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Generation of expressed sequence tags from a cDNA library of *Coleus forskohlii* for identification of genes involved in terpene biosynthesis

Y. FANG[1,2], J. HUANG[1], X. HUANG[1], S.H CHEN[2], P.C. ZOU[3], W.S. LI[3], K. YU[1,2,3]*, and Y.W. LIU[1,2]*

College of Pharmacy, Hubei University of Chinese Medicine, Wuhan 430065, P.R. China[1]
Key Laboratory of Traditional Chinese Medicine Resource and Chemistry, Hubei University of Chinese Medicine, Wuhan 430060, P.R. China[2]
Postdoctoral Research Center, Furen Pharmaceutical Co., Tongcheng 437405, P.R. China[3]

Abstract

Coleus forskohlii (syn. *Plectranthus barbatus*) is a widely used medicinal plant and its main bioactive constituents are diterpenes forskolin and isoforskolin. The present study aimed to construct a cDNA library to identify expressed sequence tags related to terpene biosynthesis in *C. forskohlii*. We constructed a high quality normalized full-length cDNA library which reached the requirements (abundance, integrity, and library content) for isolating full-length genes. A total of 4 224 cDNA clones were sequenced and 2 394 unigenes were assembled with an average unigene size of 753 bp. A total of 2 100 (87.7 %) unigenes were functionally classified using gene ontologies, and 1 716 (71.7 %) unigenes were assigned to establish pathway associations in *KEGG* mapings. Notably, 64 unigenes putatively participated in the biosynthesis of secondary metabolites, in which 17 unigenes were identified that might be involved in the biosynthesis of the terpenoid backbone and monoterpenes, diterpenes, and triterpenes.

Additional key words: forskolin, isoforskolin, terpene backbone.

Introduction

Coleus forskohlii is a perennial plant in the tropical and subtropical regions of India, Pakistan, Sri Lanka, China, south of Arabian Peninsula, east Africa, and Brazil with diverse medicinal uses (Lukhoba *et al.* 2006, Alasbahi and Melzig 2010a, Kavitha *et al.* 2010, Falé *et al.* 2012). Although forskolin, known as one of the main bioactive constituents of *C. forskohlii*, has not been isolated from the plants distributed in China (Xu and Kong 2004, Alasbahi and Melzig 2010b), another diterpenoid isoforskolin was identified for its potential biological effects (Bhat *et al.* 1983, Jin *et al.* 1990, Tian *et al.* 2011).

Although plant diterpenes have more than 12 000 different structures, they are all formed from geranylgeranyl diphosphate (GGPP) (Peters 2010). The precursors of GGPP, isopentenyl diphosphate and dimethylallyl diphosphate, are produced from the plastidal methylerythritol-4-phosphate (MEP) pathway and the cytosolic mevalonic acid (MVA) pathway in plants, with the former pathway prevailing (Chen *et al.* 2011). Diterpene synthases (diTPSs) catalyze the critical steps from GGPP to various diterpenes. Forskolin and isoforskolin belong to the labdane diterpenoids whose biosynthesis requires two monofunctional diTPSs. Class II diTPS catalyzes the protonation-initiated cyclization of GGPP to bicyclic diphosphate intermediates. Class I diTPSs then catalyze ionization-dependent cyclization and additional rearrangement reactions (Chen *et al.* 2011, Pulido and Perello 2012). Diterpene skeletons produced by diTPSs often undergo functional modification, such as oxygenation, through the activity of cytochrome P_{450}-dependent monooxygenases (Urlacher and Girhard 2012, Zerbe *et al.* 2013).

