Identification of potential genes involved in triterpenoid saponins biosynthesis in *Gleditsia sinensis* by transcriptome and metabolome analyses

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Received: 31 July 2018 / Accepted: 18 November 2018 / Published online: 13 December 2018 © The Author(s) 2018

Abstract

*Gleditsia sinensis* is widely used as a medicinal plant in Asia, especially in China. Triterpenes, alkaloids, and sterols were isolated from *Gleditsia* species. Among them, triterpenoid saponins are very important metabolites owing to their various pharmacological activities. However, the triterpenoid saponin biosynthesis pathway has not been well characterized. In the present study, we performed de novo transcriptome assembly for 14.3 Gbps of clean reads sequenced from nine tissues of *G. sinensis*. The results showed that 81,511 unique transcripts (unigenes) were constructed, of which 31,717 unigenes were annotated with Gene Ontology and EC numbers by Blast2GO against the NCBI-nr protein database. We also analyzed the metabolite contents in the same nine tissues by LS–MS/MS, and saponins including gleditsioside I were found in fruit at higher levels. Many of the genes with tissue-specific expression in fruit are involved in the flavonoid biosynthesis pathway, and many of those have UDP-glucosyltransferase (UGT) activity. We constructed a saponin biosynthesis pathway and identified two key enzyme families in the triterpenoid saponin biosynthesis pathway, cytochrome P450 and UDP-glucosyltransferase, that are encoded by 37 unigenes and 77 unigenes, respectively. CYP72A, CYP716A, and CYP88D, which are known as key enzymes for saponin biosynthesis, were also identified among the P450s. Our results provide insight into the secondary metabolite biosynthesis and serve as important resources for future research and cultivation of *G. sinensis*.

Keywords  Saponin biosynthesis · De novo transcriptome assembly · *Gleditsia sinensis* · P450 · UGT

Introduction

*Gleditsia* is a genus from the legume family (Fabaceae) and grown worldwide, especially in Asia and America. Many species in the *Gleditsia* genus have been identified, including *G. sinensis*, *G. japonica*, and *G. triacanthos*, and these and other *Gleditsia* species are used as traditional diuretics and expectorants [1]. Recent phytochemical studies have revealed the presence of triterpenoid saponins, alkaloids, sterols, and flavonoids in these species. The extracts of these compounds exhibit several important biological activities, including antitumor, anti-inflammatory, and anti-HIV activities [2–4]. Over 30 compounds of triterpenoid saponins were identified in the tissues of *G. sinensis* [1], but the biosynthesis of these physiologically active substances is not yet fully understood.

Along with advances in the technology of DNA sequencers, the genomic and transcriptomic information of medicinal plants, e.g., *Catharanthus roseus*, *Glycyrrhiza uralensis*, and *Panax notoginseng*, has been accumulating
Large international consortiums such as the 1000 Plant Project have specifically targeted medicinal plants [8]. RNA-sequencing (RNA-Seq) technology is one of the powerful approaches for identifying functional genes without genome sequences. Two RNA-Seq studies have been conducted in *G. sinensis*. Zhu et al. analyzed the de novo transcriptomes from four tissues [9] and Han et al. reanalyzed the same transcriptome data set and identified putative chalcone isomerase genes [10]. However, these studies were not sufficient to understand differences in the bioactive metabolite biosynthesis pathway among a variety of tissues and to identify key enzymes for bioactive metabolites including saponins. Therefore, in this study, we conducted de novo assembly for the transcriptome data obtained from the nine tissues of *G. sinensis* and obtained annotated unigenes. This result could provide useful information for the mining of functional genes for the stable production of bioactive compounds of *G. sinensis*.

**Materials and methods**

**Plant materials, RNA isolation, and library preparation**

The nine tissues examined were bark, branch, bud, flower, fruit, leaf, stalk, wood, and young leaf; all were harvested from natural growing plants between May and June 2013 at Inohana Campus, Chiba University, Japan (Fig. 1). All tissues were frozen with liquid nitrogen until total RNA extraction.

