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ORIGINAL ARTICLE

Transcriptome analysis reveals in vitro-cultured regeneration bulbs as a promising source for targeted *Fritillaria cirrhosa* steroidal alkaloid biosynthesis

Qi Zhao[1] · Rui Li[1] · Yang Zhang[2] · Kejia Huang[2] · Wenguo Wang[3] · Jian Li[2]

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Abstract

The bulbs of *Fritillaria cirrhosa* is wildly used in traditional Chinese medicine to treat lung-related disease, which has recently been found to have antitussive, anti-inflammatory, antihypertensive and anti-tumor activity. Steroidal alkaloids are the major effective ingredients of *F. cirrhosa*. In the current study, we demonstrated an efficient strategy for *F. cirrhosa* bulb regeneration in vitro by cytokinin/auxin induction. Our data showed that the regenerated bulbs accumulated higher alkaloid content that the wild ones. We further performed RNA-seq and bioinformatics analysis to study the gene expression profile, especially those related to alkaloids biosynthesis. KEGG pathway annotation identified genes related to "Metabolic pathways" were the most abundant (2644, 26.0%), followed by those for "Biosynthesis of secondary metabolites" (1319, 13.0%) among the 113,865 unigenes identified. Further analysis suggested MEP pathway, other than MVA pathway, might be the major route for steroidal alkaloid biosynthesis of *F. cirrhosa*, as all the key genes in this pathway were found to be unregulated in our study. We also showed that accumulation of different phytochemicals was linked to plant hormone addition. Our current study demonstrated that in vitro cultivation is a promising strategy for mass production of *F. cirrhosa* steroidal alkaloids for pharmacological industry.

Keywords *Fritillaria cirrhosa* · Transcriptome · High-throughput sequencing · In vitro cultures · Cytokinin/auxin

Introduction

Fritillaria cirrhosa D. Don (Family, Liliaceae) is a bulbous medicinal plant native to China, especially the southeastern margin of the Qinghai–Tibet Plateau region (Zhang et al. 2010). The bulbs of this plant, called Chuan Bei Mu, are commonly used to treat cough in traditional Chinese medicine (TCM) for over 2000 years. Recent studies revealed that the extracts of *F. cirrhosa* bulbs possess anti-inflammatory and anti-cancer activities (Luo et al. 2012). A series of ingredients with proved pharmacological activities have been detected in *F. cirrhosa* bulbs, including steroidal alkaloid, saponins, terpenoids and glycosides (Jian et al. 2012). Due to the lack of effective cultivation techniques, the Chuan Bei Mu has mainly been collected from natural resources (Li et al. 2012). However, *F. cirrhosa* is endangered as a consequence of long-term overexploitation (Li et al. 2012; Zhang et al. 2010).

In recent years, huge amount of efforts has been put into the development of in vitro cultivation technique for medicinal plants producing bioactive compounds (Park and Paek.

Qi Zhao and Rui Li authors contributed equally to this work.

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✉ Wenguo Wang
wangwenguo@caas.cn

✉ Jian Li
lijian01@cdu.edu.cn

[1] College of Pharmacy and Biological Engineering, Chengdu University, 1 Shiling, Chengdu 610106, China

[2] School of Medicine, Chengdu University, 1 Shiling, Chengdu 610106, China

[3] Biogas Institute of Ministry of Agriculture, Chengdu 610041, China

2014; Qi et al. 2009). Attempt of *F. cirrhosa* tissue culture suggested a promising way for mass propagation and accumulation of major phytochemicals within a short period of time (Chen et al. 1995). Efficient production of major phytochemicals requires understanding of their biosynthetic pathways, and our present knowledge to *F. cirrhosa* phytochemical biosynthesis is limited to only a few genes involved in these pathways (Sun et al. 2011).

High-throughput RNA sequencing (RNA-Seq) is capable of generating the whole transcriptome information at single transcript level, enabling the characterization of secondary metabolite biosynthetic pathways (Pal et al. 2015). Initial efforts have been made to generate expressed sequence tags (ESTs) from the bulbs of *F. cirrhosa* collected in the wild (Sun et al. 2011). However, no large-scale transcriptome information validated by expression profiling is available for in vitro cultivated bulbs of *F. cirrhosa*. The key genes regulating major phytochemicals biosynthesis of *F. cirrhosa* have never been characterized functionally.

In this study, we performed RNA-Seq to generate and compare the transcriptomes of the wild and in vitro-regenerated *F. cirrhosa* bulbs. We comprehensively analyzed the gene expression profiles of *F. cirrhosa* with emphasis on genes related to the biosynthesis of steroidal alkaloid biosynthetic pathways, which are the major bioactive ingredients responsible for the pharmacological features (Jian et al. 2012; Wang et al. 2016). Our results demonstrated that steroidal alkaloid biosynthesis-related genes were universally upregulated in the in vitro-generated bulbs. This is consistent with the observation that the total alkaloid contents are higher in the regenerated bulbs than the wild bulbs. The current study provided supporting evidence that in vitro cultivation of *F. cirrhosa* could serve as a promising source for targeted steroidal alkaloid biosynthesis.

Materials and methods

Plant material preparation and measurement of the total alkaloid

Wild bulbs (WB) were collected from 3-year-old *F. cirrhosa* plants growing in the Kangding fold–thrust belt mountains (located at 30°3′44.9″N, 101°58′3.81″E, altitude 4300 m) in Sichuan Province, China. Regenerated bulbs (RB) were induced from wild bulb explants of *F. cirrhosa* on Murashige and Skoog (MS) medium supplemented with 30 g/L sucrose, 2 mg/L 6-BA and 0.5 mg/L NAA in a 16 h light/8 h dark cycle at 20 °C. Relative transcript abundance analysis used the same plant material as for the transcriptome analysis.

