Transcriptome Analysis Reveals Key Genes and Pathways in Borneol Biosynthesis of a New Borneol-Chemotype *Cinnamomum camphora*

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Natural borneol, a valuable monoterpenoid, is primarily derived from *Cinnamomum camphora* chvar. borneol. This unique Chinese tree was studied using the high-borneol cultivar 'Ganlong 2' and common camphor trees as material. Multi-location trials over three years confirmed that 'Ganlong 2' stably exhibits high borneol content, high essential oil yield, and low camphor content, presenting an ideal system for biosynthesis research. Transcriptomic analysis identified key differentially expressed genes (DEGs), and KEGG enrichment outlined the (+)-borneol biosynthesis pathway. Critical genes, including *CcBPPS*, *CcNUDX1*, and *CcDXS1*, were highlighted, with the MEP pathway confirmed as the primary biosynthetic route. These findings advance the understanding of monoterpenoid biosynthesis regulation and provide a theoretical and genetic basis for improving natural borneol production *via* synthetic biology and breeding high-quality varieties.

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INTRODUCTION

Cinnamomum camphora chvar. borneol, an evergreen tree belonging to the family Lauraceae and genus *Cinnamomum*, is a precious aromatic species endemic to the regions south of the Yangtze River in China. Its leaves are rich in natural borneol (primarily composed of (+)-borneol), which exhibits significant pharmacological activities such as anti-inflammatory, analgesic, and antibacterial effects. As a result, it is widely used in pharmaceuticals, perfumery, and cosmetics (Huang *et al*. 2023; Huang *et al*. 2024; Zhou *et al*. 2025). The natural borneol is a white crystalline solid with a boiling point of approximately 212 ℃ at standard atmospheric pressure. It exhibits sublimation characteristics and is slightly soluble in water but readily dissolves in various organic solvents (Mei *et al*. 2023). Chemically, borneol is sensitive to light, heat, and oxygen, and improper storage conditions – such as prolonged exposure to air and light – can lead to its oxidation to camphor (Mei *et al*. 2023). Its most common chemical reactions include: oxidation, where it converts to camphor in the presence of air or oxidizing agents, which is a critical consideration during storage and processing; and esterification, in which the hydroxyl group of borneol reacts with organic acids (*e.g*., acetic acid) to form ester

derivatives such as bornyl acetate, which is a significant pathway in fragrance synthesis (Gu *et al*. 2025). These key physicochemical properties directly influence the storage conditions, processing techniques, stability, and application performance of natural borneol in pharmaceutical and fragrance products (Zhou *et al*. 2025). With the growing market demand for natural borneol, conventional extraction methods – constrained by high resource consumption and low yield – have become increasingly inadequate. There is an urgent need to enhance the production efficiency of natural borneol through the breeding of high-yielding varieties.

Significant progress has been made in understanding the biosynthetic mechanisms of plant secondary metabolites, particularly monoterpenoids. The synthesis of monoterpenes primarily involves three stages: First, the mevalonic acid (MVA) pathway in the cytoplasm and the 2-C-methyl-D-erythritol-4-phosphate (MEP) pathway in plastids synthesize the universal precursors isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP), respectively. Subsequently, these two compounds condense to form geranyl diphosphate (GPP), which is the common direct precursor of monoterpenes. Finally, bornyl diphosphate synthase (BPPS) catalyzes the cyclization of GPP to form bornyl diphosphate (BPP), which is subsequently hydrolyzed and dephosphorylated to yield borneol (Pu *et al*. 2021).

As a monoterpenoid essential oil compound, the synthesis of (+)-borneol also relies on both the MVA and MEP pathways. Numerous studies have indicated that key enzyme genes in these pathways, such as phosphomevalonate kinase (PMK), 1-deoxy-D-xylulose-5-phosphate synthase (DXS), and DXR, serve as rate-limiting factors regulating the synthesis of IPP, DMAPP, and downstream products. These genes are important targets for genetic improvement in monoterpenoid biosynthesis (Singh and Sharma 2015; Tholl 2015). For instance, overexpression of *DXS* and *DXR* genes in *Arabidopsis* and tomato significantly increased monoterpenoid production (Estévez *et al*. 2001; Enfissi *et al*. 2005). Additionally, enzyme genes such as *BPPS* and *NUDX* play crucial roles in the biosynthesis and modification of borneol. Homologs of BPPS have been reported in various plant species, including *SoBPPS* (*Salvia officinalis*), *WvBPPS* (*Wurfbainia villosa*), *LaBPPS* (*Lavandula angustifolia*), *CbBPPS* (*Cinnamomum burmannii*), and *DgTPS1* (*Dipterocarpus gracilis*) (Despinasse *et al*. 2017; Ma *et al*. 2021a,b; Tian *et al*. 2022). Furthermore, enzyme kinetics and tobacco transient expression experiments demonstrated for the first time that *WvNUDX24* from *Wurfbainia villosa* specifically and efficiently catalyzes the hydrolysis of BPP to generate BP, thereby participating in borneol biosynthesis (Yang *et al*. 2024).

