

The effects of AgNO_3 combined with temperature stress on the transcriptome of *Gentiana rigescens* and the accumulation of gentiopicroside

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Article

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Abstract

The root and rhizome of *Gentiana rigescens* are used as important traditional Chinese medicine and ethno medicine, with gentiopicroside is the major bioactive compounds. In order to explore the effects of temperature and heavy metals stress on the gentiopicroside, we investigated the accumulation of gentiopicroside and the transcriptional response of *G. rigescens* when exposed to a combination of AgNO₃ and temperature stress. AgNO₃ (100 µmol/L) was sprayed onto sterile *Gentiana rigescens* seedlings and cultured at three temperatures (22°C, 10°C and 34°C); control plants were sprayed with sterile water and cultured at 22°C. Gentiopicroside levels were determined by high-performance liquid chromatography (HPLC), and the differential expression of genes in the gentiopicroside biosynthesis pathway in response to different treatments was analyzed by an Illumina Hiseq 6000 system. Analysis showed that the levels of gentiopicroside in response to a combination of AgNO₃ and temperature treatment were significantly higher than that of those in the control; the 100µmol/L AgNO₃ and 10°C treatment group had the highest levels of gentiopicroside. A total of 135 197 unigenes were identified by transcriptome sequencing, with an average length of 1351 base pairs (bp). A total of 238 unigenes, encoding 19 key enzymes, were identified in the gentiopicroside biosynthesis pathway. The combination of 100µmol/L AgNO₃ and 10°C treatment led to the largest number of differentially expressed transcription factors when compared with the control. Our research provides key biological information for elucidating the molecular mechanisms underlying the biosynthesis of gentiopicroside in response to the combination of AgNO₃ and temperature stress.

INTRODUCTION

As a specific trigger factor, an elicitor can regulate the activity of enzymes in plant metabolism and induce a series of defense responses to stress so as to improve the synthesis of secondary metabolites [1]. These factors can be divided into biological elicitors and abiotic elicitors according to their specific sources.

Low and high temperatures may have a negative impact on plant growth and productivity. However, low and high temperatures may also exert a positive impact on secondary metabolites and are often used as abiotic elicitors. A previous study showed that the most extensive accumulation of steroidal alkaloids and glycoside alkaloids, phenolic acids and flavonoids in *Solanum viarum* occurred at 4°C and 35°C [2]. Low temperature treatment was also shown to lead to a significant increase in the anthocyanin levels of blood orange [3] and apple [4]. Furthermore, plants often show a coordinated change at the transcriptional level in response to temperature stress.

Heavy metals are another form of abiotic elicitor that belongs to the family of chemical elicitors. Heavy metals can cause disorders in the physiological and metabolic activities of plants. The excessive accumulation of heavy metals in plants can cause certain toxic effects and even death. However, appropriate concentrations of heavy metals can stimulate the synthesis of secondary metabolism in

plants. AgNO_3 , as an ethylene inhibitor, has been shown to induce a variety of plants to produce secondary metabolites. For example, 10 $\mu\text{mol/L}$ of AgNO_3 was most conducive to the synthesis of taxol in a suspension of *Taxus* cells [5]. AgNO_3 is known as the best inducer of the polysaccharide metabolic pathway and flavonoid secondary metabolic pathway in *Astragalus membranaceus* [6]. In another study, 50 mg/L of AgNO_3 was found to inhibit the growth of hairy roots in *Tripterygium wilfordii*, but significantly increased the yield of ginsenosides [7].

Gentiana rigescens Franch. ex Hemsl. is a perennial herbaceous plant belonging to the Gentianaceae Family. This plant is distributed in Yunnan, Sichuan, Guizhou, Hunan and Guangxi Province and grows in hillside meadows, shrubland, and valleys or under the pine forest at an altitude of 1 100 to 3 000 m [8]. *Gentiana rigescens* is an important authentic medicinal material in Yunnan, China, with a long history of cultivation. *G. rigescens* is officially listed in the Chinese Pharmacopoeia under the name *Gentianae radix et rhizoma* (Jianlongdan, in Chinese) for the treatment of hepatitis and cholecystitis [9]. In addition, *G. rigescens* is widely used in ethnic minorities in Yunnan for the treatment of many diseases, including headache, toothache, stomach ache, dysentery, hepatitis, hematemesis, tuberculosis, pneumonia, and Cholecystitis [10]. Dozens of Chinese medicines have been developed that are related to *G. rigescens*.

Gentiopicroside is a type of secoiridoid that exhibits diverse pharmacological activity as one of the main medicinal ingredients of *G. rigescens*, including hepatoprotective and anti-inflammatory activity, and has been shown to ameliorate abnormal glucose and lipid metabolism, and can delay aging [11–17]. *G. rigescens* is a protected plant in China and the focus of artificial production; however, the field cultivation of this plant requires significant environmental requirements. The use of plant cell, tissue, and organ cultures has been acknowledged as a potential alternative source for the more efficient production of valuable secondary metabolites.

To the best of our knowledge, there are no previous studies of the effects of elicitors on the production of gentiopicroside from *in vitro* cultures of *G. rigescens*. In this study, we evaluated the effect of a combination of low temperature (as a physical elicitor) and AgNO_3 (as a chemical elicitor) on the accumulation of gentiopicroside and the expression of functional genes in the metabolic pathway of *G. rigescens*. Our findings may provide a foundation for the artificial regulation of *G. rigescens*.

