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Modulation of Triterpene Saponin Production: In Vitro Cultures, Elicitation, and Metabolic Engineering

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Abstract Saponins are secondary metabolites that are widely distributed in the plant kingdom and are often the active components in medicinal herbs. Hence, saponins have a potential for the pharmaceutical industry as antibacterial, virucidal, anti-inflammatory, and anti-leishmanial drugs. However, their commercial application is often hindered because of practical problems, such as low and variable yields and limited availability of natural resources. In vitro cultures provide an alternative to avoid problems associated with field production; they offer a system in which plants are clonally propagated and yield is not affected by environmental changes. Additionally, treatment of in vitro cultures with elicitors such as methyl jasmonate may increase the production of saponins up to six times. In vitro cultures are amenable to metabolic engineering by targeting specific genes to enhance saponin production or drive production towards one specific class of saponins. Hitherto, this approach is not yet fully explored because only a limited number of saponin biosynthesis genes are identified. In this paper, we review recent studies on in vitro cultures of saponin-producing plants. The effect of elicitation on saponin production and saponin biosynthesis genes is discussed. Finally, recent research efforts on metabolic engineering of saponins will also be presented.

Keywords Triterpene saponins · Saponin biosynthesis · In vitro culture · Elicitation · Metabolic engineering

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Introduction

Saponins are naturally occurring surface-active glycosides. The name "saponin" is derived from the Latin word *sapo*, which means "soap" and reflects their property to form stable, soap-like foams in aqueous solutions. It is a diverse group of terpenoids characterized by their structure. Saponins are composed of a steroidal or triterpenoid aglycon and one or more sugar chains. A plant might contain several dozen saponins with closely related structures [1]. This structural diversity is the basis of their numerous physicochemical and biological properties which are exploited in a number of traditional and industrial applications.

Recent research has established saponins as the active components in many herbs and plants used in traditional medicine [2]. A well-known example is *Panax ginseng*, one of the most valuable oriental herbs [3, 4]. Dried ginseng roots are, since ancient times, used as a healing drug and health tonic in many Asian countries. Recently, ginseng has been increasingly used for different purposes, and the medicinal value of ginseng has been of vast interest worldwide. Extensive effort is still being put into investigating its pharmacological effects [5–9].

However, the production of saponins in plants is still facing many problems, such as low yields and limited availability of natural resources. Because of that, much research effort is being put in finding a way to enhance the production of the saponins or driving the expression toward one interesting saponin.

Role of Saponins in Plants

Many research articles describe the identification of saponins in plants and their biological activities. They have been reported to have antimicrobial, virucidal, or insecticidal action [10]. In this view, saponins can be considered as a part of the plant defense mechanism and can be classified in a large group of protective molecules, namely phytoprotectants [11, 12].

Several studies indicate that antimicrobial saponins confer protection against disease, and this is studied in detail in oat. Avenacins are oat root saponins that are important for broad-spectrum resistance to soil-borne pathogens, including *Gaeumannomyces graminis* which causes "take-all" disease in cereals [13]. Saponin-deficient mutants (*sad* mutants) showed compromised resistance to several pathogens, indicating that avenacins provide a chemical defense against pathogen attack [14, 15]. In addition, *G. graminis* has the capacity to detoxify the major avenacin, avenacin A-1. Fungal mutants lacking the saponin-detoxifying enzyme, avenacinase, showed increased sensitivity to avenacin A-1 and were no longer able to infect oat [16]. These studies imply that the antimicrobial saponins in oat act in defense-related processes and strengthen the relevance of these compounds as biotechnological weapons against pathogen infection.

Biosynthesis of Triterpene Saponins

Terpenes represent the largest family of natural compounds, with more than 30,000 entities being structurally identified [17]. They are derived from units of isoprene, which has the molecular formula $(C_5H_8)_n$. Most terpenes are classified by the number of C_5 isoprene units that they contain. The classes are: hemiterpenes consisting of a single C_5 unit, monoterpenes (C_{10}), sesquiterpenes (C_{15}), diterpenes (C_{20}), sesterterpenes (C_{25}), triterpenes (C_{30}), carotenoids (C_{40}), and polyterpenes consisting of long chains of many isoprene units.

Terpene biosynthesis is a complicated process mediated by two biosynthetic pathways; the mevalonate, and methyl–erythritol pathway [18], both leading to the formation of isopentenyl diphosphate (IPP) and its isomer dimethylallyl diphosphate (DMAPP), which are the precursors from which all terpenes are formed (Fig. 1). Head-to-tail addition of IPP and DMAPP leads to formation of geranyl diphosphate (GPP) and this step is mediated by GPP synthase (GPS). FPP synthase (FPS) is responsible for the addition of one IPP unit to GPP. Further on, squalene synthase (SS) will mediate the condensation of two FPP units for the synthesis of squalene, which is the precursor for both steroidal and triterpene saponins [19] (Fig. 1). Subsequently, squalene is oxidized to oxidosqualene, the common starting point for cyclization reactions in triterpene saponin biosynthesis (Fig. 2). Oxidosqualene is converted to cyclic derivatives which create a carbocation that can undergo several types of cyclization reactions. The type of cyclase that is involved in the cyclization reaction determines the skeleton that is formed. Many different kinds of cyclases have been described and their mechanisms of action are well documented; the most important ones are dammarenediol synthase (DDS), β amyrin synthase (βAS), and α amyrin synthase (αAS), which will give rise to dammarane, oleanane, and ursane type of saponins, respectively [1, 20].

Following cyclization, further diversity is obtained by modification of the products through oxidation, hydroxylation, glycosylation, and other substitutions mediated by cytochrome P450-dependent monooxygenases, glycosyltransferases, and other enzymes. However, not much is known about the enzymes required for these chemical reactions.

One common feature shared by all saponins is the presence of a sugar chain attached to the aglycon. Glycosylation is particularly important as the sugar chain is critical for the biological activity in several saponins. The oligosaccharide chains are likely to be synthesized by sequential addition of single sugar residues to the aglycon, but there is also little experimental data about the mechanism of triterpenoid glycosylation.

