INTERNATIONAL JOURNAL OF AGRICULTURE & BIOLOGY
ISSN Print: 1560–8530; ISSN Online: 1814–9596
17–1374/2018/20–5–975–980
DOI: 10.17957/IJAB/15.0585
http://www.fspublishers.org

Full Length Article

Transcriptome Analysis of *Pinellia integrifolia* and *PEBP* Genes Identification in Related Species

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Abstract

The transcriptome analysis of *Pinella integrifolia* was carried out in this study to study its bulbil development at molecular level. Approximately 44.12 Mb high-quality clean reads were generated, and 74,550 unigenes that possess an average length of 831 bp were assembled *de novo*. A total of 44,626 unigenes (59.86%) were annotated in the database, 40,900 unigenes had blasted to the Nr database, and 6,112 and 16,797 unigenes had similar hits in the Gene Ontology (GO) and Cluster of Orthologous Groups (COG) databases, respectively. Results revealed that 31,851 unigenes were assigned to 135 KEGG pathways. Based on transcriptomic data, various members of *PEBP* gene family were identified in *P. integrifolia*, *A. bulbifer*, *P. ternata* and *Colocasia esculenta*. These data could be utilized in further research such as explorations of bulbil development in *Pinellia*. © 2018 Friends Science Publishers

Keywords: *Pinellia integrifolia*; *De novo* sequencing; Bulbil development; *PEBP* gene family

Introduction

Propagule or "seed" production play crucial roles in plant biology such as in dispersal, population genetic structure, adaptation of environment, and so on. It's also the key of production for economic plant culture, such as crop agriculture. Reproductive strategy in plant could be divided two groups: sexual and asexual reproduction. Because seed is the harvesting organ for most of crop species, the sexual reproduction (seed formation) has been researched profoundly (Meng, 1995). However, the asexual reproduction includes many types: stem tuber, root tuber, corm, bulb, pseudobulb, stolon, rhizome, bulbil, *etc.* (Gao and Wang, 2003). So, researching on each kind of asexual reproduction is very limited correspondingly, especially for the bulbil reproduction. For bulbil reproduction, reported works are mainly involved in the reproductive efficiency, anatomy process, physiological conditions for production, and so on. Revealing of anatomic characteristics could promote the comprehension of organ development. Bulbil developmental process have been reported in *Dioscorea* (yam) (Dioscoreaceae, Xie *et al.*, 1993); *Titanotrichum* (Gesneriaceae, Wang and Cronk, 2003); *Allium* (Amaryllidacease, Davis *et al*., 2011); *Lilium sulphureum* (Liliaceae, Li *et al*., 2012); and *P. ternate* (Thunb.) Breit. (Araceae, Luo *et al*., 2014). Nevertheless, reports about molecular mechanism of bulbil development are still limited.

In *Titanotrichum oldhamii*, the Gesneriaceae *FLO/LFY* homolog (GFLO) was down-regulated expression during bulbil development (Wang *et al*., 2004). Class *I KNOX* genes could be detected at during bulbil formation in *Agave tequilana* (Abraham-Juarez *et al*., 2010). The *Atq MADS1, 2, 4, 6,* and *7* genes all present the same pattern of decreasing expression along with bulbil development in *A. tequilana* (Sandoval *et al*., 2012). Although several genes that were related to bulbil formation have been reported, the study is limited and little is known abouth the molecular mechanism of bulbil development. Furthermore, members of phosphatidyl ethanolamine-binding protein *(PEBP)* gene family were involved in developments of vegetative organs, such as tuber formation (González-Schain *et al*., 2012), bulb formation (Lee *et al*., 2013). However, there were not reports that discuss variation of *PEBP* gene contents in bulbil species and related species.

