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REVIEW ARTICLE

Progress in understanding of ginsenoside biosynthesis

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ABSTRACT

Ginseng is an economically important medicinal plant. The major bioactive ingredients of ginseng are ginsenosides, which are triterpene saponins. Because of difficulties in ginseng cultivation and the low productivity of ginseng cell and tissue culture, it has become important to improve ginsenoside levels by using metabolic engineering based on the biosynthetic pathway of ginsenosides. During the last decade, substantial advances have been made in biosynthesis of ginsenosides. This review is concerned with recent developments in our understanding of the biosynthesis of ginsenosides.

INTRODUCTION

Ginseng, which has been used as a herbal medicine in East Asia for thousands of years (Kiefer & Pantuso 2003), holds an important position in oriental medicine in many countries. Ginseng posseses many pharmacological activities, *e.g.* anti-cancer, anti-diabetic, neuroprotective, radioprotective, anti-amnestic and anti-aging effects. Ginsenosides are considered to account for the bioactivity of ginseng. By 2000, at least 34 ginsenosides had been isolated (Shin *et al.* 2000), and novel ginsenosides continue to be reported (Dou *et al.* 2001, 2006; Park *et al.* 2002a,b; Wang *et al.* 2004; Yoshikawa *et al.* 2007). The most extensively researched and noteworthy ginsenosides are R_{b1},R_{b2}, R_c, R_d, R_{g1}, R_{g2}, R_{g3}, R_e, R_f, R_{h1}, and R_{h2} (Helms 2004). The synthesis of ginsenosides is not restricted to the Araliaceae, such as Asian ginseng (*Panax ginseng*) (Kiefer & Pantuso 2003), American ginseng (*P. quinquefolius*) (King & Murphy 2007), Vietnamese ginseng (*P. vietnamensis*) (Tran *et al.* 2002), Indian ginseng (*P. sikkimensis* and *P. pseudoginseng*) (Mathur *et al.* 1999), *P. notoginseng* (Wang *et al.* 2006) and Siberian ginseng (*Eleutherococcus senticosus*) (Donovan *et al.* 2003), but also occurs in the Amaranthaceae, in Brazilian ginseng (*Pfaffia paniculata*) (De Souza Daniel *et al.* 2005).

Currently, ginseng is consumed throughout the world. Markets for ginseng and related products are estimated at US$3.5 billion worldwide (Hong *et al.* 2006) and continue to expand with gradual elucidation of pharmacological mechanisms of ginseng. However, due to the long duration of cultivation (6–7 years to reach maturity) for marketable ginseng (Liu *et al.* 2004) and plant diseases such as red skin and root rot (Hong *et al.* 2006), cultivation of ginseng is very difficult. Therefore, many researchers have studied the production of ginsenosides by tissue and cell cultures, such as callus tissues (Mathur *et al.* 1999), cell suspensions (Thanh *et al.* 2005, 2006), normal roots (Kim *et al.* 2004; Ali *et al.* 2006a,b) and hairy root cultures induced by *Agrobacterium rhizogenes* (Palazon *et al.* 2003; Woo *et al.* 2004). However, productivity is low, as is the ginsenoside content. Overproduction of ginsenosides by metabolic engineering is an attractive strategy to improve ginsenoside productivity. Although there are few reports regarding biosynthesis of ginsenosides, compared with studies of pharmacological effects of ginsenosides, substantial advances have been made during the last decade. In this paper, we addressed recent progress in studies on biosynthesis of ginsenosides.

GINSENOSIDES STRUCTURES AND CLASSIFICATION

The major pharmacologically active constituents of ginseng are ginsenosides belonging to the triterpene saponins

Ginsenosides	R_1	R_2	R_3
R_{b1}	$Glc^{(2-1)}Glc$–O	H	$Glc^{(6-1)}Glc$
R_c	$Glc^{(2-1)}Glc$	H	$Glc^{(6-1)}Ara(f)$
R_d	$Glc^{(2-1)}Glc$	H	Glc
R_e	H	O-$Glc^{(2-1)}$Rha	Glc
R_f	H	O-$Glc^{(2-1)}$Glc	H
R_{g1}	H	O-Glc	Glc