Saponin Production in In Vitro Cultures

Triterpenoid saponins have complex structures, making chemical synthesis an economically uncompetitive option for large-scale production. Therefore, the current supply of saponins

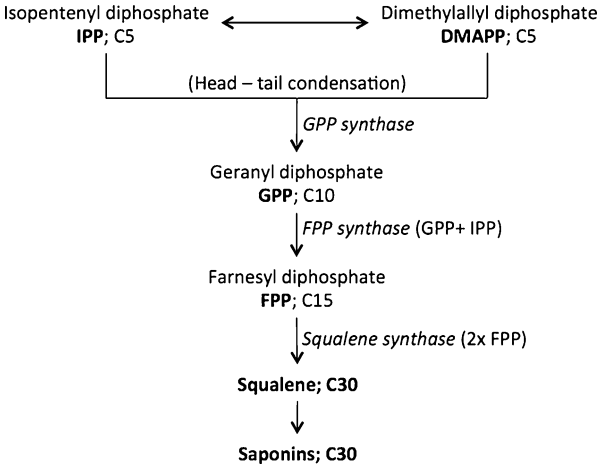

Fig. 1 Synthesis of the saponin backbone from isopentenyl diphosphate (IPP; adapted from Dubey et al. [19]). (*DMAPP* dimethylallyl diphosphate, *GPP* geranyl diphosphate, *FPP* farnesyl diphosphate)

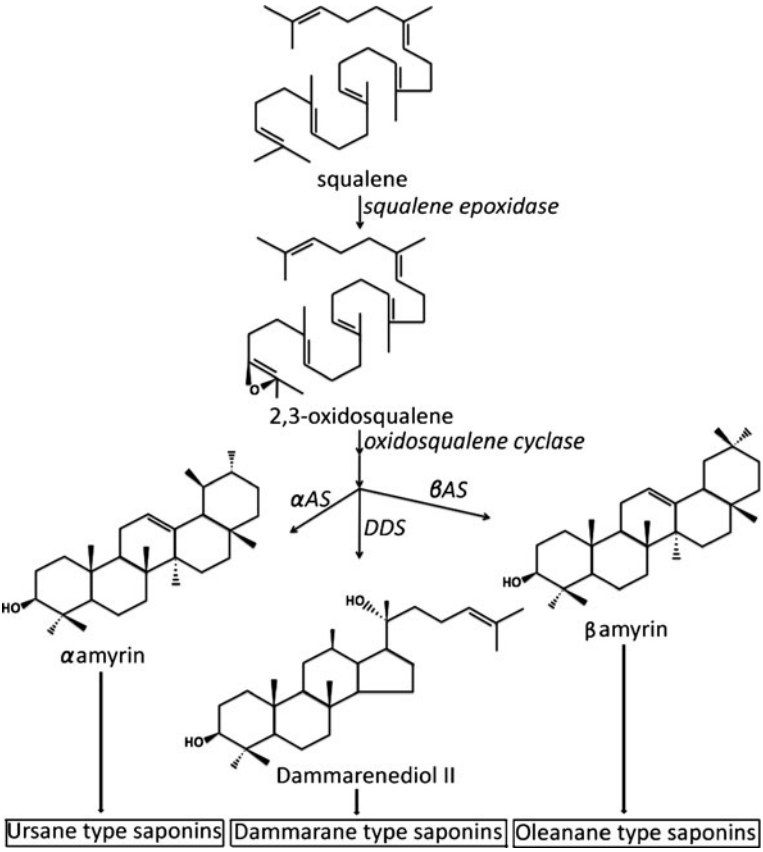

Fig. 2 Synthesis of different classes of saponins from squalene. (αAS α amyrin synthase, *βAS* β amyrin synthase, *DDS* dammarenediol synthase)

is mostly extracted from plants grown in the field, which is also accompanied with difficulties. The production and harvesting process is time-consuming and low yielding; in addition, the yield of saponins is dependent on geographical and seasonal variation. For example, *Panax* seeds must undergo a stratification process with exposure to cool–warm cycles in the preceding winter–summer seasons to promote zygotic embryo maturation and overcome dormancy. During the 18-month period of stratification and germination, there is a risk of infection by pathogens and abiotic stress which can reduce the viability of the seeds [21]. After germination, it takes a minimum of 4 years before roots can be harvested and a 6-year-old root yields only about 90 g fresh weight [22]. In addition, there are no horticulturally improved varieties, so the cultivated ginseng plants are morphologically and genetically heterogeneous, which is reflected in the variable sizes, shapes, and overall appearance of the marketed roots [21]. An additional problem for many saponin-producing plants is that they are often endangered and protected in some areas [23].

Plant cell and tissue cultures have been proposed as alternatives for the production of pharmaceutically interesting saponins. These in vitro cultures could overcome many

problems associated with saponin extraction from field-grown plants; production is not dependent on geography and season, product quality and yield are generally established, and clonal propagation methods can be used to overcome germination and plant heterogeneity issues [3]. In addition, in vitro cultures are highly suitable to manipulate and improve the production of desired compounds by using biotechnological methods, which will be discussed in detail below. There are, however, also problems with the stability, growth rate, and scaling up of in vitro cultures. So far, only ginsenosides from *Panax ginseng* are produced commercially from cell suspension cultures [24]. Because of the high cost associated with tests on a larger scale, mainly private efforts are deployed to establish cell culture technology as a means to produce medicinal compounds.

Langhansová et al. [25] established cell suspensions and adventitious root cultures of *P. ginseng* and compared the growth and saponin production in Erlenmeyer flasks with large-scale bioreactors. The total ginsenoside content as well as the production of particular ginsenosides differed in the different tissue cultures and cultivation systems tested. In adventitious root cultures, the particular ginsenoside profile was similar to that in native roots. However, the total content of saponins is only 1.8% of dry mass in Erlenmeyer flasks and 1.5% in a bioreactor, which is lower than the total content in native roots (3.3% of dry mass). On the contrary, *P. ginseng* cells produced very high yields of total saponins, 1.3% and 4.3% for callus, and cell suspension in bioreactor, respectively. In the case of cell suspensions, a completely different profile of individual saponins was found, with very high production of the ginsenosides Rb1 and Rg1. Therefore, this system would be better for the production of individual compounds. Also, due to the less-defined differentiation status of the plant cells in the culture, there were fewer problems with the extraction of the desired compounds [25]. For the important medicinal plant *Primula veris* a comparison was also made between different tissue and cell cultures. The main saponin in *P. veris*, primula acid I (PAI), is produced in the roots. The average content of PAI in in vitro seedlings, roots from micropropagated plants, and adventitious root cultures was 1.5 to two times lower than in roots of plants grown in the soil. In callus and suspension cell cultures, the content was eight times lower. Despite the lower saponin content, adventitious root cultures are a valuable alternative for the production of PAI, as *P. veris* in a highly endangered and protected plant species [23]. In vitro cultures for the production of saponins were recently also established for *Centella asiatica* [26] and *Gypsophila paniculata* [27]. For both plant species, a correlation between saponin production and expression of key enzymes in the saponin biosynthesis was found.

