Original Article
Food Science and Technology

ISSN 0101-2061 (Print)
ISSN 1678-457X (Online)

DOI: https://doi.org/10.1590/fst.122122

Transcriptome-based analysis reveals the key genes of sesquiterpene glycosylation in *Dendrobium nobile*

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Abstract

Dendrobium nobile is a traditional Chinese medicine and nourishing food in China. Alkaloids and sesquiterpene glycosides are the two main active ingredients in *D. nobile*. The previous reports showed that alkaloids and sesquiterpene glycosides have the same upstream biosynthetic pathway, starting from sesquiterpene components, part of which is converted to sesquiterpene glycosides by the action of UDP-glycosyltransferase, and part of which is further synthesized to alkaloids. Therefore, sesquiterpene UDP-glycosyltransferases play an important role in the biosynthesis of alkaloids and sesquiterpene glycosides in *D. nobile*. In this study, the key glycosyltransferase genes in sesquiterpene glycosylation in *D. nobile* were explored, predicted and validated by transcriptome technology.

Keywords: *Dendrobium nobile*; alkaloids; sesquiterpene glycosides; UDP-glycosyltransferase.

Practical Application: This investigation provides a comprehensive information for consumers and researchers to understand active ingredients and nutritional health functions in *D. nobile*.

1 Introduction

In recent years, the R&D of natural functional products from herbs has attracted more and more attention in the world (Ruiz-Cisneros et al., 2022; Wang et al., 2022a, b; Yin et al., 2022). *Dendrobium nobile* Lindl. (Orchidaceae) is a famous traditional Chinese medicine and nourishing functional food with thousands of years of history in China (Chinese Pharmacopoeia Commission, 2020). *D. nobile* is mainly distributed in Guizhou, Hainan, Guangxi, Yunnan four provinces of China and other subtropical areas (Yu et al., 2015). In traditional medicine, *D. nobile* was as a tonic to nourish Yin, clear heat, nourish stomach, and replenish body fluid (Cakova et al., 2017; Shin et al., 2017; Xu et al., 2017) and used for various diseases or as beverages (Cakova et al., 2017). In term of modern pharmacological effects, *D. nobile* exhibits various effects, such as regulating lipid metabolism, antioxidant activity, anti-immune activity, protecting the nervous system, antitumor, antifibrosis, and others (Huang et al., 2019; Lv et al., 2020).

Phytochemical investigations indicated that *D. nobile* contains many types of chemical components, including sesquiterpene glycosides, alkaloids, phenanthrenes, bibenzyls, and plysaccharides (Lam et al., 2015; Thanh et al., 2017; Wang et al., 2019; Xu et al., 2013, 2017). Among them, alkaloids and sesquiterpene glycosides were considered to be the two most important active ingredients in *D. nobile*. Alkaloids were found to be closely related to protecting the nervous system, regulating lipid metabolism (Huang et al., 2019; Lv et al., 2020). However, sesquiterpene glycosides were reported to have immune enhancing effects by stimulating the proliferation of the proliferation of B cells *in vitro* (Zhao et al., 2003). Specifically, previous reports showed that alkaloids and sesquiterpene glycosides have the same upstream biosynthetic pathway, starting from sesquiterpene components, part of which is converted to sesquiterpene glycosides by the action of UDP-glycosyltransferase, and part of which is further synthesized to alkaloids (Gong et al., 2021). Therefore, sesquiterpene UDP-glycosyltransferases play an important role in the biosynthesis of alkaloids and sesquiterpene glycosides in *D. nobile*. Our previous study found that the content of alkaloids in *D. nobile* decreases with the increase of growth years, on the contrary, the content of sesquiterpene glycosides keeps increasing, and this phenomenon also indirectly proved that alkaloids and sesquiterpene glycosides have a certain relationship (Lu et al., 2022). In the present continued study, the key glycosyltransferase genes in sesquiterpene glycosylation in *D. nobile* were explored, predicted and validated by comparing the DGEs of 1 and 3 years old *D. nobile* through transcriptome technology.

