Tree Genetics and Molecular Breeding 2022, Vol.12, No.3, 1-9
http://genbreedpublisher.com/index.php/tgmb

Research Article Open Access

Transcriptome Analysis and Differential Gene Screening of Male Flower Buds in Three Age Stages of *Ginkgo biloba*

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Tree Genetics and Molecular Breeding, 2022, Vol.12, No.3 doi: 10.5376/tgmb.2022.12.0003

Received: 17 Feb., 2022

Accepted: 24 Feb., 2022

Published: 18 Mar., 2022

Preferred citation for this article:

Yang T.T., Feng Z., Liu Y.L., Yang G., Li M., and Wang Y.Q., 2022, Transcriptome analysis and differential gene screening of male flower buds in three age stages of *Ginkgo biloba*, Tree Genetics and Molecular Breeding, 12(3): 1-9 (doi: 10.5376/tgmb.2022.12.0003)

Abstract In this paper, the key genes of flowering regulation among three age stages was screened and the molecular mechanism of the flowering pathway was revealed in *Ginkgo biloba*. It can provide scientific basis for promoting early flowering, molecular breeding and spreading planting of *Ginkgo biloba*. High throughput sequencing technology and bioinformatics tools were used to sequence the transcriptome of male flower buds of *Ginkgo biloba* in three age stages. By analyzed the sequencing data, we screened out the key differential expression genes of age pathway. A total of 57.45 Gb of raw data was generated by transcriptome sequencing. The total number of Unigenes was 35 058 and annotated in the 8 functional databases (GO, COG, KEGG, KOG, NR, Pfam, Swiss-Prot, and eggNOG). The Unigenes were classified into 55 GO categories and 126 metabolic pathways. Analysis of differentially expressed genes revealed that 37 genes were up-regulated and 75 genes were down-regulated during the flower bud undifferentiated stage vs. initial stage of flower bud differentiation. A total of 592 genes were up-regulated and 871 genes were down-regulated during the initial stage of flower bud differentiation vs. flower bud differentiation stage. The flower bud undifferentiated stage vs. flower bud differentiation stage, 961 genes were up-regulated and 1 203 genes were down-regulated. A large number of flowering related genes were discovered and finally screened out 11 key genes of flowering regulation in age pathway. including *SPL*(gene.Gb_23724, gene.Gb_03922), *AP2*(gene.Gb_00766), *MADS-box*(gene.Gb_01886, gene.Gb_15398, gene.Gb_28337, Gingko_newGene_2213), Gibberellin-regulated protein(gene.Gb_34467, gene.Gb_28606, gene.Gb_33214)and DELLAs protein(gene.Gb_34644).

Keywords *Ginkgo biloba*; Transcriptome sequencing; Age pathway; Differentially expressed genes

Ginkgo (*Ginkgo biloba* L.), also known as Gongsunshu, is the oldest relict plant of extant gymnosperms dating from dinosaurs and is recognized as 'living fossil'. Paleobotanists' studies of fossil data have shown that seed plants experienced a decline in the early Permian, but the adaptable gymnosperms such as ginkgo and cedar developed slowly in the late Triassic and peaked in the Jurassic and early Cretaceous. Trichopitys, the hairy leaves during Autunian in southern France, are recognized as reliable ancestors of *Ginkgo biloba*, and the earliest and best preserved *Ginkgo biloba* is the Yima Ginkgo in Early Jurassic discovered in China (Tralau, 1968). In the early 1990s, morphological studies on male and female flower buds of *Ginkgo biloba* were reported. Then, the transcriptome sequencing and gene cloning of *Ginkgo biloba* flowering and secondary metabolites were reported in an endless stream (Zhang et al., 2013). *Ginkgo biloba* is a dioecious plant in sex differentiation. Its male reproductive organs are also called microsporangia, like the inflorescence of Rouyi, also known as male flowers (Zhang et al., 2001). The flower buds of leaves and male flowers on short branch are called 'mixed male flower buds', or called 'male flower buds' for short (Figure 1).