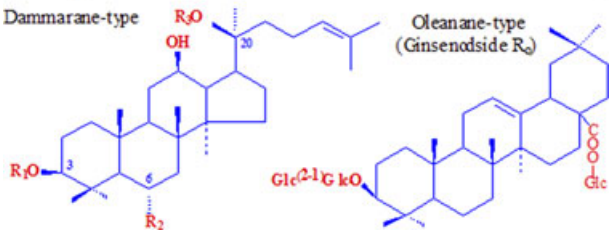

Fig. 1. Structures of representative ginsenosides of dammarane type and oleanane type. Rb1, Rc, Rd are Protopanaxadiol-type. Re, Rf, Rg1 are Protopanaxatriol-type. Abbreviations for carbohydrates are as follows: Glc, glucopyranoside; Ara(f), arabinfuranoside; Rha, rhamnopyranoside. Superscripts indicate the carbon in the glucose ring that links the two carbohydrates.

(Okazaki *et al.* 2006). Ginsenosides are named as R_x (x = 0, a-1, a-2, b-1, b-2, b-3, c, d, e, f, 20-gluco-f, g-1, g-2, h-1...X) according to the sequence of the Rf value of spots on TLC plates, from the bottom to the top (Okazaki *et al.* 2006). Ginsenosides are classified into two groups from the structures of aglycones, namely dammarane-type and oleanane-type. The major ginsenosides are of the dammarane-type, where the fundamental skeleton is tetracyclic. Dammarane-type ginsenosides are further classified into protopanaxadiol and protopanaxatriol groups (Kim *et al.* 2006), according to the positions of the carbohydrate moieties at carbons –3, –6 and –20, which can be either free or connected to sugar rings (Fig. 1). Oleanane-type ginsenosides, where the fundamental skeletons are pentacyclic, are represented by only one minor ginsenoside, R_o, where the aglycone is oleanolic acid. The representatives of ginsenoside types are presented in Fig. 1.

Currently, more than 40 kinds of ginsenoside have been isolated from white and red ginseng and most are dammarane-type, including recently newly-isolated ginsenosides from flower buds of *P. ginseng* (Yoshikawa *et al.* 2007), processed ginseng (Park *et al.* 2002a,b), and leaves of *P. ginseng* (Dou *et al.* 2001). The most extensively researched and noteworthy ginsenosides are R_{b1},R_{b2}, R_c, R_d, R_{g1}, R_{g2}, R_{g3}, R_e, R_f, R_{h1} and R_{h2} (Helms 2004). The bioactivities of the newly-discovered ginsenosides remain to be studied.

PROPOSED BIOSYNTHETIC PATHWAY OF GINSENOSIDES

Ginsenosides, secondary metabolites of ginseng, are triterpene saponins. Triterpene and sterol share the same precursor, 2,3-oxidosqualene, which is synthesised through the isoprenoid pathway. Furthermore, biosynthetic pathways leading to sterols and triterpenes are identical to each other up to the formation of 2,3-oxidosqualene and branch at its cyclization step (*Abe et al.* 1993). Isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP) are the universal precursors for all isoprenoids in all living organisms (Fig. 2). Two biosynthetic pathways lead to the isoprene unit: the MVA pathway and the methylerythritol phosphate (MEP) pathway. In the more recently disclosed MEP pathway (Hoeffler *et al.* 2002), synthesis of IPP and DMAPP start from pyruvate and D-glyceraldehyde phosphate via 1-deoxy-D-xylulose-5-phosphate, 2-C-methyl-D-erythritol-4-phosphate, 4-diphosphocytidy-l-2-C-methyl-D-erythritol, 4-diphosphocytidyl-2-C-methyl-D-erythritol 2-phosphate, 2-C-methyl-D-erythritol-2,4-cyclodiphosphate and 4-hydroxy-2-methylbut-2-enyl 1-phosphate.