2 Materials and methods

2.1 Plant materials

The stems of 1-year-old and 3-year-old *D. nobile* were randomly collected from the Dendrobium Germplasm Garden

Received 02 Nov., 2022
Accepted 12 Dec., 2022
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of Chishui Xintian Company located in Chishui City, China in October of 2019 and authenticated by Associate Professor Daopeng Tan (pharmacognosy, Zunyi medical university). All stems were processed as described previously (Li et al., 2022). Briefly, all samples were divided into two groups according to growth years and each group had three biological replicates for sequencing.

2.2 RNA extraction, cDNA library and Illumina sequencing

Total RNA of each sample was extracted by using Trizol (Invitrogen, CA, USA) on the basis of manufacturer's instructions for the creation of individual cDNA libraries and Illumina sequencing. After purification and characterization, RNA with a RIN number greater than 7.0 was next purified from total RNA (5 μg) using poly-T oligo-attached magnetic beads. And then, the mRNA was split into small fragments of approximately 200 bp using divalent cations at high temperature. The cleaved RNA fragments were then reverse transcribed to create the final cDNA library following the protocol of the mRNASeq sample preparation kit (Illumina, San Diego, USA), with an average insert size of 300bp (±50bp) for the paired-end library. Paired-end sequencing was then performed on an Illumina Nova seq™ 6000.

2.3 De novo assembly, unigene annotation and functional classification

Methods for de novo assembly, unigene annotation and functional classification were processed as previous studies (Li et al., 2022). Briefly, de novo assembly of transcriptomes was performed by Trinity 2.4.0 (Grabherr et al., 2011). Trinity groups transcripts into clusters based on shared sequence content. Such transcript clusters are very loosely referred to as "genes". The longest transcript in the cluster was selected as the "gene" sequence (aka Unigene). All assembled Unigenes were aligned using DIAMOND with the Non-Redundant (Nr) Protein Database, Kyoto Encyclopedia of Genes and Genomes (KEGG), Gene Ontology (GO), the SwissProt and eggNOG databases with a threshold E value < 0.00001.

2.4 Differentially expressed genes analysis

The expression level of each assembled unigene was calculated by TPM (Mortazavi et al., 2008), using Salmon (Patro et al., 2017). Differentially expressed genes (DEGs) were selected by the R package edgeR (Robinson et al., 2010) with the criteria of log2 (fold change) > 1 or log2 (fold change) < -1 and statistical significance (p-value < 0.05).

2.5 qRT-PCR analysis

To verify the accuracy of the transcriptome data, we selected nine differential genes (DEGs) for qRT-PCR validation. Total RNA was extracted by BIO FIT polysaccharide polyphenol bioreagent kit and cDNA was synthesized by TIANGEN reverse transcription kit. For each sample, three biological replicates were used for qRT-PCR assay with three technical replicates. Gene expression was calculated by the $2^{-\Delta\Delta Ct}$ method. The primers used for qRT-PCR were listed in Table 1.

2.6 Construction of phylogenetic tree of glycosyltransferase genes

The sequence of *Arabidopsis thaliana* glycosyltransferase protein was compared with the predicted Dendrobium glycosyltransferase by the ClustalW function in MEGA5 software, and the redundant base sequences were removed. The phylogenetic tree was constructed by using the neighbor-joining method, and the bootstrap value was set to 1,000.

2.7 Molecular docking

The crystal structures of the candidate glycosyltransferase-related proteins from Dendrobium were downloaded from the AlphaFold Protein Structure Database [AlphaFold DB, Jumper et al. (2021)] and modified using the Autodock tools 1.5.6 software (Jumper et al., 2021; Varadi et al., 2022). AlphaFold DB provides programmatic access to and interactive visualization of predicted atomic coordinates, per-residue and pairwise model-confidence estimates and predicted aligned errors. The 3D structures of sesquiterpene glycosides and their aglycones were built by ChemBioDraw Ultra14.0 and then converted to PDBQT coordinateds using AutoDockTools. The rotatable bonds in the ligand were assigned with AutoDock Tools, and the ligand docking was performed with the AutoDock Vina. Construct 2D maps of protein-ligand interactions using LigPlot software to analyze the interaction forces between protein and ligand binding. Construct 3D maps of protein-ligand interactions using PyMOL software to view the binding sites between proteins and ligands.