**Illumina sequencing**

Total RNA extraction was performed using an RNeasy Mini kit (Qiagen, Valencia, CA) and the qualities were assessed by using a Bioanalyzer 2100 (Agilent, Palo Alto, CA). The samples with an RNA integrity number (RIN) above 7.8 were used for further analysis. The cDNA library was constructed using an Illumina TruSeq Prep kit v2 according to the manufacturer’s protocol (Illumina, San Diego, CA). The resulting cDNA library was sequenced using a HiSeq 1500 system (Illumina) with 100 bp paired-end reads.

**De novo transcriptome assembly**

The quality of the raw reads was checked by FastQC [11] and then the reads were processed by Trimmomatic software version 0.36 [12] to remove adaptor sequences and unpaired reads. Reads with a sequence length less than 75 bp were also removed. We performed de novo assembly using Trinity version 2.3.2 [13] with cleaned reads from all the samples to construct the unique transcripts (contigs). To determine the gene expression levels, we used RSEM [14] and filtered out

![Fig. 1 Nine tissues of *Gleditsia sinensis* used for de novo transcriptome analysis. RNA extraction and RNA-sequencing were conducted against the nine tissues (bark, branch, bud, flower, fruit, leaf, stalk, wood, and young leaf)](image-url)
the transcripts expressed at low levels with fragments per kilobase of transcript per million mapped reads (FPKM) < 1. The longest isoforms within the remaining transcripts were selected as unigenes and used for further analyses.

**Functional annotation**

All unigenes were searched against the NCBI-nr protein database (formatted in May, 2017) with Blastx (E value cutoff of 1e−5) and annotated by Blast2GO version 4.1.9 [15] with default settings. The GO categories, EC numbers, and gene IDs of the KEGG pathways [16] were also assigned and the top 20 gene ontology terms from three categories (biological process, molecular function, and cellular component) at level 3 for unigenes and the species distribution of the top Blastx hits were visualized by Blast2GO.

**Expression analysis**

The expression levels based on counts per million (CPM) in each sample were calculated against the unigenes by Bow-tie2 in RSEM. The raw counts were normalized by trimmed-mean of M normalization (TMM) and transformed to CPM with EdgeR for further analysis [17]. Principal component analysis was conducted with R.

**GO enrichment analysis of tissue-specific gene expression**

We identified the genes specifically expressed in each of the nine tissues using the tissue specificity index tau, which ranged from 0 (expressed in all tissues) to 1 (expressed in a single tissue) [18]. Tau was calculated on TMM-normalized CPM and the threshold was set to 0.95 for the tissue-specific genes. GO enrichment analysis was performed with the goseq [19] bioconductor package using hypergeometric testing and the overrepresented p value cutoff of < 0.01.

**Selection of candidate genes associated with the saponin biosynthesis pathway**

The putative saponin biosynthesis pathway was constructed on the basis of the terpenoid backbone pathway (map00900) in KEGG, and the enzymes were mapped on the basis of the EC numbers annotated by Blast2GO. The putative open reading frames (ORFs) were obtained by Blast2GO on the basis of the top hit, and those less than 100 amino acid residues in length were excluded. The predicted protein sequences were searched against the Pfam database by HMMER version 3.1b2 (E value cutoff of 1e−5). Candidate P450s and UGTs were predicted if the unigenes were assigned to PF00067 (cytochrome P450) and PF00201 (UDP-glucuronosyl and UDP-glucosyl transferase), respectively. To select the candidate P450 involved in saponin biosynthesis, all P450s were searched against predicted P450s in three Fabaceae species (*Lotus japonicus*, *Cicer arietinum*, and *Cajanus cajanifolius*) from the cytochrome P450 homepage (https://drnelson.uthsc.edu/CytochromeP450.html) by Blastp (E value cutoff of 1e−5 and percentage identity cutoff of 40%).

**Metabolite extraction**

Each tissue of *G. sinensis* was immediately frozen in liquid nitrogen and ground to powder using a mortar and pestle. Powdered samples (100 mg) were extracted with three volumes of methanol. After two homogenizations using a TissueLyser (Qiagen) at 27 Hz for 2 min each, the homogenates were centrifuged (12,000 × g, 10 min, 4 °C). The supernatant was filtered through a C18 Spin column (GL Sciences, Tokyo), and the filtrate was used for LC-Orbitrap-MS analysis.

