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Full Length Article

Transcriptome Analysis of the Effects of Endophytic Fungi on the Biosynthesis of Essential Oils in *Cinnamomum longepaniculatum*

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Abstract

Cinnamomum longepaniculatum is an important commercial crop and main source of volatile terpenoids. Terpenoid biosynthesis constitutes a multi-step, multi-enzyme process affected by various factors. In this study two endophytic fungi (*Penicillium commune* 2J1 and *Neurospora crassa* 3J1) were used to treat the samples and transcriptome sequencing was performed to explore the differences in regulation of gene expression related to volatile terpenoid metabolism. Treatment with 2J1 and 3J1 resulted in 8315 and 6516 differentially expressed unigenes, respectively, with a greater proportion of upregulated genes in latter group. Both endophytic fungi upregulated genes involved in the monoterpene-biosynthetic pathway in *C. longepaniculatum*. Annotation of unigenes involved in plant hormone signal transduction revealed differential expression relative to the control in several key signaling pathways. *NPR1* (nonexpressor of PR-1) and *PR-1* (pathogenesis-related protein 1) were significantly upregulated following 2J1 treatment in the SA (salicylic acid) signal-transduction pathway, with *TGA* (TGA transcription factor) significantly downregulated, whereas all of these genes were upregulated after 3J1 treatment. JA (Jasmonic acid) signaling pathway genes *JAR1* (jasmonic acid resistant 1), *JAZ* (jasmonate ZIM-domain-containing protein) and *MYC2* (transcription factor MYC2) showed upregulated expression following 2J1 treatment, whereas *JAZ* expression was downregulated following 3J1 treatment. Quantitative polymerase chain reaction validated the transcriptome results for genes involved in the monoterpene-biosynthetic pathway of *C. longepaniculatum*. Collectively, these annotations of unigenes associated with secondary metabolism, plant hormone-signal transduction and terpenoid biosynthesis provide valuable insight into the endophytic fungi-regulated biosynthesis of essential oils in *C. longepaniculatum*. These findings establish a foundation for the subsequent cloning of volatile terpenoid-metabolism-related genes in *C. longepaniculatum*.

Keywords: *Cinnamomum longepaniculatum*; Endophytic fungi; Signal transduction; Transcriptome; Essential oil

Introduction

Cinnamomum longepaniculatum (Gamble) N. Chao is an endemic *Cinnamomum* tree in south of Sichuan province, China, which is planted in hills (from an altitude 300 to 800 m) with acidic or slightly acidic soil. Essential oils of *C. longepaniculatum* can be extracted from its roots, stems, leaves and seeds. Terpenoids are the main components (>85%) of the essential oils extracted from the leaves of *C. longepaniculatum* (Hu *et al*., 2012). Among them, 1, 8-cineole, γ-terpinene and α-terpilenol have been reported as economically important components (Deng *et al*., 2012; Li *et al*., 2012a; Li *et al*., 2014; Xu *et al*., 2014). The demand for these natural essential oils from *C. longepaniculatum* has increased exponentially along with continuous improvements in socioeconomic status and standards of living (Wei *et al*., 2016). However, the yield and quality of the essential oils from *C. longepaniculatum* vary due to multiple factors such as instability of the secondary metabolites and environmental constraints (Yan *et al*., 2017). Therefore, sustainable retrieval of essential oils from *C. longepaniculatum* is an important research focus for the resources of this species and essential oils.

Endophytic fungi invading host plants are capable of directly or indirectly promoting the formation and accumulation of secondary metabolites in their hosts (Du and Guo, 2009; Katoch *et al*., 2014; Jinfeng *et al*., 2017). This process can induce the expression of host defense-related genes in plant cells, trigger allergic reactions in plants and promote the biosynthesis of certain secondary metabolites in plant cells (Ebel and Scheel, 1992). Recent studies reported the effects of using endophytic fungi to activate secondary metabolic pathways in plant cells and affect the expression of plant defense genes. These fungi can regulate the transcription and expression of specialized metabolism-related genes, thereby affecting the biosynthesis and accumulation of secondary metabolites (Li *et al*., 2011; Tan *et al*., 2013; Krstić-Milošević *et al*., 2017; Ali *et al*.,

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2018). Thus, the synthesis and accumulation of volatile terpenoids, which are secondary metabolites in *C. longepaniculatum*, might also be induced and regulated by endophytic fungi. To date, most of the studies have primarily focused on the active ingredients in medicinal plants, whereas studies related to effect of endophytic fungi on the biosynthesis of essential oil in spice plants are relatively rare, especially at the molecular level (Gandhi *et al*., 2014; Vaccaro *et al*., 2014; Fouda *et al*., 2015; Chowdhary and Kaushik, 2015).

Terpenoid biosynthesis constitutes a multi-step, multi-enzyme process affected by various factors such as the environment, genetic and developmental stages. Therefore, it is not sufficient to focus on the metabolic regulation of a single enzyme gene to fully understand the regulation of biosynthesis. RNA-sequencing (RNA-Seq) technology, now enables conducting large-scale analyses of functional genes in a species through the investigation of relevant transcription factors and their regulation at multiple points (Kim *et al*., 2011; Xu *et al*., 2011; Strauss *et al*., 2012; Ward and Weber, 2012). This method has also promoted the development of new approaches for studying the regulation of terpenoid secondary metabolism (Zhao *et al*., 2012). Hence, this study was conducted to evaluate differentially expressed genes in *C. longepaniculatum* induced by different endophytic fungi to get understanding of the regulatory mechanisms associated with the involvement of endophytic fungi in biosynthesis of essential oils in *C. longepaniculatum*. This work should lay the foundation for the subsequent cloning of volatile terpenoid-metabolism-related genes in *C. longepaniculatum*.

Materials and Methods

Materials

Samples of *C. longepaniculatum* were collected from Hongyan mountain in Yibin (located at 27°50' N; 105°20' E) and were identified by Professor Qin Wei from the Yibin University. Two endophytic fungal strains, *Penicillium commune* 2J1 (hereafter 2J1) and *Neurospora crassa* 3J1 (hereafter 3J1), previously isolated from *C. longepaniculatum* plantlets (Table S1), were identified and cultured in potato dextrose agar medium for subsequent inoculations (Fig. S1 and S2). *C. longepaniculatum* seedlings were planted in pots containing sterile soil and subjected to adaptive growth in an artificial-climate chamber for 7 days. The seedlings were inoculated with both fungal strains by spraying the conidial suspensions (2.0×10^5 conidia/mL) evenly onto their leaves and seedlings sprayed with sterile water were used as controls. All inoculated seedlings were further incubated in the artificial-climate chamber for 21 days prior to the collection of leaves. The collected leaves were rapidly rinsed with sterile water, followed by removal of surface moisture. The leaves were then rapidly frozen using liquid nitrogen in a 50 mL centrifuge tube and stored at −80°C in an ultra-low temperature freezer until use.