To more expeditiously reveal the mechanism of bulbil development; more excellent researching material should be selection. Eleven species has been recorded in the genus of *P.* Tenore (Araceae) that is distributed mainly in China (Tae and Jim, 2005; Li *et al*., 2010). All species of *Pinellia* are perennial herbs. Diverse reproductive strategies are involved in this genus, including seed formation in spadix for all species, stem tuber reproduction for *Pinellia peltata* C. Pei, tubercles growth for *Pinellia polyphylla* S.L. Hu, *Pinellia tripartite* (Blume) Schot, *Pinellia yaoluopingensis* X.H. Guo and X.L. Liu, and *Pinellia pedatisecta* Schott around the tuber, bulbil or leaf tubercle development for *Pinellia cordata* N.E. Brown (2 bulbil per leaf at both the petiole and leaf blade base) and *P. ternata* (1 bulbil per leaf at the petiole) (Li *et al*., 1997, 2010). In terms of bulbil development at the leaf, there are three kinds: two bulbil

To cite this paper: Liu, D., R. Luo, H. Chen, H. Gao and L. Xia, 2018. Transcriptome analysis of *Pinellia integrifolia* and *PEBP* genes identification in related species. *Int. J. Agric. Biol*., 20: 975–980

development per leaf at both the petiole and leaf blade base for *P. cordata*, only one bulbil development per leaf at the petiole for *P. ternata*, and no bulbil development on leaves for *P. integrifolia*. Subsequently, these three species (*P. cordata*, *P. ternata*, and *P. integrifolia*) could be parallel used as researching materials to reveal them mechanism of bulbil development. Some works concerning the bulbil development have been published with *P. ternate* (Wang, 1986; Jiang *et al*., 1996; Pan, 1998; Song *et al*., 2005).

Transcriptome analysis has been extensively used to explore molecular mechanism for development of various organs (Liu *et al*., 2017; Zhou *et al*., 2017). Transcriptome sequencing also has been reported in *Pinellia* to identify genes involved in secondary metabolites biosynthesis (Zhang *et al*., 2016), or only to supply researching basement for other works (Huang *et al*., 2016). However, transcriptome sequencing for *P. integrifolia* and *PEBP* genes identification has not been reported today. Therefore, molecular works based on transcriptomes comparison could not be carried out. To facilitate further molecular works on bulbil development with *Pinellia* spp., transcriptome analysis for *P. integrifolia* and *PEBP* genes identification were reported in this study. The transcrpitome data for *P. integrifolia* also could be help to reveal characteristics of secondary metabolite biosynthesis.

Materials and Methods

Plant Materials, RNA Isolation, Library Construction, and Transcriptome Sequencing

Plant materials: *Pinellia integrifolia* N. E. Brown plants, which were collected from the Chishui (Guizhou Province, China; 28.57° N, 105.69° E) in May 2016, were cultured in laboratory with garden pots. Petiole and tubers (each sample is about 0.3 g) of young individuals were selected. The specimens were washed with pure water, dried using filter paper, and small pieces were cut on ice and frozen immediately in liquid nitrogen. All of the samples were stored at -80°C.

RNA Isolation, Library Construction and Transcriptome Sequencing

Total RNA was isolated from each sample using the improved CTAB method. To remove DNA, Total RNA was digested with DNase I. The mixture of equal amounts of RNA from each sample was prepared for mRNA-seq library construction. Subsequently, Oligo (dT) beads are used to isolate poly(A)-containing m RNA. Then mRNA mixed with the fragmentation buffer was fragmented. Using mRNA fragments as templates, the first strand cDNA was synthesized. Purification of short fragments were resolved with EB buffer. Then, reparation and single nucleotide A (adenine) addition were carried out. After that, adapters were connected with short fragments. The suitable fragments were selected for the PCR amplification. For quantification and qualification of the sample library, Agilent 2100 Bioanaylzer and ABI Step One Plus Real-Time PCR System were used. Then the library was sequenced using Illumina Hi Seq platform.

