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Identification of key genes and active anti-inflammatory ingredients in *Panax* medicinal plants by climate-regulated callus culture combined with gene-component-efficacy gray correlation analysis

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Abstract

Objective: We aimed to establish a novel strategy for identifying key genes and active anti-inflammatory ingredients in Panax medicinal plants.

Methods: First, fresh roots of 2-year-old *Panax* plants, including *P. ginseng C. A. Mey., P. quinquefolium L., P. notoginseng (Burk.) F. H. Chen, P. japonicus C.A.Mey., P. japonicus Mey. var. major (Burk.) C. Y. Wu et K. M. Feng,* were selected as explants, and callus formation was induced under three experimental temperatures (17, 24, and 30°C). Second, high-performance liquid chromatography-mass spectrometry was used to analyze the saponin content of the callus. Nitric oxide reduction efficacy was used for "component-efficacy" gray correlation analysis to find the active anti-inflammatory ingredients. Quantitative reverse-transcription polymerase chain reaction (qRT-PCR) was used to determine the inflammatory factors and verify the active ingredients' anti-inflammatory effects. Finally, qRT-PCR was used to detect the expression of key genes in the callus, and "gene-component" gray correlation analysis was used to examine the relationships between the regulatory pathway of the genes and the components.

Results: Among the three experimental temperatures (17, 24, and 30°C), the lowest temperature (17°C) is the most suitable for generating *Panax* callus. Lower-latitude native *Panax notoginseng* is more adaptable under high culture temperatures (24°C and 30°C) than other *Panax* plants. The ginsenoside contents of the callus of *P. notoginseng* and *P. japonicus* were the highest under similar climate conditions (17°C). Major anti-inflammatory components were G-Rh1, G-Rb1, G-Rg3, and G-Rh6/Floral-GKa. CYP76A47 contributed to the accumulation of anti-inflammatory components.

Conclusions: This study provides a strategy for the gene-component-efficacy correlational study of multi-component, multi-functional, and multi-purpose plants of the same genus.

Keywords: Anti-inflammatory ingredient, Gene-component-efficacy gray correlation analysis, HPLC–MS, *Panax*, qRT-PCR; Callus culture

Graphical abstract: http://links.lww.com/AHM/A38

Introduction

Many *Panax* medicinal plants with potent therapeutic or auxiliary effects are used in traditional herbal medicines worldwide[1]. The word "Panax" originated from the Latin words "pan" (all) and "zxos" (medicine treatment), indicating the practicality of these plants for the treatment of many diseases[2]. Their medicinal value is recognized for treating cardiovascular, cerebrovascular, and nervous-system diseases[3–4]. The pharmacological activities include anti-inflammatory, anti-allergic, anti-aging, and anti-tumor functions[5–6]. *Panax ginseng*, *P. notoginseng*, *P. quinquefolium*, *P. japonicas*, and *Panacis majoris* are the most common species with significant therapeutic efficacy[2]. For example, according to traditional Chinese medicine (TCM) theory, *P. ginseng* can provide vital energy and *P. notoginseng* can prevent congestion and stop bleeding, *P. japonicus* can nourish the body, and *Panacis majoris* rhizomes can maintain the lungs, while *P. quinquefolium* can provide nourishment "*qi*" and "*yin*"[7].

Panax plants have congeneric active ingredients such as ginsenosides. Ginsenosides can be divided into two groups according to the structure of the glycosides: dammarane and oleanane. The dammarane type can be further divided

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Jiao Ai and Yongshen Ren contributed equally to this work.

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into panaxadiol (PPD) and panaxatriol (PPT)[8]. The ginsenoside types and contents differ in different *Panax* plants, significantly impacting each plant's properties, efficacy, and clinical application. The variation in ginsenoside types and contents of the *Panax* plant greatly affects their properties, effectiveness, and clinical application. This should be studied further to show the component-efficacy relationship and their biosynthetic pathways. Such research could improve medication accuracy and provide a scientific basis for new drug development.

The environment plays an important role in plant secondary metabolism, especially in medicinal plants. Secondary metabolites exhibit remarkable changes with changes in the growth environment. Environmental changes lead to differences in dominant gene expression in plants; even plants of the same family and genus that are grown in different environments will exhibit significant changes in dominant gene expression, which manifests as differences in the characteristics of the plant and its secondary metabolites. Thus, controlling and changing the plant growth environment artificially, especially with congeneric plants distributed in different regions, can allow an understanding of the relationship between gene regulation and secondary metabolite synthesis[9–13].

Recently, *in vitro* plant culture, including callus culture, somatic embryo culture, cell suspension culture, hairy root culture, and adventitious root culture, has become increasingly regarded as an alternative to traditional plant cultivation. It is used for obtaining bioactive metabolites and makes it possible to investigate the gene regulation mechanism of secondary metabolite synthesis.

Existing studies have demonstrated that, compared with full light, a shaded environment is more conducive to the expression of Damenediol synthetase (DS), β-Amyrin (β-As) in ginseng[14–15] and other key enzyme genes in the process of ginsenoside synthesis, while a low temperature is more conducive to the response of Squalene synthase1, DS-II, Squalene epoxidase1, and HMG-CoA reductase2 than room temperature[16]. Gene expression changes in *Panax* plants will also change the corresponding effective components. For example, co-overexpression of the *HMGR* gene and SS gene can enhance the synthesis of triterpene saponins and phytosterols[17–18].

Liquid chromatography-mass spectrometry[19–20] is an effective method for analyzing and identifying bioactive substances quantitative reverse-transcription polymerase chain reaction (qRT-PCR)[21] is the conventional method used to identify and characterize key genes and is widely used in quantitative gene expression detection. Gray correlation analysis (GCA)[22] is a powerful method for studying the effective material basis of Chinese herbal medicines (CHMs). It can be used to analyze the complex relationships in multi-component and multi-efficacy plants by calculating and identifying the main active ingredients.

In this research, the above-mentioned methods were applied to analyze the components of *Panax* synthesized in callus culture. A phylogenetic tree[23] was constructed to analyze the homology of *Panax* plants and assess intra- and inter-generic genetic relationships. The combined application of related technologies in this study was expected to provide a new strategy for analyzing gene regulation of secondary metabolite synthesis in congeneric plants under different artificial environments. The study aimed to identify the key biosynthetic pathways underlying the main efficacious substances and to provide a scientific strategy for new drug development based on bioartificial synthesis.

Material and methods

Herb collection

The fresh roots of five species of biennial *Panax* plants were collected for callus culture. Ginseng (RS), *P. ginseng* Meyer., was collected from Fusong county, Jilin Province, China, and represents a high latitude species. American ginseng (XYS), *P. quinquefolium* L., was collected from Fusong county, Jilin province, China; it represents a high latitude species. Notoginseng (SQ), *P. notoginseng* (Burk.) F. H. Chen, was collected from Wenshan county, Yunnan province, China; it represents a low-latitude species. Panax japonicas (ZJS), *P. japonicus* C.A.Mey., was collected from Enshi county, Hubei province, China; it represents a middle latitude species. Panacis majoris rhizome (ZZS), *P. japonicus* Mey. var. major (Burk.) C. Y. Wu et K. M. Feng, was collected from Enshi county, Hubei province, China, representing a mid-latitude variety. All samples were identified by Professor Maochuan Liao of the South-Central University for Nationalities and stored in room 13304, National Medicine Research Laboratory, South-Central University for Nationalities, Wuhan, China.

Media preparation and callus culture

Preparation and sterilization of Murashige–Skoog (MS) medium. Exactly 4.74 g MS medium, 2.0 mg 2,4-D (2,4-dichlorophenoxyacetic acid), 0.1 mg KT (6-furfurylaminopurine)[24], and 30 g sucrose and 7 g agar were placed in a 1 L beaker, and 1 L water was added to the beaker. After slightly boiled, the pH was adjusted to 5.8. The mixture was separated into the culture bottles and sterilized for 20 minutes (121°C, 0.105 MPa). The sterilized containers were then cooled to room temperature.