MATERIALS AND METHODS

Plant materials and stress treatment

The seeds of *Gentiana rigescens* have been provided by yuanzheng plantation company in Dali, Yunnan, China (99°58'47"N, 25°58'47"E, 2200). Following sanitization, seeds were cultivated on MS_0 culture medium. Rootless seedlings were cut into stem segments and transferred to a propagation medium (4.42g/L of MS, 30g/L of sucrose; 5.4–5.5 g/L of agar, 0.3 mg/L of 6-BA, 0.1 mg/L of NAA; pH 5.8) for three months. Sterile seedlings with a consistent appearance and growth status were selected as

experimental materials (Fig. 1). Nine sterile seedlings were used to form three biological replicates per treatment. In vitro experiments with plants complied with accordance with the relevant institutional and national legislation.

Sterile seedlings were sprayed with 100 $\mu\text{mol/L}$ of AgNO_3 and cultured at 10°C (LT), 22°C (MT), and 34°C (HT) in an artificial climate box (Zhejiang Keheng Instrument Co., Ltd). The control group (CK) was sprayed with sterile water and cultured at 22°C. Samples were collected on the third day of treatment. Three plants were randomly selected from each treatment as one biological replicate. One half of each of the three selected plants was snap frozen in liquid nitrogen and stored at -80°C to await further analysis; the other half was dried to a constant weight at 55°C and sieved with a 60 mesh to determine the levels of gentiopicroside.

Determination of the gentiopicroside levels of *G. rigescens* under combined stress

The levels of gentiopicroside were determined by high-performance liquid chromatography (HPLC; Agilent, Santa Clara, CA, USA). The extraction and detection of gentiopicroside were conducted as described previously by Liu et al. ^[18]. In brief, 0.1 g of dried powder was extracted under ultrasonication in 10 ml of 80% methanol for 40 min (45°C; working frequency: 55 kHz), followed by centrifugation of 2000 rpm. The supernatant was aspirated through a 0.22 μm membrane with a 1 mL syringe; the filtrate represented the test solution.

The conditions for chromatography were as follows. The chromatographic column was an Agilent Intersil-C18 column (4.6 mm $\times$ 150 mm, 5 μm). The mobile phase was 0.1% formic acid aqueous solution (A) and acetonitrile (B) with a flow rate of 1 mL/min with a gradient elution of 0–2.5 min, 7–10%; 2.5–20 min, 10–26%; 20–29.02 min, 26–58.3%; 29.02–30 min, 58.3–90%, 30–34 min, and 90% B. The column temperature was 30°C, the sample size was 5 μL , and the detection wavelength was 241 nm. The levels of gentiopicroside under different treatments were determined by comparing their peak times and retention times with that of a standard. Finally, the data was processed using Excel 2016 and plotted using GraphPad Prism 6.01.

RNA-seq processing and data analysis

Total RNA was extracted from samples, purified, and tested for purity, concentration, and integrity. After the samples had been tested, we isolated and purified mRNA by Oligo (dt) in order to construct a cDNA library. Illumina NovaSeq6000 was performed after the library was prepared. The original data were filtered to remove primer and connector sequences, sequences with a fragment length < 50bp, sequences with an N base ratio > 10%, low-quality reads, and low-quality bases. Following splicing and assembly using Trinity, we were able to obtain transcripts and single gene clusters.

The DIAMOND software (v2.0.4) ^[19] was used to compare the unigene sequence with the multiple databases including Nr ^[20], Swiss-prot ^[21], Clusters of Orthologous Genes (COG) ^[22], euKaryotic Orthologous Groups (KOG) ^[23], and KEGG ^[24].

Screening for genes involved in gentiopicroside biosynthesis

The higher the transcript abundance, the higher the gene expression level. Gentiopicroside biosynthetic genes were screened by referring to species of *Gentiana*, as described previously [25–26]. Combined with the annotation results arising from GO, Nr and KEGG analysis, we mine gentiopicroside biosynthesis-related unigenes from the transcriptome data, and the expression abundance of the corresponding unigenes was expressed by FPKM (fragments per kilobase of transcript per million mapped reads)

FPKM values were calculated as follows:

$$FPKM = \frac{\text{mapped fragmentsof transcript}}{\text{TotalCountof mapped fragments (Millions)} \times \text{Lengthof transcripts (kb)}}$$

In this formula, cDNA fragments refer to the number of fragments as compared to a certain transcript, the number of double-ended reads; mapped fragments (millions) refer to the total number of fragments as compared to a transcript (in 10⁶ units), and transcript length (kb) refers to the transcript length (in 10³ bases).