**HPLC–MS analytical conditions**

HPLC–MS was performed with an Agilent 1200 system (Agilent) coupled to a Finnigan LTQ Orbitrap XL (Thermo Fisher Scientific, Waltham, MA), which was equipped with an electrospray source operating in the negative-ionization mode. Analiquot of the extracted sample (5 μL) was injected into an Ascentis Express C18 (2.7 μm, 150 × 4.6 mm; Sigma, St. Louis, MO) with mobile phases that consisted of 0.1% (v/v) aqueous formic acid (solvent A) and 0.1% (v/v) formic acid in acetonitrile (solvent B). The gradient program was as follows: 30–60% solvent B for the first 48 min, 60–95% solvent B for the next 2 min, 95% solvent B for the next 5 min, and 30% solvent B for the last 5 min, with a flow rate of 0.5 mL/min. The column oven temperature was set at 40 °C. HPLC–MS analysis was performed using electrospray ionization (ESI) in negative-ionization mode. The full scan (m/z 800–2000) used a resolution of 60,000. MS/MS were acquired on a Top5 data-dependent mode with a parent list for saponins.

**Data analysis of HPLC–MS**

All data obtained from the HPLC–MS analysis were acquired with the Xcalibur™ software (Thermo Fisher Scientific). Detection and structural prediction of saponins were determined by comparing the obtained data with the accurate mass, MS/MS spectra, and retention time from the previous data set (Supplementary Table S3) [20]. Full MS data and MS² analysis for gleditsioside I are shown in Supplementary Fig. 1.
Results

RNA-sequencing and de novo transcriptome assembly

A total of 14.3 Gbp reads from the nine tissues of *G. sinensis* were checked for quality and then applied to the transcriptome analysis. About 95% of the raw reads were of good quality (quality value (QV) > 30). The high-quality cleaned reads were used for de novo transcriptome assembly with Trinity. We obtained 230,780 contigs and estimated the expression levels with Bowtie2 in RSEM. We obtained 230,780 contigs and estimated the expression levels with Bowtie2 in RSEM. The 81,511 contigs were selected as unique transcripts (unitranscripts) after removing the genes with low gene expression of FPKM < 1. Moreover, the longest isoforms were selected from each of the unitranscripts as unigenes, because the splicing variants were included in the unitranscripts. As a result, the average, N50, and maximum lengths of the 47,855 unigenes were 1103 bases, 1952 bases, and 17,250 bases, respectively, and their GC content was 40.7% (Table 1).

Functional annotation

The Blast2GO searches successfully annotated the functions of 31,717 of the 47,855 unigenes (66.3%). The distribution of the species of the entries is shown in Fig. 2a, and 80% of the annotated unigenes were highly similar to genes of *Fabaceae* family members (*Cajanus cajan*, *Glycine max*, *Lupinus angustifolius*, and *Glycine soja*). The top 20 annotated gene ontology terms at the level 3 annotation with three categories (biological process, molecular function, and cellular component) are shown in Fig. 2b. The top five GO biological process terms were involved in metabolic processes (organic substance metabolic process (GO: 0071704), cellular metabolic process (GO: 0044237), primary metabolic process (GO: 0044238), nitrogen compound metabolic process (GO: 0006807), and biosynthetic process (GO: 0009058). Organic cyclic compound binding (GO: 0097159), heterocyclic compound binding (GO: 1901363), ion binding (GO: 0043167), transferase activity (GO: 0016740), and small molecule binding (GO: 0036094) were the top five GO molecular function terms. Intracellular (GO: 0005622), intracellular part (GO: 0044424), intracellular organelle (GO: 0043229), intrinsic component of membrane (GO: 0031224), and membrane-bounded organelle (GO: 0043227) were the top five GO cellular component terms.