During plant growth, shoot apical meristem (SAM) continuously produces lateral branch organs. According to the morphological characteristics and functions of lateral branch organs, postembryonic development of plants can be divided into vegetative stage and reproductive stage. SAM produces leaves at vegetative stage and produces flowers at reproductive stage (Poethig, 2003). Flower transition, from vegetative stage to reproductive stage, is

coordinated by multiple genetic pathways to respond to various developmental and environmental signals (Bäurle and Dean, 2006; Srikanth and Schmid, 2011). There are six genetic pathways involved in flowering regulation in *A. thaliana*, a model plant with relatively thorough research on flowering regulation. Among them, the research of photoperiod, temperature, vernalization, gibberellin and autonomous pathways is more thorough, while the research of age pathways remains to be further studied (Srikanth and Schmid, 2011). *A. thaliana* has a short growth cycle and is not strongly dependent on age pathways, but *Ginkgo biloba* has a very long life span and its childhood period is much longer than other plants, so it is particularly urgent to study the age-dependent pathway of flowering regulation. In this paper, transcriptome sequencing technology was used to conduct bioinformatics analysis of male flower buds in three age stages of *Ginkgo biloba* and screen differentially expressed genes, which can provide scientific basis for the study on age pathway of flowering regulation.

Figure 1 Ginkgo male flower bud

1 Results and Analysis

1.1 Sequencing data and quality control

A total of 57.45 Gb data were obtained by RNA-seq. The data amount of each sample reached 6.07 Gb, and the percentage of Q30 base was 94.59 % and above. The data of each sample was compared with the designated reference genome, and the comparison efficiency ranged from 85.64% to 90.19% (Table 1). The results showed that the sequencing quality is high and can be further analyzed.

Table 1 RNA-seq data statistics

Samples	Number		Total Reads	Mapped Reads (percentage)	GC Content (%)	≥Q30 (%)	Samples
	Clean reads	Clean bases					
Juvenile stem	21,260,012	6,357,478,046	42,520,024	37,747,560 (88.78%)	44.83	94.81	Juvenile stem
Apex T01-T03	21,492,957	6,424,620,892	42,985,914	38,249,203 (88.98%)	44.86	94.59	Apex T01-T03
	21,441,156	6,405,708,414	42,882,312	38,676,555 (90.19%)	44.69	94.68	
One year's	21,285,080	6,352,022,540	42,570,160	38,062,526 (89.41%)	44.89	94.65	One year's
flowering stem	21,372,065	6,386,940,100	42,744,130	36,606,834 (85.64%)	44.95	95.11	flowering stem
apex T04-T06	22,665,601	6,777,488,624	45,331,202	40,660,557 (89.70%)	44.88	95.12	apex T04-T06
Stem end of	21,395,341	6,394,514,808	42,790,682	37,703,333 (88.11%)	44.61	94.94	Stem end of
perennial f	21,008,039	6,278,849,030	42,016,078	37,790,151 (89.94%)	44.60	95.02	perennial flower
lowerT07-T09	20,298,126	6,069,357,696	40,596,252	35,752,244 (88.07%)	44.60	95.18	T07-T09

Note: ≥ Q30 means percentage of quality values of bases involved clean reads is equal or greater than 30

1.2 Functional annotation of *Ginkgo biloba* Unigene

Unigenes sequences were compared with Nr (Nun-redundant Proteins) database, COG (Cluster of Orthologous Groups of Proteins) database, GO (Gene Ontology) database, KEGG (Kyoto Encyclopedia of Genes and Genomes) database, KOG (euKaryotic Orthologous Groups) database, Pfam protein database, Swiss-Prot (Swiss-Protein) database, and eggNOG (evolutionary genealogy of genes: Non-supervised Orthologous Groups) database to obtain annotation information (Table 2). Finally, in each database, a total of 35 058 genes obtained annotation information. Among the newly discovered 1 062 new genes, the number of genes with annotation information was 681. It can be seen that a large part of genes has not been annotated, which needs further exploration and research.

