Chapter 2: Literature review

Among the quarter million plant species that have been reported, only a fraction has been chemically investigated (Brielmann et al., 2006). They produce primary metabolites that are involved in their growth and development. They also produce some metabolites known as secondary metabolites (SMs) to protect themselves from the harsh environment like extreme weather, pathogens, pests and herbivores as well as attract beneficial organisms like pollinators which help them in reproduction and thus in propagation and sustenance of the species (Bourgaud et al., 2001; Cseke et al., 2006b; Taiz and Zeiger, 2002; Dixon, 2001). Some of these SMs have been useful to humankind for various purposes and are used as medicines, health supplements, food, dyes, etc (Table 2.1). For example, artemisinin is used as an antimalarial drug (Qinghaosu Antimalaria Coordinating Research Group., 1979; Guo, 2016b; Tu, 2011), paclitaxel is used in the treatment of cancer (McGuire et al., 1989; Ramalingam and Belani, 2004), shikonins and anthocyanins are used as dyes and food colorants (Jackman et al., 1987; Khoo et al., 2017; Papageorgiou et al., 1999), linalool and limonene are used as fragrance in perfumes and personal care products (Buckley, 2007) and vanillin is used as a flavouring agent (Sinha et al.,2008).

2.1. Classification of plant secondary metabolites

Plant secondary metabolites are grouped into three major categories: terpenes, phenolics and nitrogen-containing compounds (Croteau et al., 2000; Springob and Kutchan, 2009; Taiz and Zeiger, 2002). Terpenes are the largest group of secondary metabolites and are made up of five-carbon unit called isoprene (Croteau et al., 2000). They are also called isoprenoids. Structurally, they range from a simple molecule like geraniol to a complex molecule with thousands of isoprene units like rubber. Based on the number of isoprene units, the terpenes are further classified into several types as given in table 2.2. For example, aromatic molecules like menthol, limonene and camphor are monoterpenes with two isoprene units (C_{10}), artemisinin and farnesol are diterpene with three isoprene units (C_{15}), paclitaxel and ingenol mebutate are diterpenes (C_{20}), leucosesterterpenone and leucosterlactone are sesterterpenes (C_{25}), ginsenosides and glycyrrhizin are triterpenes (C_{30}) while β-Carotene and abibalsamin A are tetraterpenes (C_{40}) (Table 2.2). Rubber and gutta-percha with numerous isoprene units are

Table 2.1 Examples of plant secondary metabolites useful to humankind

Secondary metabolites	Plant	Uses	Reference
Artemisinin	*Artemisia annua*	Malaria treatment	Qinghaosu Antimalaria Coordinating Research Group., 1979; Guo, 2016; Tu, 2011
Paclitaxel	*Taxus* species	Breast, ovarian and lung cancer treatment	McGuire et al., 1989; Nowak et al., 2004; Ramalingam and Belani, 2004;
Ingenol mebutate	*Euphorbia ingens*	Actinic keratosis (non melanoma skin cancer) treatment	Keating, 2012; Ramsay et al., 2011
Cocaine	*Erythroxylum coca, E. novogranatense*	Topical anaesthesia	Biondich and Joslin, 2016; Kennedy et al., 2004; Tayeb et al., 2017
Morphine	*Papaver somniferum*	Analgesic	Fabricant and Farnsworth, 2001; Sverrisdóttir et al., 2015
Quinine	*Cinchona ledgeriana*	Malaria treatment	Achan et al., 2011; Sverrisdóttir et al., 2015
Salicin (used for synthesis of salicylic acid)	*Salix spp.*	Analgesic, antipyretic, anti inflammatory	Brodniewicz and Grynkiewicz, 2012; Mahdi, 2010
Llimonene	*Citrus spp.*	Perfumes and personal care products	Buckley, 2007; Erasto and Viljoen, 2008
Vanillin	*Vanilla planifolia, V. tahitensis, V. pompona*	Flavouring agent	Sinha et al.,2008; Ni et al., 2015

examples of polyterpenes. Like other secondary metabolites, they serve diverse functions such as defence against herbivores and pathogens and many of them also attract insects and birds for pollination and animals for seed dispersal (Ashour et al., 2010; Taiz and Zeiger, 2002). Moreover, like other types of secondary metabolites, many terpenes are also used as medicines (Table 2.2). Some terpenes are used as falvouring agents in foods and beverages while others are used as fragrances in perfumes, soaps and toothpastes (Caputi and Aprea, 2011; Croteau et al., 2000).

Phenolics are secondary metabolites that contain phenol group in their structures (Crozier et al., 2006; Taiz and Zeiger, 2002). Phenolics range from a low molecular weight simple compounds with a single aromatic ring like *trans* – cinnamic acid and *p* – coumaric acid to a large and complex compounds like lignins and tannins (Taiz and Zeiger, 2002). Phenolics are further classified into flavonoids and non-flavonoids. Flavonoids contain two aromatic rings and a three-carbon bridge with a total of fifteen carbon atoms. They are present in leaf epidermis and fruit skins and are responsible for pigmentation, UV protection and disease resistance. They are further classified into the flavones, flavonols, anthocyanins,

Table 2.2 Types of terpenoids based on number of isoprene units

Terpene type	No. of isoprene units/No. of carbon atoms	Examples	References
Hemiterpenes	01/C_5	Isoprene, isovaleraldehyde, tiglic acid, angelic acid	Croteau et al., 2000; Cseke et al., 2006
Monoterpenes	02/C_{10}	Camphor, menthol, limonene, α-pinene	Croteau et al., 2000; Cseke et al., 2006; Taiz and Zeiger, 2002
Sesquiterpenes	03/C_{15}	Artemisinin, abscissic acid, farnesol	Cseke et al., 2006; Guo et al., 2016b
Diterpenes	04/C_{20}	Paclitaxel, ingenol mebutate, gibberellins	Cseke et al., 2006; Taiz and Zeiger, 2002; Fallen and Gooderham, 2012
Sesterterpenes	05/C_{25}	Leucosesterterpenone, leucosterlactone	Evidente et al., 2015
Triterpenes	06/C_{30}	Ginsenosides, glycyrrhizin	Kim et al., 2009c; Wang et al., 2012; Yang et al., 2016
Tetraterpenes	08/C_{40}	β-Carotene, lycopene	Cseke et al., 2006
Polyterpenes	>08/>C_{40}	Rubber, gutta-percha	Cseke et al., 2006; Karliati et al., 2014

flavanones, isoflavones and anthocyanidins (Crozier et al., 2006). Non-flavonoids are also further grouped into phenolic acids, hydroxycinnamates and stilbenes. Salicylic acid, rosemarinic acid, sinecatechins and tannins are notable examples of phenolics. Salicylic acid and sinecatechins have medical uses. Rosemarinic acid has anti-inflammatory, antibacterial, antiviral and anticarcinogenic properties (Nunes et al., 2017). Tannins are used in leather and wine industry (Ma et al., 2014; Maier et al., 2017).

Nitrogen containing compounds have nitrogen in their structures and are biosynthesized from the common amino acids (Cseke et al., 2006; Roberts et al., 2010; Taiz and Zeiger, 2002). This group includes alkaloids, cyanogenic glycosides, glucosinolates and non protein amino acids (Taiz and Zeiger, 2002). Some of the notable nitrogen containing compounds includes morphine, quinine, camptothecin, ephedrine, caffeine, nicotine and cocaine. Morphine, quinine, camptothecin, ephedrine and caffeine have medical uses while nicotine and cocaine are used for recreational purposes.

2.2. Biosynthesis of secondary metabolites

According to the broad classification, there are three basic types of secondary metabolite biosynthetic pathways: isoprenoid/terpenoid pathway, shikimate pathway and polyketide pathway (Figure 2.1) (Singh, 2011; Cseke et al., 2006; Lussier et al., 2012).

However, novel compounds are constantly being discovered and their biosynthesis is poorly understood. The precursors for these pathways are obtained from primary carbon metabolism. The cytoplasmic mevalonate (MVA) pathway and plastidial methylerythritol phosphate (MEP) pathways are the two precursor supplying pathways for the terpenoid biosynthesis (Hemmerlin et al., 2012; Vranová et al., 2013). The MEP and MVA pathways get their starting molecules from primary carbon metabolism (Hemmerlin et al., 2012). The end products of each of these pathways are IPP and DMAPP which further undergoes series of reactions to produce different types of terpenes. The shikimate pathway uses erythrose 4-phosphate of the nonoxidative branch of the pentose phosphate pathway and phosphoenolpyruvate (PEP) of the glycolysis and converts them into chorismate (Herrmann, 1999; Tzin et al., 2012). Chorismate is the precursor for numerous secondary metabolites called alkaloids and phenolics. Type III polyketide synthases (PKSs) make up the plant polyketide pathway (Flores-Sanchez and Verpoorte, 2009; Lussier et al., 2012). Bacteria and fungi have type I, type II and type III polyketide biosynthetic pathways made up by type I, type II and type III PKSs, respectively. The different types of PKSs differ on biochemical features and product structure (Flores-Sanchez and Verpoorte, 2009; Lussier et al., 2012). Acyl-CoAs are used for the polyketide biosynthesis. Some examples of plant polyketides include hypericin, hyperforin, naringenin and olivetolic acid (Flores-Sanchez and Verpoorte, 2009; Lussier et al., 2012).

The SMs are plant specific and therefore they are synthesized through specific secondary metabolic biosynthetic pathways. The focus of the present thesis is ginsenoside biosynthetic pathway which produces ginsenosides, which are triterpenoid saponins. MVA and MEP pathways supply precursors to the ginsenoside biosynthetic pathway. They are reviewed in the following sections of this chapter.