Although *C. camphora* chvar. borneol serves as a valuable source of natural borneol, research on its biosynthesis remains relatively limited. The overall regulatory network of borneol biosynthesis has not yet been fully elucidated, and systematic identification and functional characterization of key genes are still pending. In this study, the high-borneol-type 'Ganlong 2' and a common borneol camphor tree obtained through directional breeding were used as experimental materials. By integrating biochemical and transcriptomic analysis, the metabolic pathway of borneol biosynthesis in *C. camphora* chvar. borneol was constructed. For the first time, a series of key enzyme genes including *GPPS*, *BPPS*, and *NUDX* were identified. These findings lay a foundation for further deciphering the regulatory mechanisms underlying natural borneol biosynthesis and hold significant implications for promoting the sustainable development of the camphor tree industry and alleviating the supply-demand imbalance in the natural borneol market.

EXPERIMENTAL

Plant Materials and Essential Oil Determination

From 2022 to 2024, regional trials were conducted in the Ji'an, Jiujiang, and Ganzhou, areas of Jiangxi Province. The new borneol camphor variety 'Ganlong 2' was used as the test material, with common borneol camphor trees as the control. Fresh leaves were collected from November to December each year, and volatile oils were extracted using steam distillation. The essential oil yield was determined, and the borneol and camphor contents were analyzed by gas chromatography (GC) (Pragadheesh *et al*. 2013).

Statistical Analysis: All data are presented as the mean ± standard deviation. A three-factorial experimental design was employed with two cultivars ('Ganlong 2' *vs.* common *C. camphora* chvar. borneol). Additional fixed factors were the locations (Ji'an, Jiujiang, Ganzhou), and years (2022, 2023, 2024). Initially, a multi-way ANOVA was used to examine the effects of these three main factors on essential oil yield, borneol content, and camphor content, and to assess their interactions (Liland and Færgestad 2009). Where the ANOVA indicated significant differences, Tukey's Honest Significant Difference (HSD) post-hoc test was applied for multiple comparisons to identify specific differences among the treatment groups across cultivars, locations, and years (Bita and Indreica 2016). All statistical analyses were performed using R software (version 4.3.0), and the significance level was set at $p < 0.05$.

The plant materials used in this study were obtained from the Ji'an Forestry Science Research Institute. In November 2024, three healthy individuals with uniform growth vigor were selected randomly from 'Ganlong 2' and common borneol camphor trees as sampling plants. Mature leaves were collected, immediately frozen in liquid nitrogen, and stored at -80°C in an ultra-low temperature freezer for subsequent analysis.

RNA Extraction, Library Construction, and Sequencing Assembly

The total RNA extraction from samples and cDNA library construction were completed by Huazhi Biotechnology Co., Ltd., and qualified libraries were sequenced using the Illumina HiSeq platform after passing quality control. After obtaining the raw sequencing data, adapter sequences and low-quality reads (containing >5% N bases or Q<20) were first filtered out, followed by trimming bases with quality scores below 20 at read ends using a sliding window approach (window size 4bp), and finally removing reads shorter than 100 nt along with their paired reads to obtain high-quality clean reads. After completing quality control of the data, de novo transcriptome assembly was performed on the clean reads using Trinity v2.4.0 software (Haas *et al*. 2013) to obtain transcript sequences.

Unigene Functional Annotation

The Unigenes were aligned against multiple databases including NT (NCBI nucleotide sequences), NR (NCBI non-redundant protein sequences), COG/KOG (Clusters of Orthologous Groups of proteins/euKaryotic Ortholog Groups), Swiss-Prot (a manually annotated and reviewed protein sequence database), TrEMBL, GO (Gene Ontology), and KEGG (Kyoto Encyclopedia of Genes and Genomes) using NCBI Blast+ 2.14 (Altschul *et al*. 1997). Based on the annotation results from SwissProt and TrEMBL, GO annotations were obtained through Uniprot annotation information, while KEGG annotations were acquired using the KEGG Automatic Annotation Server, thereby establishing

comprehensive functional characterization of the transcriptome data through these systematic bioinformatics analyses.

qRT-PCR Validation

Using cDNA templates synthesized from three independent biological replicates per cultivar and with *Actin2* as the internal reference gene (Shen *et al.* 2022), eight candidates differentially expressed genes were selected, and specific primers were designed using Primer 3 software (Untergasser *et al*. 2012) (Table 1). The expression levels of these candidate genes in leaves of 'Ganlong 2' and ordinary *C. camphora* chvar. borneol were detected by PCR using the SYBR Green method with a 10 μL reaction system run in technical triplicates. The thermal cycling protocol consisted of initial denaturation at 95 ℃ for 2 min, followed by 40 cycles of 95 ℃ for 15 s (denaturation), 60 ℃ for 15 s (annealing), and 72 ℃ for 1 min (extension). Relative expression levels were calculated with use of the $2^{-\Delta\Delta CT}$ method to ensure accurate quantification of gene expression differences between the two varieties.

Table 1. Primer Sequence of qRT-PCR

Gene Name	Forward Primer	Reverse Primer
TRINITY_DN10187_c0_g1	AAGTAGGCAGTGGTTTTGATGTTAG	TCCCCTAGTAGAAGAGTCATCAGTG
TRINITY_DN29449_c0_g1	GCAGATTGGAGGGAAGACTC	GCTGCTTCTGCAGGAGGATA
TRINITY_DN992_c1_g1	TGTTCGGCGCATGTGAGT	TTGCTTACTCCGCTGGGC
TRINITY_DN3644_c0_g1	CTCCTTGTGCGGGCTCAA	GGCTGCTTCGTCCGTCTT
TRINITY_DN70319_c0_g2	TTCTTCGTCAGCGCACGA	ACTTCTTCGCGCACTTCCT
TRINITY_DN15539_c0_g1	GGCCCACTATCAGGTCACC	CCTGCTGGGCCATGTGTA
TRINITY_DN22089_c0_g1	GCTGCATGCTTCCTCCCA	CACTCCCCATGCAGCTCC
TRINITY_DN4150_c0_g1	AAAGCGCAGAGAACGGCT	GTGGTGCGCAATGGCAAA
Actin2	CGGGCATTCACGAGACCAC	AATAGACCCTCCAATCCAGACACT