Total alkaloid extraction was performed as previously described with minor modifications (Liu et al. 2004). UV spectrophotometer was adopted to determine the content of total alkaloids using peiminine as the standard.

RNA extraction, cDNA library construction, and sequencing

Total RNA was extracted using RNeasy® Plant Mini Kit (Qiagen) according to the manufacturer's protocol. The integrity of RNA was assessed by formaldehyde agarose gel electrophoresis. A total of 30 μg mixed RNA from three biological replicates detected by 2100 Bio-analyzer (Agilent, USA) was digested with DNase I (TAKARA), and then purified by Dynabeads® Oligo (dT)25 (Life, USA). 100 ng derived mRNAs were fragmented and reverse transcribed into first-strand cDNAs with random hexamer. The second-strand cDNAs were synthesized using a NEBNext® Ultra™ RNA Library Prep Kit for Illumina (NEB). The double-stranded cDNAs were purified and ligated to adaptors for Illumina paired-end sequencing. The cDNA libraries were sequenced on the Illumina HiSeq™ 2000 platform using the paired-end technology of Gene Denovo Co. (Guangzhou, China).

De novo assembly and annotation

Raw reads were first processed using in-house Perl scripts. The raw reads were filtered by removing adapter sequences, reads containing poly-N sequences, and low-quality sequences. Clean reads were de novo assembled using the Trinity Program (Grabherr et al. 2011). Unigenes were defined after removing redundancy and short contigs from the assembly. The unigenes were predicted by "GetORF" in the EMBOSS.

The unigenes were packaged (Rice et al. 2000) and aligned to the protein sequence database NCBI NR (non-redundant protein database), Swiss-Port (Annotated protein sequence database), KEGG (Kyoto encyclopedia of genes and genomes) and KOG (Clusters of orthologous groups of protein) by Blastp with an E-value threshold of 1×10^{-5}.

The number of unique-match reads was calculated and normalized to RPKM (reads per kb per million reads) for gene expression analysis. Comparison of unigene expression between WB and RB was performed according to DESeq as described by Abders and Huber (Anders and Huber 2010). The differentially expressed genes (DEGs) between two samples were restricted with FDR (false discovery rate) ≤ 0.001 and the absolute value of log2 Ratio ≥ 1.

To examine the expression profile of DEGs, the expression data υ between two samples were normalized to 0, log2 (υRB/WB), and then clustered by Short Time-series Expression Miner software (STEM) (Ernst and Bar-Joseph 2006). The clustered profiles with P value ≤ 0.05 were considered as significantly expressed. Then the DEGs in all or in each

profile were subjected to gene ontology (GO) classifications using WEGO (Ye et al. 2006), and KEGG (Kanehisa et al. 2000) pathway enrichment analysis.

Validation of differential expression using qRT-PCR

The cDNA was generated from 1 µg total RNA isolated from the bulbs using a Prime-Script ™ 1st Strand cDNA Synthesis Kit (TAKARA, Japan). Primers for quantitative real-time PCR (qRT-PCR) were designed using Primer Premier 5.0 software (Premier, Canada) and synthesized by Sangon Biotech (Shanghai) Co., Ltd. The 18S (Gen-Bank accession number: AY616727.1) was selected as the reference. The primer sequences are listed in Supplementary Table S1. qRT-PCR was performed on a Bio-Rad iQ5 Optical System Real Time PCR System (Bio-R ad, USA). Each reaction mixture was 20 µL containing 10 µL of SYBR Green PCR Master Mix (TaKaRa, Japan), 250 nM of each primer, and 6 µL of diluted first-strand cDNAs. The qRT-PCRs were run as follows: 50 °C for 2 min, 95 °C for 10 min, followed by 40 cycles of 95 °C for 30 s, 56 °C for 30 s, and 72 °C for 30 s in 96-well optical reaction plates. The Ct values were determined for three biological replicates, with three technical replicates for each value. Expression levels of the tested reference genes were determined by Ct values and calculated by $2^{-\Delta\Delta Ct}$.

Statistical analysis

All data were statistically analyzed by means of the SPSS with LSD to identify differences. Significant differences ($P < 0$ 0.05) between treatments were indicated by different letters.

Results and discussion

Regeneration of *F. cirrhosa* bulbs in vitro and the quantification of total alkaloid

F. cirrhosa is a medicinal plant with high pharmaceutical value, and the bulbs produce major secondary metabolites responsible for most of the pharmacological activity of this plant (Wang et al. 2016). The accumulation of secondary metabolites in alpine growing plants can be influenced by various environmental factors as well as the age of the plant at harvest (Verma and Shukla 2015). The growing demand in the pharmaceutical industry led us to search for an alternative approach for mass production of *F. cirrhosa* bulbs. In vitro cultivation techniques have been shown to be a useful tool for mass multiplication (Chen et al. 1995; Sairkar et al. 2009; Senthil et al. 2015).