Sequencing Data Processing, Assembly and Annotation

The image data outputted from the sequencing machine was transformed by base calling into sequence data (raw data/reads). The *de novo* transcriptome assembly of the short clean reads was conducted using Trinity with the fixed default k-mer size of 25 (Grabherr *et al*., 2011). Finally, all assembled unigenes were searched using BLASTX against protein databases, such as the non redundant protein database (NR), the Swiss-Prot database, the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway database (Kanehisa *et al*., 2006), and the Cluster of Orthologous Groups (COG) (Tatusov *et al*., 1997) database, with an E-value cut off lower than 10^{-5}. Gene Ontology (GO) of unigenes were carryout out by Blast2GO (Conesa *et al*., 2005). For each unigene within a GO annotation, the WEGO software (Ye *et al*., 2006) was used to obtain the GO functional classification for the unigenes. Unigenes were aligned to the InterPro database by InterProScan5 (Quevillon *et al*., 2005).

Transcriptome data of *P. ternata* (SRX484200), *A. bulbifer* (SRX182517), and *C. esculenta* (SRX290678) were download from NCBI database and were assembled and annotated similar to *P. integrifolia* to identify *PEBP* genes.

Results

Pinella integrifolia Transcriptome Sequencing and *De novo* Assembly

Sequencing: Two samples of *P. integrifolia* were collected and sequenced on the Illumina Hiseq platform. A total of 44.12 Mb high-quality clean reads were obtained after the removal of ambiguous, sequences, adaptor and low-quality reads (Q20 < 20). The Q20 and Q30 percentages were 97.88 and 95.35%, respectively (Table 1).

Assembly: The assembly *de novo* were carried out because of non reference genome existing for *P. integrifolia*. A total of 166,017 Trinity were obtained, and the mean length is 572 bp. The length distribution of transcriptomic Trinity: 200 to 500 nt, 70.68%; 600 to 1000 nt, 14.37%; 1100 to 1500 nt, 6.36%; 1600 to 2000 nt, 3.78%; 2100 to 2500 nt, 2.16%; 2600 to 3000 nt, 1.14%; and ≥ 3000 nt, 1.51% (Fig. 1a). Contigs were further assembled into 74,550 unigenes with an average length of 831 bp using paired-end joining and gap-filling methods (Table 1). The length distribution of all unigenes were listed as below: 200 to 500 nt, 52.93%; 600 to 1000 nt, 19.45%; 1100 to 1500 nt, 10.84%; 1600 to 2000 nt, 7.29%; 2100 to 2500 nt, 4.15%; 2600 to 3000 nt, 2.30%; and ≥3000 nt, 3.04% (Fig. 1b).

Table 1: Summary of the transcriptome of *P. integrifolia*

Database	Number
Total clean reads (Mb)	44.12
Q20/Q30 percentage	97.88/95.35
Clean Reads Ratio (%)	79.37
Assembly	Trinity
Number of contigs	166,017
Total length of contigs (bp)	94,985,034
Mean length of contigs (bp)	572
Contig size N50/N70/N90 (bp)	972/439/226
GC percentage	47.88%
Number of ungiese	74,550
Total length of ungiese (bp)	61,962,063
Mean length of ungiese (bp)	831
ungiese size N50/N70/N90 (bp)	1440/820/312
GC percentage	47.48%

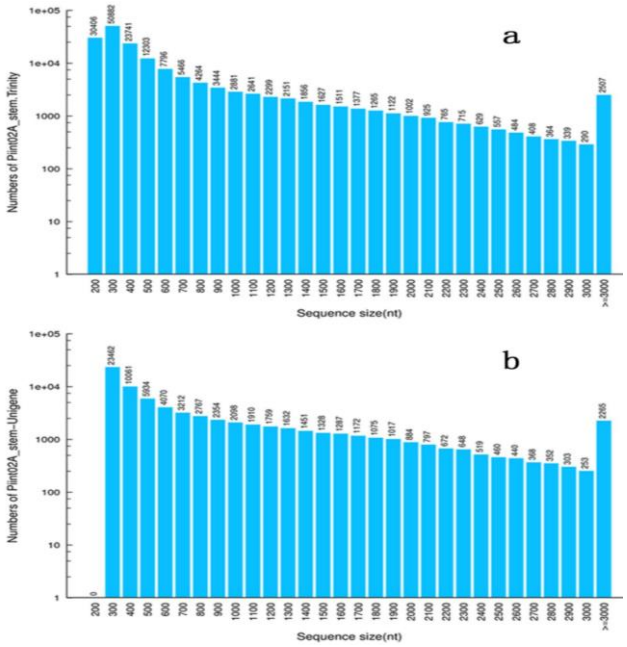

Fig. 1: Overview of *P. integrifolia* assembly. a. Size distributions of the contigs; b. Size distributions of the unigenes