RESULTS AND DISCUSSION

Analysis of Differences in Essential Oil Content between 'Ganlong 2' and Common *C. camphora* chvar. borneol

A comparative study was conducted on the essential oil yield, borneol content, and camphor content in fresh leaves of 'Ganlong 2' and common *C. camphora* across three trial sites (Ji'an, Jiujiang, and Ganzhou) from 2022 to 2024 (Table 2). The results indicated that, except for a non-significant difference in essential oil yield in Jiujiang in 2022, 'Ganlong 2' consistently demonstrated significantly higher essential oil yield and borneol content, along with significantly lower camphor content, in all other years and locations. The three-year averages showed that the essential oil yield of 'Ganlong 2' was 1.39 times that of the common variety, the borneol content was 6.8% higher, and the camphor content was only 45.9% of that in the common variety, highlighting the distinct novelty and specificity of this new cultivar. Furthermore, no significant differences were detected in the essential oil characteristics of 'Ganlong 2' across different years or geographical regions, demonstrating its high stability.

The marked contrast in borneol content between 'Ganlong 2' and common *C. camphora* chvar. borneol provides an ideal basis for transcriptome sequencing to identify key differentially expressed genes involved in the biosynthetic pathway of borneol.

Sequencing and Quality Assessment of 'Ganlong 2' and *C. camphora* chvar. borneol

To systematically understand the key signaling pathways in 'Ganlong 2' and to identify critical genes responding to natural borneol biosynthesis, 6 cDNA libraries were constructed and subjected to high-throughput sequencing, including the 'Ganlong 2' leaf sections (GL001, GL002, GL003) and common *C. camphora* chvar. borneol leaf sections (LN001, LN002, LN003). In total, approximately 38.56 Gb clean data were obtained in this study (Table 3), with an average of about 5.80 Gb clean reads per sample. The GC content for each sample ranged from 44.6% to 45.7%, and the Q30 values all were higher than 97%. These results directly illustrated that the sequencing data were authentic and reliable for further analysis. The mapping rate of reads for all samples exceeded 96.8%, with uniquely mapped reads ranging between 60.9% and 63.1%, and multiply mapped reads ranging between 34.2% and 36.1%.

Table 2. Essential Oil Content Determination Results

Variety	Location	Time (year)	Fresh Leaf Essential Oil Yield (%)	Borneol Content in Leaf Oil (%)	Camphor Content in Leaf Oil (%)
Ganlong 2	Ji'an City	2022	2.57±0.11a	89.0±1.8a	0.77±0.26b
C. camphora chvar. borneol			1.88±0.05b	81.2±2.3b	1.51±0.30a
Ganlong 2	Jiujiang City		2.58±0.10a	89.3±3.5a	0.77±0.16b
C. camphora chvar. borneol			1.95±0.06ab	81.2±1.2b	1.51±0.22a
Ganlong 2	Ganzhou City		2.62±0.17a	89.8±1.4a	0.77±0.26b
C. camphora chvar. borneol			1.87±0.12b	78.2±0.7b	1.51±0.33a
Ganlong 2	Ji'an City	2023	2.56±0.21a	91.5±1.6a	0.65±0.21b
C. camphora chvar. borneol			1.85±0.14b	82.8±2.1b	1.61±0.30a
Ganlong 2	Jiujiang City		2.60±0.18a	90.3±1.6a	0.73±0.15b
C. camphora chvar. borneol			1.82±0.06b	82.2±1.1b	1.57±0.16a
Ganlong 2	Ganzhou City		2.58±0.11a	91.6±2.4a	0.71±0.18b
C. camphora chvar. borneol			1.83±0.13b	81.8±1.9b	1.53±0.29a
Ganlong 2	Ji'an City	2024	2.54±0.20a	91.1±2.0a	0.71±0.20b
C. camphora chvar. borneol			1.82±0.06b	81.6±1.6b	1.73±0.23a
Ganlong 2	Jiujiang City		2.58±0.04a	91.5±1.7a	0.69±0.15b
C. camphora chvar. borneol			1.79±0.12b	80.9±2.4b	1.56±0.26a
Ganlong 2	Ganzhou City		2.60±0.22a	91.2±2.2a	0.72±0.10b
C. camphora chvar. borneol			1.83±0.08b	81.4±1.7b	1.57±0.27a
Ganlong 2	--	3-year average	2.58±0.14a	89.0±1.8a	0.72±0.25b
C. camphora chvar. borneol			1.85±0.11b	82.2±2.3b	1.57±0.28a

Note: *C. camphora* chvar. borneol refers to the common borneol camphor tree (hereafter the same), and 'Ganlong 2' is a new cultivar of borneol camphor.