Table 2 The database distribution of unigene and new gene functional annotation

Annotated databases	Annotated Number	Annotated New Gene Number
COG	11852	180
GO	19144	393
KEGG	12155	205
KOG	20334	310
Pfam	27178	450
Swiss-Prot	23987	449

Ginkgo biloba Unigene was compared with the NR database to understand the evolutionary relationship between *Ginkgo biloba* and other species. According to the distribution of gene matching rate between *Ginkgo biloba* Unigene and other species in NR database (Table 3), it can be seen that *Ginkgo biloba* has the highest number of homologous genes with *Picea sitchensis*, which is also a gymnosperm. Apart from the high homology, it may also be related to the in-depth study of *Picea sitchensis* and the relatively clear annotations. In addition, 11 404 Unigenes were not compared with other species, which may be unique genes of *Ginkgo biloba*.

Table 3 Species distribution after comparison between *Ginkgo biloba* Unigene and NR database

Species	Number	Ratio (%)
Picea sitchensis	10816	31.18
Amborella trichopoda	3677	10.6
*Nelumbo nucifer*a	2707	7.8
Vitis vinifera	1487	4.29
Physcomitrella patens	1206	3.48
Selaginella moellendorffii	1146	3.3
Elaeis guineensis	848	2.44
Phoenix dactylifera	699	2.02
Ginkgo biloba	695	2
Other	11404	32.88

In organisms, different gene products construct a complex regulatory network, which can eventually perform biological functions through coordination with each other. Analyzing the annotations of gene pathway is helpful to understand the process of genes performing their functions. The annotation results of KEGG (Kyoto Encyclopedia of Genes and Genomes) showed (Table 4) that the Unigene of male flower buds of *Ginkgo biloba* involved 126 metabolic pathways in three age stages, among which starch and sucrose metabolism (304 genes), plant hormone signal transduction (302 genes), ubiquitin mediated proteolysis (222 genes) and plant circadian rhythm (72 genes) may play an indispensable role in flower bud induction. Other pathways are responsible for providing energy and raw materials and maintaining normal life activities during flower bud differentiation.

1.3 GO enrichment analysis of *Ginkgo biloba* Unigene

In order to further understand *Ginkgo biloba* Unigene enrichment locating on which biological function, pathway or cell, GO enrichment analysis was performed. The results showed that all *Ginkgo biloba* sequences were divided into three categories: Biological Process, Molecular Function, and Cellular Component, and that 35 990 entries, 27 079 entries and 15 534 entries were enriched, respectively. Among them, the Biological Process is rich in Biological Regulation, Developmental Process, Reproductive Process, Signaling, Growth, Reproduction and Rhythmic Process, which may be related to the age pathway of flowering regulation that this research focuses on. Take the first 50 items for drawing (Figure 2).

Table 4 Enrichment of KEGG metabolic pathway

Code	Pathway	Pathway ID	Gene number
1	Biosynthesis of amino acids	ko01230	369
2	Carbon metabolism	ko01200	360
3	Protein processing in endoplasmic reticulum	ko04141	340
4	Ribosome	ko03010	328
5	Spliceosome	ko03040	319
6	Starch and sucrose metabolism	ko00500	304
7	Plant hormone signal transduction	ko04075	302
8	Phenylpropanoid biosynthesis	ko00940	294
9	Purine metabolism	ko00230	260
10	RNA transport	ko03013	244
11	Oxidative phosphorylation	ko00190	233
12	Ubiquitin mediated proteolysis	ko04120	222
13	Endocytosis	ko04144	221
14	Glycolysis / Gluconeogenesis	ko00010	201
15	Phenylalanine metabolism	ko00360	199
16	RNA degradation	ko03018	198
17	Pyrimidine metabolism	ko00240	195
18	Amino sugar and nucleotide sugar metabolism	ko00520	185
19	Ribosome biogenesis in eukaryotes	ko03008	184
20	mRNA surveillance pathway	ko03015	184