The majority of genes encoding enzymes of the isoprenoid pathway have been isolated from fungi, animals and plants (Rodriguez-Concepcion & Boronat 2002). In plants, the MVA pathway is found in the cytoplasm, whereas the MEP pathway is present in plastids (Seemann *et al.* 2006). In some prokaryotes, like strains of *Streptomyces*, both the MEP and the MVA pathways are sequentially utilised for synthesis of isoprenoid compounds, whereas in plants they are compartmentalised and operate in parallel (Hemmerlin *et al.* 2003). Furthermore, it is suggested that these two pathways can exchange their intermediates for isoprenoids synthesis (Hemmerlin *et al.* 2003; Rohdich *et al.* 2003). The MEP route is strongly linked to photosynthesis (Zeidler & Lichtenthaler 2001); however, the active MEP pathway is also found in plant roots (Fester *et al.* 2002; Hampel *et al.* 2005). Generally, it is suggested that, in higher plants, steroids are preferentially formed via the MVA pathway, whereas monoterpenes, diterpenes, carotenoids, phytol and plastoquinone are formed predominantly via the MEP pathway (Rohdich *et al.* 2003). However, up to now, there has been no research in the MEP pathway in ginseng. In addition, the question whether both pathways or only one of the two pathways plays an important role in ginsenoside synthesis remains to be answered.

The condensation of DMAPP with IPP to form farnesyl diphosphate (FPP) is carried out by farnesyl diphosphate synthase (*FPS*). Squalene synthase (*SS*) catalyses the first enzymatic step, FPP to squalene, from the central isoprenoid pathway toward sterol and triterpenoid biosynthesis (Abe *et al.* 1993). Squalene epoxidase catalyses the step from squalene to 2,3-oxidosqualene. Both phytosterols and triterpenes in plants are synthesised from the products of cyclisation of 2,3-oxidosqualene catalysed by oxidosqualene cyclases (*OSCs*). In *P. ginseng*, β-amyrin synthase (β*AS*), dammarenediol synthase (*DS*) and cycloartenol synthase (*CS*) belong to the oxidosqualene cyclase (*OSC*) family that is situated at the branching point for triterpene and sterol biosynthesis. *CAS* catalyses the production of cycloartenol as a precursor of phytosterols, *DS* is responsible for the synthesis of dammarenediol provid-

