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ScienceDirect
Chinese Journal of Natural Medicines 2020, **18**(6): 417-424
doi: 10.1016/S1875-5364(20)30049-2
Chinese Journal of Natural Medicines

•Review•

Advances in biosynthesis of triterpenoid saponins in medicinal plants

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Available online 20 Jun., 2020

[ABSTRACT] In recent years, biosynthesis of triterpenoid saponins in medicinal plants has been widely studied because of their active ingredients with diverse pharmacological activities. Various oxidosqualene cyclases, cytochrome P450 monooxygenases, uridine diphosphate glucuronosyltransferases, and transcription factors related to triterpenoid saponins biosynthesis have been explored and identified. In the biosynthesis of triterpenoid saponins, the progress of gene mining by omics-based sequencing, gene screening, gene function verification, catalyzing mechanism of key enzymes and gene regulation are summarized and discussed. By the progress of the biosynthesis pathway of triterpenoid saponins, the large-scale production of some triterpenoid saponins and aglycones has been achieved through plant tissue culture, transgenic plants and engineered yeast cells. However, the complex biosynthetic pathway and structural diversity limit the biosynthesis of triterpenoid saponins in different system. Special focus can further be placed on the systematic botany information of medicinal plants obtained from omics large dataset, and triterpenoid saponins produced by synthetic biology strategies, gene mutations and gene editing technology.

[KEY WORDS] Medicinal plant; Cytochrome P450 monooxygenases; Uridine diphosphate glucuronosyltransferases; Transcription factor; Triterpenoid saponins

[CLC Number] Q67 **[Document code]** A **[Article ID]** 2095-6975(2020)06-0417-08

Introduction

Medicinal plants growth is influenced by various environmental factors, which induces the diverse change of active ingredients [1, 2]. Many rare medicinal plants are difficult to cultivate and grow slowly [3, 4]. Besides, active ingredients of some medicinal plants have low contents, complex structures, and difficulties in chemical synthesis [5, 6]. But by biotechnology, the production of active ingredients has many advantages, such as a short growth cycle, standardized production processes, and controllable quality [7, 8]. Synthetic biology provides an effective strategy for the sustainable development of traditional Chinese medicine. Oleanolic acid production was improved to 606.9 ± 9.1 mg·L^{-1} by reconstruction of cellular galactose regulatory network and further fermentation optimization in *Saccharomyces cerevisiae*, which was 7.6-fold higher than the reported maximum production [9]. In *S. cerevisiae*, by repurposing UGT51, an inherently promiscuous glycosyltransferase, about 300 mg·L^{-1} of ginsenoside Rh_2 was obtained which was the highest titer reported [10]. By increasing the copy number of *Uni25647* and pairing cytochrome P450 reductases from various plant sources, the production of glycyrrhetinic acid was increased to 18.9 ± 2.0 mg·L^{-1}, which was 946.5-fold higher than previously reported data [11].

Triterpenoid saponins are most studied in medicinal plant with diverse structures skeletons of α-amyrin, β-amyrin, dammarenediol, lupeol, etc. [12-14], further reactions of diverse structures skeletons by cytochrome P450 monooxygenases (CYP450s) and uridine diphosphate glucuronosyltransferases (UGTs) induced the production of numerous triterpenoid saponins (Fig. 1). Biologically, triterpenoid saponins are considered defensive compounds against external stress [15] (Fig. 2). Because of their various pharmacological effects, they are meaningful to humans [16, 17]. Table 1 shows the summary of CYP450s, UGTs and transcription factors (TFs) involved in triterpenoid saponins biosynthesis, including their plant sources, catalytic sites and moieties [8,18-43]. Recent studies focus on CYP450s, UGTs and TFs. Different plant