Differential expression analysis

DESeq [27] was used for differential expression analysis between samples. First, we calculated the fold change (FC) of expression between two samples. Subsequently, the Benjamin Hochberg method was used to correct the hypothesis testing probability (P-value) and obtain the error detection rate (False Discovery Rate, FDR). We then used an FDR < 0.05 and a |log₂(Fold_change)| ≥ 1 as screening criteria; genes satisfying these criteria were considered as differentially expressed genes (DEGs).

qRT-PCR (Real-time polymerase chain reaction)validation

A total of nine genes were identified from the secoiridoid biosynthesis pathway using our specific screening criteria: | log₂ (FC) | ≥ 2 and FDR < 0.01. The 18S rRNA is present in the ribosomal subunit, and its encoding gene rDNA (18S rRNA/rDNA) is evolutionarily conserved. qRT-PCR was performed using the Analytik Jena-qTOWER 2.2 (Analytik Jena, Jena, Germany) with a TUREscript 1st Stand cDNA Synthesis Kit (Aidlab, Hong Kong). Gene-specific primers were designed using Primer Premier 5.0, and the primer sequences are listed in (Table 1). Relative gene expression levels were then calculated by the 2^{−ΔΔCt} method [28].

Table 1
Primers for PCR.

Gene		sequence(5'-3')	Product size
<i>HMGR</i>	F	TGACTGCAAACGAGCAGCTA	128 bp
	R	CCGGCATTTCACAACACTGG	
<i>STR</i>	F	TACGGACAGCAGCACCAAAT	198 bp
	R	CTGCTGTTTGTAGTCTCGGC	
<i>GGPPS</i>	F	ATGGGATCGTGGAAGGTCAG	127 bp
	R	CCTAAAATCGCCCCACAAGC	
<i>IDI</i>	F	GCGCCTCATGTTTGAAGACG	131 bp
	R	CGCTAAATGCCCTATGCAGC	
<i>8-HGO-1</i>	F	TGGCGGTCTTGGTCATGTAG	159 bp
	R	GTTGCAGCCTTCAGCTGTTC	
<i>8-HGO-2</i>	F	TCGTTTAGGTGCCGACTCTT	167 bp
	R	TCGGCACTCCGACAACAATA	
<i>AACT</i>	F	AAGCATACAGTTGGGCGTCA	114 bp
	R	GTATCATGCCCAAGCCGAGA	
<i>8-HGO-3</i>	F	AGAGGTTGGAAGCAAGGTGG	133 bp
	R	TGTGAGTATCTGACCGGCAC	
<i>18S</i>	F	CAACCATAAACGATGCCGA	
	R	AGCCTTGCGACCATACTCC	

RESULTS

A comparison of the gentiopicroside levels of sterile *G. rigescens* seedlings under four different conditions

In order to investigate the accumulation of the main active ingredients of *G. rigescens* in response to AgNO₃ combined with temperature stress, we determined the specific levels of gentiopicroside in each sample. Analysis indicated that the gentiopicroside levels of each stress group were significantly higher ($p < 0.01$) than those in the control group (Fig. 2). These data demonstrated that AgNO₃ induced the accumulation of gentiopicroside. Moreover, the lower the temperature, the higher the levels of gentiopicroside. Thus, the sterile seedlings of *G. rigescens* in the LT group exhibited the greatest

accumulation of gentiopicroside Thus, temperature was an effective inducer for the accumulation of gentiopicroside.

Transcriptome analysis of *G. rigescens* and database analysis

We obtained a total of 12 libraries under the four treatments (three replicates for each treatment) including high-quality read fragments, as follows: 40 778 416, 47 365 784, and 43 551 830 in the HT group; 52 397 476, 47 566 956, and 52 069 084 in the CK group; 48 611 366, 45 168 148, and 56 123 670 in the LT group; and 57 888 626, 51 289 868, and 40 637 046 in the MT group. The datasets generated and analysed during the current study are available in the SRA database (Registration number: PRJNA1041091). The similarity between the three biological repeats was represented by a distance heat map between samples, and we observed high levels of similarity between samples (Fig. 3A).

Functional annotation and transcription factors (IF)

Unigene sequences were compared by the KEGG, NR, Swiss-Prot, GO, COG/KOG, TREMBL databases using DIAMOND BLASTX software. After predicting the amino acid sequences of, unigenes, we used HMMER software to perform comparisons with the Pfam database to obtain unigene annotation information for *G. rigescens*. The annotations arising from each database are shown in (Table 2); the NR database accounted for 58.54% of the largest number of annotations, followed by the TrEMBL database, accounting for 58.08%.

Table 2
Functional annotation of the transcriptome of *G. rigescens*

Database	Number of Genes	Percentage(%)
KEGG	60 559	44.79
Nr	79 149	58.54
Swiss-Prot	59 172	43.77
TrEMBL	78 525	58.08
KOG	47 880	35.41
GO	68 298	50.52
Pfam	50 887	37.64
Annotated in at least one Database	81 701	60.43
Total Unigene	135 197	100

When comparing the unigenes of *G. rigescens* with the NR database, we found that *G. rigescens* showed the highest similarity with *Catharanthus roseus* (20.52%), *Coffea arabica* (11.81%) and *Coffea eugenoides* (6.37%) (Fig. 3B).

After analyzing the functional annotations yielded by the GO database, we found that 68 298 unigenes were assigned to the three categories (biological process, cellular component and molecular function (Fig. 3C). With regards to biological processes, unigenes were mainly clustered in cellular processes, metabolic processes, and responses to stimulus, with 47 413, 3 9421, and 18 767 annotations, respectively. For the cellular components, the most clustered taxa were cellular anatomical entities and protein-containing complexes, with 54 519 and 10 269 unigenes annotated, respectively. The three taxa that were most commonly associated with molecular function were binding, catalytic activity, and transporter activity, with 41 874, 34 405 and 4 021 unigenes annotated, respectively.