Disinfection and inoculation of explant: First, fresh roots of *Panax* plants were rinsed with running water for 30 min and transferred to a sterile operation platform (SW-CJ-1FD, Purification Equipment Co., Ltd., Tongjing, Jiangsu). After soaking in 70% alcohol for 60 s, the roots were rinsed with sterile water four times, placed into 2% NaOCl for 5 min, and repeatedly rinsed with sterile water four times. Second, the roots were placed in 0.2% mercuric chloride for 5 min, rinsed with sterile water 4 times, and the surface water was absorbed with sterile filter paper. Finally, the roots were cut into 5 mm × 5 mm × 3 mm pieces and placed into culture bottles under sterile conditions. Each bottle was inoculated with three explants.

Callus culture: The tissue culture bottles were put into three incubators (HM-430; Electronic Technology Co., Ltd. Hengmei, Shandong) dark culture with temperature settings at 17, 24, and 30°C, respectively, to simulate different growth temperature conditions. The 17°C samples were named 17ZJS, 17ZZS, 17XYS, 17RS, and 17SQ, and the *P. notoginseng* sample placed at 24°C was named 24SQ, while the one at 30°C was named 30SQ.

Ginsenoside extraction

First, fresh radicals and callus of *Panax* samples were dried in an oven (50°C) and then crushed and sifted through a 65 μm mesh sieve, respectively. The powder was weighed, and about 1.0 g was placed separately into 50 mL centrifuge tubes. Exactly 5 mL of 75% ethanol was added and extracted by ultrasonic wave three times for 30 min (65°C, 100 Hz). Second, the supernatant of the ethanol extractions was combined, and rotary evaporated to dryness (65°C, −0.1 MPa, 60 rpm). The precipitate was dissolved in 10 mL distilled water, washed with 5 mL petroleum ether three times, and then extracted with 5 mL n-butanol three times. The n-butanol layers were combined and evaporated to dryness at 80°C. Finally, the precipitates were stored at 4°C.

HPLC–MS analysis

The Ginsenosides extracts were dissolved in methanol, diluted to 10 mL to a final concentration of 0.1 g/mL of crude herb or callus, and filtered through a 0.22 μm membrane for further analysis. The HPLC–MS fingerprints were recorded on an Agilent 1200 HPLC instrument coupled with Agilent 6420 Triple Quad Mass spectrometer *via* ESI interface (Thermo Fisher, San Jose, CA). The samples were separated on high-performance liquid chromatography (Agilent 1200 HPLC system) equipped with a Welch X UPLC C_{18} column (100 mm × 2.1 mm, 1.8 μm). A two-component mobile phase was used; one composed of 0.1% formic acid aqueous solution (A) and the other acetonitrile (B). The following gradient elution program was applied: 0 to 8 min, 80% to 79% A; 8 to 9 min, 79% to 74% A; 9 to 30 min, 74% to 68% A; 30 to 44 min, 25% to 1% A. The injection volume was 10 μL, the flow rate was 0.4 mL/min, and the column temperature was set at 27°C. MS conditions: the ionization temperature was maintained at 350°C, the scanning range was 100 to 2,000 Da, the capillary voltage was 3,000 to 3,500 V, and the spray pressure was 250 kPa.

Determination of anti-inflammatory efficacy

RAW264.7 macrophages were used to study the anti-inflammatory efficacy of *Panax* medicinal plants and their callus. Macrophages were stimulated to M1 macrophages by 1 μg/mL lipopolysaccharide (LPS) *in vitro*. The ginsenosides extracts mentioned above were diluted in DMEM to 0.5 to 5 mg/mL (crude herb concentration). The concentration was assessed with the methyl thiazolyl tetrazolium assay in the early stage of the experiment and showed no cell toxicity. Subsequently, according to the Griess Kit (L-2B80, Biotechnology Co., Ltd. Jiancheng, Nanjing, China) manufacturer's instructions, NO excretion was detected 24 h after LPS stimulation and drug (Ginsenosides extracts) administration. The control group (KB) was provided with the same volume of PBS[25–26].

"Component-efficacy" relational analysis

The data processing system (DPS) V15.10 advanced edition (Information Technology Co., Ltd. Ruifeng, Hangzhou) was used for gray correlation analysis (GCA) to (1) explore the relationship between the anti-inflammatory effect and secondary metabolite synthesis, and (2) ascertain the most important components that play a regulatory role against inflammation.

qRT-PCR detection of key genes in samples

First, total RNA was extracted according to the instructions of the spin-column plant total RNA extraction and Purification Kit (B518661; Bioengineering Co., Ltd., Shanghai, China). The concentration and purity of RNA were determined with a nanodrop instrument. The cDNA was synthesized according to the instructions of the cDNA synthesis Kit (B532445-0020). Finally, SGExcel FastSYBR Mixture (B532955-0005, Bioengineering Co. Ltd.) was used as the premixed system for real-time fluorescence quantitative detection. The PCR reactions were placed in the quantitative fluorescence analyzer (Prism@ 7500; TaKaRa Bio, Shiga, Japan) for detection. The PCR reaction procedure was as follows: pre-denaturation at 95°C for 30 min, 40 cycles aof denaturation at 95°C for 5 s, and annealing at 60°C for 20 s. The primers designed by Primer 5.0 software are shown in Tables 1 and 2.

"Gene-component" relational analysis

The DPS tool was used for GCA to explore the relationship between gene regulation and secondary metabolite synthesis. GCA is very effective for finding key genes that can upregulate anti-inflammatory component expression.

Construction of the phylogenetic tree

The ginseng genome database was used to identify the UGT family mRNA sequences. BLAST was used for mRNA sequence comparison. GO was used for homologous sequence comparison. The targets and amino acid

Table 1

Gene primers in macrophages

Gene	Gene Bank ID	Forward primer (5'-3')	Reverse primer (5'-3')
GADPH	AY618569.1	CCCATGATGTCGGACCCTAA	TGTCATGAATGAACTCGGAGGTG
IL-1β	NM_008361.4	AGTTGACGGACCCCAAAAGATGAAG	TTCTCCACAGCCACAATGAGTGATAC
IL-6	M24221.1	CTTCTTGGGACTGATGCTGGTGAC	AGTGGTATCCTCTGTGAAGTCTCCTC
CXCL10	U58677.1	CCGTAGTGTAGTTGCTCCTGTATGTATC	GATGGAGGTGCCCTAGTCCTGAAG
TNF-a	NM_011609.4	AATTACCTCAGGCAGTGTCTCAGTTG	GTCTCACTCAGGTAGCGTTGGAAC
MCP-1	NM_001303244.1	CGTTTATGTTGTAGAGGTGGACTGGAC	ATGTCATCTTCTTCAGGCGTGTCAC
MMP-9	NM_013599.5	AAACCCTGTGTGTTCCCGTTCATC	AGTTTATCCTGGTCATAGTTGGCTGTG
TGF-β1	NM_011577.2	GCAACAATTCCTGGCGTTACCTTG	GAAAGCCCTGTATTCCGTCTCCTTG

Table 2

Gene primers in Panax

Gene	Gene Bank ID	Forward primer (5'-3')	Reverse primer (5'-3')
SS	AB010148	TTGGAAGCGGTTACCAGGAG	TTGGAAGCGGTTACCAGGAG
β-AS	KJ939267	GGGTTGCTGAAGATGGAATG	TTCTCCTTGACCTGGGACTT
CYP716A47	JN604537	AGGAGAACCGATGGCAATCTTGTG	CGTTGATCTGTTGTTGGCGATTCC
CYP716A53v2	JX036031	CAACATCCTTAGCAGGCGAGAA	AGCGACTCTGACATAGCGAAAG
CYP716A52v2	JX036032	AGGCTTCAGCAACACAAGACAT	GCACTTCACAGGCTACATTCCA
UGT94Q2	MH673780	ATG GATCTCTTTATCTCATCTCAA	TTAAAGCGTACAAGGTGATAGACG
GAPDH-1	KF699323	GGTGTAACCTAAGATTCCCTTGAGT	ACTGTCAGGTTGGCGAAGAAG

sequences were imported into MEGA software V 7.0 for protein sequence comparison and phylogenetic tree construction. The results were transformed into an NWK file and then visualized using the iTOL tool.