2.3. Ginsenosides

Ginsenosides are triterpene saponins found in several species of genus *Panax* and quantified in *P. ginseng* (Lee et al., 2017a, b), *P. notoginseng* (Li et al., 2005; Wang et al., 2014a), *P. quinquefolius* (Li et al., 1996; Popovich et al., 2012; Qu et al., 2009), *P. japonicus* (Kochkin et al., 2013; Morita et al., 1983), *P. vietnamensis* (Duc et al., 1994a, 1994b; Duc et al., 1993; Vo et al., 2015), *P. bipinnatifidu*s (syn. *P. sikkimensis*) and *P. sokpayensis* (Gurung et al., 2018). As of 2012, 289 ginsenosides had been reported from different species of *Panax* (Yang et al., 2014b). *P. ginseng* is known as the “king of herbs”

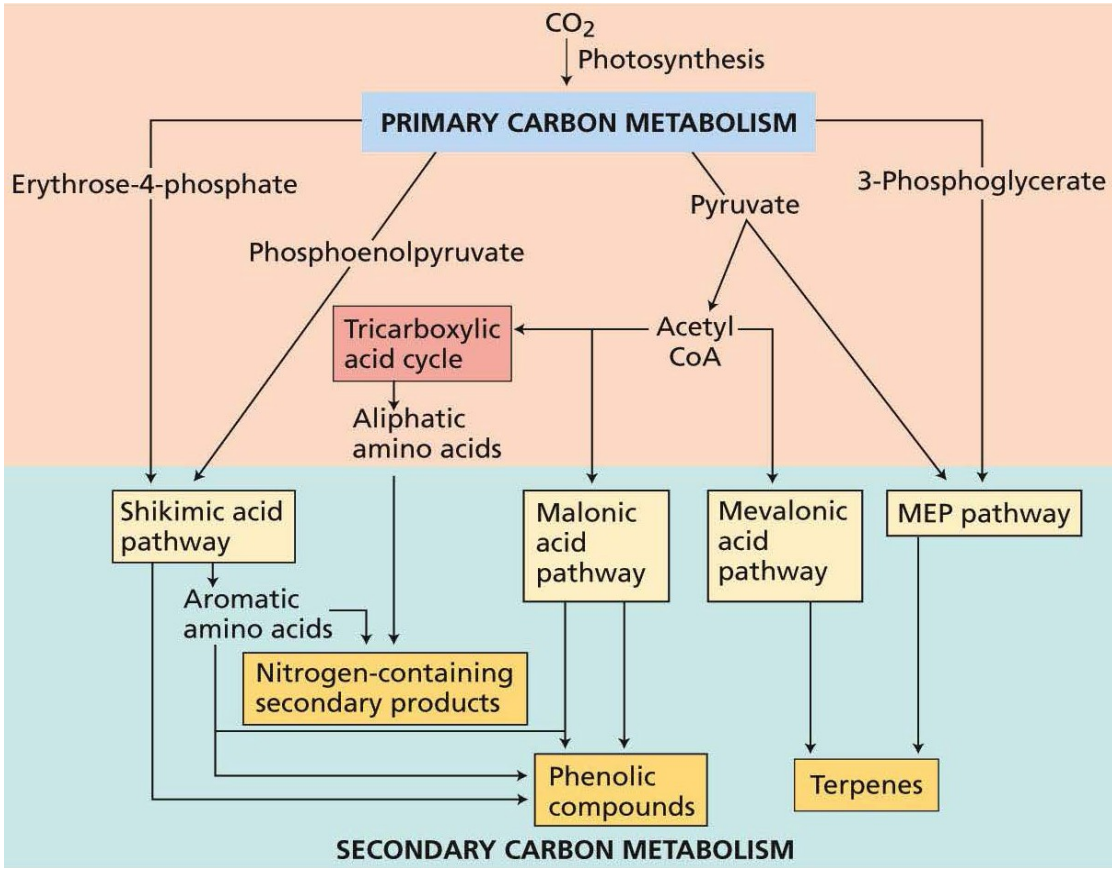


Figure 2.1 Secondary metabolic pathways in plants and their interconnections with primary metabolism (Taiz & Zeiger, 2002).

in Traditional Chinese Medicine (TCM) and has been used for over 2000 years for treating various ailments like anorexia, insomnia, hemorrhage, diabetes, impotence and palpitation (Xiang et al., 2008). *In vitro* and *in vivio* studies have found ginsenosides to possess cardioprotective, immunomodulatory, antifatigue, anticancerous, antidiabetic and antioxidant properties (Christensen, 2009; Lee and Kim, 2014). Presence of ginsenosides makes these plants commercially important in both local as well as international markets ranking them among the highly traded medicinal plants in the world. As on 2009, the world market of *Panax* species (roots plus processed goods) was estimated at $2085 million (Baeg and So, 2013). Among these *Panax* species, *P. ginseng* Meyer (Korean ginseng), *P. notoginseng* (Burk) F. H. Chen (Chinese ginseng) and *P. quinquefolius* L. (American ginseng) are the major contributors to the ginseng trade.

Ginsenosides are of two types depending on the type of aglycone that they possess, oleanane type with pentacyclic aglycone and dammarane type with tetracyclic aglycone (Kim et al., 2009c; Liang and Zhao, 2008; Wang et al., 2012). Dammarane group is further divided into two types, protopanaxadiol and protopanaxatriol types based on the number of

hydroxyl (OH) group attached to the aglycones (Kim et al., 2009c; Wang et al., 2012) (Figure 1.3A, B). In protopanaxadiol type of ginsenosides, glycosylation occurs at carbon-3 and carbon-20 whereas in the protopanaxatriol type, carbohydrate moiety is attached to carbon-6 and carbon-20 (Shin et al., 2015). Ginsenoside Ro (Figure 1.3C) is the only member reported from the oleanane type of ginsenosides till date implying that rest of all the ginsenosides reported thus far are of dammarane type (Kim et al., 2009c; Liang and Zhao, 2008; Shin et al., 2015). Major ginsenosides in the dammarane group are ginsenosides Rb1, Rb2, Rc, Rd, Re, Rf, Rg1 and Rg2 among which first four belong to the protopanaxadiol group while the remaining four belong to the protopanaxatriol group (Kim et al., 2009c; Liang and Zhao, 2008; Shin et al., 2015; Wang et al., 2012).

2.4. Biosynthesis of ginsenosides

Ginsenosides are biosynthesized through ginsenoside biosynthetic pathway and the precursors for this pathway are provided by two pathways, viz., cytoplasmic mevalonate (MVA) pathway and plastidial methylerythritol phosphate (MEP) pathway. After the synthesis of basic skeletons, further modifications by hydroxylation and glycosylation results in the formation of different ginsenosides (Figures 3A, B). Ginsenoside biosynthetic pathway genes studied in different *Panax* species are enlisted in tables 2.3, 2.4, 2.5.

The first investigation related to the ginsenoside biosynthetic pathway was done by Kushiro et al (1997) in which 2,3-oxidosqualene was successfully converted to dammarenediol using *P. ginseng* microsomes. However, the first ginsenoside biosynthetic pathway gene successfully cloned was that of β-amyrin synthase (β-AS), an oxidosqualene cyclase that converted 2,3-oxidosqualene to β-amyrin, which is an intermediate in the biosynthesis of ginsenoside Ro, the only oleanane type of ginsenoside reported so far (Kushiro et al., 1998). Since then several genes from the proposed ginsenoside biosynthetic pathway have been cloned and some of them have been used to produce functionally active corresponding proteins in heterologous systems.

2.4.1. Mevalonate Pathway

The classical mevalonate pathway in the cytosol produces IPP and DMAPP, the precursors for the ginesnoside biosynthesis. In this pathway, acetyl CoA is condensed to yield HMGCoA which is further reduced to MVA by enzyme HMGR. MVK and PMVK

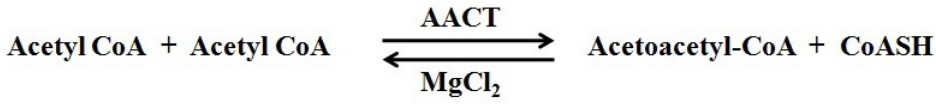
Table 2.3 Cloning and charactreization of ginsenoside biosynthetic pathway genes in different *Panax* species