For *Panax quinquefolius* (American ginseng), an in vitro propagation protocol through somatic embryogenesis was optimized. Embryogenic callus was induced on leaf and stem explants within 2 weeks. Suspension cultures were established by transferring embryogenic callus to liquid medium. Globular somatic embryos from these cultures were germinated with a success rate of 50–60%. Shoot and root development could be enhanced through the addition of gibberellic acid and 6-benzylaminopurine. Based on TLC analysis, it could be concluded that ginsenoside profiles in seed-derived plants were identical to those in plantlets derived from tissue culture. This efficient method for multiplication of American ginseng could be used to avoid problems associated with seed stratification and germination and heterogeneity of *Panax* plants [21].

Most callus and cell suspension cultures described are grown in medium with exogenously applied auxin. However, auxin can have a negative impact on the biosynthesis of specific classes of secondary metabolites like anthraquinones [28]. A solution to this problem would be the production of callus and cell cultures without exogenous application of synthetic auxins [22, 29, 30].

Elicitation of Saponins

As mentioned above, plant cell culture technology is a promising alternative to field cultivation for the production of complex plant-specific metabolites, like saponins. However, low production titer could prevent commercial application.

Saponins are phytoprotectants which are either produced upon a stimulus conveyed by the pathogen or produced in a developmentally controlled fashion. Inducible phytoprotectants are known as *phytoalexins*, while constitutive phytoprotectants are called *phytoanticipins*. Phytoanticipins occur constitutively in healthy plants before challenge by microorganisms or other stresses. Some phytoanticipins are found on the plant surface; others are sequestered as preformed compounds in vacuoles or organelles and released through a hydrolyzing enzyme after pathogen challenge [12]. In contrast, phytoalexins are not present in healthy plants but are synthesized in response to pathogen attack or stress as part of the plant defense response and are restricted to the tissue colonized by the fungus and the cells surrounding the infection site [11].

These defense responses can be activated through a signal transduction pathway via recognition of an "elicitor" by the receptors located in the plasma membrane and formation of secondary messengers, such as jasmonates, ethylene, and salicylic acid, which in turn activate the expression of defense genes, including genes that code for enzymes catalyzing the formation of secondary metabolites [31].

Elicitors

The term "elicitor" is very general and refers to chemicals from various sources, biotic or abiotic, as well as physical factors, that can trigger a response in living organisms resulting in accumulation of secondary metabolites.

Methyl jasmonate (MeJA) is a widely used elicitor which modulates many physiological events in higher plants, such as defense responses, flowering, and senescence and because of that is regarded as a new class of phytohormones. MeJA and its derivatives have been proposed to be key signaling compounds in the process of elicitation leading to the accumulation of secondary metabolites [32]. Recently, unconventional synthetic elicitors such as 2-hydroxyethyl jasmonate (HEJ) were also found to be very powerful in eliciting plant secondary metabolites in cell cultures [33]. Salicylic acid (SA) is another elicitor that is widely studied as a stress signaling molecule involved in pathogen responses in plants. The importance of salicylic acid in the signal transduction pathway of disease resistance has been well documented [34]. MeJA and SA are the most important elicitors used as inducers of triterpene saponin production. To a lesser extent, yeast extracts, phytohormones, and heavy metals are also used.

Changes in Saponin Production after Elicitation

Elicitation of triterpene saponins is studied for the greater part in *P. ginseng* (Table 1). MeJA in a concentration of 0.2 mM increases ginsenoside production four times in adventitious roots compared with the control [35] and 1.8–3.1 times in cell suspensions [33, 36–38]. In response to 0.2 mM SA, three times more saponins were produced in *P. ginseng* adventitious roots and HEJ increased ginsenoside production 2–4.4 times in cell suspensions [33, 35, 38]. In *Glycyrrhiza glabra*, elicitation was tested in the roots of whole plants, and it was found that 2 mM of MeJA and 1 mM of SA could increase saponin production 3.8 and 4.5 times, respectively (Table 1) [39]. Also for *C. asiatica,* whole plants

Table 1 Overview of elicitation strategies for triterpene saponins described in literature

Plant species	Culture system	Elicitor treatment			Saponin Increase	Reference
		Elicitor	Concentration	Duration		
P. ginseng	AR in bioreactor	MeJA	0.2 mM	7 d	4.0×	[35]
		SA	0.2 mM	7 d	3.0×	
P. ginseng	AR	IBA	0.025 mM	10 d	1.6×	[36]
P. notoginseng	CS	MeJA	0.2 mM	4 d	3.0×	[33]
		HEJ	0.2 mM	4 d	2.0×	
P. notoginseng	CS in bioreactor	MeJA	0.2 mM	4 d	2.6×	[36]
P. notoginseng	CS	MeJA	0.2 mM	15 d	2.6×	[37]
	CS in bioreactor	MeJA	0.2 mM	15 d	1.8×	
P. notoginseng	CS	MeJA	0.2 mM	14 d	3.1×	[38]
		HEJ	0.2 mM	14 d	4.4×	
G. glabra	Roots of whole plants	MeJA	2 mM	24 h	3.8×	[39]
		SA	1 mM	24 h	4.5×	
C. asiatica	Whole plants	MeJA	0.01 mM	7 d	1.5×	[41]
		YE	0.1 g/l	7 d	1.4×	
	Leaves of whole plants	MeJA	0.01 mM	36 d	3.5×	
C. asiatica	Aerial part of whole plants	MeJA	0.1 mM	35 d	2–3×	[26]
	Roots of whole plants	MeJA	0.1 mM	35 d	4–6×	

MeJA Methyl jasmonate, *HEJ* 2-hydroxyethyl jasmonate, *YE* Yeast extract, *AR* Adventitious roots, *CS* Cell suspension, *SA* Salicylic acid, *IBA* indole-3-butyric acid

were used and highest yields were reached with 0.01 mM MeJA in the leaves of whole plants and 0.1 mM in the roots of whole plants. Elicitation with 1 g/l yeast extract increased saponin production 1.4 times in whole plants of *C. asiatica,* which was approximately the same with elicitation with 0.01 mM MeJA (Table 1). For elicitation in cell cultures of the medicinal plant *Tabernaemontana catharinensis*, different biotic elicitors were tested. Addition of cell wall homogenates to the cell cultures, no matter the concentration or type of microorganism source tested, resulted in increased triterpene levels. Among elicitors tested, *Saccharomyces cerevisae* showed the best results in terms of triterpene accumulation [40].

