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高山植物黄管秦艽 cDNA 文库构建与表达序列标签 (EST) 分析

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摘要：黄管秦艽 (*Gentiana officinalis*) 是一种重要的藏药高山植物，本研究构建了该物种开花期的 cDNA 文库。经检测达到中等 cDNA 文库水平，文库滴度为 1.2×10^7 pfu ml，重组率 95.9%，插入片段平均长度大于 500 bp。对 343 个随机挑选的重组克隆进行部分测序，获得的 ESTs 经编辑后共有 181 条有效序列。经生物信息学方法分析 181 条表达序列标签 (EST) 代表 144 个单克隆序列，其中 55 个与已鉴定的基因同源，35 个序列与未鉴定的 EST 匹配，54 个未找到同源序列；后两者共有 89 个 EST 序列未发现功能相似的蛋白。对已鉴定的 EST 进行功能分析发现，相关基因主要编码以下蛋白：与蛋白表达相关的占 35%；光合作用相关的占 22%；新陈代谢相关的占 18%；抗性相关的占 11%；质膜运输和细胞分裂相关的分别占 5%；染色体变化和细胞信号转导的分别占 2%。根据有效 EST 序列设计引物，通过 RT-PCR 进一验证了所得 EST 的准确性。这些研究结果为将来研究黄管秦艽的功能基因以及该物种与相关物种的群体遗传学、进化生物学等方面提供了基础。

关键词：黄管秦艽；基因组学；cDNA 文库；表达序列标签；龙胆属
中图分类号：Q 943 **文献标识码**：A **文章编号**：0253-2700 (2009) 02-146-07

cDNA Library Construction and Expressed Sequence Tags (ESTs) Analyses of an Alpine Plant Species *Gentiana officinalis* (Gentianaceae)

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Abstract: *Gentiana officinalis*, an alpine plant, one of the widely used Tibetan traditional medicines. In this study, total RNA was extracted from the whole plant of flowering individuals of this species, and a cDNA expression library was constructed using Creator™ SMART™ cDNA Library Construction Kit. The results showed that the titer of the cDNA expression library was 1.2×10^7 pfu ml and the efficiency of recombination was 95.9%. The average length of insert fragments in the library was longer than 500 bp. A total of 181 valid ESTs were obtained from random sequencing of 343 clones. Further bioinformatic analyses suggested they represented 144 unique clonal sequences in which 55 sequences showed high homology to previously identified genes in Gentianaceae or other plants, 35 sequences matched to other uncharacterized expressed sequence tags (ESTs), and 54 sequences showed no well matches to available sequences in DNA databases. No protein matched to the latter two sorts of ESTs (89). Fifty-five ESTs with matched proteins were involved in a series of diverse functions: protein expression (35%), photosynthesis (22%), metabolism (18%), defense (11%), membrane transport (5%), cell division (5%), chromosome metabolism (2%) and signaling components (2%). At last, RT-PCR primers were designed according to the effective ESTs to amplified the cDNAs of *G. officinalis*, which further verified the accuracy

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of the ESTs. This cDNA library provided a critical basis for further analyses of functional genes and gene expression in this alpine species. In addition, these ESTs could be used to design functional nuclear primers for studying population genetics of this species and closely related species.

Key words: *Gentiana officinalis*; Genomic Research; cDNA library; Expressed Sequence Tags; *Gentiana*