Gene	Species	Functional validation	Reference
HMGS	*P. notoginseng*	-	Liu et al., 2016
HMGR	*P. ginseng*	Functional complementation of *A. thaliana HMGR1* mutant	Kim et al., 2014b
	P. notoginseng	-	Liu et al., 2016
	P. quinquefolius	-	Wu et al., 2012
MVK	*P. notoginseng*	-	Guo et al., 2012
MVD	*P. ginseng*	-	Kim et al., 2014c
FPS	*P. ginseng*	*In vitro* assay of *PgFPS* heterologously produced in *E. coli*	Kim et al., 2010a
SS	*P. notoginseng*	-	Niu et al., 2014
	P. ginseng	Functional complementation in yeast erg9 mutant lacking SS activity[1].	Kim et al., 2011[1]; Lee et al., 2004
	P. notoginseng	*In vitro* assay of *PnSS* heterologously produced in *E. coli*[1]	Jiang et al., 2017[1]; Niu et al., 2014
SE	*P. ginseng*	RNAi[1], *In vitro* assay of *PgSE* heterologously produced in *E. coli*[2]	Han et al., 2010[1]; Hu et al., 2012[2]
	P. notoginseng	-	He et al., 2008a; Niu et al., 2014
DS	*P. vietnamensis*	-	Ma et al., 2016
	P. ginseng	*In vitro* assay of *PgDS* heterologously produced in *E. coli*[1]; Functional complementation in yeast erg7 mutant lacking lanosterol synthase[2]	Hu et al., 2013[1]; Tansakul et al., 2006[2]
	P. notoginseng	-	Niu et al., 2014
β-AS	*P. quinquefolius*	*PqDS* heterologously produced in *S. cerevisiae* produced dammareneiol-II	Wang et al., 2014b
	P. ginseng	*Pqβ-AS* heterologously produced in *S. cerevisiae* produced β-amyrin[1] + RNAi[2]	Kushiro et al., 1998[1]; Zhao et al., 2015a[2]
	P. japonicus	-	Wu et al., 2017a
β-AO	*P. ginseng*	*Pgβ-AO* heterologously produced in *S. cerevisiae* produced oleanolic acid + *in vitro* enzyme assay of recombinant *Pgβ-AO*	Han et al., 2013
PPDS	*P. ginseng*	*PgPPDS* heterologously produced in *S. cerevisiae* produced protopanaxadiol + *in vitro* enzyme assay of recombinant *PgPPDS*	Han et al., 2011
	P. quinquefolius	*PqPPDS* heterologously produced in *S. cerevisiae* produced	Sun et al., 2013

		protopanaxadiol + RNAi	
PPTS	*P. ginseng*	*PgPPTS* heterologously produced in *S. cerevisiae* produced protopanaxatriol + *in vitro* enzyme assay of recombinant *PgPPTS*	Han et al., 2012
	P. quinquefolius	*PqPPTS* heterologously produced in *S. cerevisiae* produced protopanaxatriol + RNAi	Wang et al., 2014c
CS	*P. ginseng*	*In vitro* enzyme assay of *PgCS* produced heterologously in *S. cerevisiae*	Kushiro et al., 1998

catalyze the conversion of MVA to MVPP which is further decarboxylated to yield IPP. HMGR is a major point of regulation of substrate influx through this pathway. Various enzymes involved in MVA pathway are AACT, HMGS, HMGR, MVK, PMVK, MVD and IDI which are described in the following section.

2.4.1.1. Acetyl-CoA C-Acetyltransferase (AACT)



MVA pathway begins with the AACT catalyzed Claisen condensation of two acetyl CoA molecules producing acetoacetyl CoA (Chen et al., 2017; Miziorko, 2011). AACT requires $MgCl_2$ as a co-factor (Oeljeklaus et al., 2002; Vishwakarma et al., 2013). Acetoacetyl CoA serves as an important starting molecule for the natural product biosynthesis that ultimately leads to the biosynthesis of myriad of metabolites. The *AACT* gene in plants has been studied in *Arabidopsis thaliana* (Ahumada et al., 2008), *Nicotiana tabacum* (Wentzinger et al., 2012), *Helianthus annuus* (Oeljeklaus et al., 2002), *Bacopa monniera* (Vishwakarma et al., 2013), *Ginkgo biloba* (Chen et al., 2017) and *Hevea brasiliensis* (Sando et al., 2008a). qRT-PCR study found the expression of *AACT* to be higher in roots of *P. notoginseng* when compared to its leaves and flowers (Liu et al., 2015). *In silico* differential expression studies of RNA transcriptomes of different *Panax* spp. have also indicated that this gene to be differentially expressed among different organs (Rai et al., 2016) and across different developmental stages (Wu et al., 2013) as well as during elicitation by methyl jasmonate (Cao et al., 2015).

2.4.1.2. 3-Hydroxy-3-methylglutaryl coenzyme A synthase (HMGS)

HMGS catalyzes the condensation of acetyl-CoA and acetoacetyl-CoA to give 3-hydroxy-3-methylglutaryl-CoA (HMGCoA) (Chang et al., 2015; Liu et al., 2016; Miziorko, 2011). There are two isozymes of this enzyme in eukaryotes which are located in cytosol and mitochondria (Liao et al., 2014). In cytosol, it is involved in the biosynthesis of cholesterols/isoprenoids while in mitochondria it is involved in the production of ketone bodies (Hegart, 1999; Liao et al., 2014). HMGS is well characterized in animals but less studied in plants. Studies related to HMGS have been reported for *A. thaliana* (Ishiguro et al., 2010; Montamat et al., 1995), *H. brasiliensis* (Sirinupong et al., 2005; Suwanmanee et al., 2002), *Matricaria chamomilla* (Tao et al., 2016) and *Chamaemelum nobil*e (Cheng et al., 2016). *HMGS* has been recently cloned from *P. notoginseng* (Liu et al, 2016). However, its complete characterization including functional assays and studies related to its regulatory roles have not been conducted in the any of the *Panax* spp. On qRT-PCR analysis *HMGS* was found to be highly expressed in roots of *P. notoginseng* in comparison to its leaves and flowers (Liu et al., 2015) while in *P. vietnamensis* var. *fuscidiscus* it showed higher expression in young stem when compared to leaf, root and lateral roots (Zhang et al., 2015a). It was also found to be differentially expressed across different developmental stages, among different organs and when treated with methyl jasmonate indicating its probable regulatory role in ginsenoside biosynthesis (Cao et al., 2015; Rai et al., 2016; Subramaniyam et al., 2014; Wu et al., 2013).

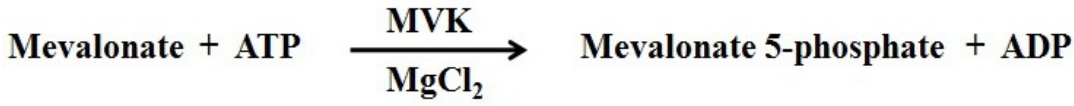
2.4.1.3. 3-Hydroxy-3-methylglutaryl coenzyme A reductase (HMGR)

$$\text{HMG-CoA} \xrightarrow[\text{NADPH}]{\text{HMGR}} \text{Mevalonate} + \text{CoASH}$$

Conversion of HMG-CoA to mevalonate is catalyzed by HMGR (Friesen and Rodwell, 2004; Miziorko, 2011). *HMGRs* from *P. ginseng* and *P. quinquefolius* have been cloned (Liu et al., 2016; Wu et al., 2012). *HMGR* from *P. ginseng* has been functionally characterized by creating transgenic *A. thaliana* and *P. ginseng* (Kim et al., 2014b). HMGR requires NADPH as a cofactor for its function (Dhar et al., 2015; Kim et al., 2014b). HMGR has also been studied in other plants like *Ocimum kilimandscharicum* (Bansal et al., 2018), *Taraxacum kok-saghyaz* (Ponciano and Chen, 2014), *A. thaliana* (Enjuto et al., 1994), *Stevia rebaudiana* (Kim et al., 1996), *G. biloba* (Shen et al., 2006) and *Gossypium* spp. (Liu et al., 2018). HMGR in plants belong to multigene family with many isforms (Enjuto et al., 1994; Friesen and Rodwell, 2004; Liu et al., 2018; Loguercio et al., 1999). HMGR in plants is a rate limiting enzyme that plays important regulatory roles in phytosterol/terpenoid

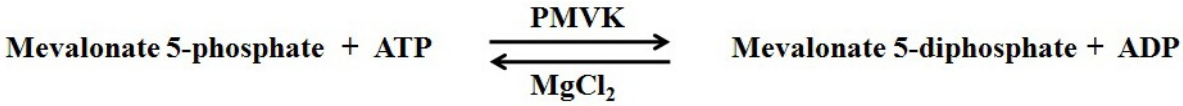
biosynthesis (Bansal et al., 2018; Lange et al., 1998; Liu et al., 2018).

2.4.1.4. Mevalonate kinase (MVK)



The phosphorylation of mevalonate to mevalonate-5-phosphate is catalyzed by MVK (Lluch et al., 2000; Miziorko, 2011). It requires a divalent cation, Mg^{2+} as a co-factor for its activity (Kumari et al., 2015; Schulte et al., 2000). This expression of this gene is regulated by methyl jasmonate suggesting its regulatory role in the secondary metabolite biosynthesis (Chen et al., 2017; Luo et al., 2014; Misra et al., 2014; Cao et al., 2015). Full length *MVK* was reported from *P. notoginseng* along with its *in silico* analysis related to secondary structures, deduced polypeptide sequence, molecular weights and other aspects (Guo et al., 2012). MVK has been studied in other plants like *A. thaliana* (Lluch et al., 2000), *Catharanthus roseus* (Schulte et al., 2000), *B. monniera* (Kumari et al., 2015), *G. biloba* (Chen et al., 2017) and *H. brasiliensis* (Sando et al., 2008a).