Expression analysis

To estimate the abundance of expression of genes in the nine tissues, all the cleaned reads were mapped to the contigs constructed by de novo transcriptome assembly using Bowtie2 in RSEM. The raw count was normalized between tissues using the trimmed-mean of M normalization (TMM) method and transformed to counts per million (CPM) using edgeR. In order to compare the gene expression patterns among the nine tissues, we performed principal component analysis (PCA) using the prcomp function in R. PCA revealed that the nine tissues were clustered along developmental organs (Fig. 3). Two major components accounted for 41% of the gene expression variance. Along the PC2 axis, the results showed that gene expressions in the leaf and young leaf (yellow and gray) were significantly different from those in the other tissues. Along the PC1 axis, the other tissues were clustered into three new groups: branch, wood, and bark (blue, pink, and red); fruit and stalk (orange and brown); and bud and flower (green and purple). These results showed that the gene expression dynamics differed among the nine tissues.

Next, we analyzed the tissue specificity of the highly expressed genes in each of the nine tissues based on the tissue specificity index (tau). The tau value ranged from 0 to 1, where 1 means that a gene is highly expressed in only one tissue [18]. We identified 3959 genes (8.2%) with tau > 0.95 as tissue-specific genes. A heatmap displayed differential expression patterns of the tissue specific genes.
Triterpenoid saponins are major secondary metabolites of *G. sinensis*. Understanding their biosynthesis pathway is important for the high-volume production of cultivated species, but the basic information included in the genome sequencing and transcriptome profile is not sufficient.
Top-Hit Species Distribution [trinity_unigene_longest]

Species
- Cajanus cajan
- Glycine max
- Lupinus angustifolius
- Glycine seja
- Arachis ipaenesis
- Cicer arietinum
- Juglans regia
- Arachis duranensis
- Phaseolus vulgaris
- Vitis vinifera
- Medicago truncatula
- Vigna angustissima
- Vigna radiata var. radiata
- Ziziphus jujuba
- Vigna angustissima var. angustissima
- Trifolium subteraneum
- Theobroma cacao
- Lotus japonicus
- Manihot esculenta
- Prunus persica
- Citrus sinensis
- Prunus mume
- Morus notabilis
- Jatropha curcas
- Ricinus communis
- Cephalotus follicularis
- Conchorus capsularis
- Malus domestica
- Conchorus olitorius
- others

GO Distribution by Level (3) – Top 20

BP
- organic substance metabolic process
- cell metabolic process
- nitrogen compound metabolic process
- single-organism metabolic process
- single-organism cellular process
- regulation of cellular process
- establishment of localization
- cellular component organization
- regulation of metabolic process
- cellular response to stimulus
- response to stimulus
- cell communication
- catalytic process
- single-organism signaling
- signal transduction
- single-organism localization
- cellular component biogenesis
- response to chemical

MF
- organic cyclic compound binding
- heterocyclic compound binding
- oxidoreductase activity
- transferase activity
- small molecule binding
- hydrolase activity
- carbohydrate derivative binding
- oxidoreductase activity
- protein binding
- transmembrane transporter activity
- substrate-specific transporter activity
- structural constituent of ribosome
- hydrolase activity
- carbohydrase activity
- transcription factor activity, sequence-specific
- lyase activity
- isomerase activity
- carbohydrate binding
- receptor activity
- enzyme regulator activity

CC
- intracellular
- intracellular organelle
- intracellular organelle
- intrinsic component of membrane
- membrane-embedded or transmembrane protein
- cytoplasm
- cell periphery
- cytoskeleton
- organelle
-cytoplasmic
cell part
- plasma membran
- protein complex
- endomembrane system
- ribonucleoprotein complex
- organelle membrane
- catalytic complex
- organelle lumen
- membrane protein complex
- whole membrane
- membrane
- plasma membrane part
- external encapsulating structure
Advanced sequencing technology helps us to discover new genes involved in secondary metabolite formation and to infer the biosynthesis pathway for medicinal plants. De novo transcriptome studies have been successfully used to infer the saponin biosynthesis pathway in plants such as *Panax japonica*, *Anemone flaccida*, *Ilex asprella*, and *Platycodon grandiflorus* [26–29].