Table 3. RNA Sequencing Results

Sample ID	Clean reads	Clean bases	Mapped reads	Unique mapped reads	Multiple map reads	Q30(%)	GC(%)
GL001	45926908	6.87 Gb	44529589 (96.96%)	28725206 (62.55%)	15804383 (34.41%)	97.33	44.59
GL002	36246520	5.42 Gb	35253793 (97.26%)	22855784 (63.06%)	12398009 (34.20%)	97.85	44.60
GL003	37787388	5.64 Gb	36627885 (96.93%)	23561154 (62.35%)	13066731 (34.58%)	97.58	45.21
LN001	48500204	7.25 Gb	46967484 (96.84%)	29839153 (61.52%)	17128331 (35.32%)	97.61	45.31
LN002	49791336	7.44 Gb	48353922 (97.11%)	31066208 (62.39%)	17287714 (34.72%)	97.47	45.00
LN003	39748570	5.94 Gb	38535302 (96.95%)	24193641 (60.87%)	14341661 (36.08%)	97.61	45.72
Total	258000926	38.56 Gb	250267975 (97.00%)	160241146 (62.11%)	90026829 (34.89%)	--	--

Note: Mapped reads refer to the number of reads aligned to the reference sequence and percentage of clean reads; Unique mapped reads denote the number of reads aligned to the unique positions of the reference sequence and percentage of clean reads; Multiple map reads represent the number of reads aligned to multiple positions of the reference sequence and percentage of clean reads; Q30 represents the proportion of clean data with a base quality accuracy rate of 99.9%.

Identification of DEGs between 'Ganlong 2' and Common *C. camphora* chvar. borneol

Based on the threshold criteria of qValue < 0.05 and $|\log_2^{(\text{fold change})}| > 1$, a total of 3,471 differentially expressed genes (DEGs) were identified between 'Ganlong 2' and common *C. camphora* chvar. borneol (Fig. 1), including 1,598 up-regulated and 1,873 down-regulated genes. Among these, expression levels of *CcFRS5* (*TRINITY_DN3028_c0_g1*), *CcBPPS* (*TRINITY_DN22089_c0_g1*), and *CcRGA1* (*TRINITY_DN12683_c0_g1*) in 'Ganlong 2' were 426.16-, 315.41-, and 166.88-fold higher, respectively, than those in common camphor. Conversely, *CcUBI4* (*TRINITY_DN15810_c0_g4*), *CcNPR4* (*TRINITY_DN22356_c0_g1*), and *CcGST23* (*TRINITY_DN9833_c0_g1*) exhibited 656.30-, 134.94-, and 78.66-fold higher expression, respectively, in common camphor compared to 'Ganlong 2'. These results indicate substantial transcriptional differences between 'Ganlong 2' and the common variety, both in the number and magnitude of gene expression changes.

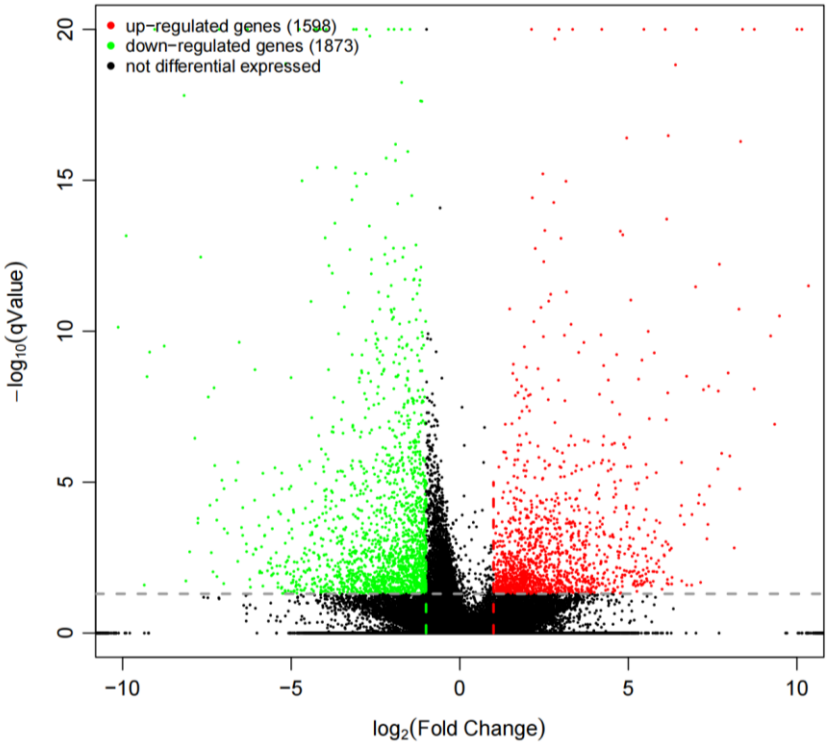

Fig. 1. Volcano plot of DEGs between 'Ganlong 2' and common *C. camphora* chvar. borneol

GO Enrichment Analysis of DEGs between 'Ganlong 2' and Common *C. camphora* chvar. borneol

Gene ontology (GO) enrichment analysis (Fig. 2) between 'Ganlong 2' and common *C. camphora chvar.* borneol revealed that the DEGs were assigned to 53 functional subcategories within three major categories: biological process, cellular component, and molecular function. In the biological process category, the DEGs were primarily enriched in terms such as cellular process (436 up-regulated and 806 down-regulated genes) and metabolic process (382 up-regulated and 714 down-regulated genes). Within cellular component, the most enriched terms included organelle (354 up-regulated and 587 down-regulated genes) and membrane (271 up-regulated and 597 down-regulated

genes). For molecular function, the dominant terms were binding (491 up-regulated and 781 down-regulated genes) and catalytic activity (414 up-regulated and 687 down-regulated genes).