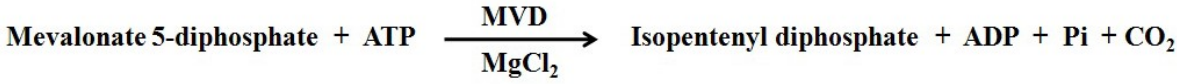
2.4.1.5. Phosphomevalonate Kinase (PMVK)



Mevalonate-5-phosphate is further phosphorylated to mevalonate-5-diphosphate by PMVK (Miziorko, 2011; Olivier et al., 1999). Mg^{2+} is required as a co-factor by PMVK for its function (Miziorko, 2011; Skilleter and Kekwick, 1971; Tsay and Robinson, 1991). PMVK has been studied in mammals like humans and rats (Chambliss et al., 1996; Olivier et al., 1999) but

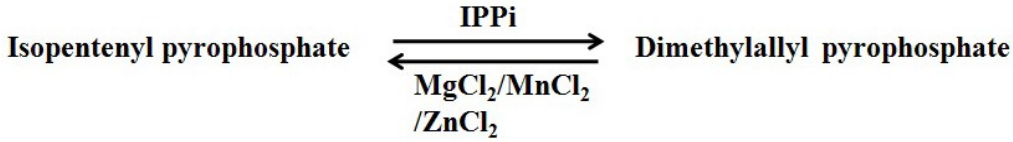
less studied in plants. In plants, cDNAs belonging to PMVK have been cloned and characterized from *M. Chamomilla* (Xu et al., 2018), *C. Roseus* (Simkin et al., 2011), *H. brasiliensis* (Sando et al., 2008a) and *C. nobile* (Yan et al., 2016). As per NCBI record, *PMVK* has been reported from plants like *Prunus persica*, *Brassica oleracea*, *Astragalus membranaceus* and *P. ginseng* but this gene has been less characterized in plants. *In silico* analysis of *PMVKs* in the transcriptome studies of *Panax* spp. have found it to be differentially expressed across different developmental stages, among different organs and when treated with methyl jasmonate indicating its probable regulatory role in ginsenoside biosynthesis (Cao et al., 2015; Rai et al., 2016; Wu et al., 2013).

2.4.1.6. Mevalonate diphosphate decarboxylase (MVD)



MVD catalyzes ATP dependent decarboxylation of mevalonate 5-diphosphate to form IPP in the last reaction of MVA pathway (Kim et al., 2014c; Miziorko, 2011). MVD has been found to use Mg^{2+} as a co-factor (Miziorko, 2011; Skilleter and Kekwick, 1971). *MVD* from *P. ginseng* has been used to create transgenic hairy roots wherein, triterpene production has been enhanced by overexpressing the *MVD* gene (Kim et al., 2014c). *MVD* has been studied in other plants like *Bacopa monniera* (Abbassi et al., 2015), *H. brasiliensis* (Wu et al., 2017) and *G. biloba* (Pang et al., 2006). *MVD* has also been reported from the transcriptome studies of *P. ginseng* (Cao et al., 2015; Chen et al., 2011; Li et al., 2013), *P. notoginseng* (Liu et al., 2015; Luo et al., 2011), *P. quinquefolius* (Sun et al., 2010a; Wang et al., 2016b; Wu et al., 2013) and *P. vietnamensis* (Zhang et al., 2015a).

2.4.1.7. Isopentenyl diphosphate isomerase (IDI)



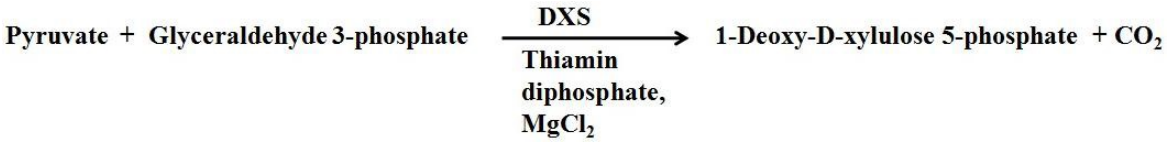
The reversible isomerization of IPP to DMAPP is catalyzed by IDI (Berthelot et al., 2012). Plant IDI requires divalent cations like Mg^{2+}, Mn^{2+} or Zn^{2+} for its enzyme activity (Berthelot et al., 2012; Ramos-Valdivia et al., 1997). IDI catalyzes isomerisation of IPP and DMAPP produced from both MEP and MVA pathways. Plant *IDI* belongs to a multigene family. Two cDNAs coding for two different IDIs have been identified in *A. thaliana, C. Roseus, Adonis aestivalis, Lactuca sativa* (Campbell et al., 1998; Cunningham and Gantt, 2000; Guirimand et al., 2012). *IDI* has been detected in multiple subcellular locations like plastids, cytosol, peroxisomes and mitochondria (Guirimand et al., 2012; Phillips et al., 2008; Sapir-Mir et al., 2008). qRT-PCR has shown *IDI* to be differentially expressed among different organs in *P. notoginseng* (Liu et al., 2015) and *P. vietnamensis* var. *fuscidiscus* (Zhang et al., 2015a). *In silico* expression studies have also found *IDI* showing differential expression patterns among the different organs, across different developmental stages and in response to methyl jasmonate suggesting its regulatory role in ginsenoside biosynthesis in different *Panax* spp. (Cao et al., 2015; Rai et al., 2016; Wu et al., 2013).

2.4.2. MEP pathway

The classical mevalonate pathway in cytosol was thought to be the only pathway

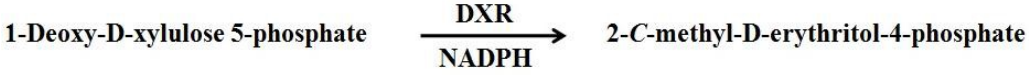
responsible for the production of IPP and DMAPP, the precursors for the isoprenoid biosynthesis. However, an alternative pathway known as non-mevalonate or MEP pathway for the production of these important precursors was discovered in bacteria (Rohmer et al., 1996). Subsequently, MEP pathway was also found to be present in plants (Lichtenthaler, 1999; Rohmer, 1999). MEP pathway has been reported to be involved in the ginsenoside biosynthesis (Zhao et al., 2014). Surprisingly, despite an enormous effort to understand the ginsenoside biosynthetic pathway, researchers have not paid an attention to studying the genes and enzymes of MEP pathway in *Panax* spp. Like the MVA and ginsenoside biosynthetic pathways, studies related to MEP pathway in *P. sokpayensis* are absent. Enzymes that make up MEP pathway are DXS, DXR, CMS, CMK, MCS, HDS and HDR which are discussed in the following section.

2.4.2.1. 1-Deoxy-D-xylulose-5-phosphate synthase (DXS)



DXS is the first enzyme of MEP pathway that catalyzes the condensation of pyruvate with D-glyceraldhyde-3-phosphate to form 1-deoxy-D-xylulose-5-phosphate (DXP) (Lichtenthaler, 1999). The DXS requires both thiamin and divalent Mg^{2+} for its activity (Banerjee et al., 2016; Kuzuyama, 2002; Lichtenthaler, 1999). It is a rate limiting enzyme in the isoprenoid/terpenoid biosynthesis (Estévez et al., 2001; Lois et al., 2000). Cloning and characterization of full length *DXS* from the genus *Panax* have not been reported yet but *DXS* have been reported from transcriptome studies of *P. quinquefolius* and *P. ginseng* (Wang et al., 2016b; Zhang et al., 2017). On *in silico* analysis, some unigenes of *DXS* were found to respond to methyl jasmonate treatment in *P. quinquefolius* (Wang et al., 2016b). DXS has been studied in many plants including *Populus trichocarpa* (Banerjee et al., 2016), *Glycine max* (Zhang et al., 2009) and Ilex cornuta (Yang et al., 2017a).

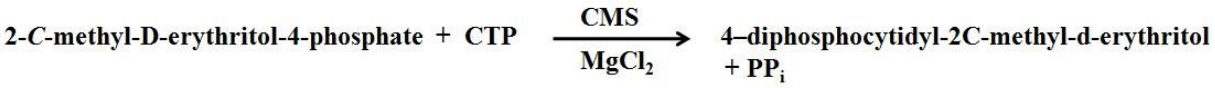
2.4.2.2. 1-Deoxy-D-xylulose 5-phosphate reductoisomerase (DXR)



The DXP is converted to 2-*C*-methyl-D-erythritol-4-phosphate (MEP) by DXR (Kuzuyama, 2002; Schwender et al., 1999). DXR requires NADPH and divalent a cation (Mg^{2+}, Mn^{2+} or Co^{2+}) for its enzyme activity (Kuzuyama, 2002; Proteau, 2004). The reaction catalyzed by the DXR is actually the first committed step of the MEP pathway

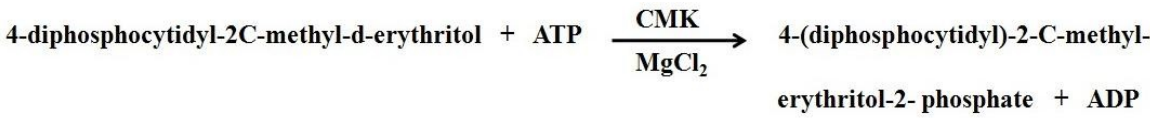
(Carretero-paulet et al., 2002; Rodríguez-concepción et al., 2001). *DXR* transcripts have been reported from RNA sequencing studies of *P. quinquefolius* and *P. ginseng* (Wang et al., 2016b; Zhang et al., 2017). *In silico* analysis of transcriptomes, the *DXR* unigenes were upregulated by methyl jasmonate treatment in *P. quinquefolius* adventitious roots (Wang et al., 2016b). However, studies related to its characterization from *Panax* spp. are missing. *DXR* has been studied in other plants like *Amomum villosum* (Yang et al., 2012), *Salvia miltiorhiza* (Shi et al., 2014) and *A. thaliana* (Schwender et al., 1999).

2.4.2.3. 2-C-methyl-D-erythritol 4-phosphate cytidylyltransferase (CMS)



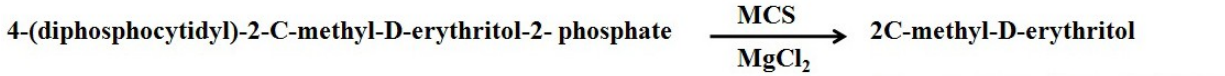
The conversion of MEP to 4–diphosphocytidyl-2C-methyl-d-erythritol (CDP-ME) is catalyzed by CMS (Kuzuyama, 2002; Rohdich et al., 2000a). Mg^{2+} is required as cofactor for the activity of CMS (Rohdich et al., 2000a). *CMS* was reported from the root transcriptome of *P. ginseng* as *isoprenoid synthase-containing protein D* (*IspD*) (Zhang et al., 2017). However, its characterization has not been reported from any of the *Panax* spp. so far. *CMS* has been studied in few species like *A. thaliana* (Okada et al., 2002; Rohdich et al., 2000a), *Rauvolfia verticillata* (Lan, 2013), *G. biloba* (Kim et al., 2006b) and *H. brasiliensis* (Sando et al., 2008b).