Gentiana (Smith, 1936), a large genus in the Gentianaceae, is widely distributed in the high mountains in the temperate regions of the world (Ho and Liu, 2001). Many species of this genus have been used as traditional Chinese medicines and or Tibetan medicines (Pharmacopoeia commission of PRC, 2000), for example, "Long Dan" and "QingJiao". These medicines were used to stimulate digestion and appetite and relieve heartburn and stomach (Van der Sluis *et al*., 1983; Tang and Eisenbrand, 1992). The main chemical constituents in these plants are comprise gentiopicroside and swertiamarin (Skrzypczak *et al*., 1993). Despite the diverse researches on the resource, taxonomy and evolution of the genus (Adams, 1995; Ho, 1985, 1988; Ho and Liu, 2001), nuclear genes of the genus received little attention. A cDNA library and expressed sequence tags (ESTs), through sequencing of randomly selected cDNA clones from cDNA library, provide an important basis for further functional analyses of nuclear genes within one species (Ewing *et al*., 1999, 2000; Nelson *et al*., 2000). In addition, EST-derived nuclear markers can be developed from such ESTs and used to evaluate genetic diversity, interspecific relationships of plants (Ma *et al*., 2006; Zhou *et al*., 2007).

In this study, we aimed to construct cDNA library and analyze ESTs characteristics of *G. officinalis*. This species occurs in the high altitude region and has been used as one of traditional Tibetan medicine. To our knowledge, this is the first time to construct a cDNA library and sequence ESTs for one alpine gentiana species.

1 Materials and Methods

1.1 Plant materials

The flowering individuals of *G. officinalis* were used to exact the total RNA. Fresh leaves, flowers and roots were collected together and dehydrated in liquid nitrogen, then stored at -80℃ for further RNA extraction.

1.2 RNA extraction and cDNA library construction

Total RNA was extracted using Trizol Reagent Kit (Molecular Research Center, Inc. USA). The following procedure was performed according to the manufacturer's recommendation of the Creator™ SMART™ cDNA Library Construction Kit (Clontech, Mountain View, CA). At first, using SMART technique, CDS III 3' primer was used to synthesize the first-strand cDNA. Long distance polymerase chain reaction (LD PCR) was used to synthesize the double-strand cDNA that was then digested by *Sfi*I and fractionated by CHROMA SP IN-400 Column. The cDNAs are longer than 0.5 kb were collected and ligated to pBNR-LIB vector. The recombinant plasmids transformed into *E. coli*-DH5α. The quality of the cDNA library was strictly checked by conventional titer determination. Twenty-one plaques were randomly picked and tested using PCR with universal primers-M13 derived from the sequence flanking of the vector.

1.3 Sequence assembly, alignment and analysis

Clones for sequencing were selected randomly from the cDNA library, and each clone was incubate at 37℃ in 1.5 ml of LB broth overnight with shaking. Plasmid DNAs of cDNA clones for sequencing were isolated with the standard alkaline lyses protocol using the Mini-plasmid kit (U-gene). The cDNA inserts were subjected to single-pass partial sequencing from the 5′ end by employing the 5′ end sequencing primer and ABI chemicals on ABI 3730 DNA sequencers (Shanghai Bioasia, PRC). We used to MEGA4.0 (Borland, America) to analyze the EST sequence. Each EST was firstly analyzed using a multimodule custom pipeline which linked sequence backup, base calling, the elimination of sequences shorter than 100 bp (and low-quality sequences), vector trimming, and sequence assembly. The resulting unisequences were compared against the nonredundant (nr) protein database at the protein level by using BLASTx with default parameters. In general, similarities with E-values $< 10^{-5}$ were considered significant. Unisequences displaying no significant similarity to known genes (BLASTx E-values $> 10^{-5}$) were searched against the dbESTest-others (non-mouse, non-human) using the tBLASTx algorithm.

1.4 RT-PCR confirmation

Reverse transcription (RT) was performed based on 2 μg of the previously isolated total RNAs of *G. officinalis*. The cDNAs were synthsized accroding to the Reverse Transcriptase M-MLV ($RNase\ H^-$) Kit (Takara Biotechnology Co., Ltd). To validate the accuracy of the ESTs, RT-PCR was performed using cDNAs as templates and a specific pair of primers designed for each selected gene. The amplification conditions were 1 cycle for 2 min

at 94℃, and 25 cycles of 94℃ for 30 s, 52℃ for 1 min, and 72℃ for 1 min 30 s. PCR products were electrophoresed on a 1.1% agarose EtBr gel.