The three groups of samples were labeled as CK, 2J1 and 3J1, respectively, where CK denotes the control *C. longepaniculatum* leaf samples treated with sterile water, and 2J1 and 3J1 denote *C. longepaniculatum* leaf samples treated with the two endophytic fungi, respectively.

Total RNA Extraction and Quality Assessment

Total RNA was extracted from *C. longepaniculatum* leaves using the Trizol method and the concentration and purity of the extracted RNA were determined using a Nanodrop 2000 spectrophotometer (Thermo Fisher Scientific, Waltham, M.A., U.S.A.). RNA integrity was assessed using agarose gel electrophoresis and the RNA integrity number was measured using an Agilent 2100 bioanalyzer (Agilent Technologies, Santa Clara, CA, USA).

Transcriptome Library Construction and Illumina Sequencing

The mRNA enriched from the total RNA samples using oligo (dT)-conjugated magnetic beads was fragmented and reverse-transcribed with reverse transcriptase using random primers to synthesize the first-strand cDNA, followed by second-strand synthesis to form stable double-stranded cDNAs. The double-stranded cDNA was joined using an end-repair kit to convert its sticky ends into blunt ends. A single adenine overhang was added to each of the 3′-ends for the ligation of Y-shaped adapters. The ligation product was then subjected to size selection *via* agarose gel electrophoresis prior to polymerase chain reaction (PCR) amplification. The constructed RNA-Seq libraries were then subjected to sequencing on the Illumina Hiseq 4000 platform (Illumina, San Diego, C.A., U.S.A.).

Data Processing and Assembly

The total raw reads obtained from sequencing were subjected to quality assessment, removal of adapter sequences and removal of empty reads due to adapter dimers. Low-quality bases (with quality scores < 20) at the 3′-end of reads were trimmed, and reads that still contained bases with quality scores < 10 after the trimming were discarded. Reads with an N ratio > 10% were also discarded. After the removal of adapters and quality trimming, reads <20-bp long were discarded to finally obtain the clean reads. Trinity software (http://trinityrnaseq.sf.net/) was used to perform *de novo* transcriptome assembly (Glowacka, 2011). In brief, reads with overlapping segments were assembled into contiguous sequences and the contigs were then assembled into unigenes by paired-end assembly and gap filling (Grabherr *et al*., 2011).

Analysis of Differentially Expressed Genes

Differential gene expression analysis was performed

using edgeR software (http://www.bioconductor.org/packages/2.12/bioc/html/edgeR.html) and differential expression was calculated from the resulting gene read-count data using RSEM software (http://deweylab.biostat.wisc.edu/rsem/), which is based on the negative binomial distribution model. Selection criteria for significant differential expression included a false-discovery rate (FDR)≤0.05, $\log_2$|fold change (FC)≥1 and $P < 0.05$. Selected differentially expressed unigenes were subjected to gene ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analyses.

Reverse Transcription Quantitative PCR (RT-qPCR) Validation

To validate the reliability of sequencing results, the expression levels of the five key enzyme genes involved in the monoterpene-biosynthetic pathway, as well as one internal reference gene (β-actin) in *C. longepaniculatum* treated with endophytic fungi for 21 days were validated by qPCR. The sequences of all primers were designed using Primer Premier 5.0 software (Premier Biosoft, Palo Alto, CA, USA) as shown in Table 1. The cDNA was synthesized using PrimeScript RT reagents and the gDNA eraser kit (Takara, Shiga, Japan) according to the manufacturer instructions. In brief, RNA samples were thawed at room temperature prior to preparing reverse transcription reaction mixtures containing 1 μg total RNA, 4 μL 5× PrimeScript buffer 2, 1 μL PrimeScript RT enzyme mix, 1 μL RT primer mix, 10 μL DNA eraser and RNase-free ddH_2O up to 20 μL. Reverse transcription was performed at 37°C for 15 min, followed by 85°C for 5 s to terminate the reaction. The Products were diluted to 100 μL with 80 μL RNase-free ddH_2O and stored at −20°C until use.

qPCRs were prepared in 0.2 mL PCR tubes with a 25 μL reaction volume consisting of 12.5 μL of 2X SYBR Green qPCR Master Mix, 0.5 μL of 10 μM reverse and forward primers, 9.5 μL of ddH_2O and 2 μL template cDNA. Reactions were carried out on an ABI 7500 fluorescence-based qPCR system (Thermo Fisher Scientific, Waltham, M.A., U.S.A.) with the following reaction conditions: initial denaturation at 95°C for 10 min, followed by 40 cycles of denaturation at 95°C for 15 s, annealing at 60°C for 1 min and extension for 40 s. Each sample was examined in triplicate and relative expression levels were calculated using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001).

Table 1: Primers used for real-time qPCR

Gene	Forward primer (5′-3′)	Reverse primer (5′-3′)
DXS	CTGGCGGAACTTGGTGCT	CAGACAGACTTCAGGGCTA
DXR	GCGAGACCGAAGACAACG	CTGGGCAGAACAGCGAGT
CMS	GTGGGCTTAGCACTGACA	GGAGGAGGATTGGGACTT
GPPS	TTGATGGATCGGCGTGAG	AAGGGATACAGAGGACAAAGGA
TPS	CGTCACAAACGGGCATTA	CACGAAAGATCGACTCACAT
β-actin	TGGACTCTGGTGACGGTGTT	CCTTGATGTCACGGACGATT

Table 2: Preprocessing of raw sequencing data

Sample	Total raw reads	Total clean reads	Total clean nucleotides	Error (%)*	Q20 (%)§	GC (%)†
CK	58564542	56857306	8331942311	0.0109	98.02	48.88
2J1	67063994	65286600	9605065489	0.0107	98.13	47.12
3J1	53869350	52407882	7713566209	0.0106	98.15	47.26