Results

Morphological analysis of *Panax* callus

The growth process of the ginseng plant callus cultures was monitored, and the cultivation status of each callus was quantified as follows. First, the induction ratio (60% weight) was evaluated based on the proportion of successfully induced callus. Second, the non-browning index (20% weight) was determined by counting the proportion of non-browning callus. Lastly, the growth status of the callus (20% weight) was determined based on its appearance, size, and growth speed. The results are recorded in Figure 1A.

The regulation of the callus growth period was recorded as follows (Figure 1B): the callus cells began to grow around the 20th day. The callus was in the rapid growth stage between the 25th to 35th day and entered the growth stagnation stage after the 35th day. The appearance of the callus remained unchanged at this stage, and the callus samples were harvested on the 40th day. The fresh *Panax* plant radixes and callus growth under different environments was recorded as shown in Figure 1C. The appearance of the *Panax* callus after 45 d is shown in Figure 1D.

The results revealed the following. First, low temperature (17°C) was more suitable for the generation of *Panax* callus. Second, lower-latitude native *Panax* specie (SQ) was more adaptable than mid-latitude *Panax* species (ZJS and ZZS) and high-latitude *Panax* species (RS and XYS) under high culture temperatures (24°C and 30°C). RS and XYS (high latitude) were more suitable for growth in low-temperature environments and were not adaptable to higher temperatures. ZJS and ZZS (middle latitude) could withstand normal temperatures (24°C) but do not tolerate high temperatures (30°C), while SQ grew well in all three environmental settings. Therefore, the lowest temperature (17°C) was selected for the further study of the callus growth of different *Panax* plants in the same environment; SQ was selected for the observation of the effect of culture temperature on callus growth.

The total yield of saponins from *Panax* crude herb and callus

The 75% ethanol crude extracts of 1.0 g *Panax* plants were as follows: 245.3 mg (ZJS), 175.1 mg (ZZS), 266.3 mg (XYS), 202.4 mg (RS), and 191.9 mg (SQ). The purified total saponins were 149.2 mg (ZJS), 158.6 mg (ZZS), 45.6 mg (XYS), 91.7 mg (RS), and 136.2 mg (SQ) and the purified total saponins yields were 14.9, 15.9, 4.6, 9.2, and 13.6%, respectively. The results showed that the total saponin content in

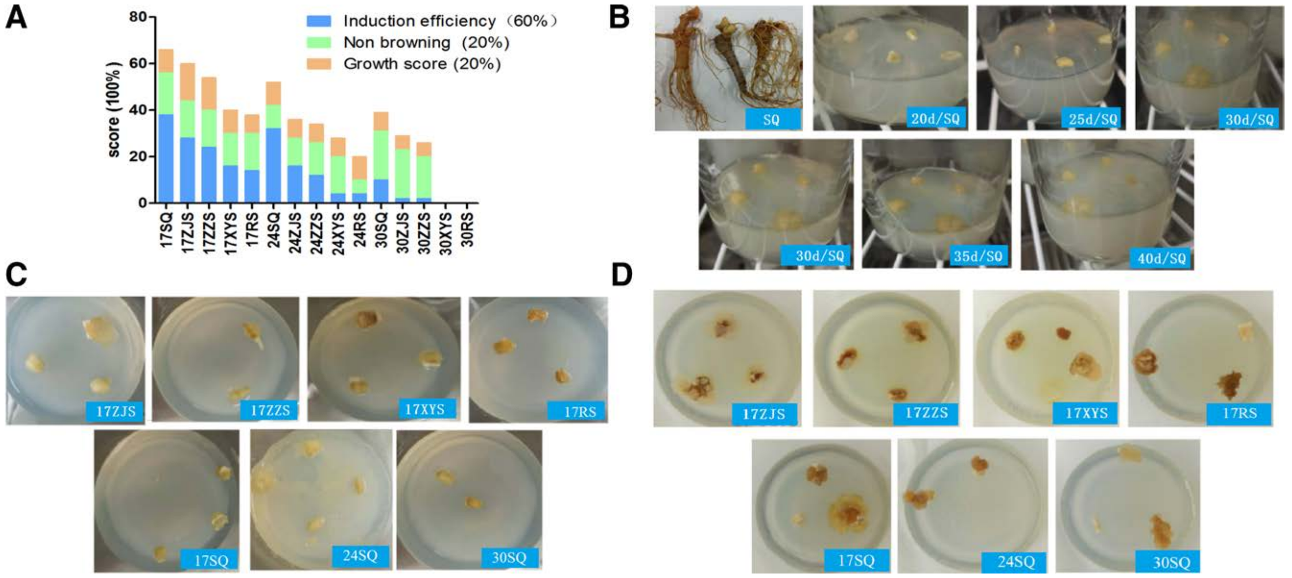

Figure 1. Morphological analysis of *Panax* callus. (A) Comprehensive score of callus; (B) appearance of *Panax* callus growth at 17°C; (C) appearance of *Panax* callus (40 d of growth); (D) appearance of *Panax* callus after 45 d of growth. ZJS: Panax japonicas; ZZS: Panacis majoris rhizome; RS:Ginseng; SQ:Notoginseng. The samples grown at 17°C were named 17ZJS, 17ZZS, 17XYS, 17RS, and 17SQ, and the P. notoginseng sample placed at 24°C was named 24SQ, while the one at 30°C was named 30SQ.

2-year-old *Panax* herbs was the highest for ZZS and ZJS, followed by SQ, while the yields from XYS and RS were comparatively lower. With the same method used, the total saponins yields from 0.1 g of 40-day-old *Panax* callus samples were as follows: 10.2 mg (17ZJS), 1.8 mg (17ZZS), 5.3 mg (17XYS), 5.4 mg (17RS), 14.0 mg (17SQ), 10.3 mg (24SQ), and 7.4 mg (30SQ), and the total saponins yields were 10.3, 1.80, 5.3, 5.4, 14.0, 10.3, and 7.4%. At 17°C, SQ grew slowly and accumulated fewer metabolites. The results showed that the ginsenosides of SQ accumulated faster, to a greater extent, at 17°C than at 24°C and 30°C, indicating that a low temperature may play a role in regulating the synthesis and accumulation of ginsenosides.

Identification of sample composition by HPLC–MS

The HPLC–MS fingerprints of the 12 samples (ZJS, ZZS, RS, XYS, SQ,17ZJS,17 ZZS, 17RS, 17XYS, 17SQ, 24SQ, 30SQ) were recorded (Figure 2). More than 23 peaks were separated by HPLC, 19 of them were identified by mass spectrometry according to literatures[27–28] and reference substances. The main peak area was recorded (Table 3).

Anti-inflammatory analysis

Studies have shown that endogenous nitric oxide (NO) can cause NF-κB nitration, reduce its binding activity with DNA[29], weaken the inflammatory response cascade, inhibit the degradation of IkB-α, indirectly inhibit the activity of NF-κB, and reduce the release of inflammatory mediators