2 Results and Discussions

2.1 Extraction and purification of total RNA

The ratio of OD260 OD280 of total RNA was 1.98. Then, the integrity of the total RNA was analyzed by agarose gel electrophoresis (Fig. 1). According to Fig. 1, the bands of 28S and 18S were obvious and the brightness of the band of the 28S was about twice of the 18S.

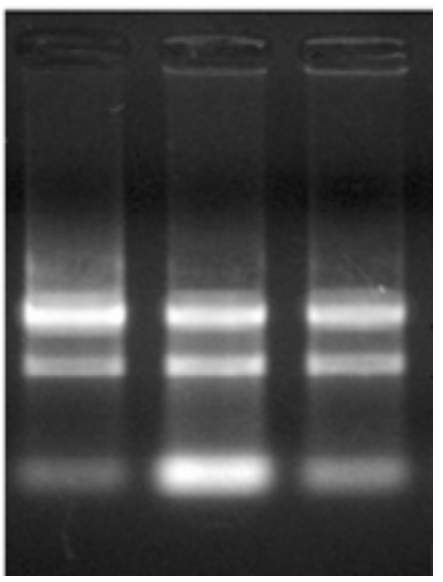

Fig. 1 Total RNA of *Gentiana officinalis*

2.2 cDNA synthesis

Using 2 μg RNAs, first-strand cDNAs were synthesized. Then, 1 5 of the 1st cDNA were used to synthesize the ds cDNAs. After 22 thermal cycles, 5 μl of 100 μl was analyzed by agarose gel electrophoresis (Fig. 2). The bands of ds cDNAs were dispersed and the length of ds cDNA was mainly bounded on 500 - 5000 bp, indicating the well quantity of the ds cDNA. This method was also previously showed to obtain a higher percentage of full-length cDNA.

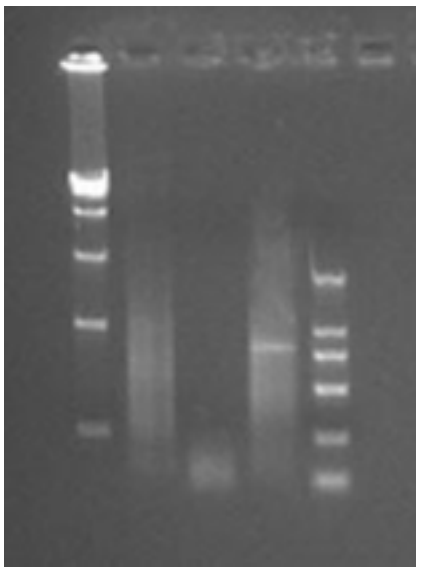

Fig. 2 ds cDNA synthesized using the SMART control reagents

2.3 The quantity of the cDNA library

The library titer was measured as 1.2×10^7 pfu ml. In order to analyze the size of the constructed library and the diversity of cDNA inserts, 21 plaques were randomly selected, and amplified with primers-M13F R (synthesised by TaKaRa Company, sense sequence: 5′- G TAAAACGACGGCCAGT - 3′ antisense 5′- AACAGCTATGACCATG - 3′) following the program: 94℃ 5 min; 94℃ 30 s, 52℃ 30 s, 72℃ 1 min for 35 cycles; 72℃ 10 min. PCR products were checked by the DNA markers (Fig. 3). The percentage of recombinants from the library was 95.9%. The average length of the inserts was 900 bp. All these analyses indicate that RNAs in the cDNA library are well represented.

2.4 General characteristics of ESTs

A total of 343 cDNA clones were selected randomly from the library and single-pass sequences were generated. After excluding those poorly sequenced and or with less than 100 bases, a total of 181 ESTs were obtained. Contigs that consist of one sequence were considered singletons, while contigs comprised of two or more sequences were classified as redundant ESTs or contigs.