2.4.2.4. 4-(cytidine 5'-diphospho)-2-C-methyl-D-erythritol kinase (CMK)



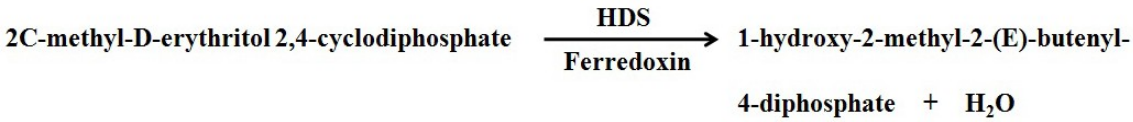
CMK is the fourth enzyme of MEP pathway that catalyzes the conversion of 4-(diphosphocytidyl)-2-C-methyl-D-erythritol into 4-(diphosphocytidyl)-2-C-methyl-D-erythritol-2- phosphate (CDP-MEP) (Rodriguez et al, 2015; Kuzuyama and Seto, 2012; Zhao
et al., 2014). Divalent co-factor, Mg^{2+} is required for the function of CMK (Rohdich et al., 2000b). *CMK*s from the *Panax* spp. have not been characterized so far though it was reported from *P. ginseng* transcriptome as *isoprenoid synthase-containing protein E* (*IspE*) (Zhang et al., 2017). *CMK* has been studied in other plants like *Lycopersicon esculentum* (Rohdich et al., 2000b), *G. biloba* (Kim et al., 2008), *Osmanthus fragrans* (Xu et al., 2016), *H. brasiliensis* (Sando et al., 2008b) and *Coleus forskohlii* (Pagoch et al., 2016).

2.4.2.5. 2-C-methyl-D-erythritol 2,4-cyclodiphosphate synthase (MCS)



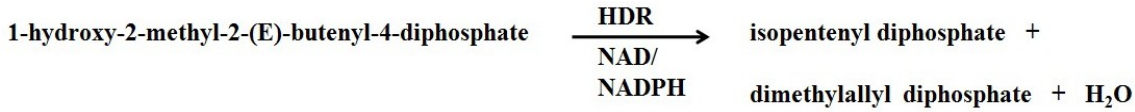
MCS catalyzes the cyclization of CDP-MEP to 2C- methyl-D-erythritol 2,4-cyclodiphosphate (MECDP) (Herz et al., 2000; Hsieh and Goodman, 2006; Kuzuyama, 2002). MCS requires Mg^{2+} as cofactor for its activity (Takagi et al., 2000). Like other genes of MEP pathway, full length *MCS* cDNAs from *Panax* spp. have not been cloned and characterized *CMK*s from the *Panax* spp. have not been characterized so far though it was reported from *P. ginseng* transcriptome as *isoprenoid synthase-containing protein F* (*IspF*) (Zhang et al., 2017). However it has been studied other plants like *A. thaliana* (Hsieh and Goodman, 2006), *H. brasilensis* (Sando et al., 2008b), *G. biloba* (Kim et al., 2006a), *S. rebaudiana* (Kumar et al., 2012a), and *taxus media* (Jin et al., 2006).

2.4.2.6. 4-Hydroxy-3-methylbut-2-en-1-yl diphosphate synthase (HDS)



The penultimate step of MEP pathway is catalyzed by HDS in which MECDP is converted to 1-hydroxy-2-methyl-2-(E)-butenyl-4-diphosphate (HMBPP) (Querol et al., 2002; Rohdich et al., 2003). The reactions catalyzed by HDS and the next enzyme, 4-Hydroxy-3-methylbut-2-enyl diphosphate reductase (HDR), of the MEP pathway are the least understood among all the reactions of the pathway (Hunter, 2007). Study on *A. thaliana* HDS has found that the electrons required for HDS catalyzed reactions is directly provided by photosynthesis via ferredoxin in the presence of light without the requirement of any reducing co-factor (Flores-Pérez et al., 2008; Seemann et al., 2006). However, ferredoxin-NADP+ reductase and NADPH system is required in the dark (Seemann et al., 2006). *In silico* transcriptome analysis of *P. ginseng* root has shown that *HDS*, which is also known as *isoprenoid synthase-containing protein* (*IspG*) was found to be upregulated in the cortex region of root when compared to its periderm and stele regions (Zhang et al., 2017). *HDS* has not been characterized in any species of *Panax* till date.

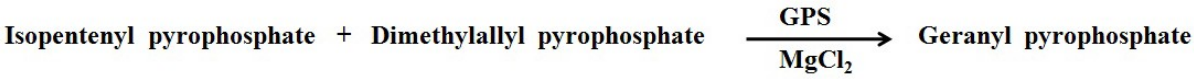
2.4.2.7. 4-Hydroxy-3-methylbut-2-enyl diphosphate reductase (HDR)



In the last step of MEP pathway, HMBPP is converted to IPP and DMAPP by HDR (Adam et al., 2002; Rohdich et al., 2002). The IPP and DMAPP are produced in 4-6:1 ratio (Peng et al., 2011; Shin et al., 2017). HDR requires NADH/NADPH as co-factor for its enzyme activity (Adam et al., 2002; Shin et al., 2017). *In silico* transcriptome analysis of *P. ginseng* root has shown that *HDR*, which is also known as *isoprenoid synthase-containing protein H* (*IspH*) was found to be upregulated in the periderm region of root when compared to its cortex and stele regions (Zhang et al., 2017). However, detailed characterization of *HDR* from the genus *Panax* has not been reported yet. Two copies each of *HDR* (*HDR1* and *HDR2*) have been reported from *A. annua* and *Huperzia serrate* suggesting that it belongs to a multigene family (Lv et al., 2015; Ma et al., 2015). *HDR* has also been studied in other plants like *S. rebaudiana*, *G. biloba* and *S. miltiorrhiza* (Hao et al., 2013; Kumar and Kumar, 2013; Shin et al., 2017).

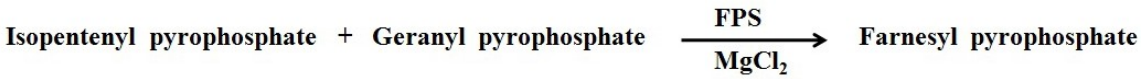
2.4.3. Ginsenoside biosynthetic pathway

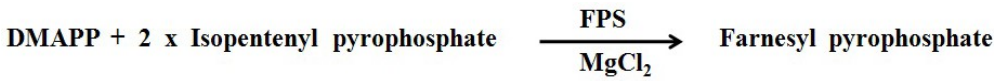
2.4.3.1. Geranyl pyrophosphate synthase (GPS)



Two C_5 isoprene isomers, IPP and DMAPP are condensed in a geranyl pyrophosphate synthase (GPS) catalyzed reaction to give a C_{10} intermediate, geranyl pyrophosphate (Kim et al., 2009c; Wang et al., 2012). *In vitro* enzyme assays have found that GPS requires Mg^{2+} as cofactor for its enzyme activity (Burke et al., 1999). *In silico* expression analysis done using the transcriptome data of methyl jasmonate treated adventitious roots of *P. ginseng* and *P. quinquefolius* have found that the expressions of unigenes representing *GPS* are affected by methyl jasmonate treatment (Cao et al., 2015; Wang et al., 2016b). *GPS* has been cloned and characterized in plants like *A. thaliana* (Bouvier et al., 2000), *Picea abies* (Schmidt et al., 2010), *Mentha piperita* (Burke et al., 1999), *H. annuus* (Oh et al., 2000) and *C. roseus* (Rai et al., 2013).

2.4.3.2. Farnesyl pyrophosphate synthase (FPS)





Synthesis of farnesyl pyrophosphate (FPP) from IPP and GPP is catalyzed by FPS (Kim et al., 2010a; Kim et al., 2009c; Wang et al., 2012). *FPS* from *P. ginseng* has been

cloned and its corresponding functional recombinant protein produced in *Escherichia coli* (Kim et al., 2010a). FPS requires Mg^{2+} for its enzymatic activity (Ferriols et al., 2015; Kim et al., 2010a; Srivastava et al., 2015). Experiments have proved that FPSs from other plants also catalyze the condensation of DMAPP and IPP to produce FPP (Ferriols et al., 2015; Srivastava et al., 2015). *FPS* from *P. sokpayensis* has not been characterized yet.

2.4.3.3. Squalene synthase (SS)

$$\text{2 x Farnesyl pyrophosphate} \xrightarrow[MgCl_2]{SS} \text{Squalene}$$

SS catalyzes the formation of a C_{30} intermediate, squalene from FPP (Wang et al., 2012). This step commits carbon for the ginsenoside biosynthesis (Sathiyamoorthy et al., 2010). *SSs* from *P. ginseng* and *P. notoginseng* have been cloned and functionally characterized (Jiang et al., 2017; Kim et al., 2011; Lee et al., 2004; Niu et al., 2014). However, *P. sokpayensis SS* (*PsSS*) is yet to be studied. It has been deduced through *in vitro* enzyme assay that SS requires Mg^{2+} as cofactor (Jiang et al., 2017).