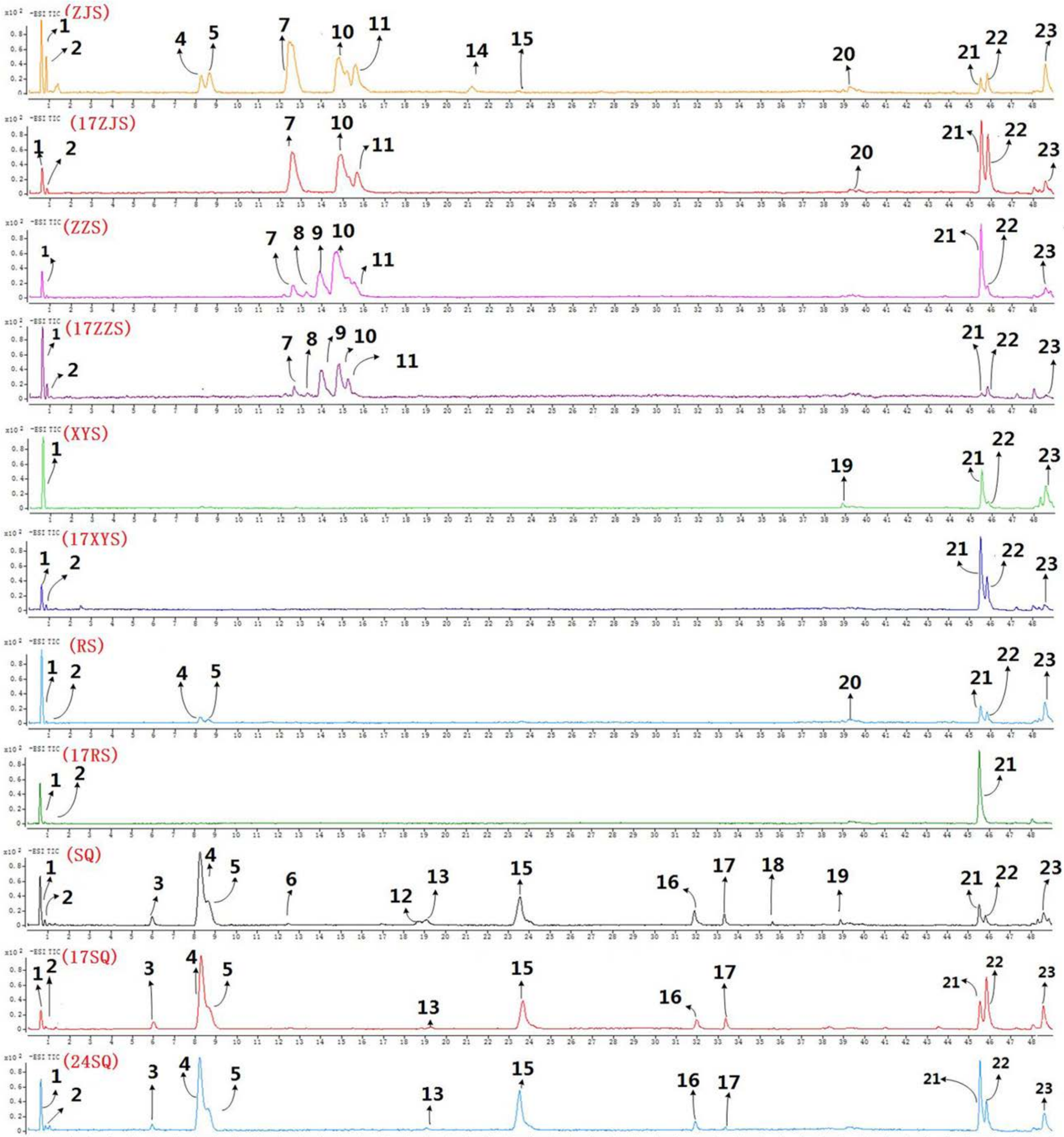

Figure 2. HPLC–MS chromatograms of experimental samples. ZJS: Panax japonicas; ZZS: Panacis majoris rhizome; RS:Ginseng; SQ:Notoginseng. The samples grown at 17°C were named 17ZJS, 17ZZS, 17XYS, 17RS, and 17SQ, and the P. notoginseng sample placed at 24°C was named 24SQ.

Table 3

Chromatographic spectral peaks area of HPLC–MS of samples P1-P23 represents 1-23 ginsenosides in the chromatogram

				Samples											
Peak area (×10^5)	Compound name	(m/z)	[M + H]$^+$	ZJS	17ZJS	RS	17RS	XYS	17XYS	ZZS	17ZZS	SQ	17SQ	24SQ	30SQ
P1	G-Rh1	638.4	683.4	28.499	10.332	47.816	30.373	82.382	12.907	12.348	17.207	36.291	16.273	23.984	17.364
P2	unknown	—	367.1	11.290	1.603	0.984	1.284	—	1.880	—	2.983	2.941	1.900	1.649	—
P3	KG-R2	769.3	771	7.594	—	—	—	—	—	—	—	1.954	—	—	—
P4	NG-R1	931.5	933.131	—	—	—	—	—	—	—	—	14.129	10.642	5.169	3.926
P5	G-Rg1	799.5	801.01	18.834	—	9.361	—	3.170	—	—	—	192.325	166.427	123.599	94.403
P6	G-Rf	799.5	801.01	26.683	—	5.400	—	2.151	—	—	—	63.060	47.661	32.105	30.825
P7	G-Ro	955.5	957.1	122.915	71.404	—	—	—	—	18.256	5.509	—	—	—	—
P8	G-Rb2/G-Rb3	1 123.6	1 124.5	—	—	—	—	—	—	7.391	1.853	—	—	—	—
P9	Pseudo-G-Rt1	925.8	926.5	—	—	—	—	—	—	60.915	25.631	—	—	—	—
P10	Chikusetsulva	925.8	926.5	93.151	84.902	—	—	—	—	185.264	28.789	—	—	—	—
P11	Chikusetsusaponin IVa	793.4	794.9	52.688	28.785	—	—	—	—	26.982	12.996	—	—	—	—
P12	unknown	—	311.1	—	—	—	—	—	—	—	—	8.944	—	—	—
P13	G-Rg2	783.3	785.01	—	—	—	—	—	—	—	—	13.972	4.574	2.164	—
P14	Pseudo-RT1	925.8	926.5	8.644	—	—	—	—	—	—	—	—	—	—	—
P15	G-Rb1	1 107.5	1 109.3	1.625	—	—	—	—	—	—	—	94.335	79.156	76.429	70.191
P16	G-Re	945.5	947.14	—	—	—	—	—	—	—	—	25.898	15.332	8.880	9.680
P17	G-Rd	945.3	947.14	—	—	—	—	—	—	—	—	14.098	13.279	2.116	3.148
P18	G-Rg3	783.3	785.01	—	—	—	—	—	—	—	—	2.426	—	—	—
P19	CS IVa	793.3	794.9	—	—	—	—	9.688	—	1.906	—	5.766	—	—	—
P20	unknown	—	339.1	6.885	2.296	5.658	—	—	—	2.377	—	—	—	—	—
P21	unknown	—	339.2	10.376	48.067	19.771	112.892	73.596	73.674	68.073	2.108	28.828	37.167	59.436	7.578
P22	Notoginsenoside R1	932.4	933.1	14.339	44.487	13.278	—	15.734	36.000	11.023	4.777	14.896	77.856	28.817	—
P23	G-Rh6/Floral-GKa	670.4	671.4	30.334	13.763	39.124	—	68.760	8.296	19.073	3.078	31.535	37.684	25.838	13.307

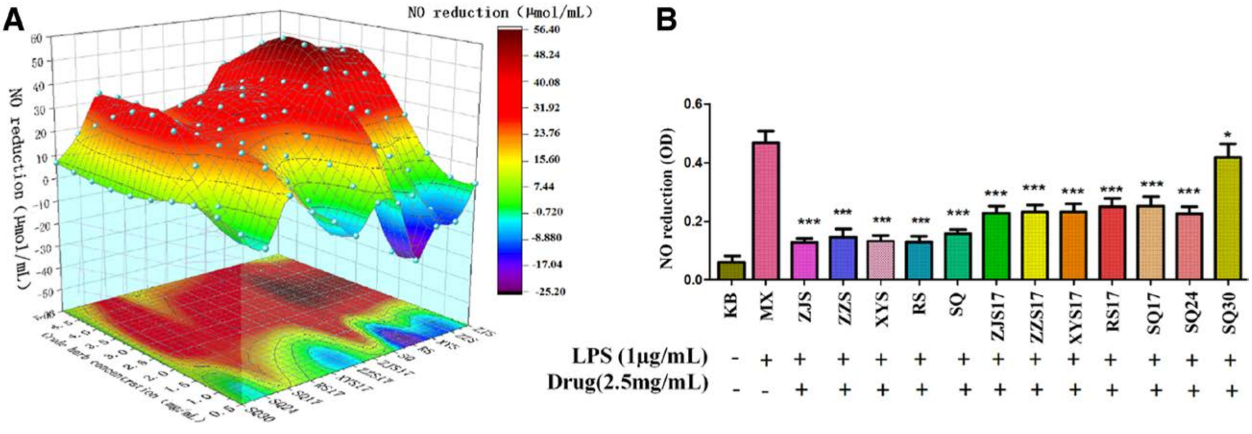

Figure 3. Anti-inflammatory activity of *Panax* plants *in vitro.* (A) Inhibitory effect of drugs on LPS-induced NO release from RAW264.7 macrophages; (B) No release was detected when concentration of crude drug was 2.5 mg/mL; $^*P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ versus the LPS group (MX). ZJS: Panax japonicas; ZZS: Panacis majoris rhizome; RS:Ginseng; SQ:Notoginseng; The samples grown at 17°C were named 17ZJS, 17ZZS, 17XYS, 17RS and 17SQ, and the P. notoginseng sample placed at 24°C was named 24SQ, while the one at 30°C was named 30SQ.