*Base error rate
†Sum of G and C bases as a percentage of the total number of bases
§Percentage of bases with Phred value > 20 as a percentage of the total number of bases

Table 3: Statistics of assembly quality

Type	Unigenes	Transcripts
Total sequence no.	82,061	105,028
GC (%)*	42.87	42.71
Largest (bp)	61022	61022
Smallest (bp)	201	201
Average (bp)	897.43	999.38
N50†	1387	1523

*Sum of G and C bases as a percentage of the total number of bases
†Assembled transcripts were sorted from largest to smallest according to length; N50 is the transcript length when the length of the transcript is half of the total length

Results

Sequencing, Raw-data Processing and Assembly

Both fungal endophyte-treated groups and one control group were subjected to high-throughput sequencing, which yielded 56,857,306, 64,286,600 and 52,407,882 trimmed, filtered, clean reads, respectively, with an average quality (Q20) score >98%. All three samples had sequencing-error rates of <0.1%, with GC percentages >45% (Table 2). These data suggested that the sequencing results were of good quality and met the threshold criteria for further analyses.

The reads were assembled into 105,028 transcripts with an average length of 999.38 bp and were joined into 82,061 unigenes with an average length of 879.43 bp and an N50 of 1387 bp (Table 3). Analysis of the length distribution of the assembled unigenes (Fig. 1) indicated that unigenes ranging in length from 1 bp to 400 bp accounted for the greatest proportion (38.15%), followed by those ranging from 601 bp to 800 bp, which accounted for 13.73% (11,267 unigenes).

Sequence Alignment

The obtained reads were aligned to their respective assembled transcriptomes using Bowtie alignment software (http://bowtie-bio.sourceforge.net/index.shtml), resulting in alignment rates > 75%, indicating a good alignment result. Specifically, 77.38% (56,857,306/43,996,176), 78.07% (65,286,600/50,967,218) and 76.01% (52,407,882/39,834,744) of the mapped reads aligned relative to the total reads for the CK, 2J1 and 3J1 treatment groups, respectively.

Differential Expression Analysis

Differentially expressed genes following treatment with endophytic fungi: 8315 and 6516 differentially expressed genes were selected in the CK *vs.* 2J1 and CK

vs. 3J1 groups, respectively, exhibiting an FDR>0.05 and $\log_2|FC| \geq 1$, including 4363 and 3952 upregulated and 3952 and 2594 downregulated genes, respectively. 509 differentially expressed genes were identified between the two fungal treatments (2J1 *vs*. 3J1), consisting of 169 upregulated and 340 downregulated genes (Fig. 2). Visualization of the differentially expressed genes between the CK *vs*. 2J1 and CK *vs*. 3J1 groups was acquired using an online tool (http://bioinfogp.cnb.csic.es/tools/venny/index.html), revealing 4896 common genes exhibiting differential expression in both groups (3419 2J1-specific and 1620 3J1-specific genes; Fig. 3). These data indicated that the 2J1 treatment of *C. longepaniculatum* resulted in a greater number of differentially expressed genes as compared with the 3J1 treatment, whereas the 3J1 treatment resulted in a greater proportion of upregulated genes relative to the 2J1 treatment. Overall, these results suggest that treatment with 3J1 more effectively upregulated the expression of genes in *C. longepaniculatum* leaves.

Annotation of differentially expressed genes associated with the terpenoid-biosynthesis pathway in *C. longepaniculatum*: Treatment with 2J1 resulted in upregulation of 11 genes, including one *CMS* (4-diphosphocytidyl-2-C-methyl-D-erythritol synthase), one *DHDDS* (ditrans,polycis-polyprenyl disphosphate synthase), *GPPS* (geranyl diphosphate synthase), one *chlP* (geranylgeranyl reductase), four *DXS*, one *DXR* (1-deoxy-D-xylulose-5-phosphate synthase reductoisomerase), one *atoB* (acetyl-CoA-C-acetyltransferase) and one *FCLY* (farnesylcysteine lyase) unigenes, as well as one downregulating of *atoB* unigene. By contrast, treatment with 3J1 resulted in upregulation of one *CMS*, one *DHDDS*, three *DXS*, one *DXR*, one *GPPS*, one *SPS* (all-trans-nonaprenyl-diphosphate synthase), one *chlP*, one *IDI* (isopentenyl diphosphate isomerase) and one *HDS* (4-hydroxy-3-methylbut-2-enyl diphosphate synthase) unigenes, as well as the downregulation of one *DXS* (c133016_g1) unigene (Table 4). Compared with the control group, treatment with 2J1 resulted in upregulation of four *a-TPS* and three (3S)-linalool synthase unigenes, which are involved in the monoterpenoid biosynthetic pathway. By contrast, treatment with 3J1 resulted in upregulation of three *a-TPS* and three (3S)-linalool synthase unigenes. However, neither treatment resulted in the downregulation of genes involved in the monoterpene-biosynthesis pathway (Table 5; Fig. S3 and S4).

Relative to the control group, treatment with 2J1 induced the upregulated expression of *CMS*, *DXR*, *DXS* and *GPPS* in the MEP (2-C-methyl-d-erythritol-4-phosphate) pathway, with *DXS*, *DXR*, and *CMS* encoding key enzymes in the upstream MEP pathway, whereas *GPPS* encodes a key enzyme in the downstream pathway (Fig. S5). Treatment with 3J1 induced the upregulation of *CMS*, *DXR*, *DXS*, *IDI*, *HDS* and *GPPS*, with *HDS* and *IDI* encoding key enzymes involved in the midstream and downstream MEP pathways, respectively (Fig. S6). Therefore, treatment with the above two endophytic fungi resulted in the highest numbers of differentially expressed genes in the MEP pathway of terpenoid biosynthesis, indicating that genes responsible for the monoterpene-biosynthesis pathway in *C. longepaniculatum* were significantly regulated by endophytic fungi. Furthermore, different genes exhibited variable responses to different fungal species. Only the *DXS* unigene annotated in the 3J1 treatment group was downregulated, which might be explained by the fact that *DXS* is a multi-copy gene that usually exists in the form of a gene family. This suggested that differently annotated *DXS* unigenes also exhibit different expression patterns.