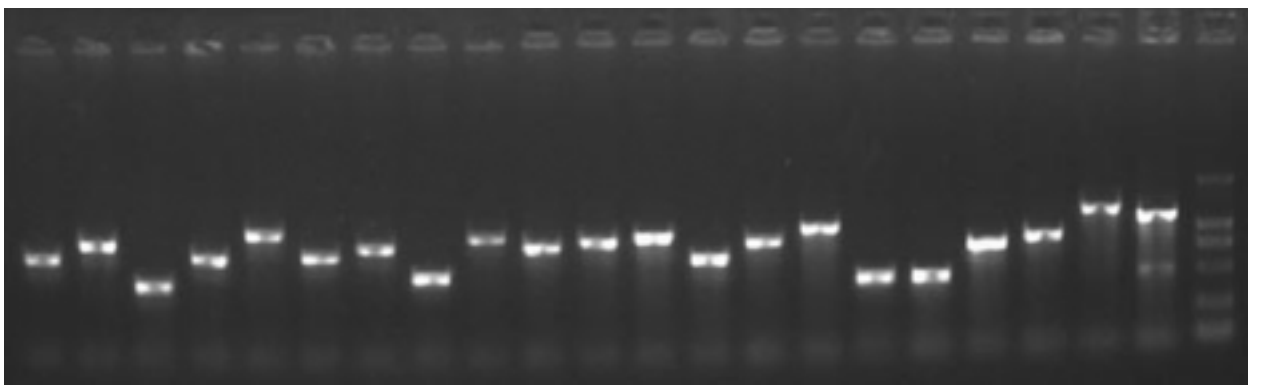

Fig. 3 Composition of cDNA fragments in the libraries

These EST sequences were clustered in 20 contigs and 124 singletons . Since the cDNA library has not been amplified and the clones for sequencing have not been subtracted, the number of ESTs basically reflect the prevalence of the corresponding mRNA (Fig . 4) .

Based on matches with available data (Table 1), among 144 unique sequences, 55 showed homology to previously identified genes in Gentianaceae or other plants, 35 matched other uncharacterized expressed sequence tags (ESTs), and 54 showed no significant matches to sequences present in DNA databases . The latter two classes of ESTs (together 89 sequences) also showed no corresponding protein match . Further analyses of the ESTs with the matched proteins showed they

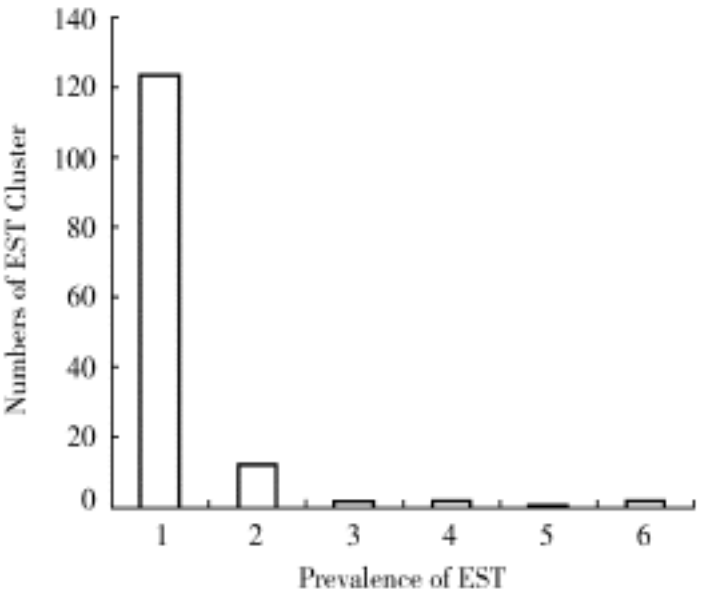

Fig . 4　Prevelence distribution of identified ESTs
(number of singletons is 124; numbers of contigs with two, three, four, five and six sequences are 12, 2, 2, 1, 2, respectively)

Table 1　Database match of *Gentiana officinalis* ESTs to the genes of the other organisms