To our knowledge there has been only one study on the transcriptome analysis of *G. sinensis* [9]. Zhu et al. constructed 142,155 contigs from four tissues based on 77.5 million reads but did not describe saponin biosynthesis. Our de novo transcriptome assembly from the nine tissues yielded 47,855 unigenes with N50 1952 bp based on 143 million reads. The 31,717 high-quality unigenes were annotated with the NCBI-nr protein database using Blast2GO. The annotation revealed that the unigenes were highly similar to those of *Glycine* spp. The ontologies significantly enriched for the genes specifically expressed in fruit were involved in saponin biosynthesis, and the contents of many saponins were high in fruit by the metabolome analysis. These results suggested that saponin biosynthesis is active mainly in the fruit. In fact, oleanane-type triterpenoid saponins, including gleditsiosides I, J, and K, were previously isolated from the fruit of *G. sinensis* [21]. On the basis of the Blast2GO annotation, we identified the 84 genes that may be involved in saponin biosynthesis, including those
Fig. 6 Metabolomic analysis for the nine tissues by LC/MS. Base peak chromatogram of a fruit extract of *G. sinensis* by LS/MS (a) and the content of the top 10 saponins in fruit (b). The elution peak (Rt=11.00 min, m/z = 1453.6849) detected and speculated as saponin of gleditsioside I which is compared to data of compound no. 0.43 of Wang et al. [20]. Glc: glucose, Ara: arabinose, Rha: rhamnose, Xyl: xylose. The saponin nos. are described in Supplementary Table S3 in detail.
Fig. 7  Putative triterpenoid backbone biosynthesis pathway (MVA and MEP) of *G. sinensis* and gene expression profile of the key enzymes.
active in the mevalonate (MVA) and methylerythritol phosphate (MEP) pathways. In the gleditsioside biosynthesis, cyclization of 2,3-oxidosqualene is the first step taken by the enzymes coding oxidosqualene cyclases (OSCs), including beta-amyrin synthase. The cyclic skeletons created by OSCs are oxidized by cytochrome P450 (P450s) to sapogenins, which are the non-sugar part of saponins. The sapogenins undergo glycosylation of the C3-hydroxyl and C28-carboxyl by UDP-dependent glycosyltransferase (UGTs) in order to increase the diversity of saponins. On the plant genomes, 1% of coding genes code P450s, which is the largest family of enzymes. The plant P450 families form a category 11 clan based on their phylogenetic tree [30]. With respect to triterpenoid saponins biosynthesis, the clans CYP51, CYP71, CYP72, and CYP85 have been reported as key enzymes. In the CYP72 clan, DN43204_c0_g4_i1, which has a high similarity to CYP72A219, is specifically expressed in fruit. In Panax ginseng, Han et al. reported that CYP72A219 encodes secologanin synthase, which catalyzes the conversion of loganin to secologanin. In the CYP85 clan, DN28231_c0_g1_i4 and DN40674_c1_g1_i1 are highly similar to CYP88D3 and beta-amyrin 28-oxidase, respectively. The function of CYP88D3 is not clear, but Seki et al. identified CYP88D6 as a beta-amyrin 11-oxidase in Glycyrrhiza plants [24]. In addition, DN42708_c1_g1_i9 was similar to UGT91A1, which was predicted to be involved in the glycosylation of flavonols or flavonol glycosides in Arabidopsis thaliana [31]. The function of these candidate P450s and UGTs involved in triterpenoid saponin biosynthesis has not been well characterized in G. sinensis, but de novo transcriptome analysis could help us to identify important candidates for future functional characterization.

## Conclusions

In this study, we performed de novo transcriptome analysis and metabolome analysis using nine tissues of G. sinensis. Our transcriptome data were more comprehensive than the data in previous reports. This data set will help to elucidate the secondary metabolite biosynthesis pathway for the breeding of medicinal plants with a high yield of saponin, and thereby accelerate the future commercial production of saponins.

## Acknowledgements

This work was supported by the Ministry of Health, Labour and Welfare of Japan [under a Health and Labour Sciences Research Grant for the enhancement of a Comprehensive Medicinal Plant Database]; and by the Japan Agency for Medical Research and Development (AMED) [under a Grant for Research on the Development of New Drugs; 17ak0101046h0002], and the Kazusa DNA Research Institute Foundation.