In general, it was also found that the elicitation of saponins was less effective in bioreactors compared with small-scale cultures. Wang et al. [37] studied the difference in ginsenoside production in *Panax notoginseng* cell cultures in flasks and bioreactors. In flasks, there was a sharp increase in total ginsenoside content from day 8 to day 15 after elicitation with a maximum on day 15, which was 2.6 times that of the control. In contrast, cells grown in bioreactors showed a slow increase in saponin production with a maximum on day 15 with 1.8-fold the concentration of the control. However, a repeated elicitation, 5 days after the first addition of MeJA, combined with sucrose feeding was suitable for the bioreactor cultivation of *P. notoginseng* cells for the hyper-production of ginsenosides [37].

Elicitation with MeJA or SA is often accompanied with a decrease in growth of the tissues. For *P. ginseng* adventitious root cultures, it was found that there was a severe decrease in biomass after incubation for longer than 9 days in 0.2 mM MeJA or SA [35]. Therefore, sometimes, phytohormones are simultaneously added with the elicitor. Kim et al. [41] proved that addition of 0.025 mg/l thidiazuron could prevent the negative effects of

MeJA on whole-plant growth and increased saponin production more than MeJA alone in whole plants. However, this increase in whole plants was probably due to a gain in biomass rather than a stimulation of secondary metabolism. Because, if only mature leaves were considered, there was no further increase in saponin content in addition to MeJA [41].

In some cases, it is also reported that elicitor treatment of in vitro cultures not only increases the saponin production but additionally alters the stoichiometry of the precursors and final products. This is the case, for example, upon elicitation in large-scale cell cultures of *P. notoginseng* with 200 μM MeJA or HEJ [37, 38].

Upregulation of Saponin Biosynthesis Genes in Response to Elicitation

Elicitation does not only have an effect on the saponin content but likewise also influences the expression of saponin biosynthesis genes. *Medicago truncatula* is a model species for plant functional genomics and produces at least five different triterpene aglycons. In addition, four putative early enzymes of triterpene biosynthesis were identified: SS, βAS, and two squalene epoxidases (SE1 and SE2). Therefore, *M. truncatula* is a good tool to study the behavior of saponin biosynthesis genes after elicitation. Suzuki and coworkers [42, 43] found that addition of abscisic acid, and SA to *Medicago* cell cultures had no effect on βAS, SS, and SE transcript level, and addition of yeast extract only had a week's effect. In contrast, increasing concentrations of MeJA resulted in an accumulation of βAS transcripts, with a maximum of over 50 times increase, 24 h after exposure to 500 μM MeJA. SS was coordinately induced. SE1 transcripts were not significantly induced, but SE2 transcript induction closely followed βAS expression profile, indicating that SE2 but not SE1 may function specifically in the formation of triterpenoids [42, 43].

Genetic studies were also performed in *P. ginseng.* In this system, SS and SE transcription levels increased nine to six times, respectively, 24 h after elicitation with 200 μM HEJ. Total ginsenoside increased from 12 h to 10 days after addition of HEJ or MeJA. Upon addition of the jasmonate inhibitor DIECA (NA-diethyldithiocarbamate), there was a decrease in both jasmonic acid production and ginsenoside content. DIECA also inhibited the HEJ-induced upregulation of SS and SE, suggesting that induction of saponins is dependent on a jasmonate signal transduction [33].

In *P. notoginseng* cell cultures, there is an increase in enzyme activity of UGRdGT, a glucosyltransferase that catalyzes the biotransformation from Rd1 into Rb1, two of the major saponins in *P. ginseng* [37]. In contrast, ginsenoside-α-arabinofuranase, which hydrolyses ginsenoside Rc into Rd was not detected. These results suggest that a biosynthesis pathway from Rd to Rb1 exists in the cell line and MeJA can activate this pathway by inducing UGRdGT activity [37]. These results were confirmed by Hu & Zhong [38], who found an increase in UGRdGT activity following elicitation with MeJA or HEJ, and this coincided with a higher content of ginsenoside Rb1.

Metabolic Engineering

Overexpression of Key Enzymes

Triterpenoid saponin backbones are synthesized via the isoprenoid pathway through a largely unidentified number of sequential enzymatic steps. Squalene is the precursor for triterpene saponins and is produced from mevalonic acid through a series of enzymatic reactions with GPS, FPS, and squalene synthase (SS) [44]. Subsequently, squalene epoxidase

transforms squalene into 2,3-oxidosqualene. The cyclization of 2,3-oxidosqualene into β-amyrin, α-amyrin, and dammarenediol is catalyzed by oxidosqualene cyclases (OSCs). More than 40 of these cyclases have been cloned from higher plants. Some of them have been functionally analyzed and characterized including β-amyrin synthase, α-amyrin synthase, and dammarenediol-II synthase [20, 45]. The triterpenoid skeleton then undergoes various modifications, such as oxidation, substitution, and glycosylation, also mediated by different enzymes. However, many of the enzymes involved in the later steps of saponin biosynthesis are not yet known or characterized. An increased production of triterpenoid saponins could be achieved by overexpression of key enzymes that interfere with saponin biosynthesis pathway.

Farnesyl Diphosphate Synthase

FPS has not previously been identified as a key regulatory enzyme in triterpene biosynthesis; however, Kim and coworkers [46] investigated the role of FPS in triterpene biosynthesis in *C. asiatica.* This plant was transformed with a construct harboring *P. ginseng FPS* (PgFPS)-encoding cDNA coupled to the cauliflower mosaic virus 35S promoter (p35S). CaDDS (*C. asiatica* dammarenediol synthase) and CaCYS (*C. asiatica* cycloartenol synthase) mRNA showed high expression in all transgenic hairy root lines when compared with the controls. However, there is no change in expression profile of CaSQS (*C. asiatica* squalene synthase), which is upstream of both CaDDS and CaCYS. The upregulation of CaDDS transcripts suggests that FPS may be an important enzyme in triterpene saponin production, which is reflected in enhanced saponin levels in transgenic lines (up to 1.5 times). Interestingly, the upregulated levels of CaCYS also correlated with increase in total sterol contents, which were time times higher than those of the controls. Therefore, these results indicated that overexpression of FPS is useful to enhance not only triterpene saponins, but also phytosterol production in plants [46].