Differentially expressed genes responsible for plant hormone signal transduction pathways: Compared with

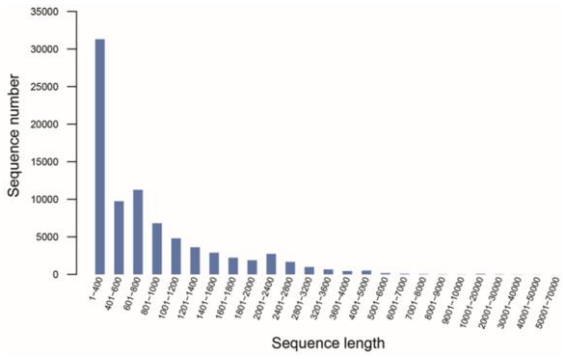

Fig. 1: Length distribution of assembled unigenes. The abscissa and ordinate show the length range of assembled unigenes and the corresponding number of unigenes, respectively

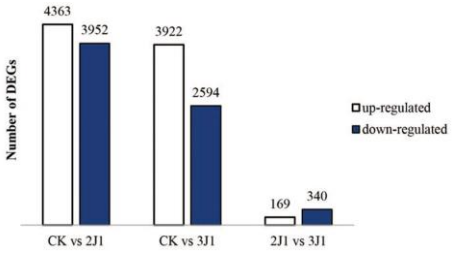

Fig. 2: Statistics associated with differentially expressed genes (DEGs)

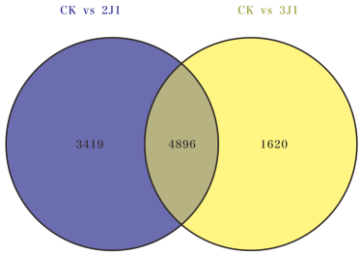

Fig. 3: Venn diagrams showing the number of distinct and common differentially expressed genes following fungal inoculation

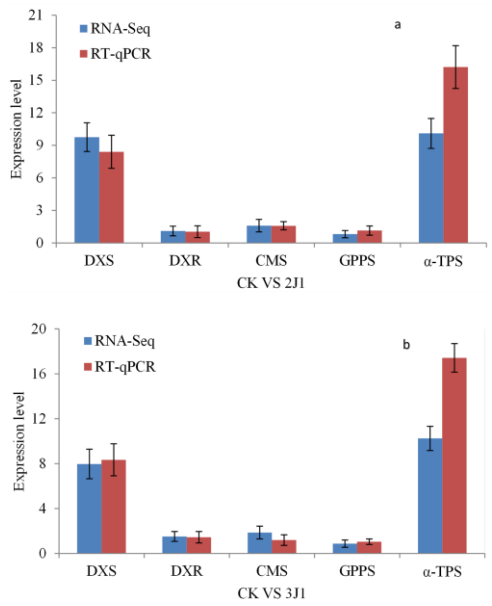

Fig. 4: qPCR validation of sequencing results related to differentially expressed genes between the (**a**) 2JI and (**b**) 3JI treatment groups

the control group, treatment with 2J1 resulted in the upregulation of most of the genes associated with plant hormone signal transduction pathways, including two upregulated *ERF1* (ethylene response factor 1) unigenes and one downregulated *EIN3* (ethylene insensitive 3) unigene involved in the ET (Ethylene) signaling pathway. In addition, two *JAZ*, one *JAR1* and five *MYC2* unigenes were upregulated, each of which encoded key proteins involved in the JA signaling pathway. Two *NPR1* and four *PR-1* unigenes were upregulated and three *TGA* unigenes were downregulated, all of which are involved in the SA signaling pathway (Table 6).

Compared with the control group, treatment with 3J1 resulted in the downregulation of one *ERF1* unigene in the ET signaling pathway, with no differential expression of *EIN3*. Upregulation of one *JAR1*, one *COI-1* (coronatine insensitive protein 1) and two *MYC2* unigenes and the downregulation of three *JAZ* unigenes were observed in the JA signaling pathway. Two *NPR1*, four *PR-1* and one *TGA* unigenes in the SA signaling pathway were also upregulated (Table 7). These results indicated that treatment with the endophytic fungi 2J1 or 3J1 upregulated the expression of a majority of key genes involved in signaling pathways that promoted the biosynthesis of respective secondary metabolites in *C. longepaniculatum* (Fig. S7 and S8).

RT-qPCR validation of the expression of genes encoding key enzymes involved in the terpenoid-biosynthesis pathway: To validate the transcriptome-sequencing results, five genes were selected to exhibit common differential expression in both the CK *vs.* 2J1 and CK *vs.* 3J1 groups for validation using RT-qPCR. All five selected genes, *DXS*, *DXR*, *CMS*, *GPPS* and *α-TPS*, were upregulated between the two treatment groups.

DXS and *α-TPS* exhibited higher expression levels and *DXR*, *CMS* and *GPPS* exhibited lower expression levels in both groups relative to those in the CK group, indicating that *DXS* and *α-TPS*, expressed downstream of *DXS*, play more significant regulatory roles in the MEP pathway relative to the other genes analyzed here. In addition, similarities between the RNA-Seq and RT-qPCR results for the five genes suggested that the sequencing results were reliable (Fig. 4).

Table 4: Differential expression of genes involved in the terpenoid-biosynthesis pathway

Group	EC Number	Name	Unigene Number	Trend
	2.7.7.60	CMS	c132275_g1	up
	2.5.1.87	DHDDS	c114877_g1	up
	2.5.1.1	GPPS	c130455_g1	up
	1.3.1.83	chlP	c135468_g2	up
CK *vs.* 2J1	2.2.1.7	DXS	c135654_g1	up
	2.2.1.7	DXS	c135654_g2	up
	2.2.1.7	DXS	c137114_g1	up
	2.2.1.7	DXS	c60365_g1	up
	1.1.1.267	DXR	c130596_g1	up
	2.3.1.9	atoB	c137615_g1	down
	2.3.1.9	atoB	c183602_g1	up
	1.8.3.5	FCLY	c34192_g1	up
	2.7.7.60	CMS	c132275_g1	up
	2.5.1.87	DHDDS	c114877_g1	up
	2.2.1.7	DXS	c133016_g1	down
	2.2.1.7	DXS	c135654_g1	up
CK *vs.* 3J1	2.2.1.7	DXS	c135654_g2	up
	2.2.1.7	DXS	c137114_g1	up
	1.1.1.267	DXR	c130596_g1	up
	2.5.1.1	GPPS	c130455_g1	up
	2.5.1.84	SPS	c135355_g3	up
	1.3.1.83	chlP	c135468_g2	up
	1.17.7.2	IDI	c99067_g1	up
	1.17.7.1	ispG/HDS	c113132_g3	up