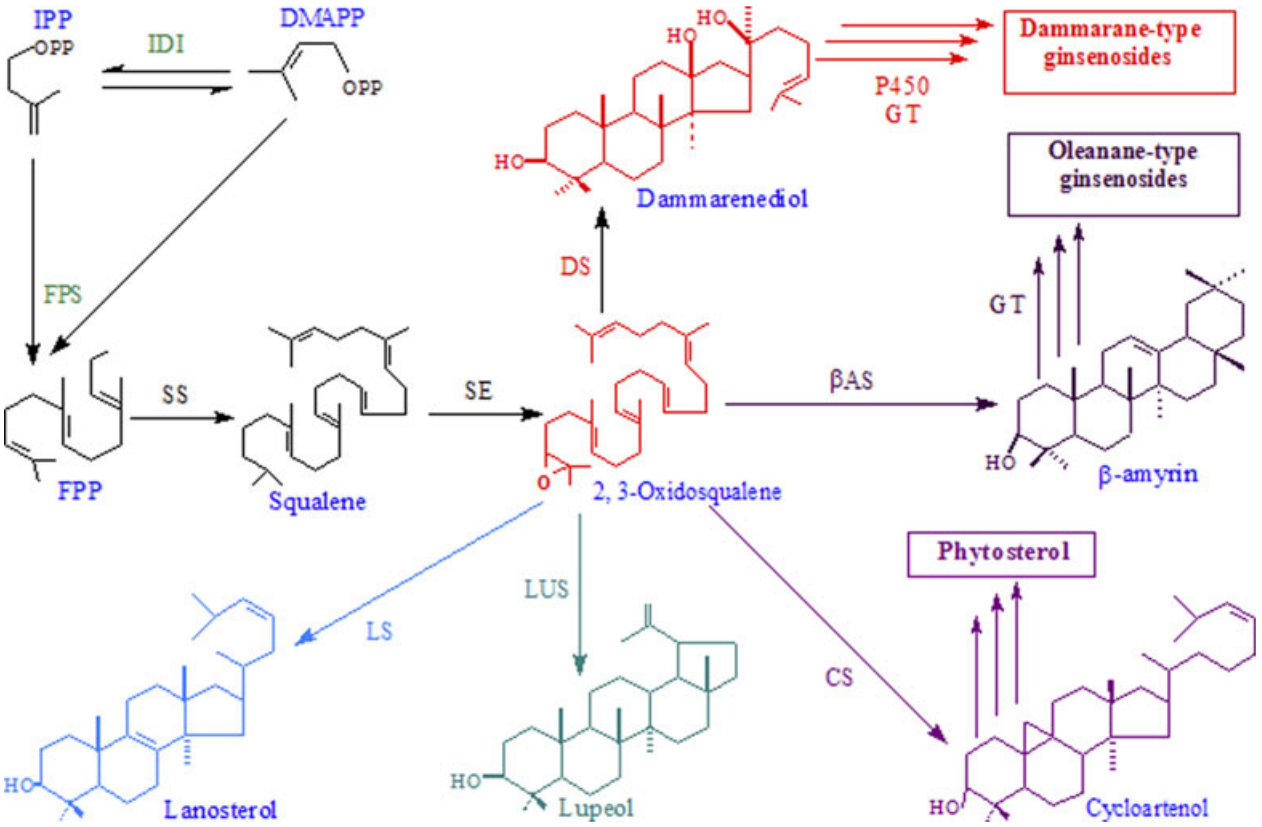

Fig. 2. The proposed biosynthetic pathay of ginsenosides.

ing tetracyclic skeletons for dammarane-type ginsenosides, and β*AS* is involved in oleanane-type ginsenoside biosynthesis (Fig. 2). The intermediates, dammarenediol and β-amyrin, are transformed into ginsenosides through a series of hydroxylation and glycosylation reactions (Kushiro *et al.* 1997, 1998). Cytochrome P450s are thought to be involved in hydroxylation of ginsenoside skeletons (Jung *et al.* 2003), and glycosyltransferases may be involved in glycosylation of ginsenoside skeletons.

So far, a complete cDNA clone for several enzymes from ginseng is available (Table 1). To our knowledge, however, only studies on *SS* (Lee *et al.* 2004; Seo *et al.* 2005), *DS* (Tansakul *et al.* 2006), β*AS* and *CS* (Kushiro *et al.* 1998) has been published. Lanosterol is the initial carbocyclic sterol precursor in animals, fungi and trypanosomatids. Although substantial labelling experiments support cycloartenol rather than lanosterol as the major plant sterol precursor (Phillips *et al.* 2006), lanosterol biosynthesis has been demonstrated in a few plants (Suzuki *et al.* 2006). Interestingly, there is one cDNA (Accession no. *AB009031*) encoding lanosterol in *P. ginseng* (Suzuki *et al.* 2006), indicating a new pathway for plant sterols in ginseng. Additionally, expressed sequence tag (EST) analysis of cDNA libraries constructed from different tissues of *P. ginseng* (Jung *et al.* 2003; Choi *et al.* 2005; Kim *et al.* 2006) revealed the candidate genes encoding enzymes involved in ginsenoside biosynthesis: *HMGR*, *FPS*, geranyl diphosphate synthase, cytochrome P450, glycosyltransferase, β-glucosidase and lupeol synthase (*LUS*). Therefore, lupeol is possibly synthesised in

Table 1. List of identified genes involved in ginsenoside biosynthesis.