GenBank accessions	Function	Score (bits)	E-Value	Closest Species	Copies
Cell division					
AY611040	NAM protein	284	3.00E-75	*Picea glauca*	1
AY639034	auxin-induced putative CP12 domain-containing protein	72.1	1.00E-10	*Arachis hypogaea*	1
BK000123	putative phytosulfokine peptide precursor	95	4.00E-17	*Solanum lycopersicum*	1
Chromosome metabolism					
EF520004	sister chromatid cohesion 2	104	6.00E-20	*Arabidopsis thaliana*	1
Defense					
AY173073	catalase	408	3.00E-128	*Hypericum perforatum*	1
DQ444292	metallothionein-like protein	61.6	6.00E-10	*Camellia sinensis*	2
DQ497591	Gentiana siphonantha isolate 1 trnS-trnG intergenic spacer	74.8	6.00E-20	*Gentiana siphonantha*	1
EU271754	osmotin	81.7	1.00E-13	*Piper colubrinum*	1
NM_111462	RCI2A (race-cold-inducable 2A)	122	3.00E-25	*Arabidopsis thaliana*	1
NM_114701	haloacid dehalogenase-like hydrolase family protein	286	1.00E-74	*Arabidopsis thaliana*	1
Membrane transport					
AF003347	ATP phosphoribosyltransferase	162	2.00E-37	*Thlaspi goesingense*	1
AF127442	ATAF1-like protein	250	1.00E-69	*Picea abies*	1
AF051222	ATAF1-like protein	297	3.00E-108	*Picea mariana*	1
Metabolism					
AB027191	isopentenyl pyrophosphate isomerase	389	8.00E-106	*Gentiana lutea*	2
AB281494	alpha beta hydrolase fold superfamily	301	2.00E-92	*Gentiana triflora*	2
AF367442	NAD-dependent malate dehydrogenase	144	4.00E-32	*Prunus persica*	1
AJ251269	geraniol 10-hydroxylase	143	1.00E-31	*Catharanthus roseus*	1
AM269122	putative glycogenin	383	5.00E-104	*Picea abies*	1
AM269142	putative homeodomain leucine zipper protein	377	4.00E-102	*Picea abies*	1
AM269256	monooxygenase	325	3.00E-87	*Picea abies*	1
AM269265	monooxygenase	225	2.00E-56	*Picea abies*	1
EU344848	plastidic aldolase	209	5.00E-64	*Solanum tuberosum*	1
LAUMTNADH	NADH dehydrogenase subunit 4	483	9.00E-134	*Lactuca sativa*	1
Photosynthesis					
AB017366	phytoene cyclase	532	2.00E-154	*Gentiana lutea*	2
AB027191	isopentenyl pyrophosphate isomerase	389	8.00E-106	*Gentiana lutea*	2
AB236868	ribulose-1, 5-bisphosphate carboxylase oxygenase small subunit	325	2.00E-89	*Panax ginseng*	2
AF034631	chlorophyll a b binding protein LHCII type I precursor	223	8.00E-58	*Panax ginseng*	1
AJ577578	PSII K protein	116	4.00E-44	*Olea europaea*	1
DQ781306	chloroplast photosystem II light-inducible protein	80.3	1.00E-19	*Pachysandra terminalis*	1
DQ887080	photosystem I psaH protein	232	2.00E-58	*Arachis hypogaea*	4
EF203260	phytoene synthase 3	385	3.00E-104	*Gentiana lutea*	2
EU308517	ribulose-1, 5-bisphosphate carboxylase oxygenase large subunit	175	3.00E-41	*Silene aegyptiaca*	1