Table 5: Differential expression of genes involved in the monoterpene-biosynthesis pathway

Group	EC Number	Name	Unigene Number	Trend
	4.2.3.111	(−)-alpha-terpineol synthase	c137256_g1	up
	4.2.3.111	(−)-alpha-terpineol synthase	c137256_g4	up
CK *vs.*	4.2.3.111	(−)-alpha-terpineol synthase	c137734_g1	up
2J1	4.2.3.111	(−)-alpha-terpineol synthase	c137734_g2	up
	4.2.3.25	(3S)-linalool synthase	c141180_g1	up
	4.2.3.25	(3S)-linalool synthase	c141180_g2	up
	4.2.3.25	(3S)-linalool synthase	c141180_g3	up
	4.2.3.111	(−)-alpha-terpineolsynthase	c137256_g1	up
CK *vs.*	4.2.3.111	(−)-alpha-terpineol synthase	c137734_g1	up
3J1	4.2.3.111	(−)-alpha-terpineol synthase	c137734_g2	up
	4.2.3.25	(3S)-linalool synthase	c141180_g2	up
	4.2.3.25	(3S)-linalool synthase	c141180_g3	up

Discussion

We sequenced the leaf transcriptome of *C. longepaniculatum* using a high-throughput sequencing technique, yielding 56,857,306 clean reads that were then assembled and merged into 82,061 unigenes. The unigenes were then queried

Table 6: Differentially expressed genes related to plant hormone-signal-transduction pathways between the CK and 2J1-treated groups

Unigene Number	Name	Trend	Unigene Number	Name	Trend
c128673_g1	AUX1	up	c137226_g1	JAR1	up
c81243_g1	AUX1	up	c135537_g7	MYC2	up
c134421_g2	ABF	up	c133182_g2	MYC2	up
c110766_g1	ARR-A	up	c127863_g2	MYC2	up
c132136_g4	ARR-A	down	c127863_g3	MYC2	up
c131636_g1	ARR-A	up	c127863_g1	MYC2	up
c121738_g2	ARR-A	up	c132839_g4	NPR1	up
c118972_g1	ARF	up	c129631_g2	NPR1	up
c130697_g2	ARF	up	c132331_g1	PP2C	up
c138196_g3	ARF	up	c137730_g1	PP2C	up
c131988_g2	ARF	up	c103366_g1	PP2C	up
c131988_g3	ARF	up	c123689_g1	PR1	up
c130697_g4	ARF	up	c115132_g1	PR1	up
c130706_g3	ARF	up	c122272_g2	PR1	up
c130706_g4	ARF	up	c122272_g1	PR1	up
c120869_g1	BKI1	up	c157961_g1	PR1	down
c120869_g2	BKI1	up	c134509_g2	PYL	down
c131708_g2	BKI1	up	c121192_g1	PYL	down
c127294_g1	CYCD3	down	c116805_g1	PYL	up
c125602_g2	DELLA	up	c139771_g5	PYL	down
c125389_g1	EIN3	down	c138717_g2	PIF4	up
c123204_g1	ERF1	up	c136723_g1	SAUR	up
c113482_g2	ERF1	up	c137995_g2	SAUR	down
c118911_g1	GH3	up	c120783_g1	SAUR	down
c124318_g1	GH3	down	c122972_g1	SAUR	down
c130561_g1	GH3	up	c96135_g1	SAUR	up
c138814_g1	GH3	up	c108406_g1	SAUR	down
c140710_g1	GID1	down	c125056_g1	SAUR	up
c136476_g1	IAA	up	c128755_g1	SAUR	down
c124734_g1	IAA	up	c94461_g1	SAUR	up
c127715_g1	IAA	up	c129795_g2	SAUR	down
c131981_g2	IAA	up	c126229_g1	SAUR	down
c134316_g2	IAA	up	c133549_g3	SNRK2	up
c131981_g3	IAA	up	c133549_g5	SNRK2	down
c117068_g1	IAA	up	c132846_g1	TGA	down
c117068_g2	IAA	up	c130277_g1	TGA	down
c129033_g1	JAZ	up	c136433_g1	TGA	down
c130330_g2	JAZ	up	c121385_g1	TCH4	down

Table 7: Differentially expressed genes related to plant hormone-signal transduction pathways between the CK and 3J1-treated groups

Unigene Number	Name	Trend	Unigene Number	Name	Trend
c130706_g4	ARF	up	c127863_g1	MYC2	up
c131988_g3	ARF	up	c139821_g1	NPR1	up
c130697_g2	ARF	up	c132839_g4	NPR1	up
c130706_g2	ARF	up	c121192_g1	PYL	down
c130706_g3	ARF	up	c134509_g2	PYL	down
c138196_g3	ARF	up	c157961_g1	PR1	up
c121050_g1	AHP	up	c122272_g1	PR1	up
c128188_g1	AHP	up	c123689_g1	PR1	up
c134421_g2	ABF	up	c115132_g1	PR1	up
c131636_g1	ARR-A	up	c136440_g1	PIF4	up
c131708_g2	BKI1	up	c136440_g2	PIF4	up
c120869_g1	BKI1	up	c138717_g2	PIF4	up
c128929_g1	COI-1	up	c132331_g1	PP2C	up
c125602_g2	DELLA	up	c135462_g1	PP2C	up
c123204_g1	ERF1	down	c137730_g1	PP2C	up
c130561_g1	GH3	up	c146860_g1	SNRK2	down
c138814_g1	GH3	up	c102925_g1	SAUR	down
c140710_g1	GID1	down	c108406_g1	SAUR	down
c117068_g2	IAA	up	c109908_g1	SAUR	down
c134316_g2	IAA	up	c94292_g1	SAUR	up
c124734_g1	IAA	up	c120783_g1	SAUR	down
c127715_g1	IAA	up	c122972_g1	SAUR	down
c131981_g2	IAA	up	c126229_g1	SAUR	down
c131981_g3	IAA	up	c128755_g1	SAUR	down
c117068_g1	IAA	up	c129795_g2	SAUR	down
c113820_g1	IAA	down	c136723_g1	SAUR	up
c128906_g1	JAZ	down	c137995_g2	SAUR	down
c133106_g3	JAZ	down	c116828_g1	SAUR	up
c126952_g1	JAZ	down	c136433_g1	TGA	up
c137226_g1	JAR1	up	c121385_g1	TCH4	down
c133182_g2	MYC2	up			

against public databases such as Gen Bank (https://www.ncbi.nlm.nih.gov/genbank/), String (http://string-db.org/), Swissprot (http://web.expasy.org/docs/swiss-prot_guideline.html), KEGG and Clusters of Orthologous Groups (https://www.ncbi.nlm.nih.gov/COG/) for further comparative analyses, which resulted in annotations for 43,106 unigenes. These results showed that treatment with 3J1 led to a greater proportion of upregulated genes as compared with 2J1 treatment, suggesting that 3J1 was more effective at inducing the upregulated expression of genes in *C. Longepaniculatum* leaves.