A total of 47 880 unigenes were annotated and 25 different KOG functional taxa were obtained from KOG analysis of the *G. rigescens* transcriptome (Fig. 3D). The number of unigenes contained for each functional group varied, of which general functional prediction only was the largest category, with 13 593 unigenes annotated, accounting for 28.38% of the total number of annotations. This was followed by post-translational modification, protein turnover, chaperones and signal transduction mechanisms, with 5 203 and 4 965 unigenes annotated, accounting for 10.86% and 10.36% of the total number of annotations, respectively. Cell motility had the fewest unigenes, (12), accounting for only 0.025% of the total number of annotations.

When considering the transcriptome data of *G. rigescens*, a total of 2 476 unigenes were identified to encode 67 transcription factor (TF) families; *bHLH* (171), *AR2/ERF-ERF* (151), *C2H2* (150), *C3H* (146), *bZIP* (140), and *MYB-related* (136) were the top six families in terms of unigene numbers (Fig. 3E). We identified 30 up-regulated DEGs in CK vs. HT, 54 in CK vs. LT, 24 in CK vs. MT, 52 in LT vs. HT, 54 in MT vs. LT, and 29 in MT vs. HT (Table 3).

Table 3
Distribution of the top six transcription factor families and DEGs

TFs Family	Number	CK vs HT	CK vs LT	CK vs MT	LT vs HT	MT vs LT	MT vs HT
<i>bHLH</i>	171	7	7	9	11	6	4
<i>AP2/ERF-ERF</i>	151	4	11	2	8	10	2
<i>C2H2</i>	150	2	15	1	4	16	4
<i>C3H</i>	146	6	7	2	8	7	3
<i>bZIP</i>	140	6	5	5	14	3	9
<i>MYB-related</i>	136	5	9	5	7	12	7

The gentiopicroside biosynthetic pathway

Gentiopicroside biosynthesis is widely considered to be completed according to several steps: intermediate generation, terpene skeleton synthesis, and post-modification ^[25–26, 29] (Fig. 4A). A total of 238 unigenes, encoding 19 key enzymes, were found to be involved in gentiopicroside biosynthesis in our transcriptome analysis (Table 4). The expression of related DEGs under different treatments was

demonstrated by heat maps (Fig. 4B-D). Based on FPKM values, we observed complex differences in gene expression across different treatments. The expression levels of different members of the same gene family were expressed differently under different treatments. For example, *8-HGO4* and *8-HGO9* were highly expressed in the CK group, *8-HGO10* and *8-HGO8* were highly expressed in the LT group, *8-HGO3* and *8-HGO5* were highly expressed in the MT group, while *8-HGO2* and *8-HGO 7* were highly expressed in the HT group.

Table 4
Genes involved in the secoiridoid biosynthetic pathway

Pathway	gene	Abbr.	Enzyme code	No of unigenes
MVA	Acetyl-CoA C-acetyltransferase	<i>AACT</i>	2.3.1.9	9
	Hydroxymethylglutaryl-CoA synthase	<i>HMGS</i>	2.3.3.10	6
	Hydroxymethylglutaryl-CoA reductase (NADPH)	<i>HMGR</i>	1.1.1.34	13
	Isopentenyl phosphate kinase	<i>IPK</i>	2.7.4.26	15
	Mevalonate kinase	<i>MK</i>	2.7.1.36	1
	Phosphomevalonate kinase	<i>PMK</i>	2.7.4.2	4
	Diphosphomevalonate decarboxylase	<i>MVD</i>	4.1.1.33	19
MEP	1-Deoxy- <i>D</i> -xylulose-5-phosphate synthase	<i>DXS</i>	2.2.1.7	6
	1-Deoxy- <i>D</i> -xylulose-5-phosphate reductoisomerase	<i>DXR</i>	1.1.1.267	6
	2- <i>C</i> -Methyl- <i>D</i> -erythritol 4-phosphate cytidyltransferase	<i>CMS</i>	2.7.7.60	8
	4-(Cytidine5'-diphospho)-2- <i>C</i> -methyl- <i>D</i> -erythritol kinase	<i>CMK</i>	2.7.1.148	1
	2- <i>C</i> -Methyl- <i>D</i> -erythritol2,4-cyclodiphosphate synthase	<i>MCS</i>	4.6.1.12	8
	4-Hydroxy-3-methylbut-2-enyl-diphosphate synthase	<i>HDS</i>	1.17.7.3	18
	4-Hydroxy-3-methylbut-2-enyl diphosphate reductase	<i>HDR</i>	1.17.7.4	7
Iridoid	Isopentenyl-diphosphate delta-isomerase	<i>IDI</i>	5.3.3.2	2
	Geranyl diphosphate synthase	<i>GPPS</i>	2.5.1.1	10
	8-Hydroxygeraniol oxidoreductase	<i>8-HGO</i>	1.1.1.324	27
	Strictosidine synthase	<i>STR</i>	4.3.3.2	34
	Geranylgeranyl pyrophosphate synthase	<i>GGPPS</i>	2.5.1.29	50

Differential gene expression analysis of the gentiopicroside biosynthetic pathway

We identified 15 807 DEGs in the LT group when compared with the HT group; of these, 7 786 DEGs were up-regulated and 8 021 were down-regulated (Table 5). These 15 807 DEGs were enriched in 143

metabolic pathways, and predominantly associated with photosynthesis-antenna proteins, benzoxazinoid biosynthesis and monoterpenoid biosynthesis (Fig. 4D).