Table 1. The information of qPCR primers.

Gene name		Primer
LOC110092682	F	ACGAGGAGGTGAAGAAGTGG
	R	CTCAGCACCTCTACCACCAA
LOC110092820	F	TCCTCAGCCATCTTCGTCTC
	R	ACCAGTTGCCAGAATCTCCA
LOC110106342	F	GCCATTTTGCGTCGGATGATAA
	R	CGAGGTGTCGGTGTCTTGAA
LOC110096304	F	CTTTGTCGCCCTCTTCAACC
	R	CAAAGCAGAGGAAGACGACG
LOC110095820	F	CTTCAAAGGCCACCAATGCT
	R	CTCATTGTAGGCGGAGAGGT
LOC110095859	F	TGAGTCCTACGCCTTCTGTG
	R	TCTCGGCAGGTGTTGATGAT
LOC110104141	F	GTCACCTTCATCACCTTCGC
	R	GCGGATGGAGACTATGGACA
LOC110093388	F	CGAACACGGCAGATGAGATG
	R	TGCCTATCGAGCCAATCCAT
LOC110100769	F	GAGGTGGAGAGCGAGTTTCT
	R	TTGTTGACATGGTGCCGAAG

2.8 Statistical analysis

R program was used to visualize the expression levels of all investigated UDP-glycosyltransferases gene expressions and profile the global changes. Principal component analysis was used for gene expression statistics, and each gene expression value of a single sample was used as a variable to construct a data matrix, which was processed by the PCA function of mixOmics in the R language open source toolkit. Differences between two groups were calculated by t-test in R, and differences between multiple groups were calculated by one-way ANOVA in R. p-values <0.05 were identified as statistically significant differences, and all data were expressed as "mean ±standard error (SEM)".

3 Results and discussion

3.1 Effect of Growth Years on UDP-Glycosyltransferase Gene in D. nobile

In this study, 184 UDP-glycosyltransferase genes in *D. nobile* transcriptome data were collected by KEGG (Kyoto Encyclopedia of Genes and Genomes). First, Principal component analysis (PCA) was used to show the difference in the expression of UDP-glycosyltransferase genes in different growth years. The results showed that 1-year-old and 3-year-old groups were completely separated in the PC1 direction, indicating that expression profiles involved in UDP-glycosyltransferase genes were significantly affected by growth years (Figure 1A). The result was also confirmed by heatmap analysis (Figure 1B). Based on the expression of these genes, a volcano plot revealed how these genes were altered by growth years. The results showed that compared with the 1-year-old samples, 35 genes of UDP-glycosyltransferase in 3-year-old *D. nobile* were up-regulated, 24 genes were down regulated, and 125 genes had no significant change (Figure 1C, D). Based on our previous research (Lu et al., 2022), the content of sesquiterpene glycosides in *D. nobile* will increase with the growth years. Therefore, we have reason to infer that the increase of sesquiterpene glycosides is caused by the upregulation of UDP-glycosyltransferase genes expression.

3.2 Validation in DEGs related to growth years with qRT-PCR

Based on the results of the exploration of DEGs related to growth years, 25 genes, which encoded UDP-glycosyltransferase, sugar transport protein, and peroxidise, were screened, and