Squalene Synthase

SS catalyzes the first committed step toward sterol and triterpenoid biosynthesis [47]. The important roles of SS genes in the regulation of triterpene and phytosterol biosynthesis have been reported in several plants such as *G. glabra* [48], *Nicotiana tabacum* [49], *P. ginseng* [50] and many other [51–54]. In *N. tabacum* and *P. ginseng*, SS transcript is mostly accumulated in apexes, compared with that in other aerial organs [49, 50]. In *G. glabra* cell cultures, SS expression during the logarithmic growth phase is higher than that in the stationary phase [48]. These reports suggest that SS is mainly expressed in organs in which cell proliferation is active.

The overexpression of *PgSS1* gene in adventitious roots of transgenic *P. ginseng* was followed by an upregulation of the downstream genes, such as squalene epoxidase, dammarenediol synthase, β-amyrin synthase, and cycloartenol synthase [50]. These results indicate that *PgSS1* is a key regulatory enzyme for both phytosterol and triterpenoid saponin biosynthesis. The *PgSS1* gene derived from *P. ginseng* has also been introduced into Siberian ginseng (*Eleutheroccous senticosus*) [53]. The transgenic *E. senticosus* plant showed enhanced production of phytosterol (β-sitosterol and stigmasterol). In addition, triterpene saponin (ciwujianosides B, C_1, C_2, C_3, C_4, D_1, and D_7) levels also increased by two- to 2.5-fold.

In contrast, triterpenes of euphol and β-amyrin were almost undetectable by over-expression of SS in *Euphorbia tirucalli* callus. On the other hand, there was a noticeable increase in the total accumulation of phytosterol. This result suggests that *EtSS* products are directly driven to the sterol-synthesis pathway [54].

Squalene Epoxidase

Squalene epoxidase (SE), also known as squalene monooxygenases, catalyzes the first oxygenation step in phytosterol and triterpenoid saponin pathway. Plants, so far examined, seem to have two or more copies of SE genes. In *Arabidopsis*, six isoforms of SE have been identified, suggesting that SE genes have different isoform-dependent functions [55]. In *Medicago truncatula* cell cultures, one squalene epoxidase gene (SE2) was upregulated by treatment with methyl jasmonate (MeJA) in contrast to SE1 [42].

The roles of two squalene epoxidase genes (*PgSQ1* and *PgSQ2*) have been studied in *P. ginseng* [56]. Amino acid sequences deduced from *PgSQ1* and *PgSQ2* share 80% homology, but the N-terminal region (first 60 amino acids) are highly divergent. *PgSQ1* was abundantly accumulated in all organs, while *PgSQ2* was weakly expressed in petiole and flower buds. RNAi of *PgSQ1* in transgenic *P. ginseng* completely suppressed *PgSQ1* expression, thus reducing ginsenoside production. Interestingly, silencing of *PgSQ1* strongly upregulated *PgSQ2* and cycloartenol synthase and resulted in enhanced phytosterol accumulation. These results indicate that expressions of *PgSQ1* and *PgSQ2* are regulated differently. Furthermore, *PgSQ1* only regulates ginsenoside biosynthesis, and not that of phytosterol in *P. ginseng*. Therefore, overexpression of *PgSQ1* could be useful to increase the production of ginsenoside in *P. ginseng*.

β-Amyrin Synthase

The most studied triterpenes found in higher plants are those from the oleanane type (β-amyrin) followed by ursane (α-amyrin) and dammarane (dammaranediol) types [20]. In addition, it is already well known that the first committed step in saponin biosynthesis in plant involves the cyclization of 2,3-oxidosqualene into the saponin types mentioned above. These conversions are catalyzed by specific OSCs including β-amyrin synthase. The genes encoding β-amyrin synthase have been cloned and characterized from different plant species such as *P. ginseng* [57], *Pisum sativum* [58], *G. glabra* [59], *Avena strigosa* [60], *M. truncatula*, and *Lotus japonicus* [61], *Betula platyphylla* [62], *Euphorbia tirucalli* [63], *Saponaria vaccaria* [64], *Aster sedifolius* [65], *Nigella sativa* [66], *Arabidospis thaliana* [20], and *Gentiana straminea* [67].

The effect of ectopic expression of *AsOXA1* cDNA, a β-amyrin synthase from *Aster sedifolius* has been evaluated on the production of saponin in *M. truncatula* [68]. Apparently, one of the four transgenic lines expressing *AsOXA1* has significantly accumulated larger amounts of triterpenoid compounds in leaves and roots compared with that in control plants. Particularly, there was a high level of bayogenin, medicagenic acid, and zanhic acid in leaves, while roots significantly accumulated bayogenin, hederagenin, soyasapogenol E, and 2β-hydroxyoleanolic acid. The increase in the total amounts of triterpenoid saponin observed in leaves of transgenic lines correlated with *AsOXA1* expression level. Furthermore, the plants expressing *AsOXA1* not only produced higher level of saponin, but also improved root nodulation. It is suggested that the high-nodulating phenotype could be associated with the significant increase in saponin production in the root. A similar effect has also been reported in *G. glabra* [69]. A high level of β-amyrin synthase mRNA has been found in the thickened main roots and root nodules. These results were consistent with high-level accumulation of soyasaponin in root nodules and that of glycyrrhizin in the thickened main roots. However, no detailed information is available on the effect of β-amyrin synthase expression on plant growth and development.

β-amyrin synthase is one of OSCs located at the branch point for sterol (primary metabolism) and triterpene saponin (secondary metabolism) biosynthesis, therefore

knowledge on the regulation of β-amyrin synthase will be critical to understand the regulation, mechanism, and physiology of secondary metabolism. So far, there is one report [68] on the manipulation of the triterpene saponin pathway based on the expression of β-amyrin synthase encoding gene. Since the specific product of β-amyrin synthase is one of the key precursors of triterpene saponin pathway, overexpression of β-amyrin synthase is likely to yield an increase saponin production.