## Author contributions

Conceived and designed experiments: HS, MY, and KS. Performed experiments: DN, SS, MN, and HS. Analyzed data: YK and HH. Contributed reagents/materials: MN, N Kawano, N Kawahara, MY, and KS. Wrote the paper: YK and HH. All authors read and approved the final manuscript.
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References

1. Zhang J-P, Tian X-H, Yang Y-X et al (2016) Gleditsia species: an ethnomedical, phytochemical and pharmacological review. J Ethnopharmacol 178:155–171. https://doi.org/10.1016/j.jep.2015.11.044
2. Yu J, Li G, Mu Y, Zhou H, Wang X, Yang P (2018) Anti-breast cancer triterpenoid saponins from the thorns of Gleditsia sinensis. Nat Prod Res. https://doi.org/10.1080/14786419.2018.1443092
3. Seo CS, Lim HS, Ha H, Jin SE, Shin HK (2015) Quantitative analysis and anti-inflammatory effects of Gleditsia sinensis thorns in RAW 264.7 macrophages and HaCaT keratinocytes. Mol Med Rep 12(3):4773–4781. https://doi.org/10.3892/mmr.2015.3936
4. Li W-H, Zhang X-M, Tian R-R, Zheng Y-T, Zhao W-M, Qiu M-H (2007) A new anti-HIV lupane acid from Gleditsia sinensis Lam. J Asian Nat Prod Res. 9(6–8):551–555. https://doi.org/10.1080/1028602060083419
5. Kellner F, Kim J, Clavijo BJ et al (2015) Genome-guided investigation of plant natural product biosynthesis. Plant J. 82(4):680–692. https://doi.org/10.1111/pj.12827
6. Mochida K, Sakurai T, Seki H et al (2017) Draft genome assembly and annotation of Glycyrrhiza uralensis, a medicinal legume. Plant J. 89(2):181–194. https://doi.org/10.1111/pj.13385
7. Chen W, Kui L, Zhang G et al (2017) Whole-genome sequencing and analysis of the Chinese herbal plant Panax notoginseng. Mol Plant. 10(6):899–902. https://doi.org/10.1016/j.molp.2017.02.010
8. Matsuoi N, Hung L-H, Yan Z et al (2014) Data access for the 1,000 Plants (1KP) project. Gigascience. 3:17. https://doi.org/10.1093/10.1093/bioinformatics/bty045
9. Chen W, Kui L, Zhang G et al (2017) Whole-genome sequencing and analysis of the Chinese herbal plant Panax notoginseng. Mol Plant. 10(6):899–902. https://doi.org/10.1016/j.molp.2017.02.010
10. Han S, Wu Z, Wang X et al (2016) De novo assembly and characterization of Gleditsia sinensis transcriptome and subsequent gene identification and SSR mining. Genet Mol Res. https://doi.org/10.4238/gmr.15017740
11. Andrews S, Fast QC (2018) A quality control tool for high throughput sequence data. https://www.bioinformatics.babraham.ac.uk/projects/fastqc/. Accessed 3 Mar 2018.
12. Bolger AM, Lohse M, Usadel B (2014) Trimmomatic: a flexible trimmer for Illumina sequence data. Bioinformatics. 30(15):2114–2120. https://doi.org/10.1093/bioinformatics/btu170
13. Grabherr MG, Haas BJ, Yassour M et al (2011) Full-length transcriptome assembly from RNA-Seq data without a reference genome. Nat Biotechnol. 29(7):564–574. https://doi.org/10.1038/nbt.1883
14. Li B, Dewey CN (2011) RSEM: accurate transcript quantification from RNA-Seq data with or without a reference genome. BMC Bioinformatics. 12(1):323. https://doi.org/10.1093/nb.11875
15. Conesa A, Götzt S (2008) Blast2GO: a comprehensive suite for functional analysis in plant genomics. Int J Plant Genom. 2008:1–12. https://doi.org/10.1155/2008/6919832
16. Kanchehia M, Furumichi M, Tanabe M, Sato Y, Morishima K (2017) KEGG: new perspectives on genomes, pathways, diseases and drugs. Nucleic Acids Res. 45(D1):D353–D361. https://doi.org/10.1093/nar/gkw1092