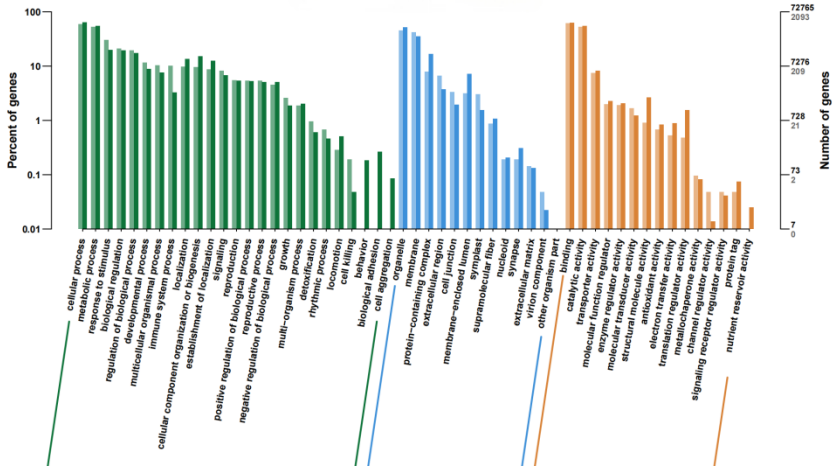

Fig. 2. Bar chart of GO classification for DEGs

KEGG Enrichment Analysis of DEGs between 'Ganlong 2' and Common *C. camphora* chvar. borneol

A total of 442 DEGs were annotated in KEGG (Fig. 3), which were mainly enriched in the pathways of plant-pathogen interaction (41 genes), neurotrophin signaling (32 genes), terpenoid backbone biosynthesis (26 genes), toll-like receptor signaling (26 genes), NF-kappa B signaling (25 genes).

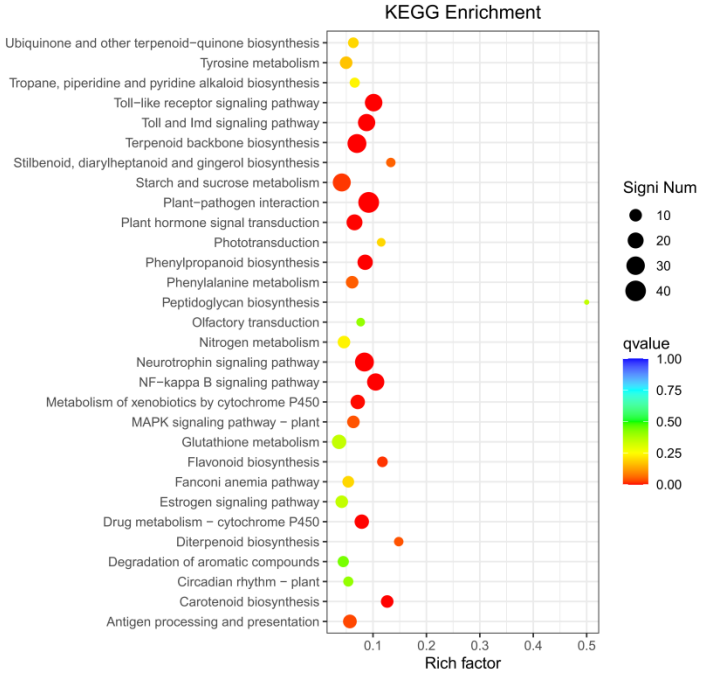

Fig. 3. KEGG enrichment scatter plot of DEGs

Analysis of the Natural Borneol Biosynthetic Pathway in *C. camphora* chvar. borneol

KEGG enrichment analysis revealed significant enrichment in the terpenoid backbone biosynthesis pathway, involving a total of 26 genes. Terpenoids, one of the most diverse classes of natural products, initiate their biosynthesis through a highly conserved terpenoid backbone assembly process (Rehman *et al*. 2016). This pathway relies on the MVA and MEP pathways to supply key precursors and employs catalytic enzymes such as GPPS (geranyl diphosphate synthase) and FPP (farnesyl diphosphate synthase) to form structurally diverse terpenoid skeletons. The biosynthesis of (+)-borneol plays a central role in this pathway. In-depth elucidation of its synthetic mechanism not only contributes to understanding the directional regulation of terpenoid metabolism but also provides a theoretical foundation for achieving efficient and sustainable production of natural borneol through metabolic engineering strategies.

This study constructed the biosynthetic pathway of natural borneol in *C. camphora* chvar. borneol and annotated and quantified the expression of key catalytic enzyme-encoding transcripts: a total of 46 transcripts from 18 key enzyme genes were annotated, of which 15 were significantly up-regulated, 7 were significantly down-regulated, and 22 showed no significant difference (Fig. 4).