Table 5
The numbers of DEGs involved in the gentiopicroside biosynthetic pathway when compared between groups

Between groups	Total number of genes	Up-regulated genes	Down-regulated genes
CK vs HT	6 812	3 375	3 437
CK vs LT	8 586	4 788	3 798
CK vs MT	3 493	1 122	2 371
LT vs HT	15 807	7 786	8 021
MT vs HT	7 628	4 327	3 301
MT vs LT	7 832	4 485	3 347

Biological functional annotation of the DEGs by KEGG analysis showed that a total of 139 metabolic pathways were enriched in 6 812 DEGs for CK vs HT, and were significantly enriched in monoterpenoid biosynthesis, benzoxazinoid biosynthesis, and zeatin biosynthesis (Fig. 5A). A total of 140 metabolic pathways were enriched in 8 586 DEGs for CK vs LT, and were significantly enriched in phenylalanine metabolism, mismatch repair, and DNA replication (Fig. 5B). A total of 132 metabolic pathways were enriched in 3 493 DEGs for CK vs MT, and were significantly enriched in photosynthesis-antenna proteins, benzoxazinoid biosynthesis, and the biosynthesis of secondary metabolites (Fig. 5C). A total of 143 metabolic pathways were enriched in 15 807 DEGs for LT vs HT, and were significantly enriched in photosynthesis-antenna proteins, benzoxazinoid biosynthesis and monoterpenoid biosynthesis (Fig. 5D). A total of 140 metabolic pathways were enriched in 7 628 DEGs for MT vs HT, and were significantly enriched in monoterpenoid biosynthesis, photosynthesis-antenna proteins, and phenylalanine metabolism (Fig. 5E). A total of 141 metabolic pathways were enriched in 7 832 DEGs for MT vs LT, and were significantly enriched in MAKP signaling pathway-plant, the biosynthesis of secondary metabolites, and metabolic pathways (Fig. 5F).

qRT-PCR validation

To further validate the RNA-seq results, we selected eight genes involved in the metabolism pathway from the candidate DEGs and analyzed these using RT-qPCR. The RT-qPCR results (Fig. 6B)for the eight genes were generally consistent with the relative transcript abundance found in the transcriptome, thus indicating the reliability of the RNA-seq data (Fig. 6A).

DISCUSSION

In this study, AgNO₃ combined with temperature stress was used to induce sterile *G. rigescens* seedlings. AgNO₃ was selected at a common concentration of 100um/L, and the lowest, highest and room temperature were selected according to the ambient temperature associated with the natural

distribution of *G. rigescens*. Our experimental results showed that the accumulation of gentiopicroside in *G. rigescens* can be increased by the combination of AgNO₃ and temperature stress. Moreover, at all three temperatures, the AgNO₃ treatment groups were significant higher than the control group, thus demonstrating that AgNO₃ could promote the accumulation of gentiopicroside. Many previous studies have shown that AgNO₃ can cause the accumulation of many types of secondary metabolites including quercetin, glycyrrhizin, phenolic compounds and tanshinone^[30–33]. In one study, AgNO₃ was used to induce *Gentiana macrophylla* seedlings; the authors found that the level of gentiopicroside was almost two-fold higher than that of the untreated plants^[34]. Metals have become one of the main abiotic stress agents for living organisms because of their increasing use in developing fields such as industry, agrotechnics; however, metals are associated with high levels of bioaccumulation and toxicity^[35]. Therefore, it is particularly important to investigate the impact of metals on the secondary metabolites of medicinal plants.

Our experiments showed that under the same concentration of AgNO₃, the lower the temperature, the higher the gentiopicroside content. When plants are exposed to high or low temperatures over long periods of time, this stress can trigger oxidative stress reactions, leading to the excessive release of reactive oxygen species (ROS) and cellular damage^[36]. However, we found that over three days of treatment, the morphology of *G. rigescens* did not change significantly; however, gentiopicroside rapidly accumulated, especially under a combination of AgNO₃ and low temperature. Therefore, both low and high temperatures may have a positive impact on the secondary metabolites of *G. rigescens*.

Terpenoids are the largest class of natural compounds which are made of MVA and MEP pathways. Plants use these compounds for growth; however, their main role is to preserve plants against a variety of biotic and abiotic stresses^[37]. In the present study, the presence of abiotic stress caused *G. rigescens* to rapidly synthesize a large amount of gentiopicroside for self-protection. Gentiopicroside is a special form of terpenoid, and one of the secoiridoid glycosides that are mainly distributed in the roots of *G. rigescens*. Gentiopicroside is the main pharmacological ingredient of over 180 traditional Chinese medicine products, including Gentiana Longlong Capsules and Longdan Xiegan Pills. Over recent years, many types of secoiridoids have been isolated from the aerial components of *G. rigescens*^[37]. In the present study, the sterile seedlings of *G. rigescens* were mainly stems and leaves; we measured high levels of gentiopicroside that were 2.5-fold higher than those of the control plants. This data demonstrates that sterile seedlings can produce gentiopicroside.