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Abbreviations: diTPS - diterpene synthase; EST - expressed sequence tag; GGPP - geranylgeranyl diphosphate; *GO* - gene ontology; *KEGG* - Kyoto encyclopedia of genes and genomes; MEP - methylerythritol-4-phosphate; MVA - mevalonic acid.
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* Corresponding authors; fax: (+86) 27 88920834, e-mail: yukun_hbtcm@163.com; fax: (+86) 27 8892083, e-mail: ywliu2008@163.com

Expressed sequence tags (ESTs) represent a snapshot of cellular transcripts in a specific tissue and/or at a specific developmental stage. Now ESTs have become a valuable tool for various genome-scale experiments to identify functional genes and reveal gene expression patterns, and can be exploited for functional genomics and comparative genomics research in a wide range of organisms (Rudd 2003, Pashley *et al.* 2006). The efficiency and economy of EST sequencing can be greatly enhanced by normalization of a cDNA library which enriches rare genes and reduces the number of abundant genes (Carninci *et al.* 2000). A normalized full-length cDNA library would be highly desirable, since EST sequencing is carried out for the purpose of gene cloning, and extensively used for gene identification (Ling *et al.* 2007).

To date, little genomic information on *C. forskohlii* is known. Mechanisms underlying the biosynthesis of diterpenes, especially forskolin or isoforskolin, remain unresolved though three enzymes (GGPP synthase, 1-deoxy-D-xylulose-5-phosphate reductoisomerase, and ent-kaurene synthase), and five diTPSs (CfTPS1-4 and CfTPS14) involved in terpene backbone and diterpene skelelon biosynthesis have been characterized from *C. forskohlii* (Engprasert *et al.* 2004, 2005, Zerbe *et al.* 2013, Pateraki *et al.* 2014). There is no report on cDNA library construction and the EST generation of *C. forskohlii*. The current study was designed to *1*) construct a normalized full-length cDNA library from *C. forskohlii*, *2*) sequence cDNA and preliminarily analyze ESTs, and *3*) identify putative genes involved in secondary metabolism, especially terpenoid biosynthesis.

Materials and methods

Coleus forskohlii Briq. (*Lamiaceae*, syn. *Plectranthus barbatus* Andr.) seeds originally collected from Huize, Yunnan Province, were sowed in an experimental field located in Tongcheng, Hubei Province. Six months after sowing, roots, rhizomes, stems, and leaves from five plants were harvested, washed, surface dried, and immediately frozen in liquid nitrogen, and then stored at -80 °C until further use.

Equal amounts of root, rhizome, stem, leaf, and inflorescence tissues (approximately 80 mg) were pooled together for RNA extraction using *Trizol* reagent (*Invitrogen*, Carlsbad, CA, USA) according to the manufacturer's instructions and subjected to a spectrophotometric analysis (*NanoDrop ND-1000*, *Thermo Fisher Scientific*, Waltham, USA).

Construction of a full-length normalized cDNA library was performed as described by Gao *et al*. (2011) with slight modifications. Full-length double-stranded cDNA was synthesized by long-distance PCR using the *SMART*™ technology (*Clontech*, Palo Alto, CA, USA) following the manufacturer's instruction. The cDNA normalization was performed using *Duplex*-specific nuclease according to the manufacturer's protocol (*Evrogen*, Moscow, Russia), then purified using a *QIAquick* PCR purification kit (*Qiagen*, Valencia, CA, USA), and ligated into a pDNR-LIB vector. For a quality control experiment, 30 clones from the cDNA library were randomly selected, and the insert fragments were amplified using M13 forward (GTAAAACGA CGGCCAGT) and M13 reverse (CAGGAAACAGCT ATGACC) primers. The PCR products were then analyzed by agarose gel electrophoresis.

For ESTs generation, cDNA clones were picked randomly and subjected to 5'-end single-pass sequencing using an *ABI 3730xl* DNA analyzer (*Applied Biosystems*, Foster City, CA, USA) in the Beijing Genomics Institute (Beijing, China). Sequence data were processed using *Phred*, *Phrap*, and *Cross_Match* software packages to obtain unigenes (Ewing and Green 1998, Ewing *et al*. 1998).

The *Blast2Go* program was used to functionally categorize unigenes to gene ontology (*GO*) terms (Conesa *et al*. 2005). Biochemical pathway assignments were performed by Kyoto Encyclopedia of Genes and Genomes (*KEGG*) mapping (http://www.genome.jp/kegg/kegg2.html).