17. Robinson MD, McCarthy DJ, Smyth GK (2010) edgeR: A Bioconductor package for differential expression analysis of digital gene expression data. Bioinformatics. 26(1):139–140. https://doi.org/10.1093/bioinformatics/btp616
18. Yani I, Benjamin H, Shmoish M et al (2005) Genome-wide mid-range transcription profiles reveal expression level relationships in human tissue specification. Bioinformatics. 21(5):650–659. https://doi.org/10.1093/bioinformatics/bti042
19. Young MD, Wakefield MJ, Smyth GK, Oshlack A (2010) Gene ontology analysis for RNA-seq: accounting for selection bias. Genome Biol. 11(2):R14. https://doi.org/10.1186/gb-2010-11-2-r14
20. Wang S, Wang Q, Qiao X et al (2016) Separation and characterization of triterpenoid saponins in Gleditsia sinensis by comprehensive two-dimensional liquid chromatography coupled with mass spectrometry. Planta Med. 82(18):1558–1567. https://doi.org/10.1055/s-0042-110206
21. Zhang Z, Koike K, Jia Z, Nikaido T, Guo D, Zheng J (1999) Triterpenoid saponins from Gleditsia sinensis. Phytochemistry. 52(4):715–722
22. Ghosh S (2017) Triterpene structural diversification by plant cytochrome P450 enzymes. Front Plant Sci 8:1886. https://doi.org/10.3389/fpls.2017.01886
23. Shibuya M, Hoshino M, Katsube Y, Hayashi H, Kushiho T, Ebijuka Y (2006) Identification of beta-amyrin and sophoradiol 24-hydroxylase by expressed sequence tag mining and functional expression assay, FEBS J 273(5):1408–1409. https://doi.org/10.1111/j.1742-4656.2006.05120.x
24. Seki H, Ohyama K, Sawai S et al (2008) Licorice beta-amyrin 11-oxidase, a cytochrome P450 with a key role in the biosynthesis of the triterpene sweetener glycyrrhizin. Proc Natl Acad Sci. 105(37):14204–14209. https://doi.org/10.1073/pnas.0803876105
25. Fukushima EO, Seki H, Sawai S et al (2013) Combinatorial biosynthesis of legume natural and rare triterpenoids in engineered yeast. Plant Cell Physiol. 54(5):740–749. https://doi.org/10.1093/pcp/pcp015
26. Rai A, Nakamura M, Takahashi H, Suzuki H, Saito K, Yamazaki M (2016) High-throughput sequencing and de novo transcriptome assembly of Swertia japonica to identify genes involved in the biosynthesis of therapeutic metabolites. Plant Cell Rep. 35(10):2091–2111. https://doi.org/10.1007/s00204-016-2211-z
27. Zhan C, Li X, Zhao Z et al (2016) Comprehensive analysis of the triterpenoid saponins biosynthetic pathway in anemone flaccida by transcriptome and proteome profiling. Front Plant Sci. 7:1094. https://doi.org/10.3389/fpls.2016.01094
28. Zheng X, Xu H, Ma X, Zhan R, Chen W (2014) Triterpenoid sap-onin biosynthetic pathway profiling and candidate gene mining of the Ilex asperella root using RNA-Seq. Int J Mol Sci. 15(4):5970–5987. https://doi.org/10.3390/ijms15045970
29. Tamura K, Teranishi Y, Ueda S et al (2017) Cytochrome P450 monoxygenase CYP716A141 is a unique beta-amyrin C-16b oxidase involved in triterpenoid saponin biosynthesis. Platyctodon grandiflorus. Plant Cell Physiol. 58(6):1119–1119. https://doi.org/10.1093/pcp/pcx067
30. Nelson D, Werck-Reichhart D (2011) A P450-centric view of plant evolution. Plant J. 66(1):194–211. https://doi.org/10.1111/j.1365-313X.2011.04529.x
31. Stracke R, Ishihara H, Hup G et al (2007) Differential regulation of closely related R2R3-MYB transcription factors controls flavon accumulation in different parts of the Arabidopsis thaliana seedling. Plant J. 50(4):660–677. https://doi.org/10.1111/j.1365-313X.2007.03078.x