ginseng species	genes encoding enzymes	accession no.
Panax ginseng	Farnesyl diphosphate synthase	DQ087959
	Squalene synthase	AB010148
	Squalene synthase *PgSS1*	AB115496
	Squalene epoxidase	AB122078
	Squalene epoxidase	AB003516
	Beta-amyrin synthase	AB122080
	Cytochrome P450	AB122079
	Cycloartenol synthase	AB009029
	Beta-amyrin synthase *PNY1*	AB009030
	Beta-amyrin synthase *PNY2*	AB014057
	Dammarenediol-II synthase	AB265170
	Lanosterol synthase *PNZ1*	AB009031
Panax quinquefolius	Squalene synthase 1	AM182456
	Squalene synthase 2	AM182457
Panax notoginseng	Squalene epoxidase	DQ457054
	Squalene epoxidase	DQ386734
	Farnesyl pyrophosphate synthase	DQ059550
	Squalene synthase	DQ186630

ginseng. Lupeol synthase has been cloned and characterised in several plant species (Herrera *et al.* 1998; Shibuya *et al.* 1999; Guhling *et al.* 2006). These studies might be useful for the research on lupeol synthase in ginseng.

CLONING AND STUDIES OF GENES ENCODING ENZYMES FOR GINSENOSIDE BIOSYNTHESIS

A full-length cDNA clone of *SS* (*PgSS1*; Accession no. *AB115496*) was isolated by EST analysis of a leaf cDNA library of *P. ginseng* and further studied (Lee *et al.* 2004). The cDNA of *PgSS1* is 1476-bp long and carries an open-reading frame of 415 amino acids, giving a predicted molecular mass of 47 kDa. *PgSS1* was thought to be consistent with a multicopy gene and/or large gene with several introns. The downstream genes in the isoprenoid pathway were transcriptionally activated by overexpression of *PgSS1*, suggesting that *SE*, β*AS* and *CAS* enzymes may be inducible enzymes or that these genes are under the control of the same regulatory elements. Overexpression of *PgSS1* enhanced activity of the *PgSS1* enzyme, which resulted in a remarkable increase in phytosterol and ginsenoside content. These results demonstrate that *PgSS1* is a key regulatory enzyme, not only for phytosterol but also for ginsenoside biosynthesis. The same results were also found by heterologous overexpression of *PgSS1* in *E. senticosus* (Seo *et al.* 2005), which revealed that phytosterols (β-sitosterol and stigmasterol) and triterpene saponin levels in transgenic *E. senticosus* increased two- to 2.5-fold (Seo *et al.* 2005). Moreover, this suggests that heterologous overexpression of other plant genes related to biosynthesis of triterpene saponins in ginseng could be employed to improve ginsenoside levels and elucidate the mechanisms of ginsenoside biosynthesis.

Two different homologous cDNA clones (*PNY1* and *PNY2*) encoding β*AS* synthase were obtained from hairy roots of *P. ginseng* (Kushiro *et al.* 1998). In addition, a further β*AS* cDNA is also available in GeneBank (Accession no. *AB122080*). This implies that β*AS* may be developed from a common ancestor by multicopy and mutation during evolution. An internal portion of the enzyme (*PNY1*) that was critical for β-amyrin formation has been identified. Intriguingly, site-directed mutation study od *PNY1* revealed a single amino acid residue, Tyr261, that is critical for product specificity. Mutation of Tyr261 resulted in synthesis of dammarene-type tetracyclic triterpenoids instead of pentacyclic triterpenosids (Haralampidis *et al.* 2002). β-Amyrin and its metabolites tend to be tissue-specific (Phillips *et al.* 2006), which may be why only one oleanane-type ginsenoside (R_o) has been identified from ginseng.

Dammarenediol synthase is considered the most important enzyme for ginsenoside biosynthesis. Kushiro *et al.* (1997) first detected the dammarenediol synthase activity from a microsomal fraction of the hairy root of *P. ginseng*. Under catalysis of this enzyme, 2,3-oxidosqualene was converted into (20S)-dammarenediol and not to (20R)-dammarenediol. The enzyme activity was highest at pH 6.0 and was not increased by addition of any detergent. Thus, this pH value might be optimal for ginsenoside biosynthesis in ginseng tissue or cell culture. Excitingly, dammarenediol-II synthase cDNA was recently cloned by homology-based RT-PCR and was confirmed by heterologous expression in a lanosterol synthase-deficient *Saccharomyces cerevisiae* strain *GIL77* (Tansakul *et al.* 2006). This *DS* contains an ORF of 2310 bp encoding a polypeptide of 770 amino acids, with a predicted molecular mass of 88.3 kDa. RNA interference of *DS* in transgenic *P. ginseng* resulted in silencing of *DS* expression, which leads to a reduction of ginsenoside production to 84.5% in roots (Han *et al.* 2006). These results indicate that *DS* is a key enzyme involved in ginsenoside biosynthesis; therefore, substantial improvement in ginsenoside biosynthesis could be expected by overexpression of *DS*. In addition, some other cDNA clones of enzymes possibly involved in ginsenoside biosynthesis have also been obtained (Table 1); however, these genes remain to be characterised.