Results

The mixture of various tissues from *C. forskohlii* plants was used as starting material for cDNA library construction. Total RNA electrophoresis on an agarose gel (1.2 %, m/v) showed distinct 28S and 18S ribosomal RNA bands (Fig. 1*A*). Ratios of absorbances A_{260}/A_{280} and A_{260}/A_{230} of total RNA were 2.01 and 2.13, respectively, indicating little contamination with polysaccharides and proteins. The double-strand cDNA was amplified by long-distance PCR and appeared as a 0.5 - 3 kb smear (Fig. 1*B*). The titer of the original library was approximately 1.6×10^6 cfu cm^{-3} with a recombination percentage of 93.05 %. The quality of the resulting library was assessed by electrophoresis of the PCR products of randomly picked 30 clones. The insert size of cDNAs ranged from 1 to 3 kb suggesting relatively long cDNAs contained in the library (Fig. 1*C*).

A total of 4 224 cDNA clones were randomly selected and sequenced from the 5' end by the Sanger dideoxy-

termination method, and 3 263 high-quality ESTs were obtained after base calling and vector sequence removal with an average length of 580 bp (Table 1). All 3 263 EST sequences were deposited in GenBank with accession numbers of JZ729631 to JZ732893. A total of 2 394 unigenes including 486 contigs (20.3 %) and 1 908 singletons (79.7 %) were assembled with an average size of 753 bp. Most (79.7 %) of the unigenes were comprised of only one EST suggesting a relatively low redundancy rate of the normalized cDNA libraries (Table 2). The unigenes were submitted to the *TSA* database of Genbank at *NCBI* with an accession number of GBTO00000000.

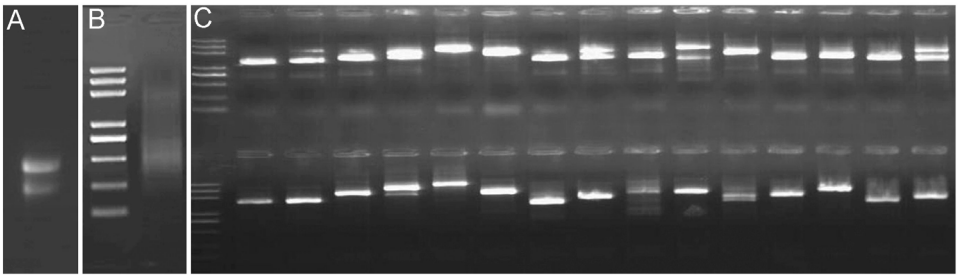

Fig. 1. Construction of the cDNA library of *C. forskohlii*: *A* - agarose gel (1.2 %, m/v) electrophoresis of total RNA; *B* - agarose gel electrophoresis of long-distance PCR products; *C* - 30 clones of the cDNA library selected randomly to evaluate their insert sizes. DNA size markers from 5000 to 100 bp are shown.

Table 1. Overview of EST sequencing from the cDNA library of *C. forskohlii*.

Description	Number/length
Number of clones sequenced	4224
Number of high-quality ESTs	3263
Average length per EST [bp]	580
Number of unigenes	2394
Number of contigs	486
Number of singletons	1908
Average unigene length [bp]	753

The unigenes were then annotated with *GO* terms using the *Blast2GO* program, and 2 100 (87.7 %) unigenes were assigned to one or more *GO* terms. A total of 12 121 *GO* terms were extracted and classified in three main *GO* categories, *i.e.*, a biological process, a molecular function, and a cellular component (Table 1 Suppl.). In the main category of a biological process, subcategories (second level *GO* terms) of metabolic processes (*GO*:0008152, 52.4 %) and cellular processes (GO:0009987, 53.3 %) accounted for large proportions. For metabolic processes, 14 subcategories (third level *GO* terms) were assigned with cellular metabolic processes (*GO*:0044237, 42.7 %) and primary metabolic processes (*GO*:0044238, 42.3 %) showing dominance. Among these subcategories, the secondary metabolite processes (*GO*:0019748) accounted for 3.1 % of the annotations. Under the main category of a molecular function, a subcategory of a catalytic activity (*GO*:0003824, 44.9 %) and binding (*GO*:0005488, 45.5 %) were mostly assigned. For the cellular component class, the most evident matches were within a cell (*GO*:0005623, 54.8%) and an organelle (*GO*:0043226, 40.2 %).