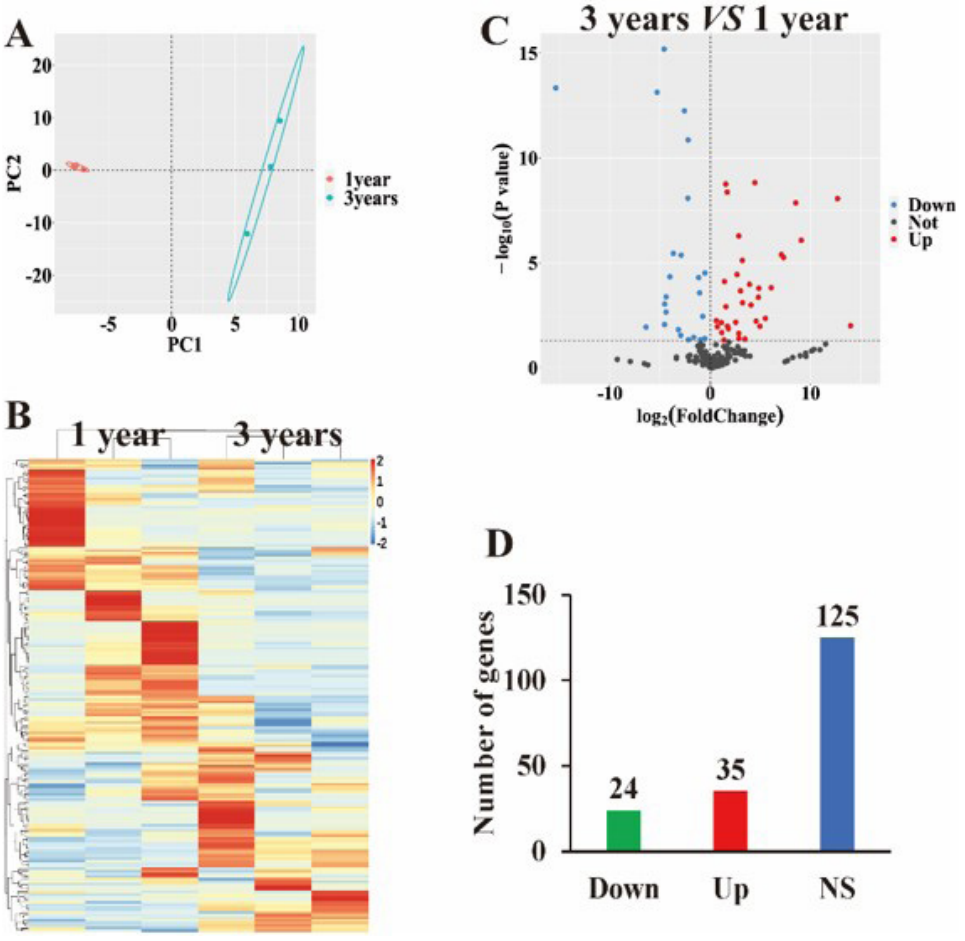

Figure 1. Alteration of UDP-glycosyltransferase genes induced by growth years. (A) PCA analysis of UDP-glycosyltransferase genes; (B) Heatmap analysis of UDP-glycosyltransferase genes; (C) Volcano plot showing UDP-glycosyltransferase genes altered by growth years; (D) Numbers of UDP-glycosyltransferase genes expression changes.

the expression of these genes differed vastly both in 1-year-old and 3-year-old groups. To confirm the dependability of the UDP-glycosyltransferase gene expression profiles for DEGs, 9 genes with the largest upregulation fold were validated by real-time quantitative RT-PCR using gene-specific primers (Table 1). Based on the qRT-PCR results, 7 of 9 candidate genes, including LOC110092682, LOC110093388, LOC110095820, LOC110095859, LOC110096304, LOC110100769, and LOC110104141, consistent with the gene expression levels obtained from RNA-seq data (Figure 2)

3.3 Construction of phylogenetic tree of UDP-glycosyltransferase genes

The UDP-glycosyltransferase genes of *Arabidopsis thaliana*, which is a model plant, have been well studied for their classification and functional annotation. Therefore, 20 UDP-glycosyltransferase genes from different families of *Arabidopsis thaliana* were selected for multiple sequence alignment with the above-mentioned six UDP-glycosyltransferase genes from *D. nobile*, and a phylogenetic tree analysis was constructed for functional annotation (Figure 3). The results showed that all the six Dendrobium UDP-glycosyltransferase genes were located in the branch of UDP-glycosyltransferase family 1. The donor molecule of glycosyltransferase family 1 is uridine diphosphate glucose, which plays the role of glucose glycosylation of ligand molecules, which is consistent with the fact that sesquiterpene glycosides in *D. nobile* are mainly glucose glycosides.