[Received on] 12-Oct.-2019
[Research funding] This work was supported by the Central Significant Increase or Decrease Program of China (No. 2060302-1604-05) and NSFC (No. 81703639).
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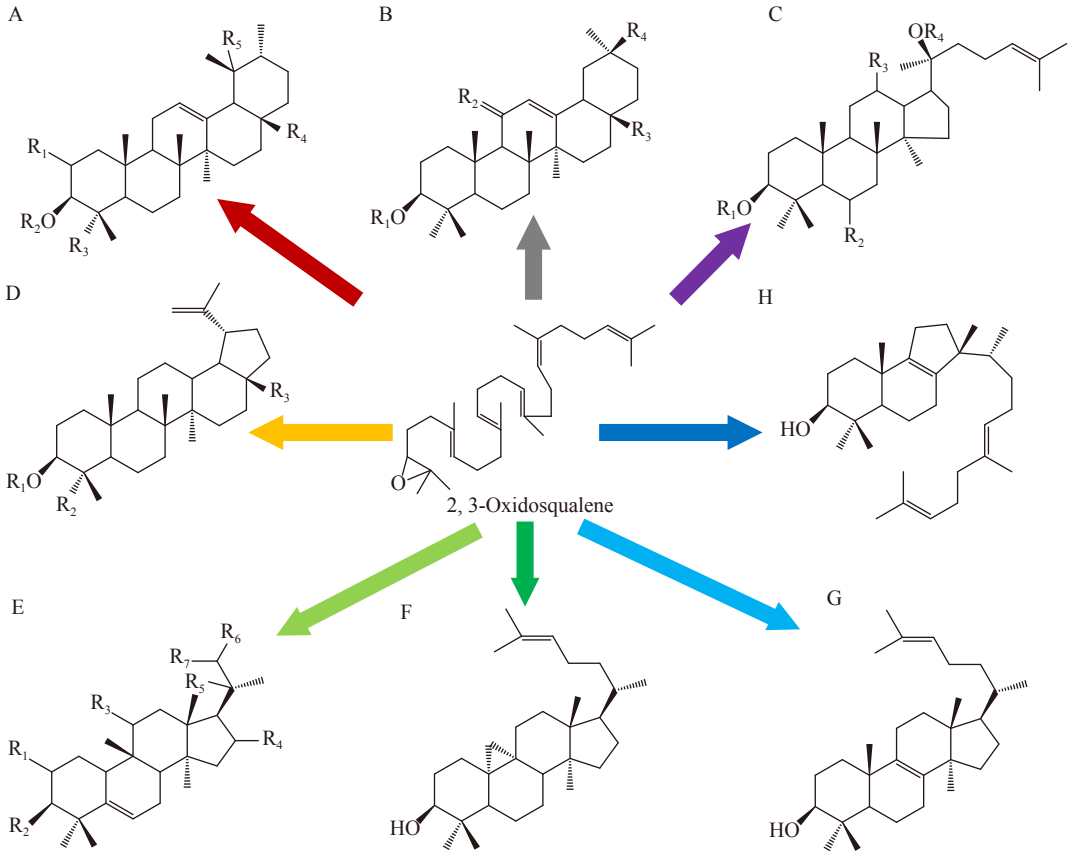
These authors have no conflict of interest to declare.



A	R_1	R_2	R_3	R_4	R_5
Ursolic acid	−H	−H	$-CH_3$	−COOH	−H
Asiatic acid	−OH	−H	$-CH_2OH$	−COOH	−H
Ziyuglycoside I	−H	−Ara	$-CH_3$	−COOGlc	−H

B	R_1	R_2	R_3	R_4
Oleanic acid	−H		−COOH	$-CH_3$
Glycyrrhizic acid	−GlcA-GlcA	=O	$-CH_3$	−COOH
Ginsenodide R_0	−Glc-GlcUA		−COOGlc	$-CH_3$

C	R_1	R_2	R_3	R_4
Protopanaxadiol	−H	−H	−OH	−H
Protopanaxatriol	−H	−OH	−OH	−H
Gininsenodide Rh_1	−H	−OGlc	−OH	−H
Gininsenodide Rh_2	−Glc	−H	−OH	−H
Notoginsenodide R_1	−H	$-Oglc\text{-}xy_1$	−OH	−Glc

D	R_1	R_2	R_3
Betulinic acid	−H	$-CH_3$	−COOH
Pulchinenoside C	−Ara-Rha	$-CH_2OH$	−Glc-Glc-Rha