Cytochrome P450

The aglycons of triterpene saponins are C30 compounds produced by the cyclization of 2,3-oxidosqualene. Subsequent oxidations of the triterpene skeleton produce structural diversity, and these oxidations are thought to be catalyzed by cytochrome P450 monooxygenases (P450s). Numerous P450s have been suggested to be involved in the biosynthesis and metabolism of triterpenoid saponin [70]. Among them are CYP93E1, from *Glycine max* with dual β-amyrin-24-hydroxylase and sophoradiol-24-hydroxylase activities [71] and CYP88D6, from *G. glabra* with β-amyrin-11-oxidase activity [72]. The 24-hydroxylase activity from CYP93E1 transformed both β-amyrin and sophoradiol into olean-12-ene-3β,24-diol and soyasapogenol B, respectively. Soyasapogenol B from *G. max* was also found to be synthesized from sophoradiol by hydroxylation of the C-24 methyl group by CYP93E1 and hydroxylation of olean-12-ene-3β,24-diol at the C-22 position catalyzed by other P450, probably an enzyme similar to CYP93E1 (Fig. 3a). In addition, CYP88D6 catalyzed two-step reactions from β-amyrin to 11-oxo- β-amyrin through C-11 oxidation. Later on, glycyrhetinic acid is produced by oxidation at C-11 and C-30 (Fig. 3a).

Several studies have been performed to elucidate the avenacin pathway, triterpene saponins produced by oats (*Avena* spp.). Genetic analysis of saponin-deficient (*sad*) mutants revealed nine loci involved in avenacin biosynthesis, eight of which are clustered and only *Sad 4* that is unlinked [15]. Qi and coworkers [73] have investigated the *sad2* mutant and showed that it is a cytochrome P450 designated CYP51H10. The CYP51H10 enzyme catalyzes the conversion of β-amyrin to avenacin (Fig. 3a) and its expression is restricted to the root epidermis [73]. This result correlated with the synthesis of avenacin A-1, which is tightly regulated and is restricted to the epidermal cell of root tips. However, the catalytic function of CYP51H10 has not been clearly characterized. The CYP51H10 belongs to the CYP51 sterol demethylase family. This enzyme is highly conserved in the synthesis of essential sterols, only found in monocots and is regarded as the most ancient cytochrome P450 family. These data indicated that, in oats, CYP51H10 has been evolved from CYP51 family to adapt to triterpene molecules.

In *P. ginseng*, ginsenosides are presumably synthesized from dammarenediol-II after hydroxylation by cytochrome P450 [71]. Cytochrome P450 members in *P. ginseng* are suggested to be involved in the hydroxylation of the C-12 position of dammarenediol for protopanaxadiol synthesis and the C-6 position of protopanaxadiol for protopanaxatriol synthesis (Fig. 3b). Both of these compounds are used as backbones for ginsenosides.

The advancement of DNA sequencing techniques has generated a great opportunity to identify a multitude of candidate P450 gene sequences. The genomic studies of model plant system provide a future basis for discovering novel P450 functions. In comparison to the availability of sequence information, only limited biochemical characterization has been achieved so far [70, 74]. Eventually, the objective of these P450s studies should be a global annotation that describes the enzymatic activity of each gene.

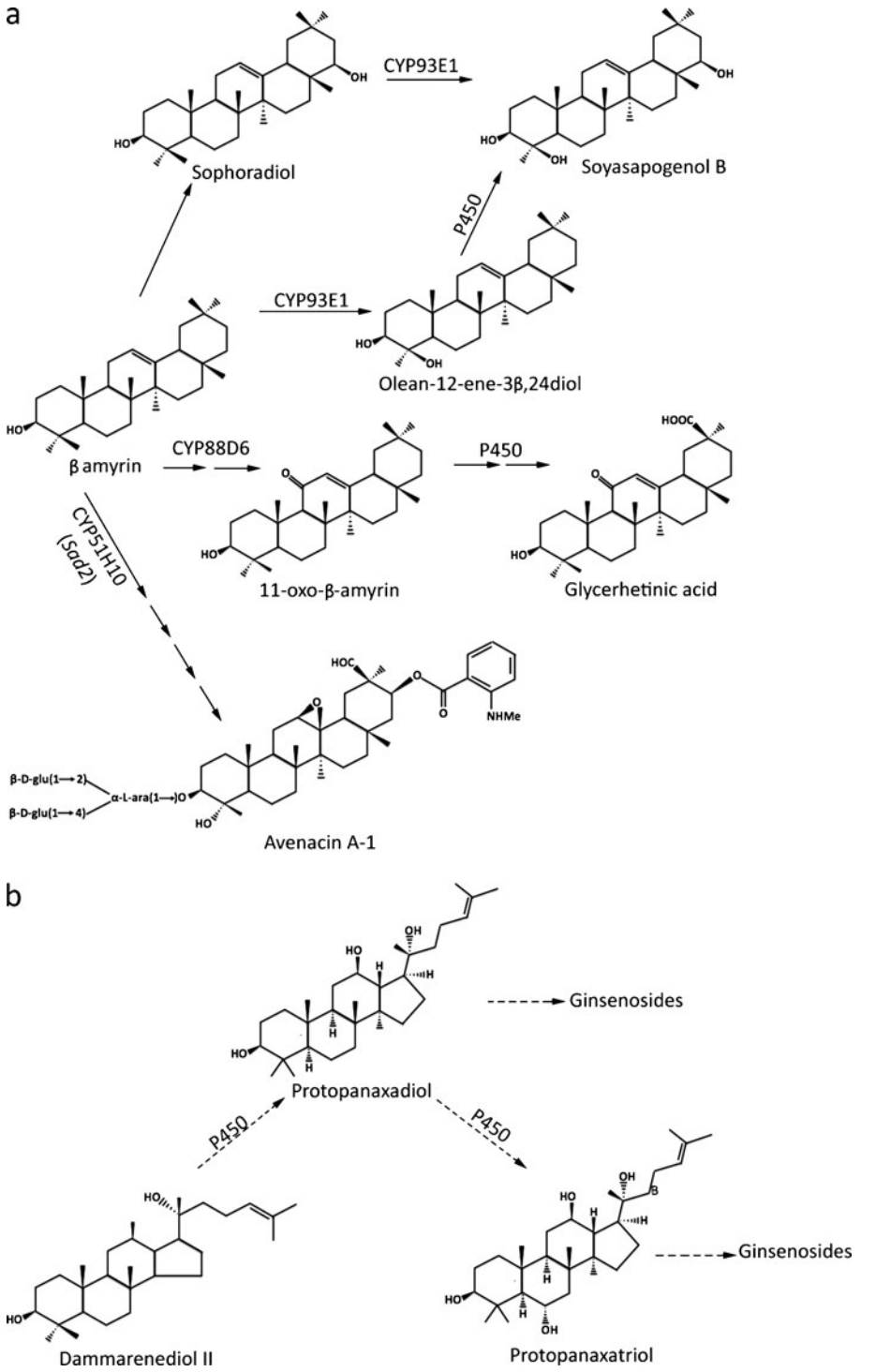

Fig. 3 The role of P450s in triterpene saponin biosynthesis. (*Sad2* saponin-deficient 2, *Glu* glucose, *Ara* arabinose)