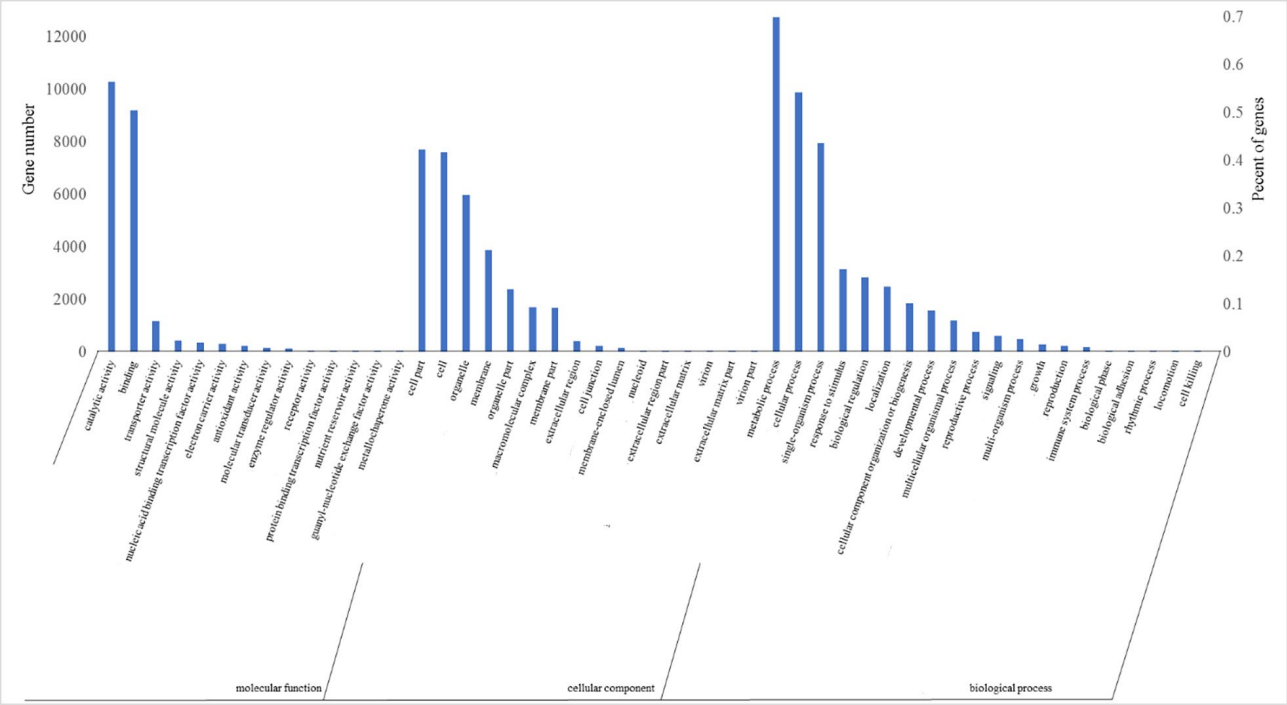

Figure 2 Go function classification

1.4 Screening of differentially expressed genes in age pathway of flowering regulation of *Ginkgo biloba*

The differential expression among sample groups was analyzed by edgeR (Figure 3). There are fewer up-regulated genes (37) and down-regulated genes (75) in junior male flower buds of *Ginkgo biloba* than in one-year male flower buds. While there are more up-regulated genes (592) and down-regulated genes (871) in junior male flower

buds than in several-year male flower buds and more up-regulated genes (961) and down-regulated genes (1203) in one-year male flower buds than in several-year male flower buds.