3.4 Molecular docking analysis

To further predict the glycosylation catalytic activity of candidate UDP-glycosyltransferases on the sesquiterpenes of *D. nobile*, molecular docking was used to simulate the

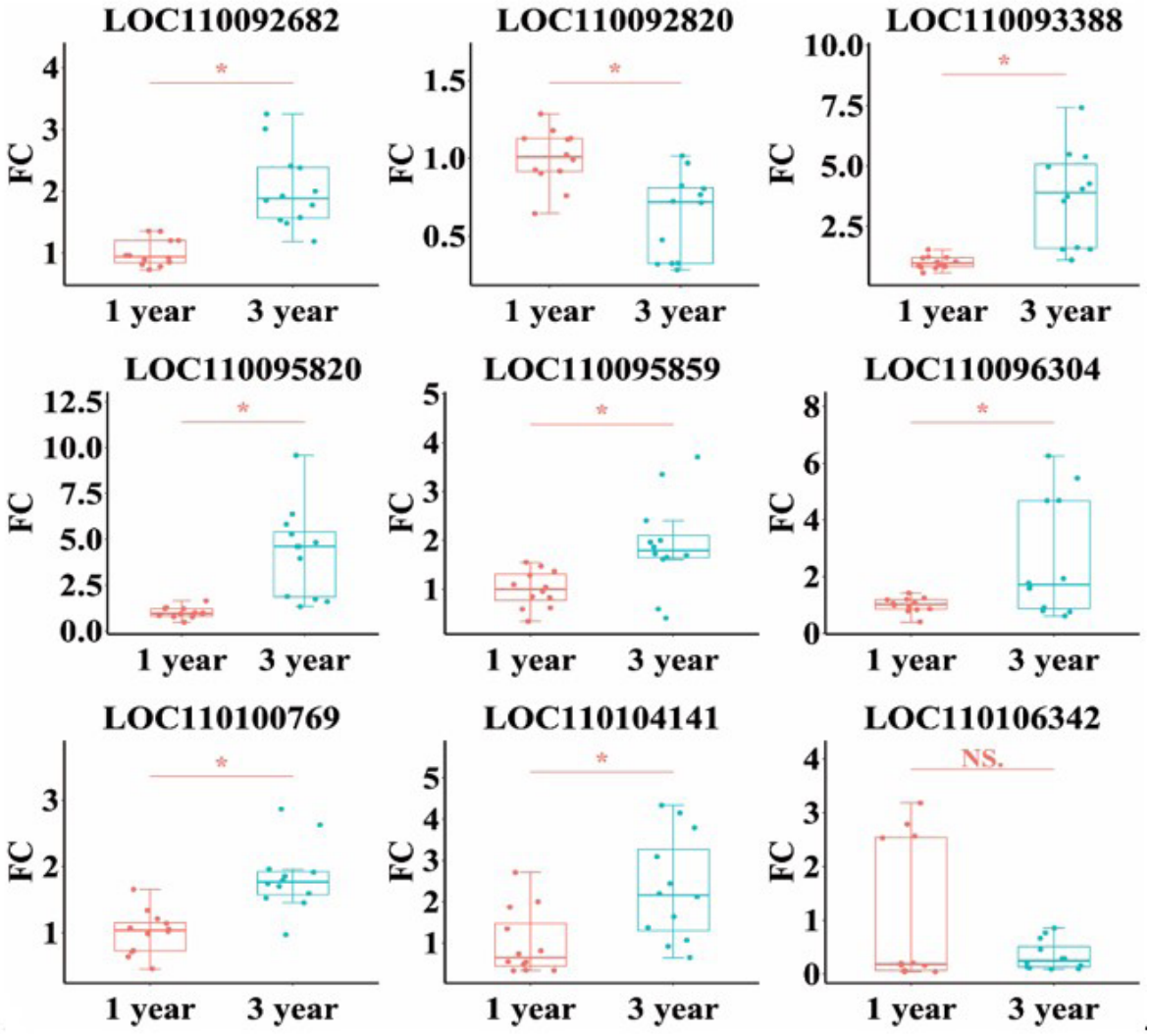

Figure 2. Quantitative RT-PCR validation of differential gene expression under growth years. Error bars represent the standard deviation based on three replicates.