E	R_1	R_2	R_3	R_4	R_5	R_6	R_7
Mogrol	−H	−OH	−OH	−H	−H	−H	$-CH_2CHOH\text{-}C(CH_3)_2OH$
Mogroside	−H	−Glc-Glc	−OH	−H	−H	−H	$-CH_2CH(Glc)_3\text{-}C(CH_3)_2OH$
Calebassine	−OH	=O	=O	−OH	−OH	=O	$-CH{=}CH\text{-}C(CH_3)_2OC{=}OCH_3$

F
Cycloartenol

G
Lanosterol

H
Thalianol

Fig. 1 Triterpenoid saponins most studied in medicinal plant with diverse structures skeletons of *α*-amyrin (A), *β*-amyrin (B), dammarenediol (C), lupeol (D), cucurbitadienol (E), cycloartenol (F), lanosterol (G) and thalianol (H), respectively

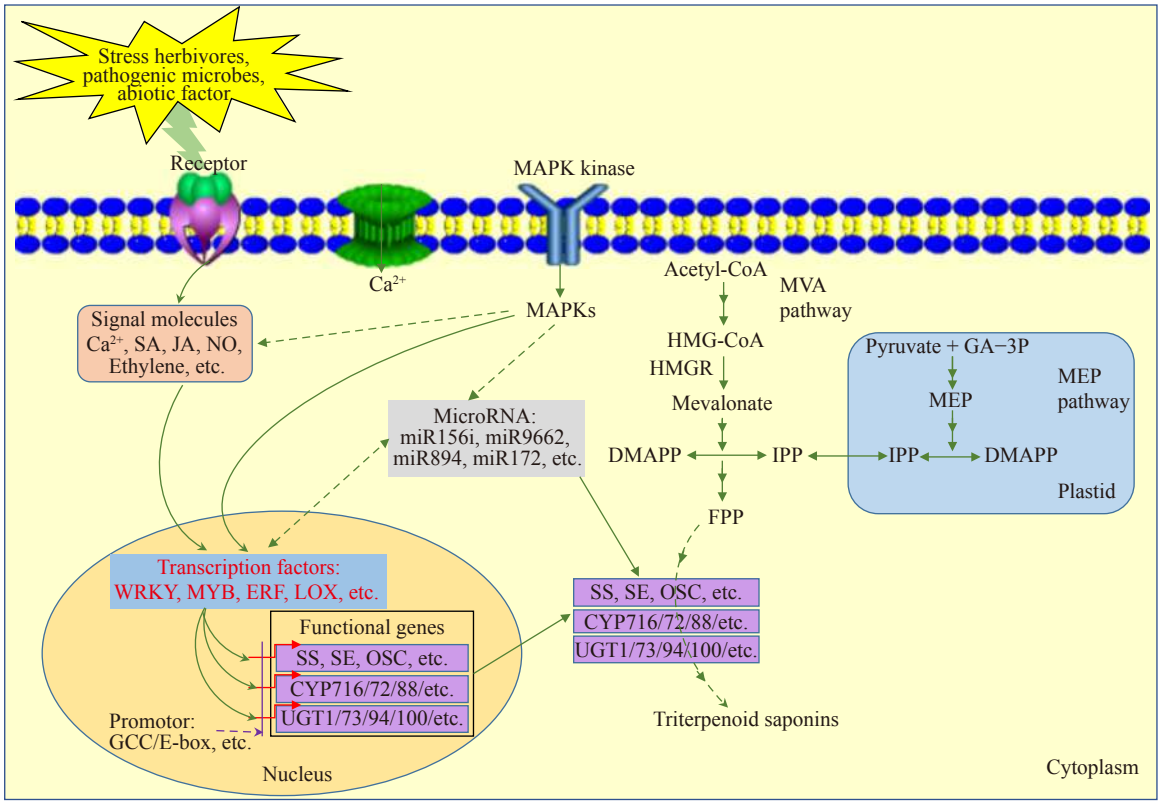


Fig. 2 The regulation of triterpenoid saponins biosynthesis in medicinal plant. Triterpenoid saponins are synthesized *via* the cytosol MVA pathway and plastid MEP pathway. Different stress affects the change of signal molecules and MAPKs, then actives the expression of transcription factors, microRNA. Transcription factors and microRNA further regulates the functional genes expression. HMG-CoA, 3-hydroxy-3-methylglutaryl-CoA; MEP, 2-C-methyl-Derythritol 4-phosphate; HMGR, 3-hydroxy-3-methylglutaryl-CoA reductase; SS, squalene synthase; SE, squalone epoxidase; OSC, oxidosqualene cyclase; UGT, UDP-glycosyltransferases. Dotted lines with multiple arrows represent multiple enzymatic catalyzed steps