Recent years, cytokinin/auxin combinations media have been successfully exploited for the induction of organogenesis (Khanam et al. 2000). In our current study, treatments were compared for the effects of cytokinin/auxin combinations (6-BA and NAA) on the induction and multiplication from WB explants. As shown in Fig. 1b, c, the regenerated bulbs were induced successfully from bulb explants of *F. cirrhosa*, as observed by swelling and enlargement of immature bulbs 45–60 days after culture initiation, and subsequent direct bulb formation during the fourth month (Fig. 1d). We then used decocting method to determine the total alkaloid content of WB and RB. As show in Fig. 2, the total alkaloids were more abundant in RB than that in WB. The results of these in vitro cultivation assays indicated that cytokinin/auxin treatment was efficient in inducing the initial proliferation of *F. cirrhosa*.

RNA-Seq and de novo assembly

RNA-seq provides a global overview of the gene expression at the transcriptome level. To understand the impact of in vitro cultivation on the genes expression profile of *F. cirrhosa*, especially those genes related to the phytochemical biosynthesis, we performed RNA-Seq with the wild and regenerated *F. cirrhosa* bulbs. We obtained 73,632,020 and 34,583,794 cleaned high-quality reads from the WB and RB, respectively (Table 1), which were remarkably more than the previously reported ESTs analysis with 1343 unique transcripts (Sun et al. 2011). All clean reads were pooled together and then de novo assembled by Trinity to generate a unique transcript library. 113,865 unigenes with a mean size of 528 bp and N50 number of 617 bp were assembled. The length of the genes ranged from 231 to 16,461 bp (Table 1).

Functional annotation of the assembled transcripts

The annotation for *F. cirrhosa* unique sequences was based on BLASTP searches of four public databases. A final number of 34,101, 25,255, 20,329 and 10,176 unigenes (*E* value $< 1e^{-5}$) had significant matches in NR, Swiss-prot, KOG and KEGG databases, respectively (Fig. 3a). Based on the NR annotation and the *E* value distribution, 79.8% of the mapped sequences showed strong homology ($E < 10^{-20}$) and 56.6% showed very strong homology ($E < 10^{-50}$) to the available plant sequences (Fig. 3b). With respect to species, 25.5 and 20.8% of the unique sequences had top matches to sequences from *Elaeis guineensis* and *Phoenix dactylifera*, respectively, with additional hits to *Musa acuminata subsp. malaccensis* (10.7%), *Oryza sativa Japonica Group* (5.7%), *Nelumbo nucifera* (3.3%), *Theobroma cacao* (2.9%), etc. (Fig 3c).

We used GO assignments to classify the functions of the predicted unigenes. GO annotated unigenes were

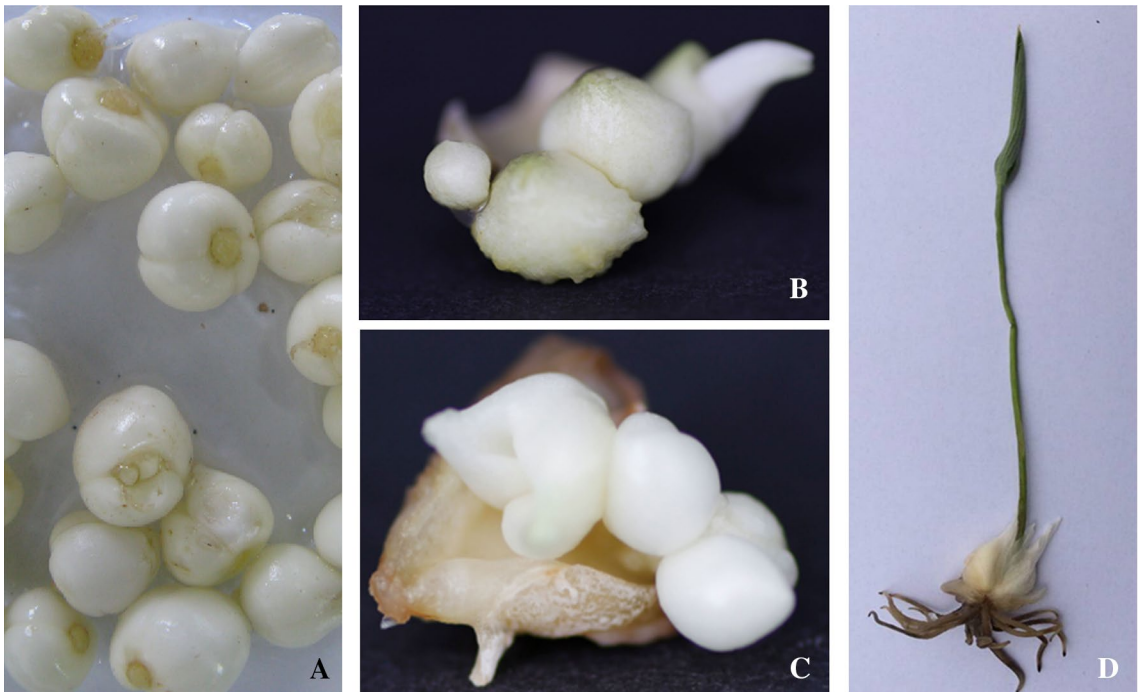

Fig. 1 In vitro propagation and plantlet regeneration from *F. cirrhosa* bulbs explants. **a** Wild bulbs; **b** and **c** induced callus from explants of wild bulb; **d** regenerated plantlet with bulb