KEGG pathway annotation and GO enrichment analysis were performed on differentially expressed genes, and 11 genes with significant expression differences that may be related to flowering regulation were further screened out (Table 5), and hierarchical clustering analysis was performed (Figure 4). After bioinformatics analysis, 11 differentially expressed genes were finally screened. The expression of *SPLs* (gene.Gb_23724, gene.Gb_03922) continued to decline during the transition of *Ginkgo biloba* from juvenile to adult, while *AP2* (gene.Gb_00766) showed a continuous upward trend, which was consistent with the expression of miR156 and miR172. The expression patterns of the four genes with *MADS-box* domains (gene.Gb_01886, gene.Gb_15398, gene.Gb_28337, Gingko_newGene_2213) are not the same. *SOC1* (gene.Gb_01886) is up-regulated and the rest is down-regulated. It indicated that multiple *MADS-box* genes may have redundant functions to regulate flowering in age pathway of flowering regulation of *Ginkgo biloba*. GA regulatory protein (gene.Gb_34467, gene.Gb_33214) is continuously down-regulated, and another GA regulatory protein (gene.Gb_28606) is up-regulated, indicating that in *Ginkgo biloba*, multiple regulatory proteins work together to regulate GA levels. The DELLA protein (gene.Gb_34644) regulated by GA signal showed a downward trend. It can be seen that GA-DELLAs played a certain regulatory role in the transition of *Ginkgo biloba* from non-flowering juvenile to flowering adult.

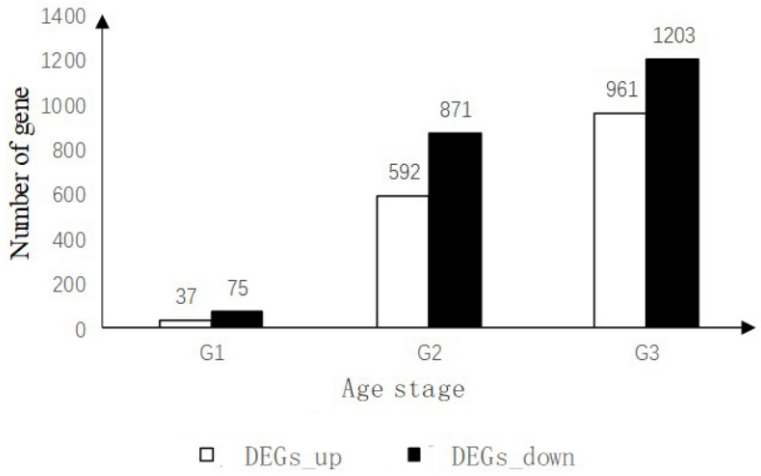

Figure 3 Differentially expressed genes in three age stages of *Ginkgo biloba* shoot apex
Note: G1: Junior vs. flowering annual; G2: Junior vs. Bloom for several years; G3: Flowering annual vs. Bloom for several years

Table 5 Differential expression gene annotation

Gene ID	Annotation
gene.Gb_23724	*SBP3* Squamosa promoter-binding-like protein 3
gene.Gb_03922	*SBP6* Squamosa promoter-binding-like protein 6
gene.Gb_00766	AP2 domain
gene.Gb_01886	*GbMADS6* MADS-box transcription factor
gene.Gb_15398	*GbMADS4* MADS-box transcription factor
gene.Gb_28337	*GbMADS8* MADS-box transcription factor
Gingko_newGene_2213	MADS-box protein GGM13
gene.Gb_34467	Gibberellin-regulated protein 8
gene.Gb_33214	Gibberellin regulated protein
gene.Gb_28606	Protein GAST1
gene.Gb_34644	DELLA protein RGA2