sources are studied, such as *Artemisia annua*, *Panax ginseng*, *Medicago sativa*. Catalytic sites and moieties of various CYP450s and UGTs also have been studied, including 3-carbonyl, 6-hydroxyl, 23-hydroxyl, 28-carboxyl, 3-glucose, 3-galactose, etc. In dammarane-type saponins biosynthesis, protopanaxadiol is synthesized from hydroxylation C-12 of dammarenediol-II by CYP716A47 [44]. Pq3-O-UGT1 from *P. quinquefolius* was identified to transfer a glucose moiety into the C-3 glucose in ginsenoside Rh_2 [45]. However, there is no report on the biosynthesis of notoginsenosides catalyzed by uridine diphosphate xylosyltransferase. TFs that participate in the biosynthesis of triterpenoid saponins usually belong to the family of bHLH [46], ERF [41], MYB [39, 40], and WRKY [43]. The mechanisms of TFs on triterpenoid saponins biosynthesis still need further study.

This review summarizes the recent progress in triterpenoid saponins biosynthesis in medicinal plants including their sources, key biosynthesis genes, etc. Gene mining, gene screening, gene function verification, catalyzing mechanism of key enzymes, and the regulation of triterpenoid saponins biosynthesis are also summarized. Perspectives on further discovery of medicinal plants biosynthesis and synthetic biology strategies for microbial production of triterpenoid saponins derived from plant are also discussed.

Gene Mining by Omics-based Sequencing

Omics-based research provides crucial insights into individual biological components (such as gene expression, protein modification, metabolite profiles) levels. Through statistical tools and data mining, the integration of omics datasets provides a blueprint of relationships between different biological components. These analyses assist in devising strategies to produce metabolites of interest by generating bacterial or plant-based systems [47].

In recent years, some important medicinal plants have been subjected to omics sequencing, such as *A. annua* [48], *Gastrodia elata* [49], *G. uralensis* [50], *P. notoginseng* [51], which draws the blueprint of medicinal plants and realizes sustainable production of pharmacological triterpenoid saponins. In *P. notoginseng*, genes potentially related to ginsenoside biosynthesis were discovered by a draft genome and comparative transcriptome, which provides the foundation and makes it possible to genetic improvement of *P. notoginseng* [52]. These potential key genes in triterpenoid saponins biosynthesis need to be further screened by genes screening in order to discover the key one.

Gene Screening

In recent years, many triterpenoid saponins such as gin-

Table 1 Overview of medicinal plant CYP450s, UGTs and transcription factors in triterpenoid saponins biosynthesis

	Gene	Position	Plant sources	Reference
CYP450s	CYP716A14v2	3-carbonyl	*Artemisia annua*	[18]
	CYP716A53v2	6-hydroxyl	*Panax ginseng*	[19]
	CYP716A47	12-hydroxyl	*Panax ginseng*	[19]
	CYP716A52v2	28-carboxyl	*Panax ginseng*	[20]
	CYP716A12	28-carboxyl	*Medicago sativa*	[21]
	CYP88D6	11-carbonyl	*Glycyrrhiza uralensis*	[22]
	CYP72A154	30-hydroxyl	*Glycyrrhiza uralensis*	[23]
	CYP716A94	28-carboxyl	*Kalopanax septemlobus*	[24]
	CYP72A397	23-hydroxyl		
UGTs	UGT73C10	3-glucose	*Barbarea vulgaris*	[25]
	UGT73C11			
	UGT74AE2	3-glucose	*Panax ginseng*	[26]
	UGT94Q2	3-glucose(2-1) glucose	*Panax ginseng*	[26]
	UGT74AC1	3-glucose	*Siraitia grosvenorii*	[27]
	GuUGAT	3-glucuronic acid	*Glycyrrhiza uralensis*	[28]
	UGT71A27	20-glucose	*Panax ginseng*	[29]
	UGTPg1	20-glucose	*Panax ginseng*	[8]
	UGTPg100	6-glucose	*Panax ginseng*	[8]
	UGTPg101	6-glucose	*Panax ginseng*	[30]
	UGTPg100	28-glucose	*Panax ginseng*	[8]
	UGTPg101	28-glucose	*Panax ginseng*	[30]
	UGT74M1	28-glucose	*Saponaria vaccaria*	[31]
	UGT73AD1	28-glucose	*Centella asiatica*	[32]
	UGT73P2	3-galactose	*Glycine max*	[33]
	UGT91H4	3-rhamnose	*Glycine max*	[33]
	UGT73P12	3-glucuronic acid	*Glycyrrhiza uralensis*	[34]
	UGT73F4	22-xylose	*Glycine max*	[35]
	UGT73F2	22-xylose	*Glycine max*	[35]
Transcription factors	PgWRKY1-9		*Panax ginseng*	[36]
	PgWRKY1		*Panax ginseng*	[37]
	PgLOX6		*Panax ginseng*	[38]
	PgMYB1		*Panax ginseng*	[39]
	PgMYB1-5		*Panax ginseng*	[40]
	PnERF1		*Panax notoginseng*	[41]
	PqWRKY1		*Panax quinquefolium*	[42]
	WsWRKY1		*Withania somnifera*	[43]