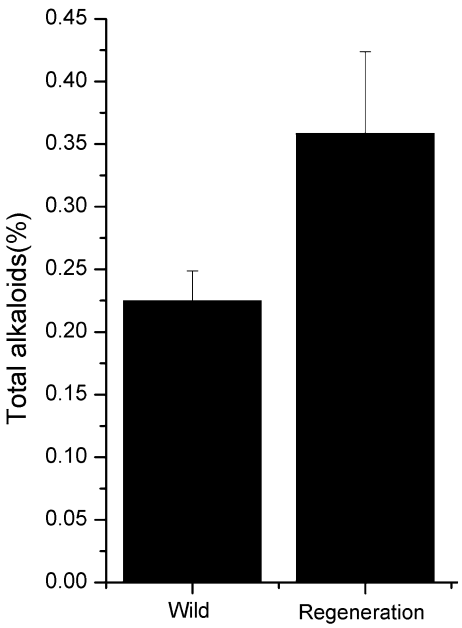

Fig. 2 Determination of *F. cirrhosa* bulbs' total alkaloid content

categorized into three ontologies: biological process, cellular component, and molecular function. The biological process category included the largest number of unigenes (33,664), followed by cellular component (26,160), and then molecular function (12,142) (Supplementary Figure S1). Within the biological process category, "metabolic process," "cellular process," and "single-organism process" were the most enriched, while proteins related to "cell," "cell part," and "organelle" were enriched in the cellular component category. Among the molecular function category, "catalytic activity" and "binding" accounted for most of the unigenes (Supplementary Figure S1). Transcripts related to GO term "metabolic process" was the most abundant in the biological process category, which is similar to the previously reported GO annotation of medicinal tissues (Han et al. 2013; Tang et al. 2016). Furthermore, 35,970 unigenes with annotations were assigned to KOG classifications. Among the 25 KOG categories, the cluster for "general function prediction only" represented the largest category (6398, 17.8%), followed by the "post-translational modification, protein turnover, chaperones" (4611, 12.8%), "signal transduction mechanisms" (3305, 9.2%), and "RNA processing and modification" (2379, 6.7%) (Supplementary Figure S2).

We then performed KEGG pathway annotation to identify the biological function and gene interaction. 10176 unigenes with annotation could be distributed into 123 KEGG pathways. Genes related to "Metabolic pathways" were found to be the most abundant in number (2644, 26.0%), followed by those for "Biosynthesis of secondary metabolites" (1319, 13.0%) and "Ribosome" (562, 5.5%) (Supplementary Table S2). Of the transcripts assigned to secondary metabolite biosynthetic pathways, a large pool with 210 (2.06%) members was mapped to phenylpropanoid biosynthesis with

Table 1 Summary of the RNA-Seq data of the two libraries

Libraries	No. of raw reads	No. of clean reads	No. of assembled unigenes	Average of unigene length (bp)	N50 of unigene length (bp)	Min of unigene length (bp)	Max of unigene length (bp)
WB	76,711,942	73,632,020	113,865	528	617	231	16,461
RB	35,324,402	34,583,794					