2.4.3.4. Squalene epoxidase (SE)

$$\text{Squalene} \xrightarrow[FAD,\ NADPH]{SE} \text{2,3-oxidosqualene}$$

2,3-oxidosqualene is formed after a SE catalyzed oxidation of squalene (Kim et al., 2009c; Wang et al., 2012). 2,3-oxidosqualene undergoes two types of cyclisation catalyzed by two different oxidosqualene cyclases to form backbones for dammarane and oleanane types of ginsenosides. *SE*s from *P. ginseng* and *P. notoginseng* have been cloned and functionally characterized (Han et al., 2010; He et al., 2008a; Hu et al., 2012; Niu et al., 2014). Three *SE* isoforms (SE, SE2 and SE3) from *P. vietnamensis* have been cloned and they were found to be differentially expressed in different tissues as well as in response to methyl jasmonate (Ma et al., 2016). Studies on prokaryotic and eukaryotic SEs have shown that they require FAD and NADPH as co-factors (Guo et al., 2016a; Laden et al., 2000; Nakano et al., 2007). Studies on cloning and characterization of *SE* from *P. sokpayensis* have not been reported yet.

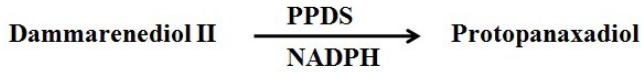
2.4.3.5. Dammarenediol synthase (DS)

$$\text{2,3-oxidosqualene} \xrightarrow[?]{DS} \text{Dammarenediol II}$$

DS is one of the two oxidosqualene cyclases of the ginsenoside biosynthetic

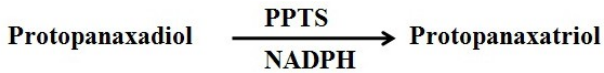
pathway, the other one being β-AS. It cyclizes 2,3 oxidosqualene to dammarenediol II which is an intermediate for the formation of dammarane type ginsenosides (Wang et al., 2012). *DS*s have been studied in *P. ginseng*, *P. notoginseng* and *P. quinquefolius* (Hu et al., 2013; Niu et al., 2014; Tansakul et al., 2006; Wang et al., 2014b). To the best of our knowledge, *in vitro* enzyme assay of purified DS after its production in heterologous hosts has not been reported yet. Hence, the co-factor required for the enzyme activity of DS has not been determined. *DS* has not been investigated in *P. sokpayensis*.

2.4.3.6. Protopanaxadiol synthase (PPDS)



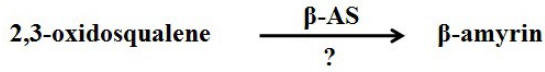
PPDS is a cytochrome P450 hydroxylase that catalyzes hydroxylation of dammarenediol II to produce protopanaxadiol (Han et al., 2011). *PPDS*s from *P. ginseng* and *P. quinquefolius* have been cloned and functionally characterized (Han et al., 2011; Sun et al., 2013). PPDS requires NADPH as a co-factor for its activity (Han et al., 2011). The gene coding for PPDS is yet to be cloned and characterized from *P. sokpayensis*.

2.4.3.7. Protopanaxatriol synthase (PPTS)



PPTS, like PPDS, is a cytochrome P450 hydroxylase that further hydroxylates protopanaxadiol to give protopanaxatriol (Han et al., 2012). *PPTS*s from *P. ginseng* and *P. quinquefolius* have been cloned and functionally characterized (Han et al., 2012; Wang et al., 2014c). PPTS requires NADPH as a co-factor for its activity (Han et al., 2012). However, PPTS gene has not been studied in *P. sokpayensis*.

2.4.3.8. β-amyrin synthase (β-AS)



β-AS catalyzes the cyclization of 2,3-oxidosqualene to β-amyrin diverting a part of 2,3-oxidosqualene and thus portion of carbon flux towards the synthesis of oleanane type of ginsenoside (Kim et al., 2009c; Wang et al., 2012). The *P. ginseng* gene coding for β-AS has been cloned and functionally characterized (Kushiro et al., 1998; Zhao et al., 2015a). However, the co-factor required for the enzyme activity of *β-AS* has not been reported so far. *β-AS* cloned from *P. japonicus* showed differential expression patterns among the different tissues (Wu et al., 2017a). *P. sokpayensis β-AS* is yet to be cloned and

characterized.

2.4.3.9. Glycosylation of ginsenoside aglycones

Glycosylation is a process in which the glycosyl moiety is transferred from uridine diphosphate (UDP)-sugar donor like UDP-glucose, UDP-galactose, UDP-arabinose, UDP-rhamnose, UDP-xylose or UDP-glucuronic acid to a metabolite aglycone catalyzed by UDP- glycosyltransferases (UGTs) (Seki et al., 2015; Tiwari et al., 2016). The glycosylation is one of the key players along with hydroxylation, methylation and acylation that are responsible for generating a host of structural diversity of plant secondary metabolites including triterpenoid saponins (Seki et al., 2015).

Table 2.4 Study of UDP glycosyltransferases related to ginsenoside biosynthesis in different *Panax* species

Glycosyltransferase	Species	Reaction	Reference
PPDS	*P. ginseng*, *P. quinquefolius*	Dammarenediol II to protopanaxadiol	Han et al., 2011; Sun et al., 2013
PPTS	*P. ginseng*, *P. quinquefolius*	Protopanaxadiol to protopanaxatriol	Han et al., 2012; Wang et al., 2014c
UGTPg1	*P. ginseng*	PPD to Compound K, Rh2 to F2, Rg3 to Rd, PPT to F1	Yan et al., 2014; Wei et al., 2015
UGTPg100	*P. ginseng*	PPT to Rh1, F1 to Rg1	Wei et al., 2015
UGTPg101	*P. ginseng*	PPT to F1, F1 to Rg1, PPD to Compound K (CK), Rh2 to F2, Rg3 to Rd	Wei et al., 2015
UGTPg45	*P. ginseng*	PPD to Rh2, Compound K to F2	Wang et al., 2015
UGTPg29	*P. ginseng*	Rh2 to Rg3, F2 to Rd	Wang et al., 2015
PgUGT74AE2	*P. ginseng*	PPD to Rh2, Compound K to F2	Jung et al., 2014
PgUGT94Q2	*P. ginseng*	Rh2 to Rg3, F2 to Rd	Jung et al., 2014
Pg3-O-UGT2	*P. quinquefolius*	Rh2 to F2, Rg3 to Rd	Lu et al., 2017

UGT – Uridine diphosphate glycosyltransferase; PPD – Protopanaxadiol; PPT – Protopanaxatriol

The presence of large number of ginsenosides with glycosylated aglycones in the *Panax* spp. suggests that UGTs play key roles in the final steps of ginsenoside biosynthesis. The UGTs glycosylate both oleanane and dammarane type aglycones to produce respective type of ginsenosides (Kim et al., 2009c; Liang and Zhao, 2008; Wang et al., 2012). However, very few UGTs responsible for the backbone modifications have been characterized till date and all of them have been reported recently. Thus, discovery and characterization of genes coding for UGTs is an important and latest area of interest in understanding ginsenoside biosynthesis. The *UGT*s that have been reported till date to

glycosylate ginsenoside aglycones (protopanaxadiol and protopanaxatriol) as well as certain ginsenosides themselves to further synthesize other ginsenosides are given in table 2.4. The transcripts of almost all of these UGTs have been reported from *P. ginseng* with only one *UGT* reported from *P. quinquefolius* (Table 2.4). *UGT*s from *P. sokpayensis* have not been reported yet.

2.5. Regulation of gene expression of ginsenoside biosynthetic pathway genes

Experiments have shown that the gene expressions of several ginsenoside biosynthetic pathway genes are regulated by various substances of biotic and abiotic origin called elicitors. These elicitors also enhance the ginsenoside production suggesting that the above genes play regulatory roles in the ginsenoside biosynthesis. Examples of such studies are given in table 2.5. Jasmonates (JAs) such as jasmonic acid (JA) and its derivative Methyl jasmonate (MeJA), and salicylic acid are plant hormones that are also effective elicitors of plant secondary metabolites (Giri and Zaheer, 2016; Ramirez-Estrada et al., 2016). MeJA, SA and JA have all been shown to have a positive effect on the ginsenoside production through the regulation of ginsenoside biosynthetic pathway genes, among which MeJA has been the most popular elicitor thus far (Table 2.5). Yeast extract (YE) and fungal cell wall extract were also found to positively regulate the ginsenoside production and the genes belonging to its biosynthetic pathway (Table 2.5) (Rahimi et al., 2014; Xiaojie et al., 2005). Other chemicals like vanadate and N,N′-dicyclohexylcarbodiimide (DCCD), and a detergent like tween 80 have also been found to elicitate ginsenoside accumulation through upregulation of regulatory genes of ginsenoside biosynthetic pathway (Table 2.5)(Huang et al., 2013; Huang and Zhong, 2013; Liang et al., 2015). All the above chemicals and strategies can be used to achieve higher quantities of ginsenosides either *in vitro* or *in vivo* to meet the ever rising demand of this important triterpenoid. Moreover, the understanding of the regulatory roles of the genes of the ginsenoside biosynthetic pathway can help the investigators to target those genes for up-scaling ginsenoside biosynthesis.

The ginsenoside biosynthetic pathway genes have also been found to be spatially regulated (Table 2.6). Both ginsenoside type and quantity are known to vary among the different organs of *P. ginseng* (Lee et al., 2017b; Oh et al., 2014; Shi et al., 2007). These suggest that the ginsenoside contents in different organs are regulated by organ specific differential expression of the ginsenoside biosynthetic pathway genes.