binding modes between 5 sesquiterpene glycosides and their sapogenins obtained from *D. nobile* in the previous period (Tan et al., 2023) and 6 candidate UDP-glycosyltransferase including LOC110092682, LOC110093388, LOC110095820, LOC110095859, LOC110096304, and LOC110104141. After docking, the conformational cluster with the lowest binding free energy is shown in the complex of the docked compounds with UDP-glycosyltransferases (Table 2). Comprehensive consideration of the binding ability of proteins to sesquiterpene glycosides and their aglycones, the results indicated that LOC110104141 had a better binding effect with Dendromoniliside D (DmD), Dendronobiloside A (DnA), Dendroside G (DG) and its aglycones, and LOC110092682 had a better binding effect with Dendronobiloside C (DnC), Dendronobiloside D (DnD) and its aglycones (Figure 4). Therefore, UDP-glycosyltransferases LOC110104141 and LOC110092682 presumably play a key role in the glycosylation of sesquiterpenes in *D. nobile*.

Table 2. Molecular docking binding energies of sesquiterpene glycosides and their aglycones with candidate proteins (kcal/mol).

Candidate protein	DmD	DmD aglycone	DnA	DnA aglycone	DnC	DnC aglycone	DnD	DnD aglycone	DG	DG aglycone
LOC110095820	-6.7	-6.6	-6.9	-6.6	-7.5	-6.3	-7.6	-6.0	-6.7	-6.1
LOC110095859	-7.4	-6.4	-7.6	-6.6	-7.1	-7.8	-7.4	-6.3	-7.5	-7.7
LOC110104141	-9.6	-7.8	-8.7	-7.5	-9.0	-7.2	-9.1	-6.9	-9.0	-7.9
LOC110093388	-8.5	-6.6	-8.3	-7.2	-9.9	-7.0	-10.4	-6.7	-8.6	-7.2
LOC110096304	-9.5	-7.5	-8.5	-7.7	-8.7	-7.5	-9.0	-6.3	-9.2	-7.8
LOC110092682	-9.0	-7.9	-6.8	-8.5	-9.1	-8.2	-9.5	-8.3	-7.6	-7.8