In order to investigate the reasons for the induction of gentiopicroside synthesis by AgNO₃ when combined with a low temperature, we investigated the transcriptome and eight genes associated with the biosynthesis pathway of *G. rigescens* under different treatment conditions. A large number of DEGs were identified between different treatment conditions. The low temperature group and the high temperature group exhibited the largest number of DEGs; however, the accumulation of gentiopicroside was not significantly correlated with these DEGs. This may be due to the fact that the DEGs were not primarily associated with the biosynthetic pathway of gentiopicroside. The low temperature group

presented with the largest accumulation of gentiopicroside, while not fully consistent with DEGs. This may be due to the fact that the DEGs encode for enzymes were not completely involved in the biosynthetic pathway for gentiopicroside.

Transcription factors (TFs) are important regulatory factors that mediate the signal transduction of environmental factors, modulate the expression of multiple genes in a specific metabolic pathway, and regulate the production of target metabolites ^[39–41]. Many TFs help to regulate the biosynthesis and accumulation of secondary metabolites ^[42–45]. In this study, we identified 2 476 unigenes which encode 67 families of TFs, including *bHLH* (171), *AR2/ERF-ERF* (151), *C2H2* (150), *C3H* (146), *bZIP* (140), and *MYB-related* (136). The DEGs of TFs identified between each group ranged from 21 to 54; of these, the CK vs. LT (the highest gentiopicroside content group) comparison presented the largest number of up-regulated DEGs.

Though we did not identify the specific relationships between stress, transcription factors, genes, and gentiopicroside, our data provide a basis for shedding light on the complex regulatory networks involved.

CONCLUSION

In summary, we found that AgNO₃ could induce the accumulation of gentiopicroside in *Gentiana rigescens*. The combination of AgNO₃ with low temperature stress may be the best approach with which to induce the accumulation of gentiopicroside. Further, we identified many DEGs between the four treatments. A total of 2 476 unigenes were identified which encode 67 TF families. The up-regulated DEGs ranged from 24 to 30 across the different groups. Transcriptomic analysis identified a total of 238 unigenes, encoding 19 key enzymes, which were found to be involved in the biosynthesis of gentiopicroside.

Declarations

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AUTHOR CONTRIBUTIONS:

T.Z. Sterile seedling culture, writing original draft, data curation. C.Y.L. HPLC, draft, visualization. Y.X.G. Sterile seedling culture, software. M.Q.W. HPLC, Software. X.H.Z. conceptualization, project administration, editing. X.L.L. conceptualization, supervision, project administration, editing, Language polishing. All authors have read and agreed to the published version of the manuscript.

DECLARATION OF INTEREST

The authors declare no competing interests.

Data Availability Statements

The data that support the findings of this study are available within the paper and its supplementary files. RNA-seq data associated with this study have been deposited in the NCBI(<https://www.ncbi.nlm.nih.gov/>) with number PRJNA1041091.

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Figures

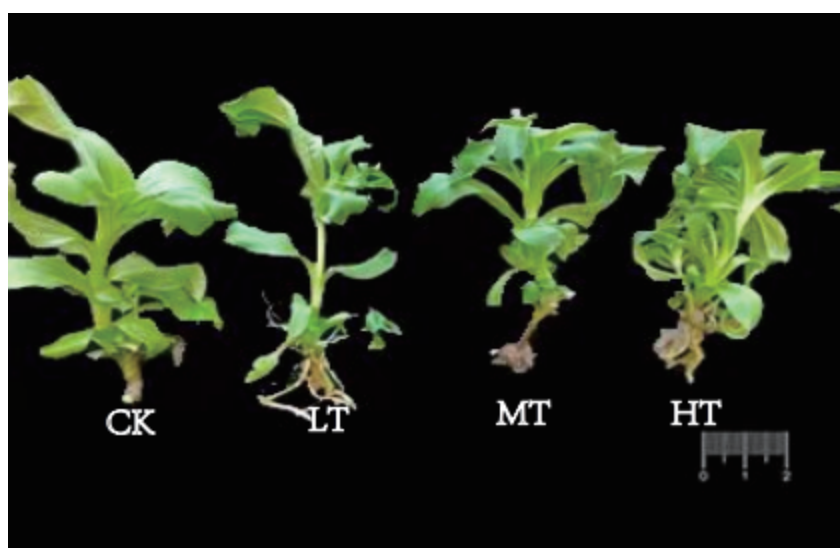


Figure 1

Sterile *G. rigescens* seedlings from four treatments (CK, the control group; LT, 100 µmol/L of AgNO₃ combined with 10°C culture; MT, 100 µmol/L of AgNO₃ combined with 22°C culture; HT, 100 µmol/L of AgNO₃ combined with 34°C).