Continue table 1

GenBank accessions	Function	Score (bits)	E-Value	Closest Species	Copies
X66727	*P. taeda* gene for protochlorophyllide reductase	85.8	8.00E-43	*Pinus taeda*	1
X95987	PSII polypeptide	184	4.00E-49	*Solanum lycopersicum*	1
Protein expression					
AB236868	ribulose-1, 5-bisphosphate carboxylase oxygenase small subunit	325	2.00E-89	*Panax ginseng*	1
AB236868	ribulose-1, 5-bisphosphate carboxylase oxygenase small subunit	332	2.00E-88	*Panax ginseng*	1
AB237912	ribosomal protein S12	153	3.00E-36	*Nicotiana sylvestris*	2
AF127593	putative 60S ribosomal protein L13a	412	4.00E-122	*Picea abies*	6
AF479180	26S ribosomal RNA	189	1.00E-45	*Exacum affine*	1
AF479180	26S ribosomal RNA	245	2.00E-62	*Exacum affine*	1
AJ316582	ribosomal protein S12	135	7.00E-36	*Atropa belladonna*	1
AM111313	Plantago major mRNA for histin H3	293	9.00E-77	*Plantago major*	1
AP009123	Ribosomal protein S12	259	9.00E-74	*Gossypium barbadense*	5
AP009374	ribosomal protein S12	319	1.00E-88	*Lepidium virginicum*	1
DQ176643	Vitis pseudoreticulata clone EST-423 23S ribosomal RNA	132	1.00E-29	*Vitis pseudoreticulata*	4
DQ673255	ribosomal protein S12	116	1.00E-36	*Jasminum nudiflorum*	1
DQ673255	ribosomal protein S12	116	7.00E-39	*Jasminum nudiflorum*	1
DQ629362	large subunit ribosomal RNA	348	2.00E-93	*Sabia* sp. Qiu 91025	1
EF207443	ribosomal protein S19	250	2.00E-64	*Cercidiphyllum japonicum*	1
EF207453	ribosomal protein L2	347	4.00E-93	*Peridiscus lucidus*	1
EU118126	ribosomal protein S12	182	4.00E-44	*Ipomoea purpurea*	1
EU301782	Daucus carota 26S ribosomal RNA gene	192	5.00E-47	*Daucus carota*	1
EU431223	ribosomal protein S12	349	1.00E-93	*Carica papaya*	1
Signaling components					
AY936336	NdhC	261	5.00E-67	*Operculina aequisepala*	1
Unclassified					
AC139600	unknown	244	4.00E-62	*Medicago truncatula*	1
AC187538	unknown	329	1.00E-87	*Solanum lycopersicum*	1
AK224216	unknown	309	2.00E-81	Oryza punctata	1
AK224613	unknown	137	5.00E-30	*Solanum lycopersicum*	1
AK246203	unknown	159	2.00E-36	*Solanum lycopersicum*	1
AK246262	unknown	280	2.00E-75	*Solanum lycopersicum*	1
AK246799	unknown	230	1.00E-61	*Solanum lycopersicum*	1
AK246799	unknown	229	5.00E-61	*Solanum lycopersicum*	1
AK251177	unknown	242	5.00E-62	*Hordeum vulgare*	1
AL606457	unknown	105	5.00E-49	*Oryza sativa* Japonica Group	1
AM425978	unknown	241	5.00E-61	*Vitis vinifera*	1
AM454485	unknown	183	1.00E-43	*Vitis vinifera*	1
AM462697	unknown	48.7	3.00E-27	*Vitis vinifera*	1
AM482227	unknown	61.1	4.00E-12	*Vitis vinifera*	1
AP004898	unknown	199	3.00E-54	*Lotus japonicus*	2
AP008212	unknown	155	6.00E-59	*Oryza sativa* Japonica Group	1
AP008218	unknown	115	4.00E-23	*Oryza sativa* Japonica Group	1
AY142543	unknown	238	2.00E-66	*Arabidopsis thaliana*	1
AY142543	unknown	238	2.00E-66	*Arabidopsis thaliana*	1
CT831892	unknown	376	1.00E-101	*Oryza sativa* Indica Group	1
CU223189	unknown	177	2.00E-49	*Populus tremula*	1
CU224065	unknown	143	2.00E-31	*Populus tremula*	1
CU224528	unknown	54.7	3.00E-22	*Populus tremula*	1
DQ226906	unknown	79	2.00E-12	*Boechera divaricarpa*	1
EF085796	unknown	199	2.00E-49	*Picea sitchensis*	1
EF087806	unknown	69.3	4.00E-09	*Picea sitchensis*	1
EF146998	unknown	90.9	1.00E-36	*Populus trichocarpa*	1
EF147066	unknown	72.5	3.00E-17	*Populus trichocarpa*	1
EF148586	unknown	142	3.00E-31	*Populus trichocarpa*	1
EF534108	unknown	105	2.00E-22	*Beta vulgaris*	1
NM_001050318	unknown	113	6.00E-23	*Oryza sativa* Japonica Group	1
NM_001050861	unknown	91.3	4.00E-27	*Oryza sativa* Japonica Group	1
NM_111205	unknown	139	6.00E-39	*Arabidopsis thaliana*	1
NM_124490	unknown	65.7	2.00E-22	*Arabidopsis thaliana*	1
Y08501	unknown	62.5	7.00E-14	*Arabidopsis thaliana*	1