This study resulted in the annotation of a large number of unigenes associated with terpenoid biosynthesis, thereby providing a large amount of data for preliminary studies of the molecular mechanisms associated with the effect of endophytic fungi on secondary metabolic processes such as terpenoid biosynthesis in *C. longepaniculatum*. Among these processes, terpenoid-backbone biosynthesis pathways are important secondary metabolic pathways in plants and play an important role in the biosynthesis of terpenoids in plants (Sapir-Mir *et al*., 2008). There are two major terpenoid-biosynthesis pathways in plants: (1) the mevalonate (MVA) pathway that operates in the cytoplasm and (2) the MEP pathway that operates in plastids (Wang *et al*., 2013; Cocetta *et al*., 2015; Keilwagen *et al*., 2017). The MVA pathway primarily synthesizes sesquiterpenes (Fig. S9); whereas the MEP pathway synthesizes monoterpenes and diterpenes (Schwender *et al*., 2001). Compared with the control group, treatment with both 2J1 and 3J1 induced the upregulated expression of *CMS*, *DXR*, *DXS* and *GPPS*, which are involved in the MEP pathway; *CMS*, *DXR* and *DXS* encode key enzymes in the upstream MEP pathway, whereas *GPPS* encodes a key enzyme in the downstream pathway. Strain 3J1 further induced the upregulation of *HDS* and *IDI*, which encode enzymes involved in the midstream and downstream portions of the MEP pathway, respectively. Therefore, treatment with the two endophytic fungi resulted in the highest number of differentially expressed genes in the MEP pathway of terpenoid biosynthesis. However, the expression levels of some annotated key enzymes in the MVA pathway, such as acetoacetyl-CoA transferase and hydroxy methylglutaryl-CoA synthase, were not significantly changed by fungal treatment. These results indicated that monoterpene biosynthesis in *C. longepaniculatum* mainly relies on the MEP pathway and that enzymes involved in this pathway can be significantly regulated by endophytic fungi.

A large number of unigenes were annotated to involve in plant hormone signal transduction. SA, JA and ET are suggested to be the major signaling molecules that induce plant defense responses (Pauwels *et al*., 2009; Zipfel, 2013; Dempsey and Klessig, 2017). SA is a widely distributed plant phenolic hormone involved in various plant physiological processes (Glazebrook, 2005; Cao *et al*.,

2015). SA-mediated signal transduction pathways are regulated by multiple genes, including *NPR1*, which represents the key gene in the SA signaling pathway and is located upstream of this pathway. *NPR1* can also participate in JA-mediated signal transduction and serves as a "node" in both the SA and JA pathways (Studham and MacIntosh, 2012). The results indicated that treatment with 2J1 significantly upregulated the expression of *NPR1*, as well as that of its downstream-regulated gene *PR-1*, in *C. longepaniculatum*, whereas the expression of *TGA* was downregulated. Treatment with 3J1 led to the upregulated expression of these three key enzymes, suggesting that the SA signaling pathway was more responsive to 3J1 treatment in *C. longepaniculatum*. JA is an important plant signaling molecule that also plays an essential role in the biosynthesis of secondary metabolites in plants. *JAR1* catalyzes the ATP-dependent adenylation of JA to form JA-Ile, which subsequently initiates the expression of downstream genes and eventually leads to JA-induced physiological responses (Liu *et al*., 2012; Sun *et al*., 2016). There are four important genes in the JA signaling pathway: *JAR1*, *COI-1*, *JAZ* and *MYC2* (Guo *et al*., 2012; Wasternack and Hause, 2013). *JAZ* is a negative regulator of the pathway, whereas the other genes encode positive regulators (Yan *et al*., 2013; Wasternack, 2014). The results showed that treatment with 2J1 upregulated the expression of *JAR1*, *JAZ* and *MYC2*, whereas treatment with 3J1 downregulated the expression of *JAZ* and upregulated *JAR1* and *MYC2* expression, indicating that the JA signaling pathway is more responsive to 3J1. However, our preliminary study found that 2J1-treated *C. longepaniculatum* produced a greater amount of essential oils as compared with the 3J1-treated leaves, indicating that the synthesis and accumulation of secondary metabolites is a complex process that might involve other signal transduction pathways and regulatory mechanisms.

Five key enzyme genes (*DXS*, *DXR*, *CMS*, *GPPS* and *α-TPS*) responsible for monoterpene-biosynthesis pathways in *C. longepaniculatum* were further selected to validate the sequencing data by qPCR. The effects of endophytic fungi were analyzed on the expression of these genes. A previous RNA-Seq analysis of *Taxus chinensis* cells yielded a total of 46,581 unigenes, consisting of 13,469 differentially expressed genes responsible for the taxane-biosynthesis pathway and 10 of these genes were validated with qPCR showing consistent results (Li *et al*., 2012b). In this study, the RNA-Seq and qPCR results were also similar for the five differentially expressed genes analyzed, suggesting the reliability of the sequencing results. The findings indicated that *DXS* is involved in the MEP pathway, and that *α-TPS* is involved in the monoterpene-biosynthesis pathway based on their higher expression levels and greater regulatory roles relative to the other genes analyzed. These genes are therefore suggested to play critical roles in the biosynthesis of essential oils. Furthermore, the data indicated that endophytic fungi affect essential oil biosynthesis in *C. longepaniculatum* primarily through the differential expression of these signal transduction genes, which alter the gene expression levels of transcriptional factors, thereby regulating the expression of genes responsible for secondary metabolic processes such as terpenoid biosynthesis and metabolism. The overexpression of *DXS*, *DXR*, *CMS*, *GPPS* and *α-TPS* in *C. longepaniculatum* will be analyzed for the regulation of terpenoids, especially the monoterpenoids, which utilize *DXS*, *DXR*, *CMS*, *GPPS* and *α-TPS* genes to provide more theoretical basis for the metabolic engineering regulation of terpenoids in *C. longepaniculatum*.