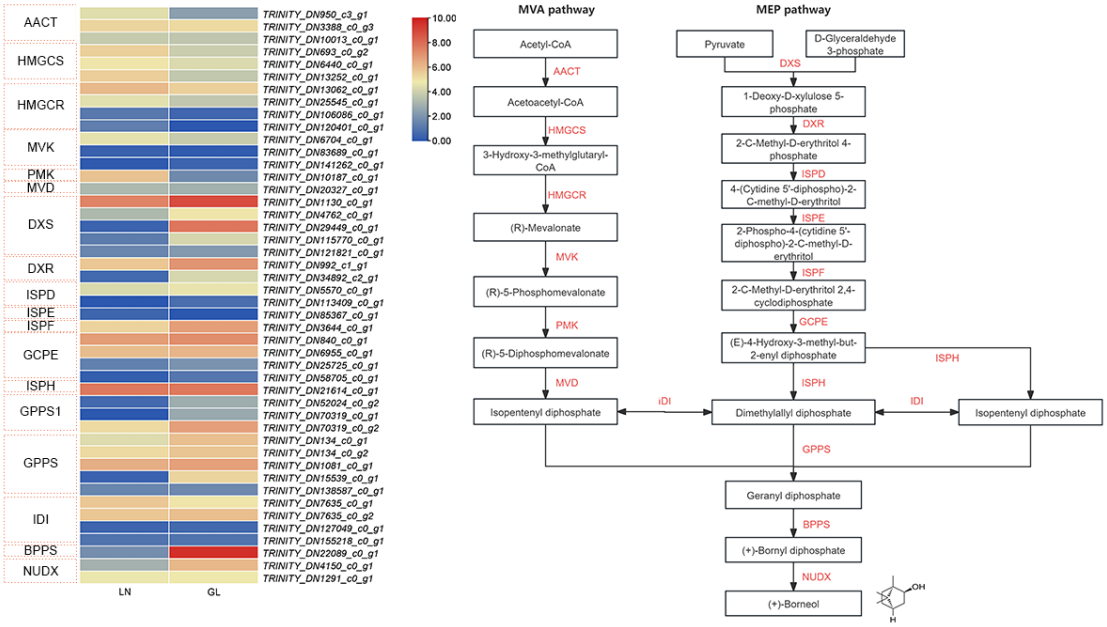

Fig. 4. Natural borneol biosynthetic pathway and heatmap in *C. camphora* chvar. borneol

Notably, 15 transcripts in the MVA pathway generally exhibited a down-regulation trend. For instance, the expression levels of *CcAACT1* (*TRINITY_DN950_c3_g1*), *CcPMK* (*TRINITY_DN10187_c0_g1*), and *CcHMGCS1* (*TRINITY_DN693_c0_g2*) in common *C. camphora* chvar. borneol were 5.07-fold, 25.54-fold, and 3.13-fold higher, respectively, than those in 'Ganlong 2'. Conversely, 16 transcripts in the MEP pathway were generally up-regulated, with the expression levels of *CcDXS1* (*TRINITY_DN29449_c0_g1*), *CcDXR1* (*TRINITY_DN34892_c2_g1*), and *CcISPF* (*TRINITY_DN3644_c0_g1*) in 'Ganlong 2' being 683.77-fold, 34.18-fold, and 2.55-fold higher, respectively, than those in common *C. camphora* chvar. borneol. Furthermore, key downstream genes in borneol biosynthesis (including *GPPS1*, *GPPS*, *IDI*, *BPPS*, and *NUDX1*) were also overall up-

regulated; particularly, the expression changes of *CcBPPS* (*TRINITY_DN22089_c0_g1*), *CcNUDX1* (*TRINITY_DN4150_c0_g1*), and *CcGPPS1a* (*TRINITY_DN70319_c0_g2*) in 'Ganlong 2' were most significant, being 315.41-fold, 10.00-fold, and 2.77-fold higher, respectively, than those in common *C. camphora chvar.* borneol. These results indicate that the enhancement of the MEP pathway is a crucial molecular basis for the high accumulation of borneol in the 'Ganlong 2' cultivar.