Functional Annotation

All of the unigenes were blasted to the seven public databases (NR, NT, Swiss-Prot, KEGG, COG, Inter pro and GO) by BLASTN ($E<10^{-5}$). 59.86% of unigenes (44,626) were annotated in the seven public databases: 54.86% (40,900) unigenes in Nr database, 44.59% (33,241) unigenes in Nt database, 38.46% (28,669) unigenes in Swiss-Prot database, 42.68% (31,851) unigenes in KEGG database, 22.53% (16,797) unigenes in COG database, 36.27% (27,037) unigenes in Interpro database, 8.2% (6,112) unigenes in GO database.

Nr Annotation

Almost 54.86% (40,900) of the unigenes were annotated using the Nr database. For the species distribution of aligned hits, 33.16% to sequences from *Phoenix dactylifera*, 13.29% to sequences from *Nelumbo nucifera*, 11.52% to sequences from *Musa acuminata subspp*. *Malaccensis*, 7.45% to sequences from *Vitis vinifera* and 34.57% of hits had top matches to sequences from other (Fig. 2).

COG Classification

A total of 16,797 unigenes were annotated to 25 functional categories using COG (Fig. 3). Among the 25 COG categories, the largest cluster was "General function prediction only"(25.36%), followed by "translation, ribosomal structure, and biogenesis" (17.25%), "transcription" (16.85%), "replication, recombination, and repair" (13.78%), "Cell cycle control, cell division, and chromosome partitioning"(13.32%), "Function unknown"(11.76), "posttranslational modification, protein turnover, chaperones" (11.66%), "signal transduction mechanisms" (10.73%), "amino acid transport and metabolism" (10.76%). The smallest groups were "nuclear structures" (6 unigenes) and "extracellular structures" (5 unigenes).

GO Classification

A total of 6112 unigenes were assigned three main GO categories and 53 subcategories (functional groups) (Fig. 4). In the "biological processes" category, a high percentage of genes were involved in "metabolic processes" (3,331 unigenes) and "cellular processes" (2,999 unigenes), followed by "single-organism processes" (2,272 unigenes) and "response to stimulus" (861 unigenes), least "rhythmic process" (3 unigenes) and "behavior" (1 unigene). For the "cellular components" category, the dominant terms were "cells" (2,687 unigenes), and "cell parts" (2,687 unigenes); followed by "organelle" (2,126 unigenes) and "membrane" (1,465 unigenes), "extracellular region part" contained only 1unigene. In the "molecular function" category, the main terms were "binding" (2,828 unigenes) and "catalytic activity" (3,228 unigenes), followed by "transporter activity" (379 unigenes) and "structural molecule activity" (224 unigenes).

KEGG Pathway Mapping

135 KEGG pathways with 31,851 unigenes were found (Fig. 5). The Global and overview maps pathway containing 7733 unigenes is the largest one, followed by Translation (3,950 unigenes), carbohydrate metabolism (2,707 unigenes), Transport and catabolism (2,164 unigenes), Folding, sorting and degradation (2,191 unigenes), Teanscription (1,874 unigenes), Environmental adaptation (1,742 unigenes). Only six unigenes were assigned to "Drug resistance".