Glycosyltransferase

Saponin diversity is generated by a sequence of three biosynthetic reactions, the cyclization of a common precursor, oxidosqualene, followed by oxidation, and glycosylation. In fact, glycosylation generates more of structural diversity of triterpene saponin in plant than does cyclization and oxidation [75, 76]. However, identification of sugar transferases involved in saponin biosynthesis remains a problem due to large number of candidate genes and the structural complexity of the sugar chain present in triterpene saponins.

Recently, six glycosyltransferases (GTs) have been functionally characterized: UGT71G1, UGT73K1 [77], and UGT73F3 [78] from *M. truncatula*; UGT74M1 from *S. vaccaria* [64]; and UGT73P2 (GmSGT2) and UGT91H4 (GmSGT3) from *G. max* [79]. UGT71G1 has specificity for medicagenic acid and also some flavonoids, while UGT73K1 specifically has shown glucosyltransferase activity for hederagenin and soyasapogenol B and E. Genetic loss of function analysis of UGT73F3 has indicated that glucoside produced by the action of UGT73F3 on hederagenin as sugar acceptor is a C-28 ester. In addition, analysis of an expressed sequence tags library of *S. vaccaria* revealed that UGT74M1 is a triterpene carboxylic acid glucosyltransferase. UGT74M1 is expressed in roots and leaves and appears to be involved in vaccaroside biosynthesis in *S. vaccaria*. UGT71G1, UGT73K1, UGT73F3, and UGT74M1 are primary GTs that transfer one sugar molecule to a triterpene aglycon to yield a triterpene saponin bearing a monosaccharide (Fig. 4a).

Further study was conducted on *G. max* to unravel how the sugar moiety of triterpene saponins with two or more saccharides is configured [79]. Identification of UGT73P2 (UDP-galactose/soyasapogenol B monoglucuronide (SBMG)-galactosyltransferase) and UGT91H4 (UDP-rhamnose/soyasaponin III-rhamnosyltransferase) showed that these GTs are involved in soyasaponin biosynthesis and they can yield triterpene saponins bearing a disaccharide and trisaccharide, respectively. Remarkably, this is the first report of GTs that transfer the second and third sugar to the triterpene saponin biosynthesis. The results also indicate that the sugar chain moiety of triterpene saponins is biosynthesized by successive sugar transfer reactions to aglycons (Fig. 4b).

Typically, the identification of GTs is based on the presence of the plant secondary product glucosyltranferase (PSPG box) sequence, a conserved carboxy-terminal sequence involved in binding of the UDP moiety of the sugar nucleotide to the enzyme [80]. However, there are no reliable methods to identify substrate specificity of plant GTs based on sequence similarity alone [81]. Recently, Naoumkina and coworkers [78] have determined the candidate GTs for triterpene saponin biosynthesis by comprehensive clustering analysis of transcript and metabolite profiles. They have focused on the transcript profiles of a large number of GTs genes tightly clustered with β-amyrin synthase in regards to both tissue-specific and elicitor-inducible expression. These results will be very helpful to identify and to select the remaining of additional GTs involves in saponin biosynthesis.

Combinatorial Biosynthesis

Current development of plant metabolic engineering indicates that combining the genes from different organisms could be important to generate the production of valuable compounds. The approach to combine genes from different organism for the production of new and interesting metabolites is referred to combinatorial biosynthesis. This is a new strategy for the generation of novel secondary metabolites, as well for the production of rare natural products [82–85]. Combinatorial biosynthesis is not limited to the introduction of a