such as IL-1 and IL-6[30]. Therefore, the amount of released NO is positively correlated with the inhibition of the inflammatory response. While *Panax* plants have similar anti-inflammatory effects, the effects may vary according to the species and growth environment. The results recorded in Figure 3A show the inhibitory effect of the drugs on LPS-induced No release from the RAW264.7 macrophages. In contrast, Figure 3B showed that with the same concentration of crude drug (2.5 mg/mL), all 12 samples effectively inhibited NO synthesis in macrophages after LPS induction. The anti-inflammatory effect among the *Panax* plants was RS > ZJS > XYS > ZZS > SQ, and the anti-inflammatory effect on callus grown in the same environment was 17ZJS > 17ZZS ≈ 17XYS > 17RS > 17SQ. The anti-inflammatory effect of *P. notoginseng* callus under different temperatures was as follows: 24SQ > 17SQ > 30SQ. These results indicated that under natural growth environments, the anti-inflammatory effect of RS was more potent than that of the other species when cultured at the same temperature of 17°C. ZJS, ZZS, and XYS callus exhibited the strongest anti-inflammatory effects, which may be due to the suitable growth conditions for these three herbs. RS may prefer a lower temperature and SQ a higher temperature, based on their latitude distribution. The results indicated that the best for growth of SQ callus is 24°C. Higher or lower temperatures are not conducive to its growth, thus reducing its anti-inflammatory effect.

"Component-efficacy" relational analysis

The differences in the anti-inflammatory effects within the same environment and between different backgrounds are mainly due to the differences in metabolite accumulation caused by environmental changes. TCM is complex, with multiple components and targets. Multiple components regulate the overall efficacy of TCM; thus, gray correlation theory is suitable for revealing the effects of the ingredients in TCM. The GCD of "component-NO reduction efficacy" is recorded in Table 4. According to the results, the components with GCD higher than 0.8 are P1 (G-Rh1), P21 (unknown), P2 (unknown), P23 (G-Rh6/Floral-GKa), P20 (unknown), P15 (G-Rb1), and P18 (G-Rg3).

Regulation of Ginsenosides on inflammatory factor gene

The specific components G-Rh1, G-Rb1, and G-Rg3 were administered to RAW264.7 cells induced by LPS at a 50 μg/mL concentration. Twenty-four hours later, the gene expression levels of inflammatory factors and cytokines were determined by qRT-PCR. The internal reference gene was GADPH, and the blank group served as the control group. The growth trend of $-\triangle\triangle$Ct was used to represent the trend in gene expression. The results were recorded in Figure 4.

Figure 4 shows that compared with the LPS group (MX), G-Rg3, G-Rb1, and G-Rh1 in *Panax* plants can inhibit the gene expression of the pro-inflammatory mediators (IL-1β, IL-6, TNF-α), chemokines (MCP-1, CXCL10), and cytokines (TGF-β1), or inhibit the expression of the proteolytic enzyme MMP-9 from maintaining strong anti-inflammatory activities. In summary, these results fully verify the effectiveness of the three selected components.

qRT-PCR analysis

From the above experiments, we deduced that P1, P21, P2, P23, P20, P15, and P18 led to the differences

Table 4

The result of "component- NO reduction" gray correlation analysis

Peak	P1	P2	P3	P4	P5	P6	P7	P8	P9	P10	P11	P12
GCD	0.8665	0.8482	0.7963	0.7798	0.7618	0.7753	0.7934	0.7988	0.7809	0.7877	0.7960	0.7804
peak	P13	P14	P15	P16	P17	P18	P19	P20	P21	P22	P23	
GCD	0.7966	0.7849	0.8131	0.7932	0.7971	0.8031	0.7874	0.8262	0.8650	0.7992	0.8424	

GCD: Gray correlation degree.

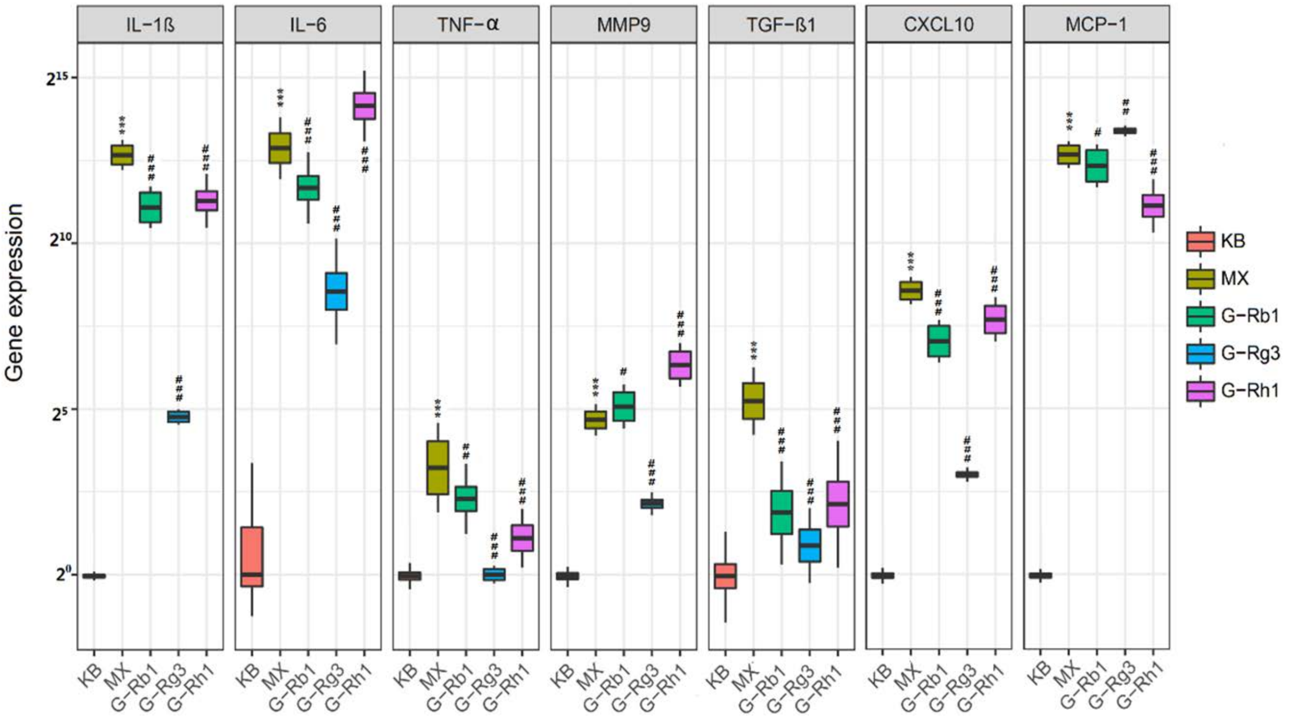

Figure 4. Inhibitory effect of drugs on LPS-induced expression of inflammatory cytokines and chemokines in RAW264.7 macrophages. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ versus the control group (KB). $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, and $^{\#\#\#}P < 0.001$ versus the LPS group (MX).