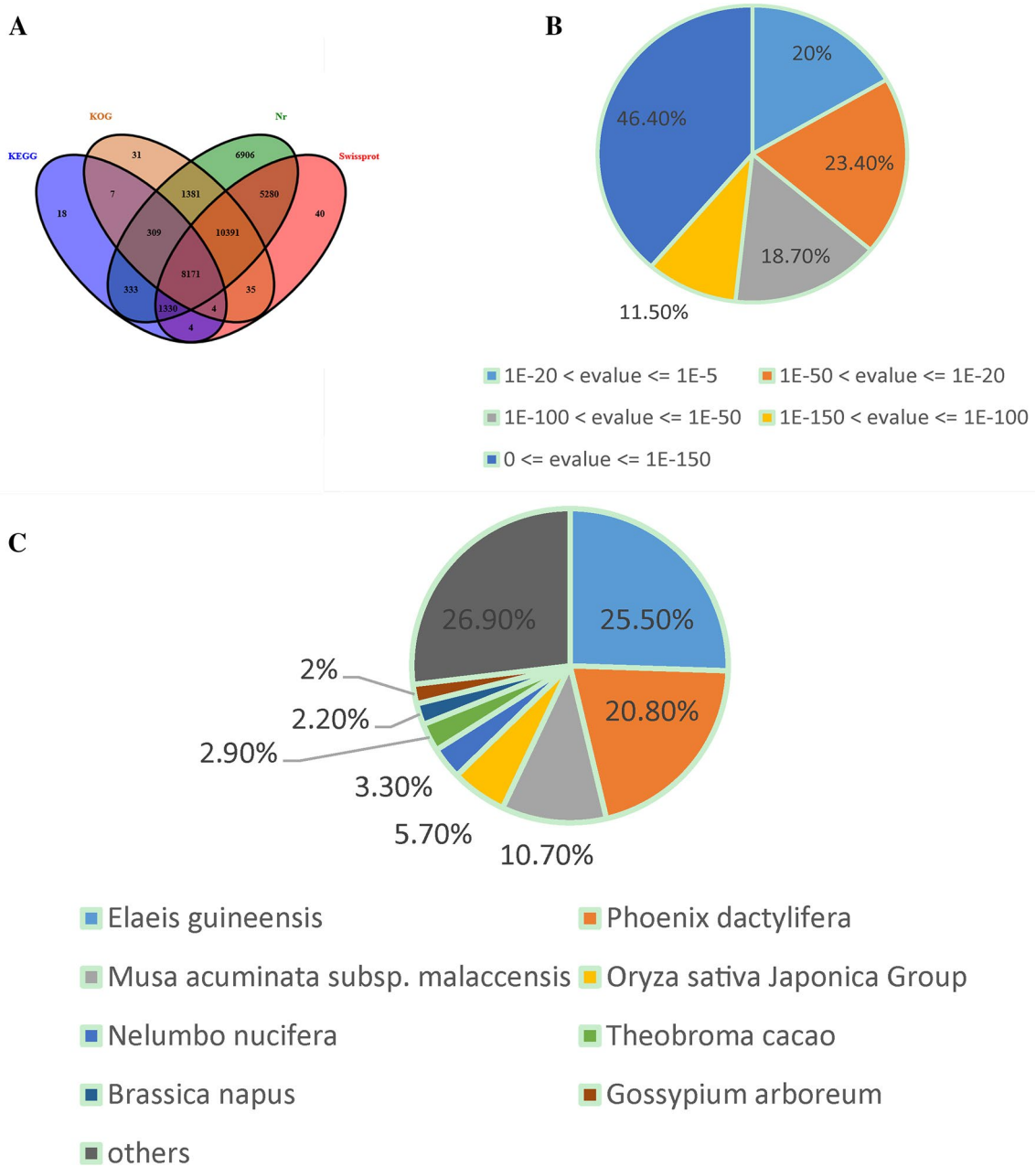

Fig. 3 Characteristics of homology search of *F. cirrhosa* unigenes. **a** Venn diagram of the number of unigenes annotated by BLASTx with an E-value threshold of 10^{-5} against protein databases. The number in the circles indicates the number of unigenes annotated by single or multiple databases. **b** *E* value distribution of the top BLASTx hits against the Nr database for each unigene. **c** Percentage of unigenes matching the top eight species using BLASTx in the Nr database

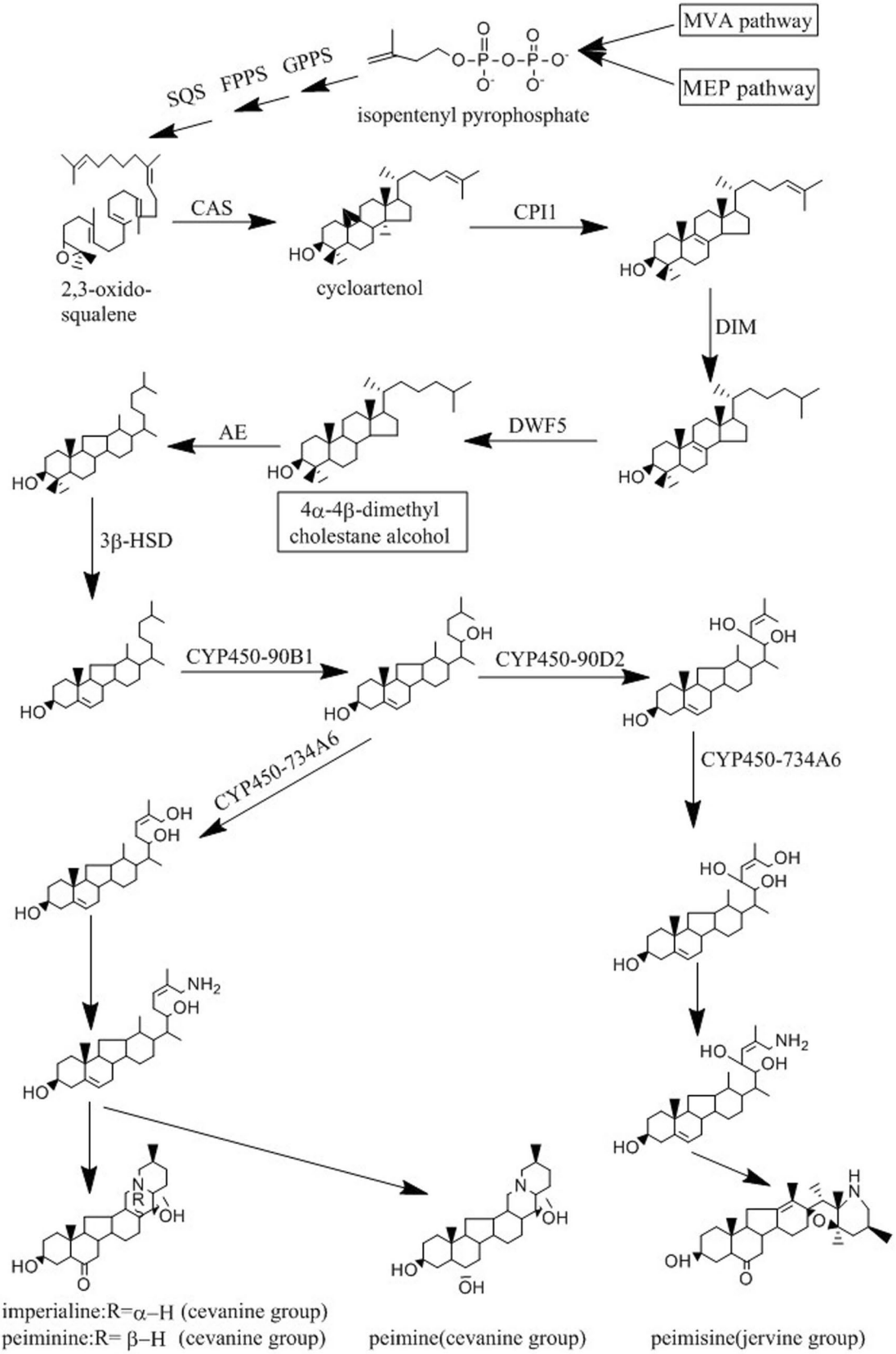

Fig. 4 Proposed *F. cirrhosa* steroidal alkaloid biosynthetic pathways

terpenoid backbone biosynthesis (81, 0.8%), and N-Glycan biosynthesis also included many transcripts (74, 0.73%) (Supplementary Table S3). The overall annotation of the *F. cirrhosa* transcriptome provided a valuable resource for investigating specific processes, functional descriptions and pathways. On the basis of the applied criteria (fold change ≥ 2, FDR < 0.001), 7045 genes were identified as significantly DEGs between the WB and RB, of these, 3509 were up-regulated and 3536 were down-regulated.