are involved in protein expression (35%), photosynthesis (22%), metabolism (18%), defense (11%), membrane transport (5%), cell division (5%), chromosome metabolism (2%) and signaling components (2%) (Fig . 5) .

2.5　Verification of the ESTs′accuracy using RT-PCR

To verified the accuracy of ESTs, we designed 12 pairs of RT-PCR primers (Table 2) accroding to the effective EST sequences selected randomly from Table 1 . These primers were used to amplify the cDNAs . The expected products were obtained for each pairs of primers (Fig . 6) .

In conclusion, we successfully constructed the cDNA library of *G. officinalis* . This library will provide a basis for cloning functional genes of this species in the future . In addition, ESTs can be further used to design a series of primers to amplify nuclear fragments of this species and closely related species during studying population genetics of these species and interspecific relationships at genomic level .

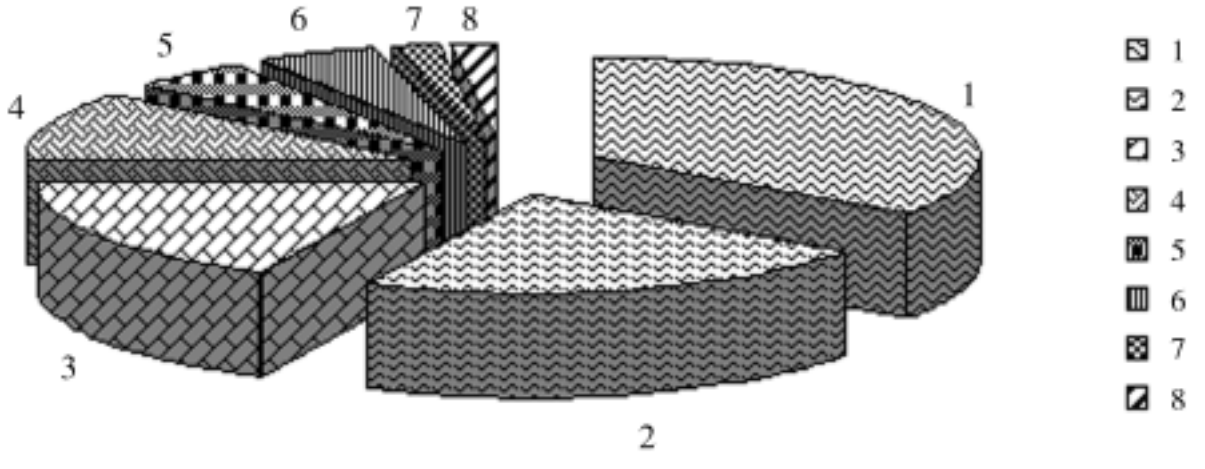

Fig . 5　Functional classification of ESTs according to the tBLASTx search results