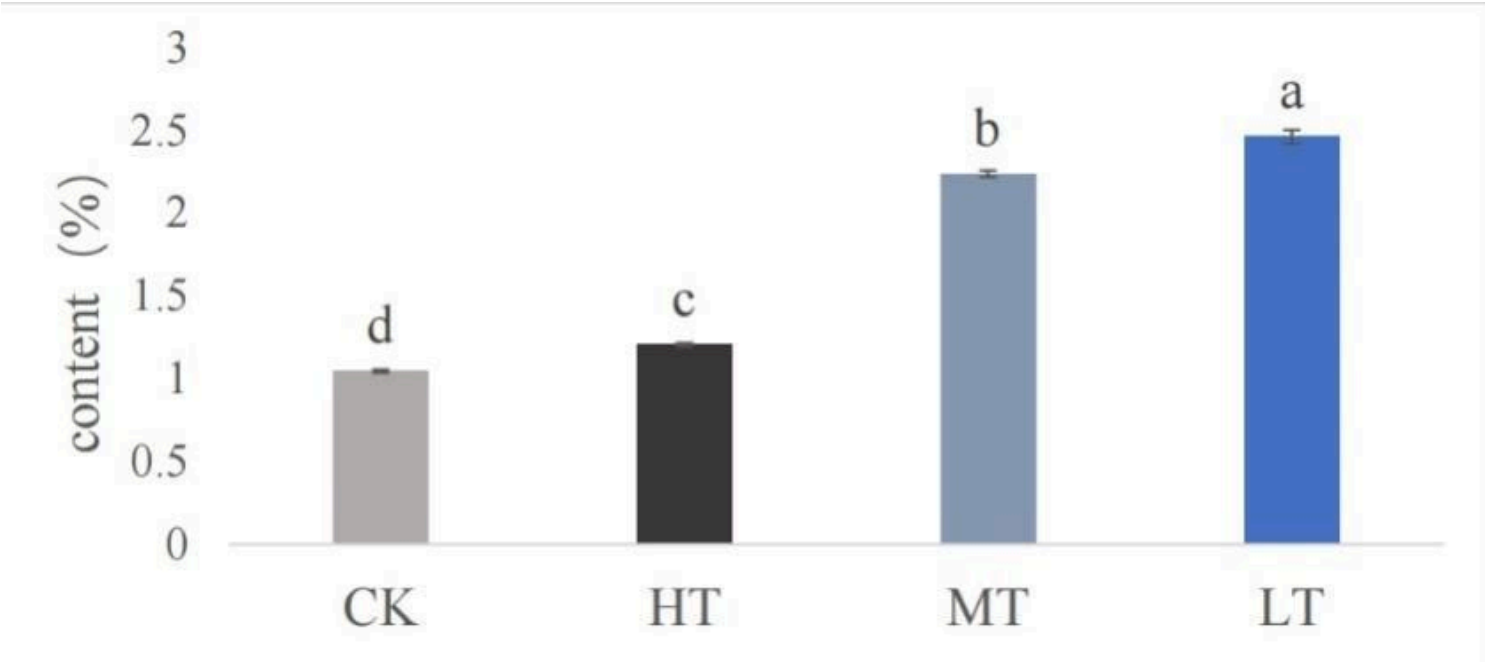


Figure 2

Changes of gentiopicroside level in *G. rigescens* under different treatments.