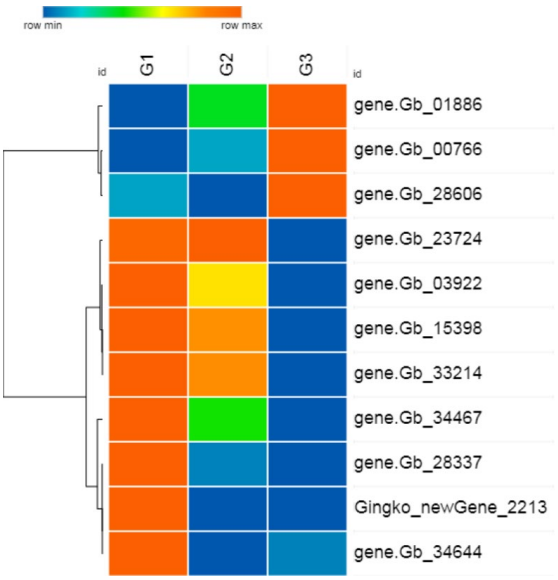

Figure 4 Cluster thermogram of differentially expressed genes

2 Discussion

In *A. thaliana*, *SPLs* with miR156 target sites are divided into two categories. One type encodes smaller proteins, such as *SPL3*, *SPL4* and *SPL5*, which control flowering time and flowering period changes. The other type encodes larger proteins, including 8 genes such as *SPL9* and *SPL15*, which promote leaf germination and flower formation (Cardon et al., 1997). In the stem end, both *SPL3* and *SPL9* can induce flowering by activating *MADS-box* genes including *API*, *LFY*, *FUL* and *SOCl* (Yamaguchi et al., 2009). And previous studies have shown that whether it is in short-day or long-day conditions, the overexpression of *SPL3* or *SPL9* of specific promoter in the stem end can lead to an early flowering phenotype (Wang et al., 2009).

AP2 transcriptional regulator is the other most important component of the age pathway. Studies have shown that *AP2* can directly inhibit *FT* which up regulated by sunlight change (Castillejo and Pelaz, 2008). According to the difference of structural domains and binding sites, proteins containing *AP2* domains can be divided into two types. One type is TEMPRANILLO1 (TEM1) and TEM2 proteins, which contain sites encoding *AP2* domains and B3 type DNA binding domains, and have been shown to inhibit redundant flowering by inhibiting FT transcription (Castillejo and Pelaz, 2008). The other type is mainly targeting protein of miR172, including *TARGET OF EAT* (*TOE*) genes (*TOE1* to *TOE3*), *AP2*, *SCHLAFMU¨TZE* (*SMZ*) and its homologous gene *SCHNARCHZAPFEN* (*SNZ*) (Aukerman and Sakai, 2003). *AP2* can not only directly bind to *SOC1* and *AGAMOUS*, the key genes for flowering regulation, and inhibit their transcription, but also can inhibit flowering and flower development by binding to miR172 gene locus and inhibiting its transcription (Yant et al., 2010). *AP2* also acts as a dual-function transcription factor, directly binding to the genomic sites of the *MADS-box* family genes *AGAMOUS-LIKE15* (*AGL15*) (Adamczyk et al., 2007) and miR156 (Wu et al., 2009), and directly activating other plant inhibitory factors, thereby regulating plant flowering.

Gibberellins (GA) is one of the most important plant hormones, which participates in many biological processes such as plant growth and development. It also plays an indispensable role in the flowering process of plants. Under short-day conditions, the addition of GA is a key factor to induce flowering of GA1 (GA REQUIRING 1) mutants (Wang, 2014). Under long-day conditions, GA also plays an important role in the flowering process (Wang et al., 2011). Between GA and miR156-SPL9, the florescence regulation is achieved by degrading the DELLAs protein (Dill et al., 2001). Under the specific promoters in leaf or stem end, the forced expression of GA-insensitive RGA or GA catabolism genes results in the late flowering phenotype (Galvão et al., 2012; Porri et al., 2012). The combination of RGA and *SPL9* interferes with the activity of *SPL9* transcriptome on the upstream of *miR172*, *SOC1* and *FUL*. Therefore, DELLAs protein inhibits the phase transition process from growth to

reproduction by inhibiting *SOCl* and *FUL* in the stem end. On the one hand, DELLA protein inhibits the expression of *SOC1*, transmits GA signals, and regulates flowering (Wilson et al., 1992). On the other hand, GA regulates *MYB* transcription factor by inhibiting the expression of miR159 through DELLA protein (Griffiths et al., 2006).

Because *SOC1*, *FT*, and *LFY* are regulated by multiple flowering pathways, they are also called flower integrons (Kardailsky et al., 1999; Kobayashi et al., 1999). As a downstream target gene of *AP2*, *SOC1* is not only inhibited by *AP2* in the age pathway, but also can be positively regulated by *SPL9* (Samach et al., 2000). It can also receive signals from GA and is negatively regulated by the DELLA protein (Simpson and Dean, 2002).