The unigenes were subjected to analysis of biochemical pathways using *KEGG* mapping. A total of 1 716 (71.7 %) unigenes were assigned to six main *KEGG* biochemical pathways including metabolism (704 unigenes), genetic information processing (205 unigenes), environmental information processing (177 unigenes), cellular processes (128 unigenes), organismal systems (260 unigenes), and human diseases (329 unigenes) (Table 2 Suppl.). A total of 64 unigenes were included in the biosynthesis of secondary metabolites (Table 3 Suppl.). Sugar metabolism, infectious diseases, signal transduction, and amino acid metabolism were the 4 most represented pathways (> 100 unigenes).

Table 2. Distribution of unigenes.

EST number of each unigene	Number of unigenes	Percentage of total unigenes
1	1908	79.70
2	316	13.20
3	99	4.14
4-5	47	1.96
6-10	19	0.79
11-20	4	0.17
21-50	1	0.04
51-100	0	0
>100	0	0
Maximal unigene	22	

Eleven identified unigenes, corresponding to 11 EC numbers (2.3.1.9, 1.1.1.34, 2.7.1.36, 2.2.1.7, 1.1.1.267, 1.17.7.1, 5.3.3.2, 2.5.1.1, 2.5.1.10, 2.5.1.29, and 2.1.1.100) were possibly involved in terpenoid backbone biosynthesis according to *KEGG* mapping (Table 3).

Among enzymes identified in the cDNA library, three enzymes were involved in diterpene biosynthesis (ent-copalyl diphosphate synthase, ent-kaurenoic acid hydroxylase, and CYP82G1), two enzymes were monoterpene synthases (myrcene/ocimene synthase and neomenthol dehydrogenase), and one enzyme (β-amyrin synthase) was involved in triterpenoid biosynthesis (Table 3).

Table 3. List of identified unigenes possibly involved in terpene biosynthesis according to KEGG mapping in the cDNA library of *C. forskohlii*.

Enzyme name	EC number	Unigene number
Terpenoid backbone biosynthesis		
Acetyl-Coa C-acetyltransferase	2.3.1.9	1
Hydroxymethylglutaryl-Coa reductase	1.1.1.34	2
Mevalonate kinase	2.7.1.36	1
1-deoxy-D-xylulose-5-phosphate synthase	2.2.1.7	1
1-deoxy-D-xylulose-5-phosphate reductoisomerase	1.1.1.267	1
(*E*)-4-hydroxy-3-methylbut-2-enyl-diphosphate synthase	1.17.7.1	2
Isopentenyl-diphosphate delta-isomerase	5.3.3.2	1
Geranylgeranyl diphosphate synthase	2.5.1.1, 2.5.1.10, 2.5.1.29	1
Protein-S-isoprenylcysteine *o*-methyltransferase	2.1.1.100	1
Monoterpene biosynthesis		
Myrcene/ocimene synthase	4.2.3.15	1
Neomenthol dehydrogenase	1.1.1.208	1
Diterpene biosynthesis		
Ent-copalyl diphosphate synthase	5.5.1.13	1
Ent-kaurenoic acid hydroxylase	1.14.13.79	1
CYP82G1	1.14.-.-	1
Triterpene biosynthesis		
β-amyrin synthase	5.4.99.39	1

Discussion

As the only known natural source of forskolin and isoforskolin, *C. forskohlii*, has attracted a high interest because of diverse medicinal uses of its bioactive constituents. Due to the scarcity of genomic information, the identities of relevant enzymes participating in the biosyntheses of forskolin and isoforskolin are largely unknown. In this study, we constructed a normalized full-length cDNA library and used genomics and bioinformatics approaches to generate EST sequences and identify putative genes involved in terpene biosynthesis in *C. forskohlii*.