CK HT MT LT

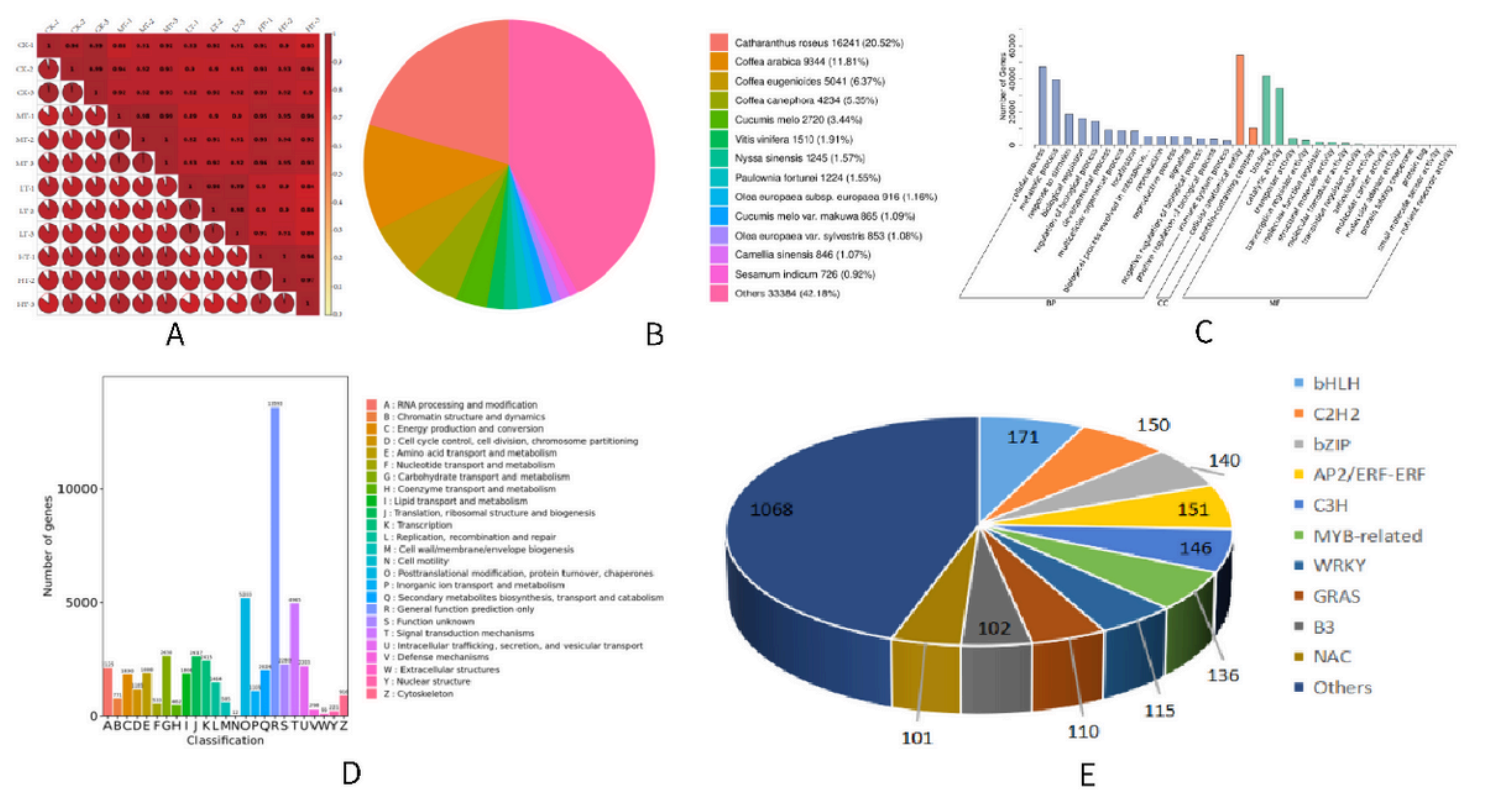


Figure 3



Figure 5

Scatter plot of DEG-based enrichment as determined by KEGG analysis.

(A) CK vs. HT, (B) CK vs. LT, (C) CK vs. MT, (D) LT vs. HT, (E) MT vs. HT and (F) MT vs. LT

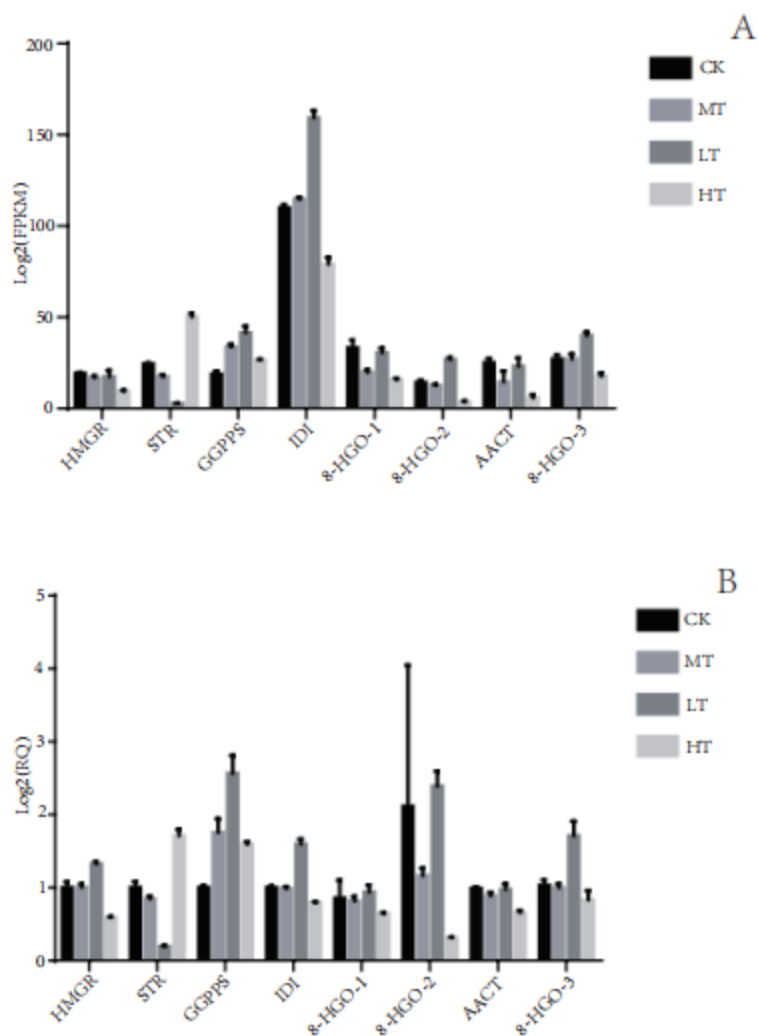


Figure 6

RNA-seq analysis of *G. rigescens* and the qRT-PCR validation of eight genes associated with the gentiopicroside biosynthesis pathway. (A) RNA-seq data of DEGs related to the biosynthesis of the secoiridoids pathways. (B) qRT-PCR validation of DEGs related to the biosynthesis of secoiridoids pathways.

Supplementary Files

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