senoside, notoginsenoside and glycyrrhizin are mostly extracted from native roots and tissue cultures of medicinal plant [28, 53, 54]. The related biosynthesis genes have been discovered [55, 56], but lots of key genes have not been explored. For example, xylosyltransferase genes in notoginsenoside biosynthesis of *P. notoginseng* have not been clearly studied.

A UGT gene, *Pq3-O-UGT2* was obtained from *P. quinquefolius* by genome-wide searching. The BLASTP search results revealed its deduced amino acid sequence had 99.55% similarity with that of PgUGT94Q2 in *P. ginseng*. The gene of *Pq3-O-UGT2* also showed a close evolutionary relationship with ginseng UGTs by phylogenetic analysis [55]. In *W. somnifera*, by phylogenetic analysis, WsWRKY1 showed close similarity to AaWRKY1, CrWRKY1 from *A. annua*, *C. roseus*, respectively. WsWRKY1 was further characterized as WRKY protein, which was involved in regulating stress tolerance of *W. somnifera* [43]. By genetic screening, key genes in the biosynthesis of triterpenoid saponins could be further selected, which makes it possible to explore gene function and improve the content of bioactive compounds by

genetic manipulation.

Verification of Gene Function

Verification of gene function in vivo or in vitro

Overexpression and RNA interference (RNAi) are common methods to verify gene function *in vivo* and can perform direct verification of gene function. In *P. quinquefolius* transgenic hairy roots, overexpression of *Pq3-O-UGT1* gene led to a higher level of protopanaxadiol-group ginsenosides, which is consistent with enzymatic assay *in vitro* that Pq3-O-UGT1 could glycosylate protopanaxadiol to produce ginsenoside Rh_2 [45]. However, it is difficult to induce their genetically modified system for some plants. Thus, verification of gene function *in vitro* was very meaningful.

Verification of gene function *in vitro* was usually studied by model plant (such as *A. thaliana*, tobacco) or engineered strains (such as *S. cerevisiae*, *Escherichia coli*) transferred specific vectors with target genes. Overexpression of *PgHMGR1* from *P. ginseng* resulted in higher production of triterpenes and sterols in Arabidopsis [56]. In *S. cerevisiae*, the function of CYP716A252 and CYP716A253 from *Ocimum basilicum* was also determined [57]. Through gene function verification, key genes could be introduced in other expression system to yield corresponding triterpenoid saponins. In *P. japonicas*, *β*-Amyrin synthase was recognized as the first key enzyme in oleanane-type saponins biosynthesis. *P. notoginseng* cells transferred into *β*-Amyrin synthase gene from *P. japonicus* (*PjβAS*) produced two oleanane-type saponins, chikusetsusaponin IV and chikusetsusaponin IVa. Both saponins were only contained in *P. japonicas*, and were first discovered in transgenic *P. notoginseng* cells [58].