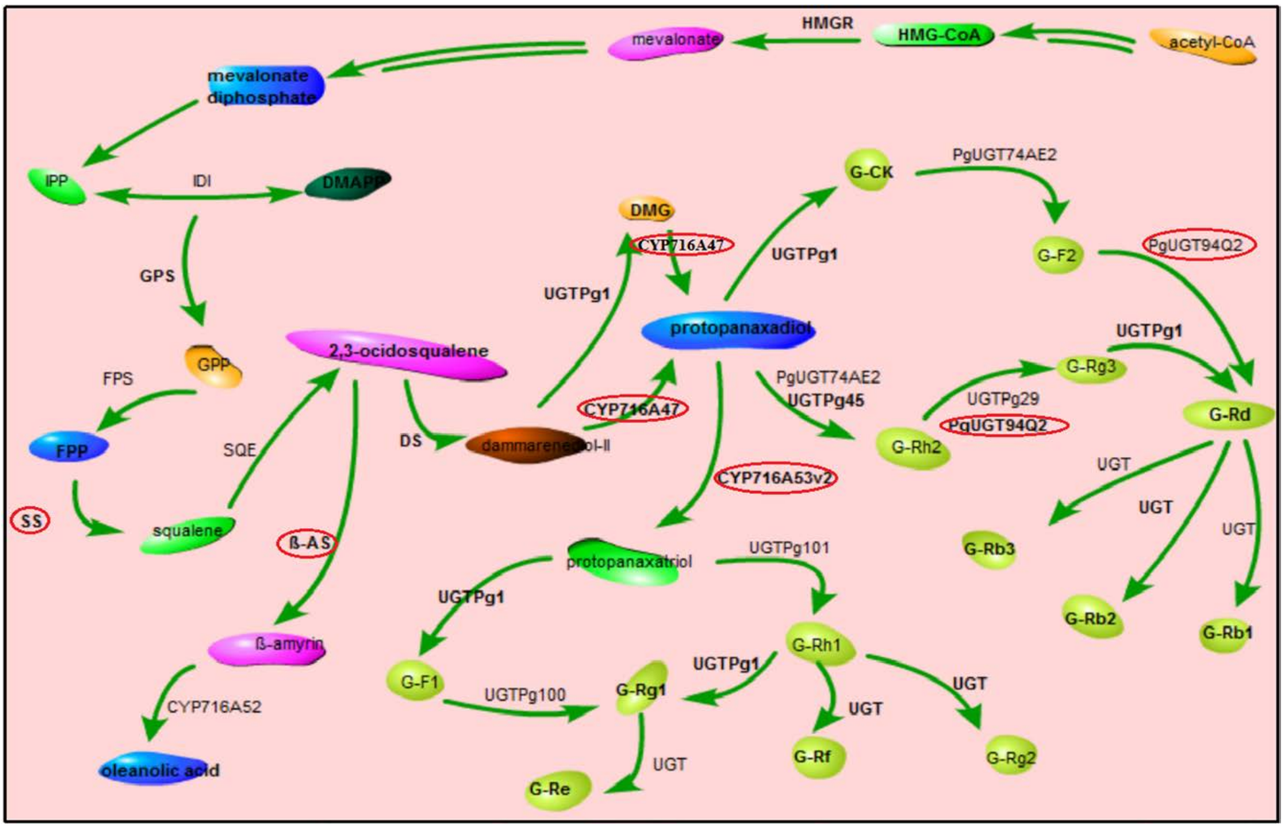

Figure 5. Biosynthesis of ginsenosides. HMG-CoA, 3-hydroxy-3-methylglutaryl-CoA; MEP, 2-C-methyl-D erythritol 4-phosphate; HMGR, 3-hydroxy- 3-methylglutaryl-CoA reductase; SS, squalene synthase; SE, squalene epoxidase; UGT, UDP glycosyltransferases. The two-way arrow indicates the reactions are reversible, and the green represents ginsenoside. The other colors represent various intermediates. Arrow represents the direction of the reaction, and the others are catalytic enzymes and transcription factors in the synthesis process.

in the anti-inflammatory efficacy of *Panax* plants. Finding genes conducive to accumulating these components may improve the anti-inflammatory effects of the plants. Previous studies have shown that ginsenoside biosynthesis is regulated by many genes (Figure 5).

This work selected five reportedly key genes in ginsenoside synthesis, i.e., *SS*, *β-As*, *UGT94Q2*, *CYP76A47*, and *CYP76A53v2*, as target *genes*. The expression of these genes was detected by a quantitative fluorescence analyzer, with SQ as the control sample and *GAPDH* as the reference gene. The results of the *Panax* herb and callus samples were recorded in Figure 6A and 6B.

The gene expression results of fresh Panax plant radixes in Figure 6A indicated that the expression of *CYP76A53v2* and *β-As* was slightly higher in ZJS than in the other samples, while the expression of *β-As* in ZZS was lower than in the others. Figure 6B showed that the expression of ginsenoside synthesis genes in the callus was higher than the Panax plant radixes in Figure 6A, especially for *SS* and *UGT94Q2*. Moreover, the gene expression of the *SS-CYP76A47-UGT94Q2/CYP76A53v2* pathway was increased under the callus culture condition, especially the upstream gene *SS* and the downstream genes *UGT94Q2* and *CYP76A53v2*. The expression of the intermediate gene *CYP76A47* increased in most samples but reduced in others, such as 17ZZS, 17XYS, and 24SQ. These results, in combination with the LC/MS results, revealed that there were hardly any significant compounds in 17XYS, indicating that *CYP76A47* is the key enzyme gene in Panax plants. The result showed that *β-As* was downregulated under the current culture conditions, and these results, combined with the LC/MS results, indicated that this condition was unfavorable for the synthesis of pentacyclic triterpene.

The gene expression patterns in the plants varied due to climatic conditions. The gene expression levels of congeneric species may also differ under the same climatic conditions. Generally, there is a "multi to multi" relationship between gene regulation and metabolism of components; this means that multiple genes may commonly regulate the biosynthetic pathway of a component, and one gene could also participate in the synthesis of multiple components. To detect the "gene regulation-component metabolizing" relationship, callus culture was adopted to amplify the gene expression and component accumulation differences under different culture conditions, and GCA was adopted to discover the potential regulation laws.

"Gene-component" gray relational analysis

The relative component contents of *Panax* herb and callus samples were correlated with *CYP76A53v2, SS, β-As, CYP76A47*, and *UGT94Q2* using the gray relational analysis method. The gray correlation degree results were recorded in Figure 7. The results showed that P1, P23, and P2 were affected most by the relative expression of *CYP76A53v2*. *CYP76A53v2* may be the key transcription factor controlling ginsenosides G-Rh1, G-Rf, and G-Re. P7, P23, P2, P4, P21, and P16 made a large contribution to the gene *β-As*, and the correlation degree of the other components was lower than 0.8. Therefore, *β-As* may control the synthesis of G-R0, G-Rh6/Floral-GKa, NG-R1, G-Re, P2, and P21 (unknown). P1, P18, P13, P16, P6, P5, P2, and P21 contributed the most to the relative expression of *UGT94Q2*, which may control the synthesis of G-Rh1, NG-R1, G-Rg3, G-Rg2, G-Re, G-Rg1, and P21 (unknown). Similarly, the synthesis of G-Rh1, P21(unknown), G-Rf, G-Rh6/Floral-GKa, NG-R1, G-Rg1, G-Re, and G-Rg3 may be controlled by *CYP76A47* and the gene SS may be the key transcription factor influencing G-Rf, G-Rg1, NG-R1, G-Re, G-Rh6/Floral-GKa, G-Rh1, G-Rg2, G-Rd, and G-Rb1. In conclusion, the "gene-component" GCA method can be used to preliminarily determine the regulatory roles of transcription factors in the synthesis of ginsenosides. This information may be useful to specifically upregulate the expression of target genes and improve the contents of active ingredients. Thus, this method provided a new analytical approach for studying fresh medicinal materials in the life sciences. The results showed multiple genes participate in the regulation of saponins, and one gene may affect multiple saponins synthesis.

