidum* CGMCC 5.616 used in this study is conspecific with *G. lingzhi* according to the molecular phylogenetic approach described by Zhou et al. (2015) (Supplementary data 1). *G. lingzhi* contains ganoderic acids (GAs), C30 lanostane-type triterpenes, considered as its active components. GAs exhibit significant pharmacological activities, including anti-tumor, anti-invasion, anti-metastasis and anti-HIV properties (Bisho et al., 2015; Li et al., 2016a,b,c; Upadhyay et al., 2014; Xu et al., 2010b). Different GAs share similar chemical structures (Fig. 1A), but exhibit various biological activities. For example, ganoderic acid (GA)-T (**1**) and GA-Me (**3**) inhibit lung cancer growth and metastasis (Tang et al., 2006; Wang et al., 2007; Xu et al., 2010). GA-S (**2**), GA-Mf (**5**), and GA-Mk (**4**) induce cervical carcinoma HeLa cell apoptosis (Liu and Zhong, 2011; Liu et al., 2012), and GA-O (**6**) elicits cytotoxic effects on 95D cancer cell line (Wang et al., 2010).

GAs are biosynthesized via the mevalonate/isoprenoid pathway in *G. lucidum* (Fig. 1B) (Shi et al., 2010; Xu and Zhong, 2015). In this pathway, 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMGR) catalyzes mevalonate formation, and squalene synthase (SQS) catalyzes condensation of two farnesyl diphosphate molecules to form squalene. Lanosterol synthase (LS) catalyzes cyclization of 2, 3-oxidosqualene (**10**) derived from squalene (**9**), as a result, lanosterol (**7**), a common intermediate of triterpene and ergosterol biosynthesis, is produced (Xu et al., 2010b). Lanosterol (**7**) is subsequently modified through different methods, such as oxidation, reduction, and acylation, to form various individual GAs (Chen et al., 2012; Xu et al., 2010b).

Although genes responsible for the steps following lanosterol (**7**) formation remain unclear, all structural genes in the upstream biosynthetic pathway of GAs in *G. lucidum* have been cloned and

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http://dx.doi.org/10.1016/j.phytochem.2016.11.006
0031-9422/

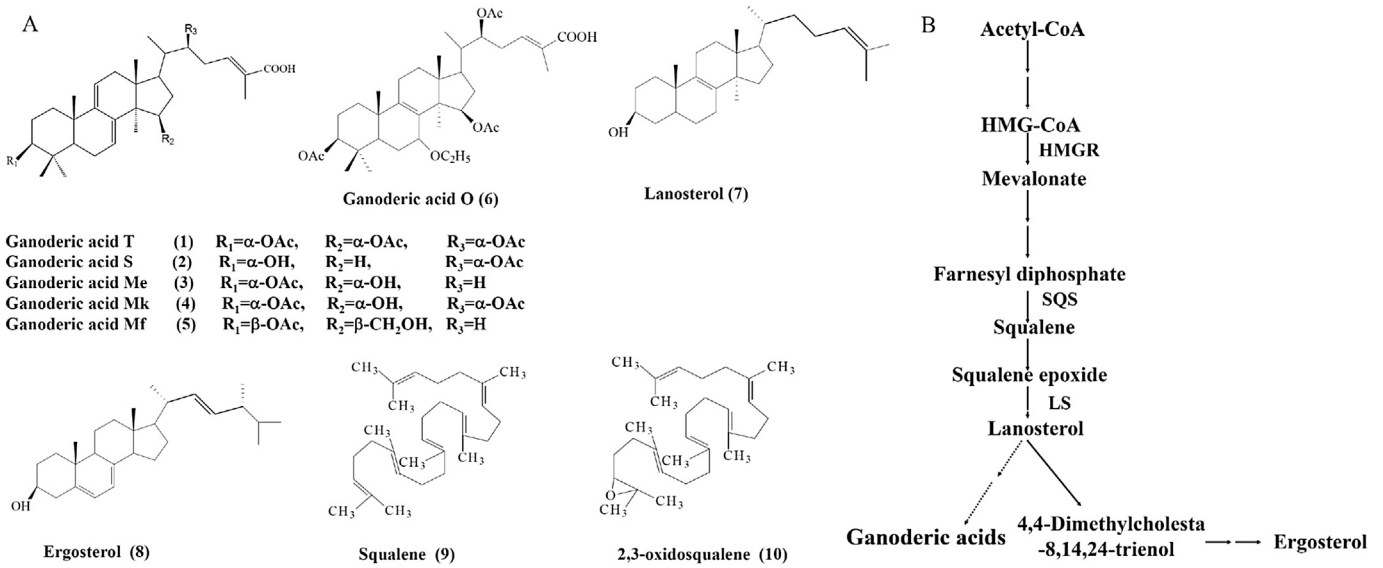

Fig. 1. Chemical structures of different individual ganoderic acids (GAs) detected in a submerged culture of *G. lingzhi* (A). The biosynthetic pathways of GAs and ergosterol (**8**) (B).