Construction and analysis of cDNA libraries are regarded as indispensable tools for a functional genomic analysis (Shao *et al.* 2009). Well-established cDNA libraries are frequently used to obtain target genes by PCR, Southern blot, or direct sequencing in various plants, *e.g.*, *Coleus blumei* (Kim *et al.* 2004), *Salvia sclarea* (Günnewich *et al.* 2013), and *Capsicum annuum* (Aza-González *et al.* 2013). The cDNA library of *C. forskohlii* constructed in the present study reached the quality index (abundance, integrity, and library content) for isolating full-length genes (Fig. 1, Table 1). The ESTs were then generated by single-pass sequencing random cDNAs from the cDNA library, which has been proven to be an efficient way of gaining information about gene expression in an organism (Wu *et al.* 2002, Hampton *et al.* 2010). Most of the assembled unigenes were functionally categorized using the *GO* and *KEGG* databases, with many of them participating in biosyntheses of secondary metabolites. These results allow us to take a glimpse of the overall picture of gene expression and facilitate a further research on the diterpene biosynthesis in *C. forskohlii*.

The secondary metabolism of *C. forskohlii* has now attracted a considerable interest (Alasbahi and Melzig 2010b). Forskolin and isoforskolin, the main bioactive constituents of *C. forskohlii*, belong to the diterpenoids whose biosynthetic pathway was made up of the terpenoid backbone biosynthesis [PATH:ko00900] and diterpenoid biosynthesis [PATH:ko00904] according to the *KEGG* pathway. A total of 11 unigenes, corresponding to 11 EC numbers, were identified and they are possibly involved in the formation of geranyl diphosphate and geranylgeranyl diphosphate which are the substrates of terpene biosynthase (Table 3). Previously reported GGPP synthase and 1-deoxy-D-xylulose-5-phosphate reductoisomerase belong to MEP and MVA pathway enzymes (Engprasert *et al.* 2004, 2005). Zerbe *et al.* (2013) recently performed transcriptome sequencing for *C. forskohlii* using high-

throughput *Illumina* and *454* sequencing platforms and identified 17 genes involved in terpenoid backbone biosynthesis by mapping the assemblies against these pathways from the *KEGG* database. They also reported a class I diTPS gene (*ent-kaurene synthase*). Five more diTPSs were further identified by database mining (Pateraki *et al.* 2014). We identified a diTPS candidate gene (*ent-copalyl diphosphate synthase*) which belongs to class II diTPS and catalyzes the formation of copalyl pyrophosphate, the substrate of ent-kaurene synthase.

Essential oil from leaves, stems, and roots of *C. forskohlii* contains nearly 100 components (Muhayimana *et al.* 1998, Kerntopf *et al.* 2002). Our results suggest the candidate genes of monoterpene synthase which might encode enzymes catalyzing the formation of essential monoterpene oil. The β-amyrin synthase is a key biosynthetic enzyme for oleanane-type triterpenoids (Yendo *et al.* 2010). A number of triterpenoids has been isolated from different tissues of *C. forskohlii* (Alasbahi and Melzig 2010a). The β-amyrin synthase gene presented herein will provide useful information for elucidating the biosynthetic pathway of triterpenoids in *C. forskohlii*.

In conclusion, the normalized full-length cDNA library which contained the most diverse genes associated with secondary metabolism was constructed from various organs of *C. forskohlii*. This study also presents a total of 3 263 ESTs and 2 394 unigenes which provides an insight in the transcriptome of this valuable medicinal plant. The large number of ESTs and unigenes will be useful resources for a functional gene analysis and molecular marker development. Many genes involved in secondary metabolism, especially terpene biosynthesis, were identified, which will contribute to uncovering the mechanisms of the biosynthesis of forskolin and isoforskolin and serve as valuable resource for genetic engineering to increase the diterpene content of *C. forskohlii*. Further studies should be performed to test the expression of candidate genes to prove their catalytic activity, identify more genes involved in biosynthesis, transport, and metabolism of forskolin or isoforskolin, and thus elucidate the underlying molecular mechanisms.

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