Homology analysis

Uridine diphosphate (UDP)-dependent glycosyltransferases (UGTs) constitute a huge hypergene family. UGTs contain the key enzyme genes of the ginsenoside biosynthesis pathway and participate in the metabolism of many species. In the ginsenoside biosynthesis pathway, UGTs mainly catalyze the connection between glycosides and ginsenosides, extend the glycopeptide chain, and transfer glycosyl. The UGT family of *Panax* is closely related to that of other plants. The phylogenetic tree is built according to the affinity of the homologous

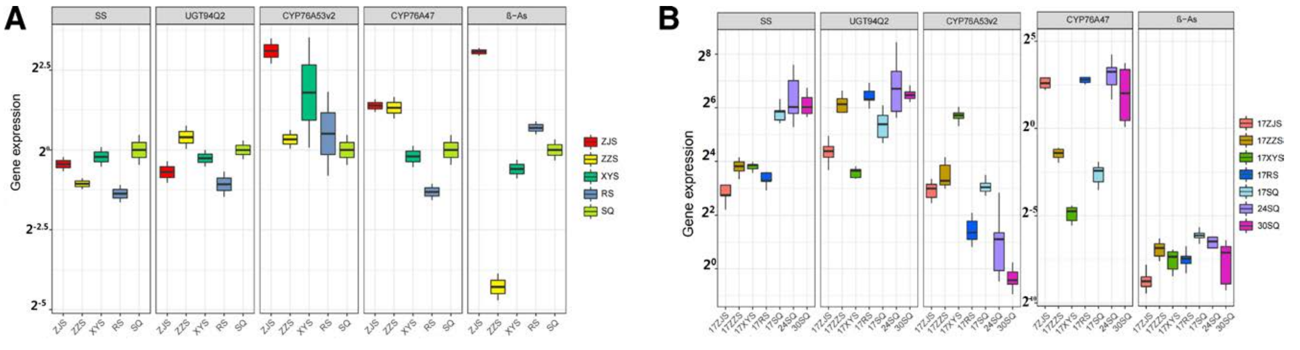

Figure 6. Effect of key gene expression on component accumulation. (A) Relative gene expression levels in fresh *Panax* plants radixes; (B) relative gene expression levels in *Panax* callus. ZJS: Panax japonicas; ZZS: Panacis majoris rhizome; RS:Ginseng; SQ:Notoginseng. The samples grown at 17°C were named 17ZJS, 17ZZS, 17XYS, 17RS, and 17SQ, and the *P. notoginseng* sample placed at 24°C was named 24SQ, while the one at 30°C was named 30SQ.

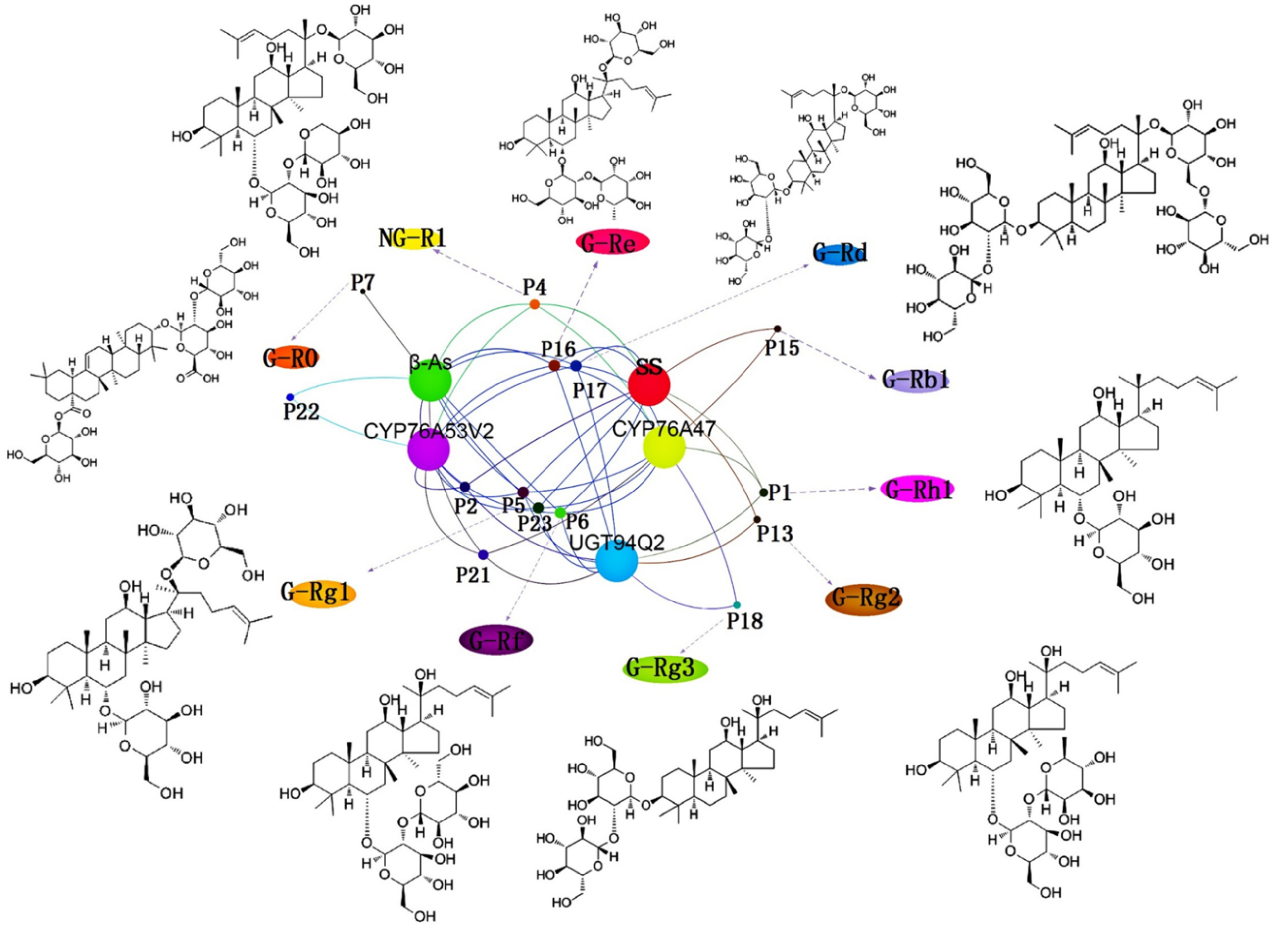

Figure 7. Correlation between key enzymes and ginsenoside synthesis.

proteins of *Panax* (pg) is closely related to other genera. This sentence is not required because it repeats the previous sentence. Figure 8 showed that 21 representative UGT metabolic enzymes of *Panax* were represented in the phylogenetic tree, each marked with blue. Each UGT gene from Panax plants was used to research other homologous UDP glycosyltransferases. These cluster into nine groups, with each group, denoted with one color. The results show that, like other plants, the UGTs in *Panax* plants are highly similar; these three UGTs are known transferases for ginsenoside synthesis. The members of the UGT family are related, and the amino acid sequences are highly similar to those of other species. After comparing the sequences of the UGT supergene family, candidate genes for saponin synthesis were screened through homology.

Discussion

The efficacy of Chinese herbal medicine (CHM) is affected by many factors, such as germplasm, origin, climate, processing factors, etc. Even species of the same genus grown in different environments will have different efficacies. For example, among the *Panax* plants used in this study, it is believed that SQ, which is distributed in low latitudes, is good at promoting blood circulation and removing blood stasis. RS and XYS, distributed at higher latitudes, are good at "tonifying *qi*." ZZS and ZJS are distributed in middle latitudes and are good at "tonifying *qi*," promoting blood circulation, and removing blood stasis. This phenomenon is known as the "climate effect efficacy theory"; just as the old saying goes, all things are born in different climates, and each has its deviation[31].