The expression patterns of *SPL3* (gene.Gb_23724) and *AP2* (gene.Gb_00766) in the stem ends of *Ginkgo biloba* in three age stages are different from those of *A. thaliana*. The expression of GA receptor GID1 (GIBBERELLIC INSENSITIVE DWARF 1) is not significantly different in *Ginkgo biloba*, but GA regulatory protein shows significant up-regulation (gene.Gb_28606) and down-regulation (gene.Gb_34467, gene.Gb_33214) trends. The expression of DELLA protein RGA2 (gene.Gb_34644) is continuously down-regulated in *Ginkgo biloba*. The previous work of this study proved that *GbMADS6* in *Ginkgo biloba* is a homologous gene of *SOC1*, which shows a continuous up-regulated expression pattern in three age stages of *Ginkgo biloba*, and promotes the flowering of *Ginkgo biloba*. In summary, it is indicated that the age-regulated flowering of *Ginkgo biloba* may be different from that of *A. thaliana*.

Based on transcriptome sequencing data, the key genes that are differentially expressed in three age stages of *Ginkgo biloba* was screened in this study. It can provide new data and theoretical basis for further cloning and functional verification of key genes, and for revealing the molecular mechanism of the flowering pathway in *Ginkgo biloba* and even other gymnosperms. It can also provide scientific basis for promoting early flowering, molecular breeding and spreading planting of *Ginkgo biloba*.

3 Materials and Methods

3.1 Materials

Juvenile male flower buds with 8 years' growth cycle, one-year-old male flower buds with 21 years' growth cycle and multi-year-old male flower buds with 45 years' growth cycle in botanical garden in Central South University of Forestry and Technology were selected as experimental materials. The flower buds were collected at the beginning of the flower bud differentiation in early June. The lower xylem of the short branches was discarded, and the green part on the short branches, namely the male flower buds, was quickly frozen in liquid nitrogen and placed in the refrigerator at -80°C.

3.2 RNA extraction and library construction

The OMEGA kit was used to extract total RNA from male flower buds at three age stages of *Ginkgo biloba*, and the RNA concentration was measured by using NanoDrop 2000 (Thermo). The RNA Nano 6000 detection kit of the Agilent Bioanalyzer 2100 System (Agilent Technologies, California, USA) was used to evaluate RNA integrity. After the samples were qualified, NEBNext UltraTM RNA Library Prep Kit from Illumina (Nebraska, USA) was used to generate a sequencing library.

3.3 Transcriptome sequencing and quality control of sequencing data

Transcriptome sequencing was completed by Beijing Biomark Biotechnology Co., Ltd. After completing the sequencing, base quality score, base content distribution and data output statistics were used to control the probability of base calling errors, the presence or absence of AT and GC separation phenomenon and the percentage of Q30 bases in each sample.

3.4 Analysis of transcriptome data

The Clean Data obtained after splicing was performed sequence alignment with the specified reference genome, and then the obtained Mapped Data was performed quality control. Structural level analysis such as alternative

splicing analysis, new gene mining and gene structure optimization was performed on Unigene. Based on the differential expression of genes, the analysis of differential expression of genes, function annotation of differential gene, functional enrichment and other expression level analysis were carried out.

3.5 Screening of differentially expressed genes

Choose edgeR Pvalue=0.01 and FC=2 as the screening criteria to draw the Venn diagram of differentially expressed genes in three age stages of *Ginkgo biloba*. On this basis, the functional annotation and enrichment analysis of the database of differentially expressed genes were carried out. The genes related to flowering were further selected, and the genes with the same or similar expression patterns were subjected to hierarchical cluster analysis.

Authors' contributions

YTT was the experiment designer and executor of this study, and she wrote the first draft of the manuscript. FZ completed data analysis. YTT and WYQ are the creators and leaders of the project, directing data analysis, manuscript writing and revision. LM and other authors also gave a lot of help in this study. All authors read and approved the final manuscript.

Acknowledgements

This study was jointly funded by National Natural Science Foundation of China (31570682) and National Key R&D Program of China (2017YFD0600701).

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