Expression of the major steroidal alkaloid biosynthesis-related genes

The major phytochemicals (steroidal alkaloid) of *F. cirrhosa* includes peimine, imperialine, peiminine, peimisine. The biosynthesis pathway of these steroidal alkaloids is still largely unknown. Steroid are synthesized via two pathways in plants: the classical mevalonate (MVA) pathway and the methylerythritol phosphate (MEP) pathway that leads to the synthesis of isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP) (Kul'Kova et al. 1999). These intermediates serve as the substrates that undergo a cascade of chemical conversions along with the formation of metabolite intermediates, such as geranyl pyrophosphate (GPP), farnesyl pyrophosphate (FPP), squalene, cycloartenol, through terpenoid backbone biosynthesis (Schaller 2010). Cycloartenol is a common intermediate for the biosynthesis of steroidal alkaloid (Dev 1984). The steroidal alkaloids of *F. cirrhosa*, which have a cevanine or jervine-type framework, are considered to arise from cycloartenol by catabolic processes with nitrogen incorporation or hydroxylation reactions (Hao et al. 2013). The possible biosynthetic pathway

Table 2 Genes identified by transcriptome in putative alkaloid biosynthetic pathways

Pathway	Annotation	Gene ID	RPKM		Trend
			Wild	Regeneration	
MVA	AACT(acetyl-CoA acetyltransferase)	Unigene0050126	128.618	121.771	–
	HMGS(hydroxymethylglutaryl-CoA synthase)	Unigene0048264	270.704	289.662	–
	HMGR(3-hydroxy-3-methylglutaryl-coenzyme A reductase)	Unigene0052102	376.287	414.101	–
	MK(mevalonate kinase)	Unigene0022250	10.8035	5.6103	↓
	PMK(phosphomevalonate kinase)	Unigene0050127	11.2354	28.5526	↑
	MVD(diphosphomevalonate decarboxylase-like)	Unigene0052125	109.969	37.8945	↓
MEP	DXS(1-deoxy-D-xylulose-5-phosphate synthase 2)	Unigene0039155	12.0899	49.5867	↑
	DXR(chloroplast 1-deoxy-D-xylulose-5-phosphate reductoisomerase)	Unigene0048178	63.5768	77.0203	–
	CMS(2-C-methyl-D-erythritol 4-phosphate cytidylyltransferase)	Unigene0020765	0.001	0.4418	↑
	CMK(4-diphosphocytidyl-2-C-methyl-D-erythritol kinase)	Unigene0019637	0.1256	9.988	↑
	MCS(2-C-methyl-D-erythritol 2,4-cyclodiphosphate synthase)	Unigene0017850	0.001	0.4999	↑
	HDR(4-hydroxy-3-methylbut-2-enyl diphosphate reductase)	Unigene0017845	0.0598	13.6742	↑
	HDS(4-hydroxy-3-methylbut-2-en-1-yl diphosphate synthase)	Unigene0048148	49.4889	80.3542	↑
	IDI(isopentenyl diphosphate isomerase)	Unigene0045079	521.259	261.7421	↓
Downstream	GPPS(geranyl pyrophosphate synthase)	Unigene0038524	10.1704	95.2013	↑
	FPPS(farnesyl pyrophosphate synthase)	Unigene0037411	0.0197	44.9723	↑
	SQS(squalene synthase)	Unigene0029799	2.5466	30.0693	↑
	CAS(cycloartenol synthase)	Unigene0038300	36.3476	87.6244	↑
	CPI1(cyclopropyl sterol isomerase1)	Unigene0033621	5.104	12.0916	↑
	DIM(delta(24)-sterol reductase)	Unigene0048260	46.8104	171.8682	↑
	DWF5(7-dehydrocholesterol reductase)	Unigene0030355	22.8576	140.7592	↑
	3β-HSD(3β-hydroxysteroid decarboxylase)	Unigene0036411	0.1364	0.9741	↑
	C-22 hydroxylase(cytochrome P450-90B1)	Unigene0037699	59.1627	362.3193	↑
	C-23 hydroxylase(cytochrome P450-90D2)	Unigene0041552	132.8696	55.9507	↓
	C-26 hydroxylase(cytochrome P450-734A6)	Unigene0040793	25.2658	57.0467	↑
Hormone	AUX1(gibberellin receptor GID1C)	Unigene0028406	0.001	19.3756	↑
	ARF(auxin response factor 11)	Unigene0028137	0.001	2.9979	↑
	GH3(indole-3-acetic acid-amido synthetase)	Unigene0033714	1.0939	8.6828	↑
	CYCD3(cyclin-D3)	Unigene0038335	4.0042	31.6655	↑
	TCH4(xyloglucan endotransglucosylase)	Unigene0020230	0.157	3.0823	↑
	MYC2(transcription factor MYC2)	Unigene0030546	2.8326	15.746	↑

of *F. cirrhosa* steroidal alkaloids was proposed by analyzing the chemical structures of different types of steroid alkaloids and the function of their biocatalytic enzymes in the functionalization of steroid skeleton (Fig. 4).