Conclusion

Annotations of unigenes associated with secondary metabolism, hormone signal transduction and terpenoid biosynthesis provided a valuable insight into endophytic fungi-regulated biosynthesis of essential oils in *C. longepaniculatum*. Few annotated genes related to metabolic pathways were associated with synthesis of volatile terpenoids from transcriptome data, with some of these enzymes characterized as multifunctional and unstable. Future studies are needed to focus non-annotated genes to screen functional gene candidates that are potentially involved in terpenoid biosynthesis of *C. longepaniculatum* and quantify downstream terpenoids, thereby combining their changes in content to make a comprehensive analysis.

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References

Ali, H., M.A. Khan, N. Ullah and R.S. Khan, 2018. Impacts of hormonal elicitors and photoperiod regimes on elicitation of bioactive secondary volatiles in cell cultures of *Ajuga bracteosa*. *J. Photochem. Photobiol. B Biol*., 183: 242–250

Cao, H., M. Nuruzzaman, H. Xiu, J. Huang, K. Wu, X. Chen, J. Li, L. Wang, J.H. Jeong, S.J. Park, F. Yang, J. Luo and Z. Luo, 2015. Transcriptome analysis of methyl jasmonate-elicited *Panax ginseng* adventitious roots to discover putative ginsenoside biosynthesis and transport genes. *Intl. J. Mol. Sci*., 16: 3035–3057

Chowdhary, K. and N. Kaushik, 2015. Fungal endophyte diversity and bioactivity in the Indian medicinal plant *Ocimum sanctum* Linn. *Plos One*, 10: e0141444

Cocetta, G., M. Rossoni, C. Gardana, I. Mignani, A. Ferrante and A. Spinardi, 2015. Methyl jasmonate affects phenolic metabolism and gene expression in blueberry (*Vaccinium corymbosum*). *Physiol. Plantarum*, 153: 269–283

Dempsey, D.A. and D.F. Klessig, 2017. How does the multifaceted plant hormone salicylic acid combat disease in plants and are similar mechanisms utilized in humans? *BMC Biol*., 15: 23

Deng, S., Z.Y. Liang and L.L. Zhang, 2012. Three kinds of antibacterial activity of essential oil. *Guizhou Norm. Univ*., 28: 30–32

Du, S.J. and X.H. Guo, 2009. The impact of endophytic fungi on plant secondary metabolites. *Mod. Agric. Sci*., 16: 17–19

Ebel, J. and D. Scheel, 1992. Elicitor recognition and signal transduction. *In: Genes Involved in Plant Defense*, pp: 183–205. Boller, T. and F. Meins (Eds.). Springer-Verlag, New York, USA

Fouda, A.H., S.D. Hassan, A.M. Eid and E.D. Ewais, 2015. Biotechnological applications of fungal endophytes associated with medicinal plant *Asclepias sinaica* (Bioss.). *Ann. Agric. Sci*., 60: 95–104

Gandhi, S.G., V. Mahajan and Y.S. Bedi, 2014. Changing trends in biotechnology of secondary metabolism in medicinal and aromatic plants. *Plantarum*, 241: 303–317

Glazebrook, J., 2005. Contrasting mechanisms of defense against biotrophic and necrotrophic pathogens. *Ann. Rev. Phytopathol*., 43: 205–227

Glowacka, K., 2011. A review of the genetic study of the energy crop *Miscanthus. Biomass Bioener*., 35: 2445–2454

Grabherr, M.G., B.J. Haas, M. Yassour, J.Z. Levin, D.A. Thompson, I. Amit, X. Adiconis, L. Fan, R. Raychowdhury, Q. Zeng, Z. Chen, E. Mauceli, N. Hacohen, A. Gnirke, N. Rhind, F.D. Palma, B.W. Birren, C. Nusbaum, K. Lindblad-Toh, N. Friedman and A. Regev, 2011. Full-length transcriptome assembly from RNA-Seq data without a reference genome. *Nat. Biotechnol*., 29: 644–652

Guo, J., Y.Z. Chen, M.S. Li, L. Shi and X.F. Yan, 2012. Does MYC2 really play a negative role in jasmonic acid-induced indolic glucosinolate biosynthesis in *Arabidopsis thaliana*? *Russ. J. Plant Physiol*., 60: 100–107

Hu, W.J., H.D. Gao and X.M. Jang, 2012. Analysis on constituents and contents in leaf essential oil from three chemical types of *Cinnamum camphora*. *Centr. South. Univ. Technol*., 32: 186–194

Jinfeng, E.C., M.I.M. Rafi, K.C. Hoon, H.K. Lian and C.Y. Kqueen, 2017. Analysis of chemical constituents, antimicrobial and anticancer activities of dichloromethane extracts of *Sordariomycetes* spp. endophytic fungi isolated from *Strobilanthes crispus*. *World J. Microb. Biotechnol*., 33: 5

Katoch, M., G. Singh, S. Sharma, N. Gupta, P.L. Sangwan and A.K. Saxena, 2014. Cytotoxic and antimicrobial activities of endophytic fungi isolated from *Bacopa monnieri* (L.) Pennell (Scrophulariaceae). *BMC Complem. Altern. Med*., 14: 1–8

Keilwagen, J., H. Lehnert, T. Berner, H. Budahn, T. Nothnagel, D. Ulrich and F. Dunemann, 2017. The Terpene Synthase Gene Family of Carrot (*Daucus carota* L.): identification of QTLs and candidate genes associated with terpenoid volatile compounds. *Front. Plant Sci*., 8: 1–18

Kim, KH., Y.J. Kang, D.H. Kim, M.Y. Yoon, J.K. Moon, M.Y. Kim, K. Van and S.H. Lee, 2011. RNA-Seq analysis of a soybean near-isogenic line carrying bacterial leaf pustule-resistant and -susceptible alleles. *DNA Res*., 18: 483–497

Krstić-Milošević, D., T. Janković, B. Uzelac, D. Vinterhalter and B. Vinterhalter, 2017. Effect of elicitors on xanthone accumulation and biomass production in hairy root cultures of *Gentiana dinarica*. *Plant Cell Tiss. Org*., 130: 631–640