CONCLUSION AND PROSPECTS

Ginsenosides are triterpene saponins synthesised from IPP and DMAPP. The MVA pathway was accepted as the only biosynthetic route to IPP and DMAPP until the MEP pathway was elucidated in bacteria and plants (Paseshnichenko 1998). The MEP pathway has now been demonstrated in many plants; however, more research is required on the MEP pathway in ginseng.

A combination of molecular biology and enzymology has been effectively employed to unravel the mechanism of ginsenoside biosynthesis. More and more complete cDNA sequences of genes and candidate genes encoding enzymes involved in ginsenoside biosynthesis have been obtained (Table 1). Although relatively few studies of these genes have been carried out, progress in this area is rapidly accelerating. The genes encoding *SS*, β*AS* and *DS* have been identified and confirmed by homology-based strategies and heterologous expression in yeast. Furthermore, *SS* and *DS* have been used to generate transgenic ginseng with altered levels of ginsenosides by overexpression and gene silencing, respectively (Lee *et al.* 2004; Seo *et al.* 2005; Han *et al.* 2006). These transgenic experiments suggest that *SS* and *DS* play an important role in ginsenoside biosynthesis.

Moreover, based on EST strategies, substantial inroads have been made into the cloning and characterisation of genes required for squalene synthesis (*HMGR*, *FPS*, geranyl diphosphate synthase, *SE*), and the later steps of ginsenoside synthesis (cytochrome P450, glycosyltransferase, β-glucosidase). The development of a methyl jasmonate-inducible ginseng tissue culture system (Choi *et al.* 2005; Thanh *et al.* 2005), which can enrich transcripts of genes related to ginsenoside biosynthesis, will undoubtedly facilitate further unraveling of the ginsenoside biosynthetic pathway. Furthermore, the discovery of candidate genes for lupeol and lanosterol synthases in ginseng only reinforces

the current limits to our knowledge of the metabolic pathway for oxidosqualene in ginseng. Traditionally, the roots of ginseng are considered as the major tissue for ginsenoside biosynthesis. However, the expression of *DS*, which is responsible for biosynthesis of the majority of ginsenosides, is highest in ginseng flower buds (Han *et al.* 2006). This suggests that flower buds of ginseng may be an ideal material for further dissecting the biosynthetic pathway of ginsenosides. Interestingly, ectopic overexpression of *P. ginseng SS* gene in *E. senticosus* can enhance triterpene saponin accumulation (Seo *et al.* 2005), implying that heterologous expression of corresponding available genes from other plants could be employed to further elucidate ginsenoside synthesis and to produce transgenic ginseng with altered ginsenoside levels.

So far, the strategies for discovery of genes encoding enzymes involved in ginsenosides are mainly homology-based RT-PCR (Kushiro *et al.* 1998) and EST analysis (Luo *et al.* 2003; Jung *et al.* 2003; Choi *et al.* 2005; Kim *et al.* 2006). Recently, aiming at the genomics of ginseng, a bacterial artificial chromosome library for *P. ginseng* has been constructed (Hong *et al.* 2004). Undoubtedly, this is a most useful resource not only for discovery of genes involved in ginsenosides but also for elucidating the regulatory mechanisms of expression of these genes. Although substantial progress has been made in unravelling the synthetic pathway of ginsenosides, most of the related enzymes have not yet been studied at the catalytic level. Furthermore, the later steps in ginsenoside biosynthesis remain to be elucidated. There is still a long way to go in dissecting ginsenoside biosynthesis; however, in view of the economic and pharmacological importance of ginseng, this is clearly an important area worth our efforts.

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