To validate the above proposed *F. cirrhosa* steroidal alkaloid biosynthetic pathways and establish a complete expression profile for genes involved in this process, we first extracted 6 and 8 unigenes annotated as different enzyme-coding genes from the MEP and MVA pathways, (Table 2). As showed in Table 2, expression levels of unigenes annotated as mevalonate kinase (MK) and diphosphomevalonate decarboxylase-like (MVD) of the MVA pathway were down-regulated, while unigene annotated as phosphomevalonate kinase (PMK) was up-regulated in the RB. For the MEP pathways, the expression levels of the majority unigenes (6 in 8) were significantly up-regulated in the RB. Therefore, unlike MVA pathway-associated unigenes that showed mixed expression trend, the expression of unigenes associated with MEP pathways showed unilateral upward trend comparing the WB and RB. These results were consistent with the qRT-PCR analysis of the MEP and MVA pathway genes identified (Fig. 5a). A previous study showed that the steroidal backbones were synthesized via the MVA pathways, and not via MEP pathways in plants (Suzuki and Muranaka 2007). While our current results are not sufficient to draw a conclusion regarding *F. cirrhosa* steroid synthesis, our data at least suggested that MEP pathway is the main route to the production of steroidal backbones for this particular plant. It is critical to confirm this result in future studies to understand *F. cirrhosa* sterol alkaloid biosynthesis comprehensively. Using chemical inhibitors to the rate-limiting enzymes on the two pathways might provide valuable information to answer this question (Bach and Lichtenthaler 1982; Zeidler et al. 1998).

Meanwhile, all the 11 enzyme-coding genes (downstream pathway) known to be involved in the alkaloid downstream biosynthetic pathways were identified based on the functionalization of steroidal backbones (Table 2) (Kutchan et al. 2008). Among these enzymes, cyclase, oxidases, isomerases, hydroxylase and aminotransferases may take part in the conversion of cholesterol to sterol alkaloids. The sterol alkaloid biosynthesis starts with squalene oxidation and cyclization by different forms of (S)-2, 3-oxidosqualene cyclase. CAS catalyzes the biosynthesis of cycloartenol, which serves as a substrate for the production of phytosterols (Kutchan et al. 2008). Cytochrome P450s (CYP450) perform the multiple

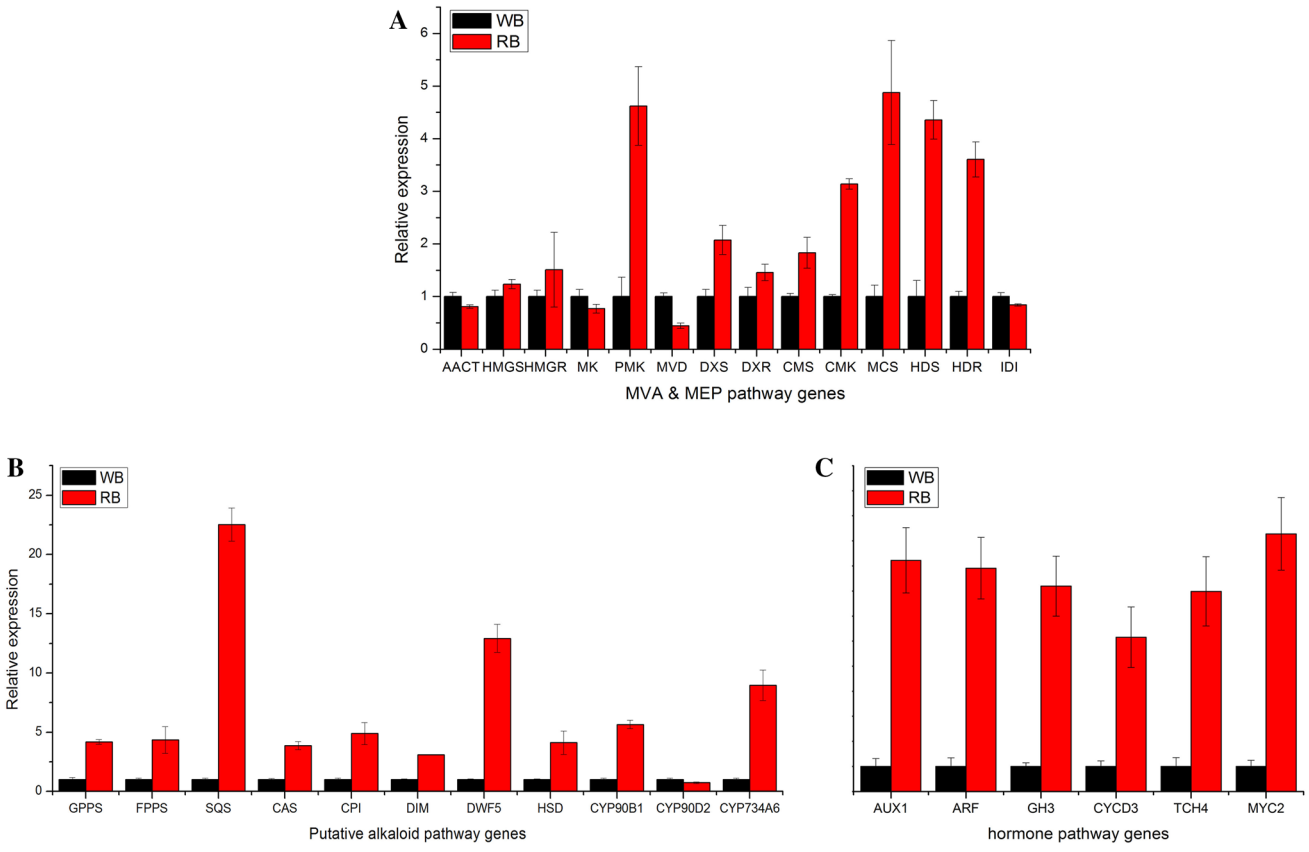

Fig. 5 qRT-PCR analysis of the expression levels of MVA & MEP-related (**a**), putative alkaloid biosynthesis-related (**b**) and hormone signal transduction-related (**c**) genes

hydroxylation reactions of a wide variety of natural compounds, which is an important mechanism in secondary metabolism (Morant et al. 2003).