Li, N., Y.G. Zu and W. Wang, 2012a. Antibacterial and antioxidant of celery seed essential oil. *Chin. Condiment*., 37: 28–30

Li, S.T., P. Zhang, M. Zhang, C.H. Fu, C.F. Zhao, Y.S. Dong, A.Y. Guo and L.J. Yu, 2012b. Transcriptional profile of *Taxus chinensis* cells in response to methyl jasmonate. *BMC Genom.*, 13: 295

Li, L., W.L. Zheng and Q.Y. Zhong, 2014. Antibacterial activity of leaf essential oil and its constituents from *Cinnamomum longepaniculatum*. *Intl. J. Clin. Exp. Med*., 7: 1721–1727

Li, P.Q., Y. Mou, T.J. Shan and G.Z. Li, 2011. Effects of polysaccharide elicitors from endophytic *Fusarium oxysporium* Dzf17 on growth and diosgenin production in cell suspension culture of *Dioscorea zingiberensis*. *Molecules*, 16: 9003–9016

Liu, Q.X., M.S. Li and J. Guo, 2012. Regulation of jasmonic acid biosynthesis and jasmonic acid signaling pathway. *Plant Physiol*., 48: 837–844

Livak, K.J. and T.D. Schmittgen, 2001. Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta CT}$ method. *Methods*, 25: 402–408

Pauwels, L., D. Inzé and A. Goossens, 2009. Jasmonate-inducible gene: what does it mean? *Trends Plant Sci*., 14: 87–91

Sapir-Mir, M., A. Mett, E. Belausov, S. Tal-Meshulam, A. Frydman, D. Gidoni and Y. Eyal, 2008. Peroxisomal localization of *Arabidopsis* isopentenyl diphosphate isomerases suggests that part of the plant isoprenoid mevalonic acid pathway is compartmentalized to peroxisomes. *Plant Physiol*., 148: 1219–1228

Schwender, J., C. Gemünden and H. Lichtenthaler, 2001. Chlorophyta exclusively use the 1-deoxyxylulose 5-phosphate/2-C-methyl-erythritol-4-phosphate pathway for the biosynthesis of isoprenoids. *Plantarum*, 212: 416–423

Strauss, T., R.M.V. Poecke, A. Strauss, P. Römer, G.V. Minsavage, S. Singh, C. Wolf, A. Strauss, S. Kim, H.A. Lee, S.I. Yeom, M. Parniske, R.E. Stall, J.B. Jones, D. Choi, M. Prins and T. Lahaye, 2012. RNA-seq pinpoints a *Xanthomonas* TAL-effector activated resistance gene in a large-crop genome. *Proc. Natl. Acad. Sci. USA*, 109: 19480–19485

Studham, M.E. and G.C. MacIntosh, 2012. Phytohormone signaling pathway analysis method for comparing hormone responses in plant-pest interactions. *BMC Res. Notes*, 5: 392

Sun, Y., J. Xiao, X. Jia, P. Ke, L. He, A. Cao, Y.F. Wu, X.Q. Gao and X. Wang, 2016. The role of wheat jasmonic acid and ethylene pathways in response to *Fusarium graminearum* infection. *Plant Growth Regul*., 80: 69–77

Tan, Y., R. Jia, J.H. Tao, N.Y. Yang, J.A. Duan and D.W. Qian, 2013. Regulation on biosynthesis of active constituents in medicinal plants by endophytic fungal elicitor. *Chin. Trad. Herbs Drugs*, 44: 2004–2008

Vaccaro, M., N. Malafronte, M. Alfieri, N.D. Tommasi and A. Leone, 2014. Enhanced biosynthesis of bioactive abietane diterpenes by overexpressing AtDXS or AtDXR genes in *Salvia sclarea* hairy roots. *Plant Cell Tiss. Org*., 119: 65–77

Wang, L.J., X. Fang, C.Q. Yang and X.Y. Chen, 2013. Biosynthesis and regulation of secondary terpenoid metabolism in plants. *Sci. Sin. Vitae*, 43: 1030–1046

Ward, J.A. and C.A. Weber, 2012. Comparative RNA-seq for the investigation of resistance to *Phytophthora* root in the red raspberry 'Latham'. *Acta Hortic*., 946: 67–72

Wasternack, C., 2014. Action of jasmonates in plant stress responses and development-applied aspects. *Biotechnol. Adv*., 32: 31–39

Wasternack, C. and B. Hause, 2013. Jasmonates: biosynthesis, perception, signal transduction and action in plant stress response, growth and development. *Ann. Bot*., 111: 1021–1058

Wei, Q., Y.Y. Tan and Q. Li, 2016. Effects of fungal endophytes on cell suspension culture of *Cinnamomum longepaniculatum*. *Guihaia*, 36: 923–929

Xu, L., L. Zhu, L. Tu, L. Liu, D. Yuan, L. Jin, L. Long and X. Zhang, 2011. Lignin metabolism has a central role in the resistance of cotton to the wilt fungus *Verticillium dahliae* as revealed by RNA-Seq-dependent transcriptional analysis and histochemistry. *J. Exp. Bot*., 62: 5607–5621

Xu, S., Q.Y. Zhong and Q. Wei, 2014. Anti-hepatoma effect of safrole from *Cinnamomum longepaniculatum* leaf essential oil *in vitro*. *Intl. J. Clin. Exp. Pathol*., 7: 2265–2272

Yan, J., H. Li, S. Li, R. Yao, H. Deng, Q. Xie and D. Xie, 2013. The *Arabidopsis* F-Box Protein CORONATINE INSENSITIVE1 Is stabilized by SCFCOI1 and degraded *via* the 26S proteasome pathway. *Plant Cell*, 25: 486–498

Yan, K., F. Chen, Q. Wei, R.Z. Feng, W.H. Zhou and M. Zhou, 2017. Effect of endophytic fungi on its essential oil accumulation and physiological-biochemical characteristic of *Cinnamomum longepaniculatum*. *Biotechnol. Bull*., 33: 138–143

Zhao, H.W., F. Ge, Y. Sun, D.Q. Liu and C.Y. Chen, 2012. Transcription factors involved in plant terpenoid biosynthesis and their application prospect. *Chin. Trad. Herbs Drugs*, 43: 2512–2519

Zipfel, C., 2013. Combined roles of ethylene and endogenous peptides in regulating plant immunity and growth. *Proc. Acad. Sci. U.S.A.*, 110: 5748–5749

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