When gene function is verified, these genes could be introduced into engineering strains and their corresponding product could be synthesized by synthetic biology strategies. Furthermore, gene mutations and gene editing technology could vary gene expression causing a change in active product content. By repurposing UGT51, an inherently promiscuous glycosyltransferase, about 300 $mg \cdot L^{-1}$ of ginsenoside Rh_2 was obtained from *S. cerevisiae* [10]. Metabolic switching of astringent and beneficial triterpenoid saponins in soybean could be achieved by a loss-of-function mutation in cytochrome P450 72A69 [59]. Verification of gene function is the significant step, and it provides a basis for synthetic biology to obtain expected compounds.

Subcellular localization

Subcellular localization was commonly used to reveal the expression and distribution sites of expressed protein [43, 60]. PgCYP736B was discovered to localize in the plasma membrane, which indicated PgCYP736B could express and function on endoplasmic reticulum [61]. UGTs are generally localized in the cytosol of medicinal plants. Subcellular localization of GFP-tagged UGT71C5 showed UGT71C5 distributed throughout the protoplast [62], similar to the cytosolic localization of the other reported UGTs [63]. A MYB gene, *PgMYB1* was cloned from *P. ginseng* C.A. Meyer. PgMYB1-mGFP5 fusion protein was discovered to specifically localize in the nucleus by subcellular localization [39]. Overall, subcellular localization of CYP450s, UGTs and TFs contributes to exploring their functional site and makes it possible to obtain the corresponding protein by directed transformation.

Catalyzing Mechanism of Key Enzymes

The discovery of catalyzing mechanism of key enzymes helps further verify gene function. It promotes the exploration of specific substrate, key catalytic sites of key enzymes [26, 28, 45]. Furthermore, it contributes to regulate triterpenoid saponins biosynthesis in transformed cell lines or engineered strains with recombinant vectors constructed by specifically optimized key enzymes and efficient substrate in large scale. The catalytic function of GuUGAT was determined by an *in vitro* enzyme assay, which showed GuUGAT could catalyse glycyrrhetinic acid to produce glycyrrhizin. Site-directed mutagenesis revealed key catalytic sites of glucuronosylation as Gln-352, and Gln-392, Glu-375, His-22, Trp-370, respectively [28].

With the development of gene engineering, gene mutation makes it possible to obtain specific functional genes in triterpenoid saponins biosynthesis. Gene mutation is essential to discover the key amino acids of catalyzing enzyme, and help to improve enzyme activity. By structural modeling and site-directed mutagenesis, Wei *et al*. found key amino acids of UGTPg1, UGTPg100 and UGTPg102 that could play essential roles in determining their bioactivities [30]. The discovery of catalyzing mechanism of key enzymes makes it possible to further change enzyme activity on their key catalytic sites.

Gene Regulation of Triterpenoid Saponins Biosynthesis in Medicinal Plants

The biosynthesis of triterpenoid saponins is affected by gene regulation in medicinal plants. Gene expression varies in different plants. Even in the same genus, genes expression is different. Both *P. ginseng* and *P. notoginseng* belong to Araliaceae family, but their saponins composition and concentration show a big difference [64, 65]. This difference may finally attribute to the various gene regulations of triterpenoid saponins biosynthesis genes between *P. ginseng* and *P. notoginseng*. Artificial changes in gene regulation by increasing the gene copy number or knocking out negative branch pathway help to synthesize expected compounds.

Key genes

Overexpression is a common method that could help verify and regulate key genes in triterpenoid saponins biosynthesis. In *P. quinquefolius* transgenic hairy roots, *Pq3-O-UGT1* overexpression induced Pq3-O-UGT1 mRNA accumulation and a higher level of protopanaxadiol-group ginsenosides, which is consistent with enzymatic assay *in vitro* that Pq3-O-UGT1 could catalyze protopanaxadiol to produce ginsenoside Rh_2 [45]. RNAi also is performed to regulate the spe-

cific functional genes in triterpenoid saponins biosynthesis. In *I. indigotica*, transcription factor Ii049 was characterized to play important role in lignan/lignin biosynthesis regulation. In order to verify its function, Ii049 was knocked down by RNAi approach, which caused a remarkable reduction of expression levels of genes involved in lignan/lignin biosynthesis and lignan/lignin contents. The result suggested Ii049 positively regulated the biosynthesis of lignan [60]. These results from overexpression and RNAi could directly reflect the regulation of genes on triterpenoid saponins biosynthesis.