Table 2.5 Effects of various elicitors on the ginsenoside biosynthetic pathway gene expression and ginsenoside accumulation

Gene	Treatment (s)	Gene expression	Effect on ginsenoside accumulation	References
PgSS1, *PgSE*, *PgbAS*	10 mM methyl jasmonate (MeJA) added in culture medium of adventitious roots of *P. ginseng*	Upregulated after 12 h	Not measured	Lee et al., 2004
PgSS1, *PgSE*	10 µM MeJA added in culture medium of adventitious roots of *P. ginseng*	Upregulated after 24 h	4 fold increase after 5 weeks of culture	Han et al., 2006a
PgSQE1, *PgSQE2*	10, 20, 30 ,50 µM MeJA added in culture medium of adventitious roots of *P. ginseng*	*PgSQE1* upregulated after 24 h whereas *PgSQE2* was downregulated	Not measured	Han et al., 2010
PgFPS	0.1 mM MeJA added in culture medium of hairy root culture of *P. ginseng*	Upregulated after 12 h reaching maximum after 24 h	Not measured	Kim et al., 2010a
PgPPDS (*CYP716A47*)	Adventitious roots treated with 10 µM MeJA	Upregulated after 12 h till 48 h	Not measured	Han et al., 2011
PgPPDS (*CYP716A47*)	Adventitious roots treated with 10 µM MeJA	Upregulated after 12 h till 24 h then decreased after 48 h and 72 h but still greater than the control	Not measured	Han et al., 2012
PnFPS, *PnSS*, *PnSE1* and *PnDS*	Four years old *P. notoginseng* leaves were continually sprayed with 200 µM MeJA for 10 min	*PnFPS* and *PnDS* remarkably upregulated at 6 h while *PsSS* slightly upregulated. *PnSE1* upregulated at 24 h after MeJA treatment.	Not measured	Niu et al., 2014
PqGPS, *PqFPS*, *PqSS*, *PqSE*, *Pqβ-AS*, *PqDS*, *CYP716A47* (*PqPPDS*), *CYP716A53v2*(*PqPPTS*), *UGT74AE2*, *UGT94Q2*, *UGTPg100*, *c52571-g3*	*P. quinquefolium* adventitious roots medium was supplemented with 5.0 mgl^{-1} MeJA after pre-cultivation for four weeks	Upregulated	Increased from 43.66 mg/g compared to 8.32 mg/g in control group in 12 days	Wang et al., 2016b
PvfSE1, *PvfSE2*, *PvfSE3*	*P. vietnamensis* var. *fuscidiscus* leaves were sprayed with 250 µM	*PvfSE1* was weakly upregulated, *PvfSE2* was strongly regulated	Not measured	Ma et al., 2016

	MeJA	increasing 19.5 fold at 24 h, *PvfSE3* registered 3.5 fold increase at 24 h.		
PgSQS, PgSE1, PgSE2, PgDS	0.1 mM MeJA added to culture medium of adventitious roots after 5 weeks of pre-cultivation.	*PgSQS* increased 3.1 fold at 24 h and then decreased to 2.89 fold at 120 h. *PgSE1* was upregulated 6.03 fold at 24 h and 14.57 fold at 120 h. *PgSE2* reached 6.66 fold at 120 h from 3.21 fold at 24 h. *PgDS* was upregulated at 24 h reaching to 5.42 fold at 120 h.	Increased ginsenoside content reaching 14.75 mg/g dry weight	Um et al., 2017
PgSS, PgSE, Pgβ-AS	Fungal cell wall extract	Induced the expressions of *PgSS, PgSE, Pgβ-AS*	Ginsenoside content increased 3 folds	Xiaojie et al., 2005
PgFPS, PgIPPI	Salicylic acid (SA) (200 µM) and yeast extract (YE) (3 g/l) were added to the growth medium of 30 days old subcultured *P. ginseng* adventitious roots.	*PgIPPI* and *PgFPS* expressions were upregulated after 6 h of SA treatment, decreased slightly after 48 h and increased again in 72 h. In response to YE treatment, *PgIPPI* expression peaked at 12 and 72 h, while that of *PgFPS* peaked at 12 and 48 h.	Highest level of mono- and sesquiterpenoids were seen at 24 h of SA and YE treatments while the control failed to produce the same.	Rahimi et al., 2014
PgFPS, PgSS, PgSE, PgDS	0.1 mM MeJA was added to the P. ginseng hairy roots medium.	*PgFPS, PgSS, PgSE, PgDS* were upregulated after at 12 and 24 h.	Protopanaxadiol type ginsenosides content was increased 5.5 – 9.7 times whereas protopanaxatriol type increased 1.85 – 3.82 times after 7 days of elicitation.	Kim et al., 2009b
PnSS, PnSE	200 µM MeJA to *P. notoginseng* cell culture medium	*PnSS, PnSE* were upregulated 6 h post elicitation.	Ginsenoside content (Rb1+Rg1+Rd+Re) increased from 12 h to 10 days after elicitation.	Hu and Zhong, 2008
PgSS, PgSE	10 µM JA was added to the	*PgSS, PgSE* were upregulated	2.4 fold increase	Hu et al., 2003

	suspension culture medium of *P. ginseng*			
PgDS, *PgPPDS*, *PgPPTS*	*P. ginseng* hairy roots were treated with 1.2 % w/v Tween 80 for 25 days.	*PgDS*, *PgPPDS*, *PgPPTS* were upregulated	Saponin content increased 3 fold	Liang et al., 2015
PgSS, *PgSE*, *PgDS*	50 µM vanadate added *P. ginseng* cell culture medium.	*PgSS*, *PgSE*, *PgDS* were upregulated within 6 h of vanadate treatment.	4.4 fold increase in total ginsenoside content	Huang and Zhong, 2013
PgSS, *PgSE*, *PgDS*	10 µM N,N′-dicyclohexylcarbodiimide (DCCD)	*PgSS*, *PgSE*, *PgDS* were upregulated withn 6 h of treatment.	3 fold increase in total ginsenoside content	Huang et al., 2013

Table 2.6 Differential expression of ginsenoside biosynthetic pathway genes in different organs

Gene	Gene expression	References
PgSQE, *PgSQE2*	Studied in different organs of 4 years old *P. ginseng*. The expression of *PgSQE1* was as follows: leaf > petiole > flower ≈ root body > fine root. The expression of *PgSQE2* was as follows: petiole > flower > root body > leaf > fine root	Han et al., 2010
PqHMGR	Studied in different organs of 4 years old *P. quinquefolius*. leaf > flower ≈ root > stem	Wu et al., 2012
PgHMGR	2weeks seedling *PgHMGR1*: petiole > root > leaf; *PgHMGR2*: petiole > root > leaf 3years plant *PgHMGR1*: Main root > lateral root > leaf > flower > stem; *PgHMGR2*: Main root > lateral root > stem > leaf > flower 6 years plant *PgHMGR1*: Main root > lateral root > leaf > flower > stem; *PgHMGR2*: Main root > lateral root > stem > leaf ≈ flower	Kim et al., 2014a
PnFPS, *PnSS*, P*nSE1*, *PnSE2*, *PnDS*	Studied in different organs of 4 years old *P. notoginseng*. *PnFPS*: flower > leaf ≈ stem > root; *PnSS*: flower > leaf ≈ stem > root; *PnSE1*: flower > leaf > stem > root; *PnSE2*: flower > root > stem > leaf; *PnDS*: flower > leaf > stem > root	Niu et al., 2014
PvfSE1, *PvfSE2*, *PvfSE3*	*PvfSE1*: stem > leaf > lateral root > root; *PvfSE2*: leaf > root > stem > lateral root; *PvfSE3*: leaf > stem > root≈ lateral root	Ma et al., 2016

2.6. Metabolic engineering of ginsenoside biosynthesis

Considering the huge market demand, several groups have attempted to increase the production of ginsenosides or their intermediates using either heterologous expression in *E. coli* or yeast or through the creation of high yielding transgenic lines. Several ginsenoside biosynthetic pathway genes like *FPS, SS, DS, PPDS, PPTS* and many *GTs* from different *Panax* species have been successfully used to produce the respective functional enzymes in microbial hosts (Table 2.7). Some groups have utilized the upstream phytosterol biosynthetic pathway of the host, *S. cerevisiae* and introduced several genes of the ginsenoside biosynthetic pathway to produce important intermediates of ginsenosides (Table 2.7). For example, *PgDS*, *PgPPDS* from *P. ginseng* and *NADPH-cytochrome P450* from *A. thaliana* were used to produce 10.94 mg/g dry cell weight (1548 mg/l) dammarenediol-II and 8.40 mg/g DCW (1189 mg/l) protopanaxadiol in yeast (Dai et al., 2013). Using recombinant DNA technology and metabolic engineering, a novel ginsenoside called compound K (CK) that has shown anti – inflammatory, hepatoprotective, anti-diabetic and anti-cancerous properties in *in vitro* and *in vivo* animal models and which is yet to be detected in *Panax* plants was also synthesized (Wei et al., 2015; Yan et al., 2014). Studies related to the heterologous expression of the ginsenoside biosynthetic pathway enzymes have been summarised in table 2.7.