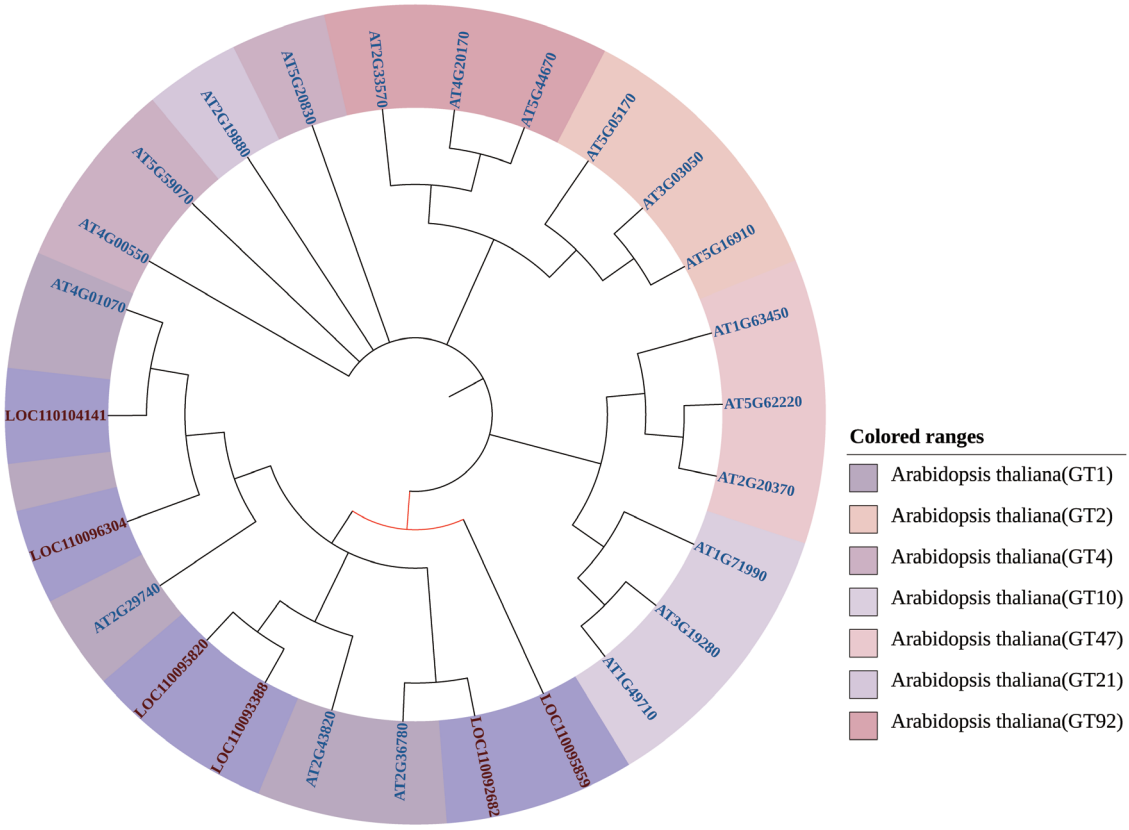

Figure 3. Phylogenetic analysis of 6 UDP-glycosyltransferase genes discovered from *D. nobile*.