Transcription factors

Transcription factors (TFs), also known as trans-acting factors, are DNA-binding proteins. They specifically interact with cis-acting elements in the promoter region of eukaryotic genes, inducing the expression of other related proteins, or inhibiting downstream genes transcription. They play an essential role in secondary metabolites biosynthesis [46, 66].

In *P. ginseng* C.A. Meyer, eight WRKY genes were cloned, characterized and their encoding WRKY proteins were assigned to WRKY Group II by phylogenetic analysis [36]. In PnbHLH1 transgenic cells, the expression levels of key triterpenoid saponins biosynthesis genes were higher than those in control. Similarly, the total saponin contents were increased compared with the control. These results suggested that in *P. notoginseng*, the PnbHLH1 is a positive regulator in triterpenoid saponins biosynthesis [46].

MicroRNA

MicroRNA (miRNA), a class of endogenous small noncoding RNA, have been discovered to play an important role in regulating gene expressions in plants [67]. In *P. ginseng*, transcriptome analysis discovered potential miRNAs could regulate ginsenosides biosynthesis by function on their target of TFs and other proteins [68]. There were four miRNAs (miR5021, miR5163, miR5293 and novel_miR_27) predicted to target genes involved in the terpenoid backbone biosynthesis pathway in *P. notoginseng* [69].

Target genes and target proteins exploration

In recent years, accumulating evidence suggests that certain regulatory factor, such as TFs, microRNA regulate secondary metabolites production by regulating genes involved in metabolic pathway [43, 69-71]. Yeast one-hybrid (Y1H) assay, electrophoreticmobility shift assay (EMSA) and chromatin immunoprecipitation assay were performed to discover the interaction between protein and DNA [43, 72]. The yeast one-hybrid assay revealed that AaGSW1 of *A. annua* only bound to one of the W-box motifs in CYP71AV1 promoter. AaGSW1 positively regulates CYP71AV1 expression. Moreover, overexpression of AaGSW1 significantly improves artemisinin content. These results revealed AaGSW1 was a positive regulator in the biosynthetic pathway of artemisinin [53]. The EMSA demonstrated that PnERF1 might bind to the promoter regions which contained GCC-box and regulate corresponding genes expression [41]. The binding interaction between PgMYB2 protein and dammarenediol synthase (PgDDS) promoter was found by Y1H assay. Moreover, EMSA identified their binding site. Based on the key role of PgDDS gene in ginsenoside synthesis, it is reasonable to believe that PgMYB2 has the potential to improve the ginsenoside production through genetic and metabolic engineering [73]. The protein-protein interactions were explored by yeast two-hybrid assay, GST pull-down, dual-luciferase assay, coimmunoprecipitation, bimolecular fluorescence complementation. The exploration of target genes and target proteins was meaningful to regulate triterpenoid saponins biosynthesis by synthetic biology strategies, gene mutations and gene editing technology in order to further improve the yield of triterpenoid saponins.

Future Perspectives and Conclusions

In this review, we systematically summarize the identification and verification of key genes in triterpenoid saponins biosynthesis in medicinal plants including their plant sources, catalytic sites and moieties. Although several triterpenoid saponins have been successfully synthesized and their yield have great improvement by biomanufacturing [10, 54, 74], there are still huge challenges in triterpenoid saponins industrial production. For some essential triterpenoid saponins (such as notoginsenoside R1), their biosynthesis catalyzed by uridine diphosphate xylosyltransferase is needed. Furthermore, the biosynthesis pathway of triterpenoid saponins is not clear and their regulation mechanism is not very clear. Future study can focus on clarification the biosynthetic pathway of triterpenoid saponins and discovering their regulatory mechanism in order to regulate it. In addition, high-yield mutant strain/plant reconstructed by synthetic biology strategies, gene mutations and gene editing technology was meaningful to further increase triterpenoid saponins production.

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Cite this article as: YAO Lu, LU Jun, WANG Juan, GAO Wen-Yuan. Advances in biosynthesis of triterpenoid saponins in medicinal plants [J]. *Chin J Nat Med*, 2020, **18**(6): 417-424.