characterized (Shi et al., 2010; Zhou et al., 2014). Such genes can be subjected to molecular cloning to investigate the regulation of GA biosynthesis and the improvement of GA yield in *G. lucidum* through genetic engineering. The expression of some structural genes involved in the early biosynthetic pathway of GA is induced by acetic acid, methyl jasmonate, calcium, phenobarbital, and nitrogen limitation (Liang et al., 2010; Ren et al., 2013, 2014; Xu and Zhong, 2012; Zhao et al., 2011). GA hyperproduction is associated with higher transcription levels of GA biosynthetic genes, such as HMGR, SQS, and LS (Li et al., 2016a,b,c; Ren et al., 2010; Xu et al., 2010a; Zhang et al., 2010). OE of the HMGR gene increases the total crude amounts of GAs in *G. lucidum* twofold (Xu et al., 2012). The contents of four major individual GAs in transgenic *G. lucidum* overexpressing homologous SQS gene are 1.3−2.8 times higher than those in the wild-type strain (Zhou et al., 2014). OE of HMGR and SQS genes indicates that they play an important role in GA biosynthesis regulation. However, genetic manipulation of GA biosynthesis should be further investigated to elucidate GA biosynthesis regulation and to enhance GA production (Qin et al., 2016; Xu and Zhong, 2015).

LS, an oxidosqualene cyclase, is located at the branch point of GAs and ergosterol in *G. lingzhi*. It is responsible for the conversion of 2.3-oxidosqualene (**10**) to lanosterol (**7**), which is the lanostane ring skeleton of GAs (Xu et al., 2010b). In *Medicago truncatula*, ectopic expression of the oxidosqualene cyclase gene increases the total triterpene content (Confalonieri et al., 2009). In *Panax ginseng*, RNA interference-mediated downregulation of the genes encoding oxidosqualene cyclase, dammarenediol synthase, and β-amyrin synthase, reduces the production of dammarene-type and oleanane-type ginsenosides, respectively (Han et al., 2006; Takagi et al., 2011). These results indicate that oxidosqualene cyclase is a pivotal regulatory point of triterpene biosynthesis in plants. In *G. lucidum*, the transcription level of the LS gene is positively correlated with the content of triterpenes during fruiting body formation (Shang et al., 2010). However, the role of LS in the regulation of GA biosynthesis through OE has yet to be evaluated, and whether GA production can be enhanced through modification of the LS gene in *G. lingzhi* has yet to be determined.

In this study, the LS gene was cloned and overexpressed in *G. lingzhi*. The OE effects on GA biosynthesis were investigated in *G. lingzhi*. This study helps examine LS function and GA biosynthesis regulation in *G. lingzhi*.

2. Results and discussion

2.1. Generation of transgenic G. lingzhi overexpressing the homologous LS gene

The LS gene was successfully amplified from the *G. lingzhi* genome, and its genome sequence was submitted to the GenBank nucleotide sequence database under accession no. KX452685. The entire gene of *G. lingzhi* LS is 2983-bp long and has an open reading frame of 2181-bp, encoding a protein with 726 amino acids, predicted molecular mass of 82. 7 kDa, and pI of 6.26. *G. lingzhi* LS gene includes seven introns, and its nucleotide sequence is 59 bp longer than that of a previously registered LS gene for *G. lucidum* HG strain. (GQ169529). Previous work also reported that the SQS gene of *G. lucidum* CGMCC 5.616 differed in size from that of *G. lucidum* HG strain (Zhou et al., 2014). *G. lingzhi* LS showed high identity with LSs from *G. lucidum* HG (99%), *Dichomitus squalens* (88%), *Trametes versicolor* (78%), *Taiwanofungus camphoratus* (72%), and *Hypsizygus marmoreus* (70%). *G. lingzhi* LS has a VSDCTGE motif and six highly conserved QW motifs that are characteristic of the oxidosqualene cyclase family (Fig. 2) (Godio and Martin, 2009; Lin et al., 2015).

LS catalyzes the cyclization of 2, 3-oxidosqualene (**10**) to form lanosterol (**7**), a common precursor for both GA and ergosterol (**8**) biosynthesis. To explore the regulatory function of LS in GA biosynthesis, the gene encoding LS of *G. lingzhi* was overexpressed. Plasmid pJW-EXP-LS (Fig. 3A), which carries the *G. linghzi* LS gene that contains seven introns, was constructed and used to transform *G. lingzhi* using the previously reported genetic transformation method (Li et al., 2016a,c; Xu et al., 2015; Yu et al., 2014). The *G. lingzhi* glyceraldehyde-3-phosphate dehydrogenase gene (*gpd*) promoter was used to constitutively OE the LS gene. Candidate transformants were screened on complete yeast medium (CYM) medium with 2 mg/L carboxin (Sigma, St. Louis, MO) antibiotics after five rounds of growth on fresh CYM medium. Those transformants stably maintaining carboxin resistance were checked by genome PCR, followed by sequencing of the PCR products. Genome PCR analysis showed the presence of one band, representing the fusion *gpd* promoter and LS gene fragment (3.45 kb), in the lanes of the LS transformant and positive control, whereas no