Table 2.7 Successful production of functional ginsenoside biosynthetic pathway enzymes in heterologous hosts using recombinant DNA technology/metabolic engineering

Gene	Ectopic hosts	Remarks	References
PgDS	*S. cerevisiae*	-	Tansakul et al., 2006
PgFPS	*E. coli*	-	Kim et al., 2010a
PgSS1, *PgSS2*, *PgSS3*	*S. cerevisiae*	-	Kim et al., 2011
PgPPDS	*S. cerevisiae*	-	Han et al., 2011
PgDS	*S. cerevisiae*	493 μgl^{-1} dammarenediol was produced	Liang et al., 2012
PgPPTS	*S. cerevisiae*	-	Han et al., 2012
PgDS	*E. coli*	-	Hu et al., 2013
PgDS, *PgPPDS* from *P. ginseng* and *NADPH-cytochrome P450* from *A. thaliana*	*S. cerevisiae*	10.94 mg/g DCW* (1548 mg/l) dammarenediol-II and 8.40 mg/g DCW (1189 mg/l) protopanaxadiol were produced	Dai et al., 2013
PqPPDS	*S. cerevisiae*	-	Sun et al., 2013
PqCYP6H (*PqPPTS*)	*S. cerevisiae*	-	Wang et al., 2014c
UGTPg1	*S. cerevisiae*	PPD to Compound K, Rh2 to F2, Rg3 to Rd	Yan et al., 2014
PgDDS, *PgPPDS*, *PgPPTS*, *PgCPR1*, *UGTPg100*	*S. cerevisiae*	All these genes were used together to produce Rh1. *PgCPR1* (Supplementary information, Data S1, Figure S11, Yan et al., 2014)	Wei et al., 2015; Yan et al., 2014
PgDDS, *PgPPDS*, *PgPPTS*, *PgCPR1*, *UGTPg1*	*S. cerevisiae*	All these genes were used together to produce F1	Wei et al., 2015; Yan et al., 2014
UGTPg100	*E. coli*	PPT to Rh1, F1 to Rg1	Wei et al., 2015
UGTPg101	*E. coli*	PPT to F1, F1 to Rg1, PPD to CK, Rh2 to F2, Rg3 to Rd	Wei et al., 2015
UGTPg45	*S. cerevisiae*	1.45 µmol/g DCW of Rh2 was produced	Wang et al., 2015
UGTPg29	*S. cerevisiae*	3.49 µmol/g DCW of Rg3 was produced	Wang et al., 2015
UGTPg45	*E. coli*	PPD to Rh2, Compound K to F2	Wang et al., 2015
UGTPg29	*E. coli*	Rh2 to Rg3, F2 to Rd	Wang et al., 2015
UGT51 (yeast glycosyltransferase)	*S. cerevisiae*	*UGT51* introduced into yeast chassis strain ZD-PPD-016(URA3$^-$) (Dai et al., 2013) containing *PgDS*, *PgPPDS* (*P. Ginseng*) & *NADPH-cytochrome P450* (*A. thaliana*)	Zhuang et al., 2017

*Dry Cell Weight

2.7. Transgenic plants for enhanced production of ginsenosides

Ginsenoside content has also been enhanced by creating transgenic hairy roots, adventitious roots, cell lines and complete plants by either overexpressing ginsenoside biosynthetic pathway genes or RNA interference (RNAi) based silencing of genes that divert metabolic flux away from the ginsenoside biosynthesis (Table 2.8). By overexpressing such genes, several groups have been able to increase the total ginsenoside and phytosterol content in different *Panax* species (Deng et al., 2017; Kim et al., 2014c; Lee et al., 2004; Wang et al., 2014b). Ginsenoside intermediate, dammarenediol II have also been successfully produced in transgenic tobacco using *P. ginseng DS* (Han et al., 2014; Lee et al., 2012). Similarly, phytosterol content of *C. asiatica* was also enhanced by creating its transgenic hairy roots containing *P. ginseng FPS*. RNAi based transgenic lines have also been used to study the regulatory roles of certain genes. For example, RNAi silenced *P. ginseng* and *P. quinquefolius PPDS* (*CYP716A47*) reduced the total ginsenoside production in the respective transgenic hairy roots indicating the regulatory role of *PPDS* in the ginsenoside production (Sun et al., 2013). Similarly, RNAi silenced *PgSQE1* in the transgenic *P. ginseng* adventitious roots resulted in less total ginsenoside production while enhancing the phytosterol content (Han et al., 2010). A double transgenic *P. notoginseng* cell line overexpressing *PnFPS* and RNAi silenced *CS*, a gene coding for a key enzyme cycloartenol synthase that channelizes metabolic flux towards phytosterol biosynthesis have also been created to enhance ginsenoside production by increasing the metabolic flux towards ginsenoside biosynthesis (Yang et al., 2017b). This approach resulted in the total triterpene increase by 1.46 – 2.46 folds. Table 2.8 gives a list of different types of transgenic lines created using different approaches to achieve different purposes.

Table 2.8 Use of ginsenoside biosynthetic pathway genes to create transgenics

Gene	Description of transgenic product	Outcome	References
PgFPS, *PgMVD*	Separate transgenic lines of *P. ginseng* hairy roots were establised using *PgFPS* and *PgMVD* separately through *Agrobacterium rhizogenes* mediated transformation	Transgenic lines overexpressing *PgFPS* showed 2.4 fold higher total ginsenoside content (36.42 mg/g DW) compared to control. Transgenic lines overexpressing *PgMVD* showed 4.4 fold higher stigmasterol content (1.39 mg/g DW) compared to control.	Kim et al., 2014c
PgPPTS (*CYP716A53v2/ protopanaxadiol 6-hydroxylase*)	Two *P. ginseng* transgenic lines, one overexpressing *PgPPTS* and the other with silenced *PgPPTS* gene (through RNAi) were created by co-cultivating *P. ginseng* cotyledon with *Agrobacterium tumefaciens* containing the desired construct.	Transgenic lines overexpressing *PgPPTS* produced higher total protopanaxatriol type ginsenosides (Rg1, Re, Rf) and lower protopanaxadiol type ginsenosides (Rb1, Rc, Rb2, Rd) than the wild type. The transgenic lines had higher total ginsenoside content (11.01 mg/g DW) than the wild type (6.30 mg/g DW). *CYP716A53v2*-RNAi transgenic lines produced lower total protopanaxatriol type ginsenosides and higher protopanaxadiol type ginsenosides than the wild type.	Park et al., 2016
PgDDS	Transgenic *N. tabacum* plants were created using *P. ginseng DS* (*PgDS*) through *A. tumefaciens* containing the *PgDDS* overexpressing construct.	The transgenic cell suspension produced 573 µg/g DW of dammarenediol – II after three weeks of culture (equivalent to5.2 mg/l).	Han et al., 2014
PgDDS	Transgenic *N. tabacum* plants were created using *P. ginseng DS* (*PgDS*) through *A. tumefaciens* containing the *PgDDS* overexpressing construct.	20 – 30 µg/g DW of dammarenediol – II was produced in the leaves of transgenic *N. tabacum*. The transgenic tobacco conferred tolerance to *tobacco mosaic virus* (TMV).	Lee et al., 2012
PgFPS	*Agrobacterium rhizogenes* harboring *PgFPS* was used to transform *Centella asiatica* leaves. The transformed leaves were used as explants to induce hairy roots.	Transgenic *C. asiatica* hairy roots produced 3 and 1.3 folds more sterol and cholesterol than the wild type, respectively.	Kim et al., 2010b
PgSS1	Transgenic *P. ginseng* plants were created using *PgSS1* through *A. tumefaciens* containing the *PgSS1* overexpressing construct.	Adventitious roots of transgenic *P. ginseng* produced ~2 fold and 1.6 – 3.0 fold higher phytosterol and triterpene, respectively than the wild type.	Lee et al., 2004
PnFPS	*PnFPS* over-expression vector and *PnCAS*-RNAi vector were introduced into *A. tumefaciens* separately. Then the *P. notoginseng in vitro* cultured cells were transformed by *PnCAS*-RNAi vector containing *A. tumefaciens*. These transgenic cells were further transformed by *A. tumefaciens* containing *PnFPS* over-expression vector to obtain double transgenic cell lines.	Transgenic cell lines produced higher total triterpene saponins (1.46 – 2.46 folds) and lower phytosterols (0.58 – 0.88 fold) when compared to the wild type.	Yang et al., 2017b

PqCYP6H (*PqPPTS*)	*Agrobacterium rhizogenes* containing *CYP6H*-RNAi and *CYP6H*- overexpression constructs were used to transform *P. quinquefolius* hairy roots.	The content of protopanaxadiol type ginsenosides (Rb1, Rb2, Rc and Rd) were slightly enhanced while protopanaxatriol type ginsenosides (Re, Rf anf Rg1) were significantly reduced in RNAi transgenic hairy roots. The effect was opposite in the transgenic lines overexpressing *CYP6H*.	Wang et al., 2014c
PqDS	*PqDS* overexpression construct was introduced into *P. quinquefolius* hairy roots through *A. rhizogenes*.	Increased the production of ginsenosides in transgenic hairy roots.	Wang et al., 2014b
PgPPDS (*PgCYP716A47*) and *PqD12H* (*PqPPDS*)	*P. ginseng PgCYP716A47*-RNAi and *P. quinquefolius PqD12H*-RNAi transgenic hairy roots were created through *A. rhizogenes*.	Total ginsenoside contents in both the transgenic lines were lower than their respective wild types.	Sun et al., 2013
PgSQE1	*PgSQE1-RNAi* construct was introduced into *A. tumefaciens* which was co-cultivated with *P. ginseng* cotyledon to created transgenic adventitious roots.	Total ginsenoside production was reduced while the phytosterol content was enhanced when compared to the wild type.	Han et al., 2010
PnSS and *PnHMGR*	Transgenic *P. notoginseng* cell line co-overexpressing *PnSS* and *PnHMGR* was created through *A. tumefaciens* mediated transformation.	The saponin content was higher in the transgenic cell lines in comparison to the control.	Deng et al., 2017