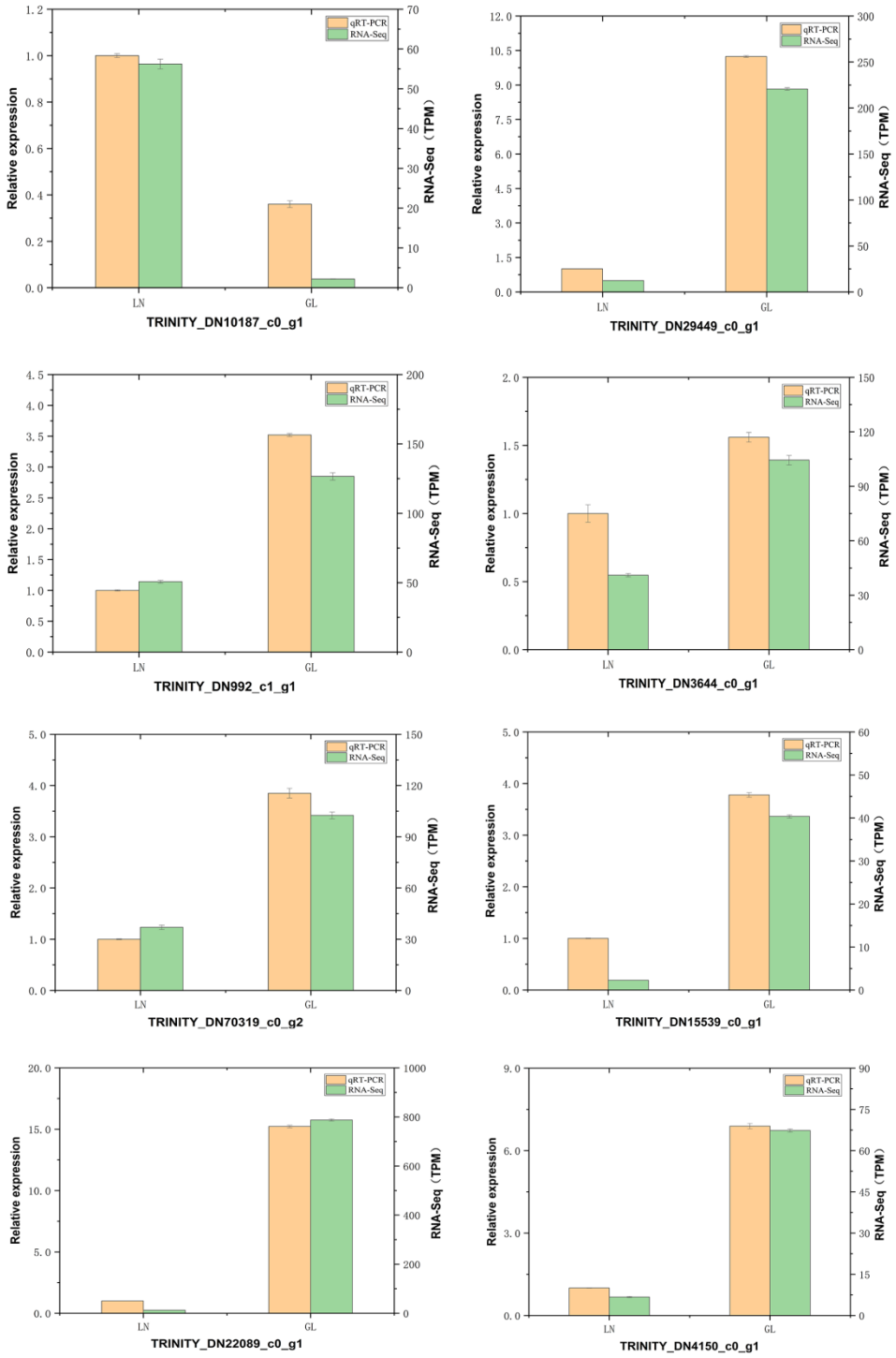

Fig. 5. Relative expression levels of DEGs

Validation of Transcriptome Data by qRT-PCR Analyses

Eight differentially expressed candidate genes, including *phosphomevalonate kinase* (*PMK*, *TRINITY_DN10187_c0_g1*), *1-deoxy-D-xylulose-5-phosphate synthase 1* (*DXS1*, *TRINITY_DN29449_c0_g1*), *1-deoxy-D-xylulose-5-phosphate reductoisomerase 1* (*DXR1*, *TRINITY_DN992_c1_g1*), *2-C-methyl-D-erythritol 2,4-cyclodiphosphate synthase* (*ISPF*, *TRINITY_DN3644_c0_g1*), *geranyl diphosphate synthase 1a* (*GPPS1a*, *TRINITY_DN70319_c0_g2*), *geranyl diphosphate synthase a* (*GPPSa*, *TRINITY_DN15539_c0_g1*), *bornyl diphosphate synthase* (*BPPS*, *TRINITY_DN22089_c0_g1*), and *NUDIX hydrolase 1* (*NUDX1*, *TRINITY_DN4150_c0_g1*), were selected for qRT-PCR validation. The results showed that except for the PMK gene, the expression levels of all other genes in the 'Ganlong 2' were higher than those in common *C. camphora* chvar. borneol, which was basically consistent with the transcriptome results, demonstrating the reliability of the transcriptome data.

Natural borneol, a high-value monoterpenoid, holds broad application prospects in pharmaceuticals, fragrances, and other industries. Although its biosynthetic pathway has been characterized in several plant species, the synthetic mechanisms vary among species, particularly in the expression regulation of key enzyme genes, metabolic flux partitioning, and secondary modifications, which require further elucidation. Studies in *Lavandula angustifolia*, *Wurfbainia villosa*, and *Salvia officinalis* have identified several enzyme genes, such as BPPS and NUDX, that are associated with borneol synthesis (Despinasse *et al*. 2017; Yang *et al*. 2024). However, the functional differentiation and expression patterns of these genes across species remain unclear. Systematic analysis of the biosynthetic network in *C. camphora*, a major natural source of borneol, is still relatively limited.

'Ganlong 2', a new variety obtained through directed breeding, demonstrated stable high essential oil yield, high borneol content, and low camphor content in trials conducted from 2022 to 2024 in Ji'an, Jiujiang, and Ganzhou. Data over three years showed that its fresh leaf essential oil yield was on average 1.39 times that of common *C. camphora* chvar. borneol, with borneol content 6.8% higher and camphor content significantly reduced (only 45.9% of that in common variety). Therefore, 'Ganlong 2' not only exhibits significant advantages in yield and quality but also shows strong environmental stability, providing a solid foundation for its further promotion and industrial development.

This study systematically analyzed the key metabolic pathways of borneol biosynthesis in *C. camphora* chvar. borneol, revealing overall upregulation of MEP pathway genes and partial suppression of MVA pathway genes in 'Ganlong 2'. Specifically, the expression levels of key MEP pathway genes *CcDXS1*, *CcDXR1*, and *CcISPF* in 'Ganlong 2' were 684-fold, 34.2-fold, and 2.55-fold higher, respectively, than those in the common variety. In contrast, MVA pathway genes *CcAACT1*, *CcPMK*, and *CcHMGCS1* were significantly downregulated. These results indicate that the MEP pathway is the primary contributor to the high accumulation of borneol in 'Ganlong 2'. This observation aligns with the subcellular compartmentalization of terpenoid biosynthesis in plants: the MEP pathway, located in plastids, primarily synthesizes precursors for monoterpenes (C10) and diterpenes (C20), whereas the MVA pathway, situated in the cytoplasm, mainly participates in the production of sesquiterpenes (C15) and triterpenes (C30) (Pu *et al*. 2021). As a monoterpene, (+)-borneol synthesis more directly relies on the plastid-localized MEP pathway, which may offer higher carbon flux efficiency and substrate specificity. This predominant role of the MEP pathway in monoterpene precursor supply appears to be conserved within Lauraceae species, as