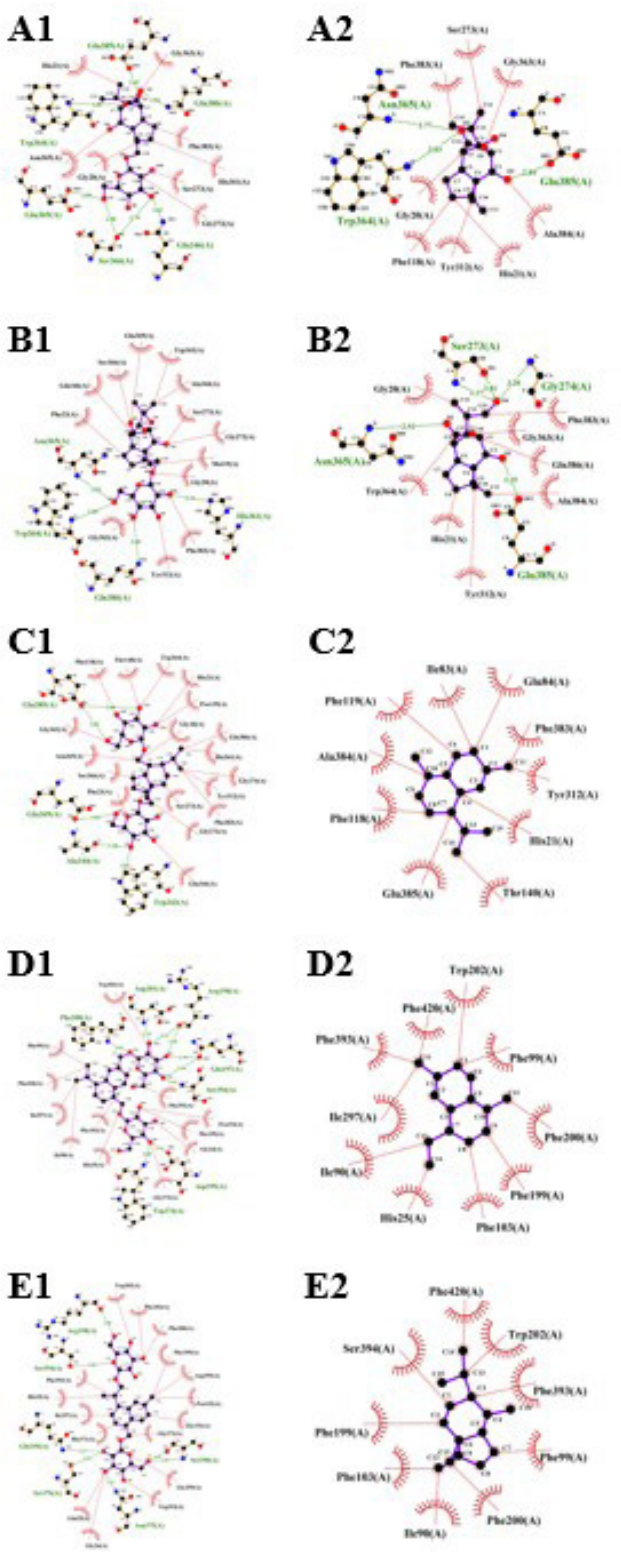

Figure 4. Sesquiterpene glycosides (1) and their aglycones (2) in *D. nobile* was docked to the binding pocket of UDP-glycosyltransferases. (A) Dendroside G (DG) and its aglycones docking with LOC110104141; (B) Dendromoniliside D (DmD) and its aglycones docking with LOC110104141; (C) Dendronobiloside A (DnA) and its aglycones docking with LOC110104141; (D) Dendronobiloside C (DnC) and its aglycones docking with LOC110092682; (E) Dendronobiloside D (DnD) and its aglycones docking with LOC110092682.

4 Conclusion

Alkaloids and sesquiterpene glycosides are the two main active ingredients in *D. nobile*. Our previous study found that the content of alkaloids in *D. nobile* decreases with the increase of growth years, on the contrary, the content of sesquiterpene glycosides keeps increasing. The previous reports showed that alkaloids and sesquiterpene glycosides have the same upstream biosynthetic pathway, starting from sesquiterpene components, part of which is converted to sesquiterpene glycosides by the action of UDP-glycosyltransferase, and part of which is further synthesized to alkaloids. Therefore, sesquiterpene UDP-glycosyltransferases play an important role in the biosynthesis of alkaloids and sesquiterpene glycosides in *D. nobile*. In our continued study, 5 sesquiterpene glycosides were isolated and elucidated from *D. nobile*. In the present study, the key UDP-glycosyltransferase genes in sesquiterpene glycosylation in *D. nobile* were explored, predicted and validated by transcriptome technology. The results indicated that UDP-glycosyltransferase genes LOC110104141 and LOC110092682 presumably play an important role in the glycosylation of sesquiterpenes in *D. nobile*, however, this result requires further functional validation.

Conflict of interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Author contributions

Daopeng Tan: Investigation, Writing-original draft. Jianmei Wang: Data curation, Supervision. Ligang Cao: Manuscript checking. Di Wu and Qingjie Fan: Manuscript checking and data analysis. Yongxia Zhao, Xingdong Wu and Yanliu Lu: Writing–review and editing. Lin Qin: Funding acquisition. Yuqi He: Funding acquisition and supervision writing.

Acknowledgements

This work was financially supported by the Department of Education of Guizhou Province (QJHKY [2021]049), Department of Science and Technology of Guizhou Province (Nos. QKHZC [2023] general 426, QKHZC[2021]420, QKHZC [2020]4Y072, QKHPTRC [2018]5772-001), Guizhou Engineering Research Center of Industrial Key-technology for Dendrobium Nobile (QJJ [2022]048 and QJJ [2022]006) and the Science and Technology Innovation Action Plan of Domestic Science and Technology Cooperation Projects in Shanghai (20025800400), Zunyi City of China (ZSKHHZ[2021]188, ZSKXX[2020]1 and ZSKXX[2020]2).

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