(1 . Protein expression 35%; 2 . Photosynthesis 22%; 3 . Metabolism 18%; 4 . Defense 11%; 5 . Membrane transport 5%; 6 . Cell division 5%; 7 . Chromosome Metabolism 2%; 8 . Signaling components 2%)

Table 2　Semi-quantitative RT-PCR primers designed according to the *G. officinalis* ESTs

Primers	Match Accessions	Product length	Sequences of primers
G_1	AB281494	632	S　5′CGTTATGGAAGAGTACACAGCA 3′ A　5′TCACTCCCCTCAAACATAACAC 3′
G_2	EF520004	1230	S　5′CCTCACATTACACCTTGCTACA 3′ A　5′GCGAATAAGCAAGCCAAGAC 3′
G_3	DQ226906	1047	S　5′AATGGTTCACCGATTCCCCC 3′ A　5′CTTTACATAAACTCGACAGG 3′
G_4	AF051222	900	S　5′TCGTCAAACAAGTCAATCCG 3′ A　5′GCTTTCCAGTATCCAGAACCAG 3′
G_5	AF003347	1163	S　5′CAAATACCATTTTACAAAATTC 3′ A　5′GGCACAAGGCAAGAAGGCGA 3′
G_6	AB027191	755	S　5′TTTGCTGCATAGAGCGTTTAGC 3′ A　5′ATGTCAGTAGCTTCTTGGAGGG 3′
G_7	EF203260	692	S　5′GCGTCACACATAAATCCAACCG 3′ A　5′ACTCCTTTCTCTGCATCATCATAG 3′
G_8	AB017366	975	S　5′GTGCGTGCGCTGAGATATTA 3′ A　5′CACGCTCCATAGAATCGTCATC 3′
G_9	AB017366	1145	S　5′GTGCGTGCGCTGAGATATTA 3′ A　5′CACGCTCCATAGAATCGTCATC 3′
G_{10}	AF034631	601	S　5′GTCTCTTCTTCCTTCTCAGT 3′ A　5′TTTATTTGATGGAGTTCGAA 3′
G_{11}	EU853017	985	S　5′GC TACGCCAGGTATTACCCA 3′ A　5′GA TTTCCCCGTTCTCTCTCC 3′
G_{12}	EU344848	1167	S　5′AATGGTTCACCGATTCCCCC 3′ A　5′CTTTACATAAACTCGACAGG 3′

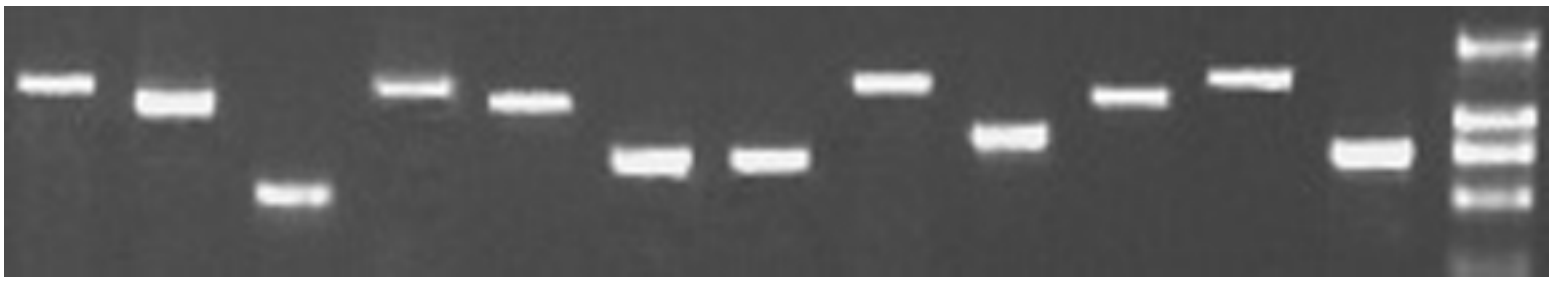

Fig . 6 RT-PCR Results

(from right to left, are 2000 marker and the products by G_1 to G_{12} pairs of primers)

Reference:

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