This study used callus cultures to clarify the influence of climatic conditions on the efficacy of *Panax* plants. Callus cultures of *Panax* plants were grown under different and the same conditions. Subsequently, the chemical components were detected (HPLC–MS), the anti-inflammatory activity of each sample was evaluated (qRT-PCR), and GCA and homology analysis was performed to observe the differences and similarities in drug efficacy, chemical components, and gene expression.

Plant tissue culture is the process by which plant tissue is grown aseptically in a nutrient-rich matrix in a climate-controlled incubator. This approach ensures the plant growth environment is controllable and reduces environmental diversity. It is an effective way to obtain ginsenosides quickly[32]. For callus induction, growth, and metabolism of *Panax* require a suitable environment[33–34], an environmental change will inevitably lead to a change in the plant's medicinal properties because *Panax's* growth depends on the climate to a great extent[35]. Moreover, the climate and development of the plant affect the synthesis of the bioactive components in *Panax*. To cope with changes in the local ecological environment, plants of the same genus from different habitats will selectively express relevant dominant genes.

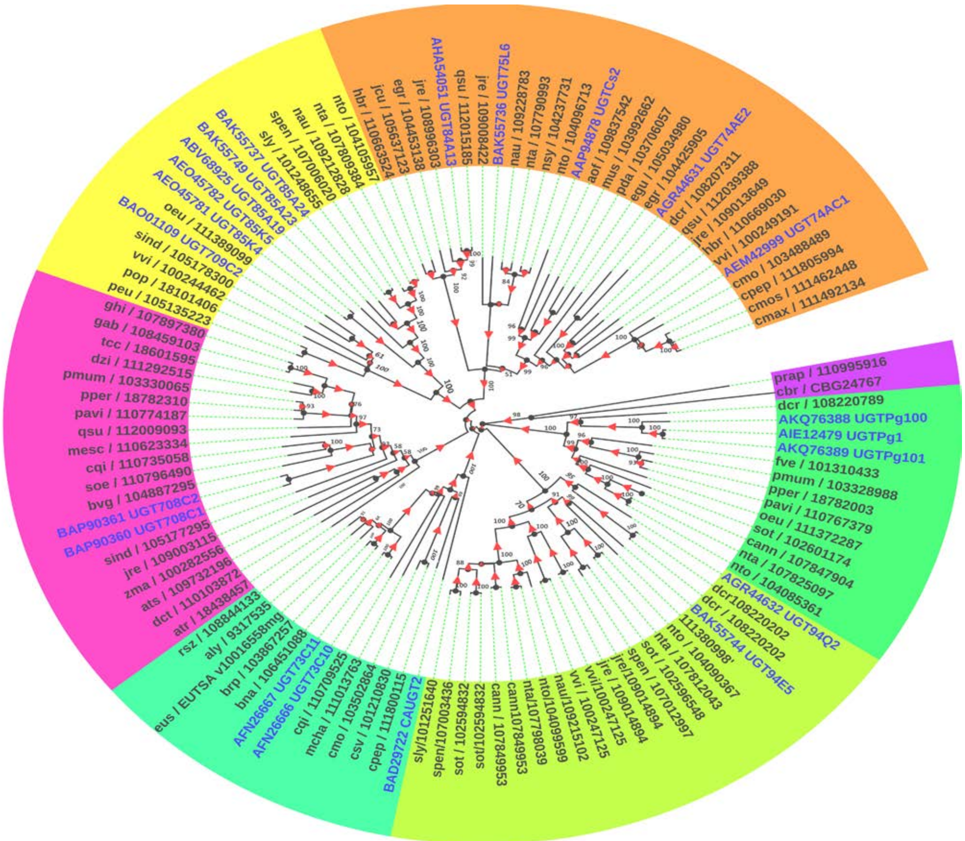

Figure 8. Phylogenetic tree of UGT gene family in *Panax*. The accession numbers of ginsenoside synthesis gene UGT family are as follows: CAUGT2 (BAD29722), UGT708C1 (BAP90360), UGT708C2 (BAP90361), UGT709C2 (BAO01109), UGT73C10 (AFN26666), UGT73C11 (AFN26667), UGT74AC1 (AEM42999), UGT74AE2 (AGR44631), UGT75L6 (BAK55736), UGT84A13 (AHA54051), UGT85A19 (ABV68925), UGT85A23 (BAK55749), UGT85A24 (BAK55737), UGT85K4 (AEO45781), UGT85K5 (AEO45782), UGT94E5 (BAK55744), UGT94Q2 (AGR44632), UGTCs2 (AAP94878), UGTPg1 (AIE12479), UGTPg100 (AKQ76388), UGTPg101 (AKQ76389).The regions with the same color represent a group of proteins with close homology, and the numbers on the phylogenetic tree represent interspecific relationships. The larger the number is, the closer their genetic relationships are Dcr, jre, and vvi represent Org codes of carrot, walnut, and grape, respectively.

Plant tissue culture technology can reduce the difficulty of comparative analysis of plants of the same genus from different areas[36–38].

Genes that played key roles in ginsenoside synthesis, including *SS, β-As, UGT94Q2, CYP76A47,* and *CYP76A53v2*, were chosen for this study. For example, *SS* was synthesized by two farnesyl diphosphate molecules, and *β-As* participates in the mevalonate pathway of ginsenoside biosynthesis[39]. *UGT94Q2* was the regulatory enzyme of ginsenoside biosynthesis[40]. *CYP76A47* could oxidize C-12 of dammarenediol to produce PPD, and then further hydroxylate C-6 through *CYP76A53v2* to produce PPT[41–45].

More than 190 types of ginsenosides have been found in *Panax* plants[46]. There are 226 UGTs in RS and 127 in SQ[47–48]. However, only 34 have been confirmed to participate in the biosynthesis of ginsenosides[49]. For example, the ginsenoside Rg3, with strong anticoagulant activity, is produced by *PgUGT94Q2* and its homologs catalyzing ginsenoside Rh2 to transfer glucose on C-3 in G-Rh2 to the C-2′ hydroxyl group[50]. *UGT71A29* has a high similarity (96.47%) with *UGTPg1* at the amino acid level[51] and can glycosylate G-Rh1 and G-Rd to produce G-Rg1 and G-Rb1 respectively[52]. While they all belong to the UGT family, their functions differ. Exploration of how UGT family members participate in regulating ginsenoside synthesis is a crucial but difficult task. Thus, to address this difficulty, the qRT-PCR/LC–MS GCA method may become a new screening method for life science research.

Conclusions

The anti-inflammatory efficacy/LC–MS correlation results showed that G-Rh1, G-Rb1, G-Rg3, G-Rh6/Floral-GKa, P2, P20, and P21 (unknown) contributed the most to the anti-inflammatory effect of *Panax* plants. It was inferred that they might be the main effective components. The gene-component correlation results showed that the expression of *CYP76A47* was affected most by the accumulation of the anti-inflammatory components and then improved the anti-inflammatory efficacy of *Panax* plants. Subsequently, to identify other enzymes that may regulate saponin synthesis, a phylogenetic tree was constructed for homology analysis. Therefore, compared with experimental screening, "anti-inflammatory component-efficacy" correlation analysis can effectively screen the pharmacodynamic substances, and "gene-component" correlation analysis can effectively filter the regulatory components of characteristic enzymes. At the same time, phylogenetic tree analysis helps find other enzymes regulating ginsenoside synthesis. This research established a novel and effective strategy for identifying key genes and active anti-inflammatory ingredients in

Panax medicinal plants. The study used climate-regulated callus culture combined with gene-component-efficacy GCA. This analysis could be used as a reference for assessing the medicinal properties of other herbs.

Conflict of interest statement

The authors declare no conflict of interest.

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Author contributions

Yongshen Ren conceived and designed the experiments. Jiao Ai, Linlin Chen, Long Cheng, Majing Jiao, Yanqiu Li, Yingzhou Wang, and Xing Deng performed the experiments. Jiao Ai and Yongshen.Ren analyzed the data. Jiao Ai and Yongshen Ren wrote and reversed the paper.

Ethical approval of studies and informed consent

Not applicable.

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