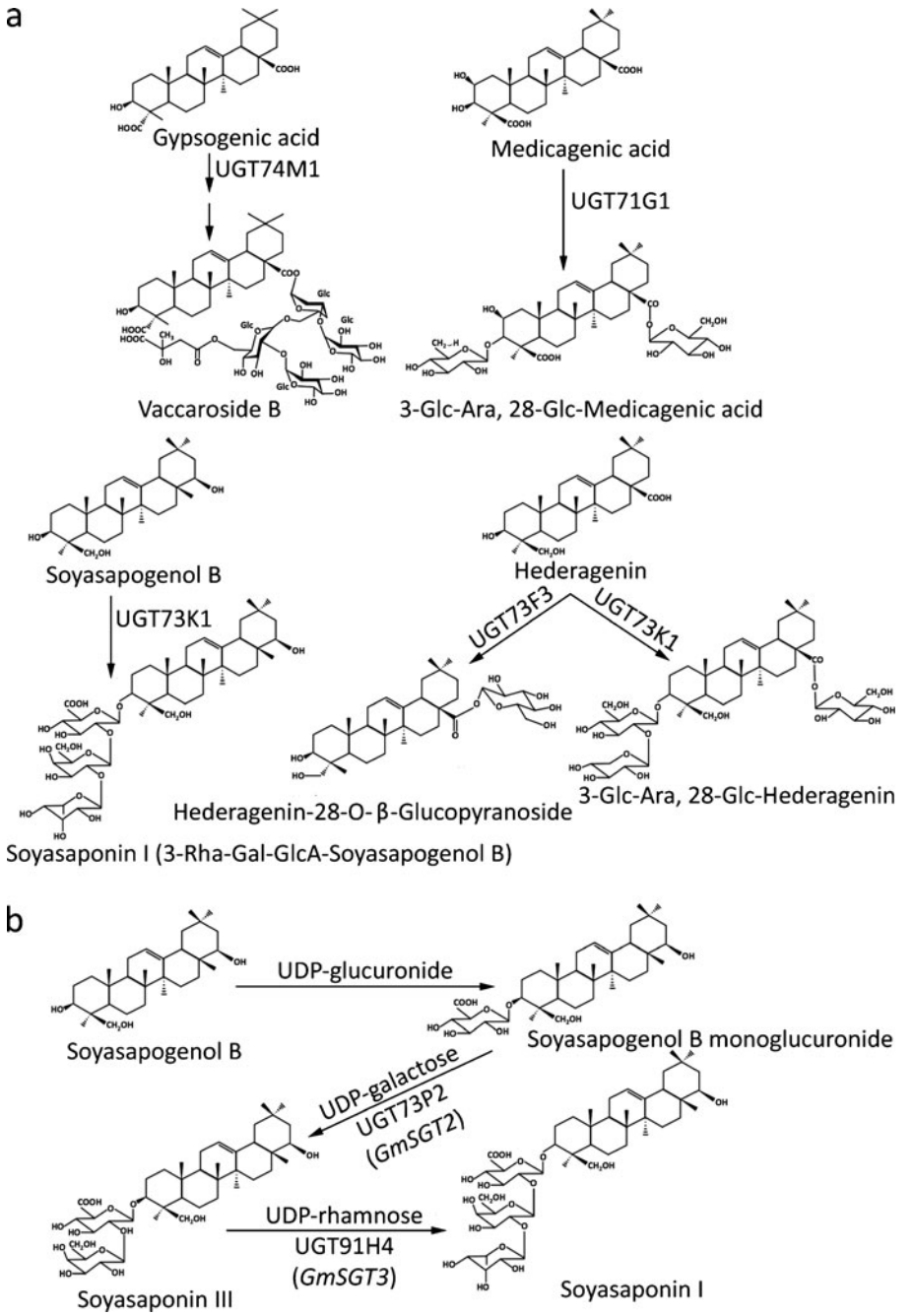

Fig. 4 Attachment of sugar moieties to the saponin backbone by glucosyltransferases (*GT*). (*Glc* glucose, *Ara* arabinose, *Rha* rhamnose, *Gal* galactose, *UDP* uridine diphosphate, *GlcA* glucuronic acid)

single gene but can also include a series of genes with the aim to reconstruct a complete biosynthesis pathway that can impinge on an existing biosynthetic pathway in the host.

The introduction of building blocks can be achieved by recruiting different biosynthesis steps for the assembly of engineered pathways in host system of choice, using new enzyme-substrate combinations. A combinatorial biosynthesis approach in microorganisms did prove successful to generate novel antibiotics. Such a strategy has not yet been implemented into a higher plant system because of the poor background knowledge of secondary metabolism and because of practical hurdles such as a slow life cycle compared with microorganisms. At the moment, combinatorial biosynthesis of plant secondary metabolites focuses on the reconstruction of the basic pathways into microbial hosts. Recent achievements with the plant polyketide biosynthesis by microorganisms, especially in *Escherichia coli*, demonstrated the utility of combinatorial biosynthesis [86].

There are not yet reports about new structure of triterpene saponins based on combinatorial biosynthesis in plants. However, there are ample possibilities by which one could modify the basic structure of saponins in a given host species. As a hypothetical example, by introducing the dammarenediol synthase from *P. ginseng* (*PgDDS*) into *M. truncatula*, a hybrid-type saponin would be created (Fig. 5). Provided that combinatorial saponins are produced, the identification of saponin biosynthesis genes from different species will become more and more important in the future.

Concluding Remarks

The vast increase in saponin-related literature reflects the growing interest in these secondary metabolites. However, despite the many beneficial pharmacological properties of

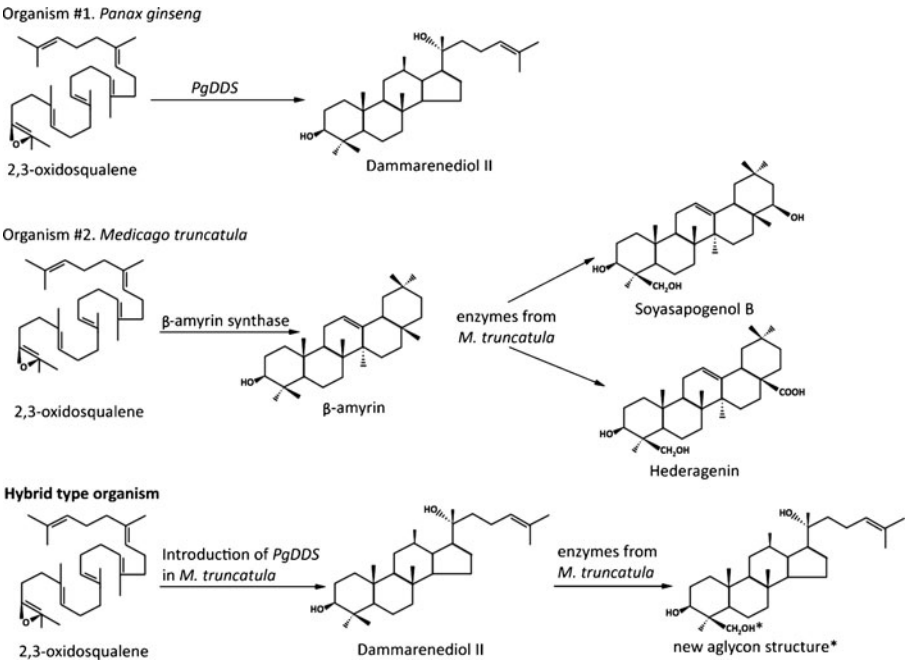

Fig. 5 Example of a hypothetical combinatorial biosynthesis strategy for triterpene saponins. (*PgDDS P. ginseng* dammarenediol synthase)

saponins, commercialization of novel saponin structures is still rare. One of the main problems is that extraction from wild plants is difficult because saponin yields are often low and dependent on many environmental factors. In vitro culture is a valuable alternative because plants can be propagated clonally and are grown under controlled conditions. Metabolite yield in in vitro plants, tissue, or cell cultures can be increased through optimization of the culture medium or through the addition of elicitors. In particular, methyl-jasmonate has been shown to increase saponin production; yet, in most plants, the synthesis of saponins is developmentally regulated.

Recent research effort is being put in elucidation of the saponin biosynthesis pathway. The genetic identification of genes and the functional analysis will provide the information required to modulate saponin production in a much more selective and profound way. Overexpression of key enzymes has already been shown to allow higher saponin production levels. Additionally, gene identification and characterization will provide novel opportunities to engineer structure function relationships of medically important saponins.

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