evidenced by the significant upregulation of a specific DXS gene (*CbDXS9*) in the high-borneol chemotype of *Cinnamomum burmannii* (Yang *et al*. 2020). This mechanism is also supported by evidence from *Blumea balsamifera* (Guan *et al*. 2024), where methyl jasmonate (MeJA) treatment specifically induced upregulation of MEP pathway genes (*e.g*., *DXS* and *DXR*), correlating positively with borneol accumulation, while the MVA pathway showed minimal response, further affirming the dominant role of the MEP pathway in borneol biosynthesis.

Furthermore, downstream synthetic genes, including *CcBPPS*, *CcNUDX1*, and *CcGPPS1a,* were significantly upregulated in 'Ganlong 2', collectively enhancing borneol biosynthesis capability. Notably, the expression level of *CcNUDX1* increased by 10-fold, suggesting a potential key role in hydrolyzing BPP to generate borneol, similar to *WvNUDX24* in Wurfbainia villosa (Yang *et al*. 2024). The functional characterization of such Nudix hydrolases across different plant taxa, including members of Lauraceae, is crucial for elucidating the final catalytic step in borneol biosynthesis. It is worth noting that natural borneol can be converted to camphor *via* catalysis by borneol dehydrogenase (BDH) (Lin *et al*. 2023); however, transcriptome data did not indicate significant differential expression of *BDH* genes, suggesting that the high borneol accumulation in 'Ganlong 2' is not achieved by suppressing camphor synthesis but rather through optimized precursor supply and enhanced dedicated borneol synthesis steps. This metabolic strategy contrasts with the scenario in borneol-type *C. camphora*, where a highly efficient BDH (*CcBDH3*) actively converts (+)-borneol to (+)-camphor (Ma *et al*. 2021b). The lack of significant BDH upregulation in 'Ganlong 2' highlights a distinct mechanism for maintaining high borneol content, primarily through pathway precursor enhancement rather than competition at the final oxidation step. Particularly noteworthy is the compensatory regulatory mechanism observed between the MVA and MEP pathways in 'Ganlong 2': while the MVA pathway is partially suppressed, the MEP pathway is significantly enhanced, thereby maintaining or even improving overall borneol synthesis levels. Such intricate inter-pathway regulation underscores the metabolic plasticity within the *Cinnamomum* genus. Comparative transcriptomics in *C. burmannii* also identified candidate transcription factors potentially involved in coordinating terpenoid metabolism (Yang *et al*. 2020), suggesting that complex regulatory networks fine-tuning the MEP/MVA balance may be a shared feature among borneol-producing Lauraceae species. This mechanism not only deepens the understanding of regulatory networks in plant terpenoid metabolism but also provides new insights for future metabolic engineering strategies aimed at coordinately optimizing multiple pathways.

From an industrial perspective, 'Ganlong 2' demonstrates significant potential for commercial development. Its stable phenotype of high essential oil and high borneol content provides a germplasm foundation for establishing large-scale, standardized raw material bases for natural borneol production, which could alleviate the current market instability and heavy reliance on synthetic alternatives. In the pharmaceutical sector, its substantially reduced camphor content is critically important, as camphor acts as a competitive component and excessive levels may induce neurotoxic side effects. Consequently, the high-purity, low-camphor natural borneol derived from 'Ganlong 2' better complies with stringent pharmaceutical quality standards, providing superior raw material for developing safer traditional Chinese medicine injections, oral preparations, and topical applications. In the biotechnology field, the key genes identified in this study (such as *CcDXS1* and *CcNUDX1*) offer valuable genetic resources for reconstructing and optimizing the borneol biosynthetic pathway in microbial cell factories through synthetic

biology strategies. For instance, building upon metabolic engineering successes in producing rare and valuable terpenoids (such as artemisinic acid and ginsenosides) in yeast, introducing efficient genetic elements from 'Ganlong 2' into engineered yeast strains holds promise for achieving green and efficient fermentative production of natural borneol (Paddon and Keasling 2014), thereby reducing traditional dependence on plant resources. Furthermore, its leaves serve as ideal materials for direct borneol production through plant cell suspension culture. Related plant cell culture technologies have been successfully applied in the industrial production of secondary metabolites such as paclitaxel, and this approach is equally applicable to borneol production, enabling precise process control and year-round operation (Wilson and Roberts 2012). Collectively, the promotion of 'Ganlong 2' and its integration with relevant biomanufacturing technologies will jointly advance the upgrading and sustainable development of the natural borneol industry chain.

CONCLUSIONS

1. A multi-site, three-year comparative study confirmed that 'Ganlong 2' consistently exhibited high borneol content, high essential oil yield, and low camphor content, providing valuable material for investigating efficient natural borneol biosynthesis.
2. Transcriptomic analysis revealed the borneol biosynthetic pathway in borneol camphor trees, identifying key genes (including *CcBPPS*, *CcNUDX1*, and *CcDXS1*) and establishing the MEP pathway as the major biosynthetic route.
3. These findings advance the understanding of monoterpene biosynthesis and offer a genetic basis for synthetic biology approaches to improve borneol production and guide the breeding of high-yield, high-purity borneol camphor varieties.

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