In the present study, out of 11 unigenes which were reported to be the rate-limiting enzymes in sterol biosynthesis (Morant et al. 2003; Suzuki and Muranaka 2007) showed up-regulated trend in the RB except for the cytochrome P450-90D2 (Table 2, Fig. 6). The upregulation of these genes were in parallel with the qRT-PCR results (Fig. 5b), and coinciding with the increased accumulation of alkaloid in the RB. There may be a positive correlation between high expression of the genes for the alkaloid downstream biosynthetic pathways and relatively higher accumulation of alkaloids in vitro bulbs. As the RB was induced by cytokinin/auxin combination, we further compared the expression level of six genes participating in plant hormone signal transduction between WB and RB (Table 2). The expression of these genes was significantly higher in RB than WB (Figs. 5c, 6). The expression level of transcription factor MYC2, which is responsible for regulating ORCA gene expression, which further affects the expression of a cascade of alkaloid biosynthesis genes (Zhang et al. 2011), was up-regulated in RB according to the RNA-seq and qRT-PCR results. Therefore, in *F. cirrhosa*, a series of genes required for the hormone signal transduction may activate some undetermined pathway (MVA or MEP) involved in steroidal alkaloid biosynthesis and finally affect the accumulation of major phytochemicals such as peimine, imperialine, and verticine.

Conclusion

The current work demonstrated that the *F. cirrhosa* bulbs could be regenerated from callus induction with cytokinin/auxin combination and RB showed higher alkaloid accumulation than the wild bulbs. Our transcriptome analysis identified 113,865 unique genes. Among these unigenes, KEGG pathway annotation identified genes related to "Metabolic pathways" were the most abundant (2644, 26.0%), followed by those for "Biosynthesis of secondary metabolites" (1319, 13.0%). Further analysis suggested MEP pathway might be the one responsible for the steroidal alkaloids biosynthesis of *F. cirrhosa,* as all the key genes in this pathway were found to be unregulated in our study. We also showed that accumulation of different phytochemicals was linked to plant hormone addition. Altogether, our work provided valuable information on the gene expression profile of in vitro-generated *F. cirrhosa* and led to a proposal regarding the steroidal alkaloid biosynthetic pathway. This work demonstrated that the in vitro cultivation is a promising strategy for mass production of *F. cirrhosa* steroidal alkaloids for pharmacological industry.

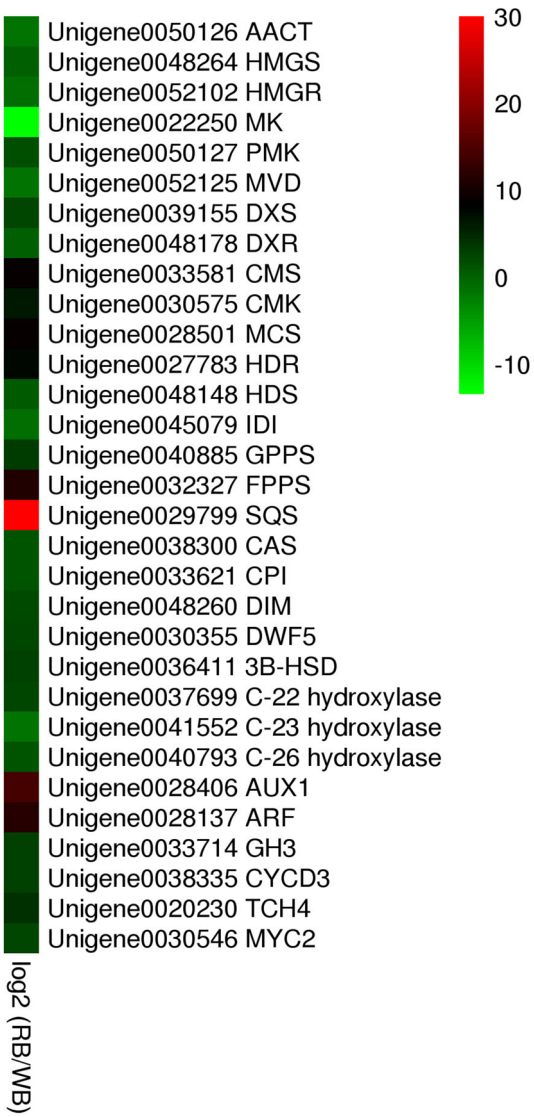

Fig. 6 Heat map analysis of the expression levels of MVA & MEP-related (**a**), putative alkaloid biosynthesis-related (**b**) and hormone signal transduction-related (**c**) genes

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Author contributions Conceived and designed the experiments: WGW, JL. Performed the experiments: QZ, RL, KJH, YZ. Contributed reagents/materials/analysis tools: WGW, JL. Wrote the paper: QZ, RL, WGW, JL. All authors read and approved the final manuscript.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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