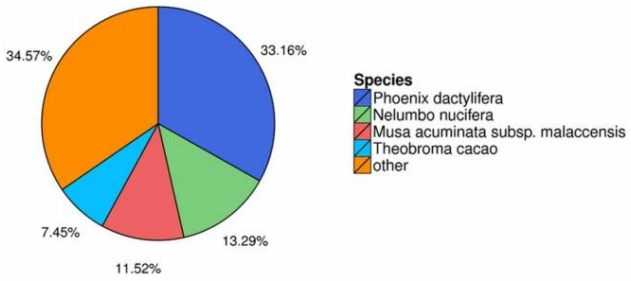

Fig. 2: Number of unigenes matching the top four species using BLASTx in the Nr database

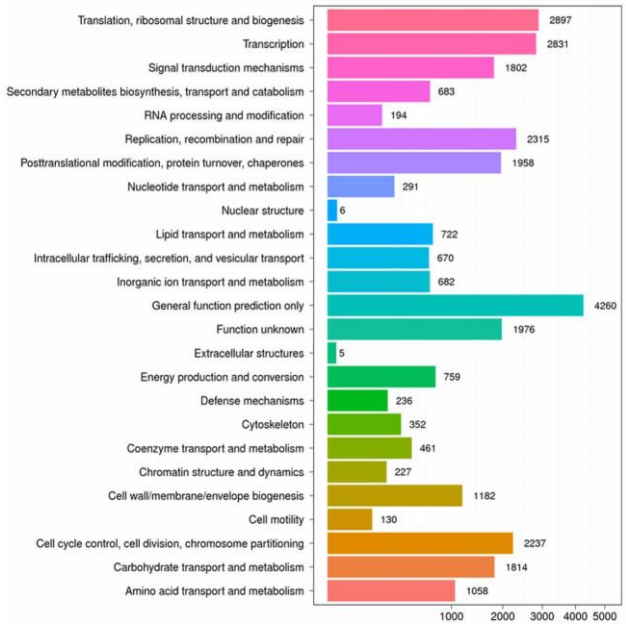

Fig. 3: Clusters of COGs functional classification of the *P. integrifolia*

Members of *PEBP* Genes

A total of 10 unigenes were identified as members of *PEBP* gene family in *P. integrifolia*: 8 unigenes for *flowering locus T (FT)* subfamily, 1 for *Terminal flowering 1(TFL1)* subfamily, and 1 for *Mother of FT and FTL1 (MFT)* subfamily. There are 6 unigens of *FT* subgene family were identified in *A. bulbifer*. A total of 4 unigenes in *P. ternata: 3* unigenes for *FT* subfamily and 1 for *MFT* subfamily. However, only 2 unigenes identified as membes of *PEBP* gene family in *C. esculenta*: 1 ungene for *FT* subfamily, 1 for *MFT* subfamily (Table 2).

Discussion

The transcriptome sequencing in this study produced 166,017 contigs and 74,550 unigenes after assembly. The number of unigenes obtained from *P. integrifolia* was lower than that obtained from *P. ternata* (89,068)

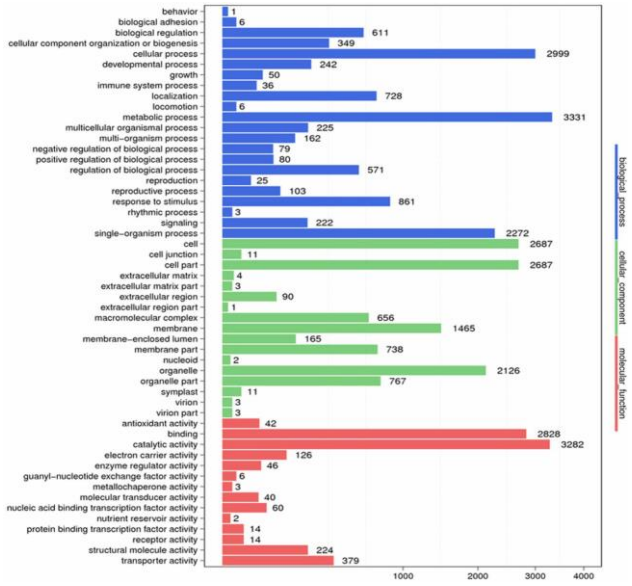

Fig. 4: Clusters of GO classification of the *P. integrifolia*

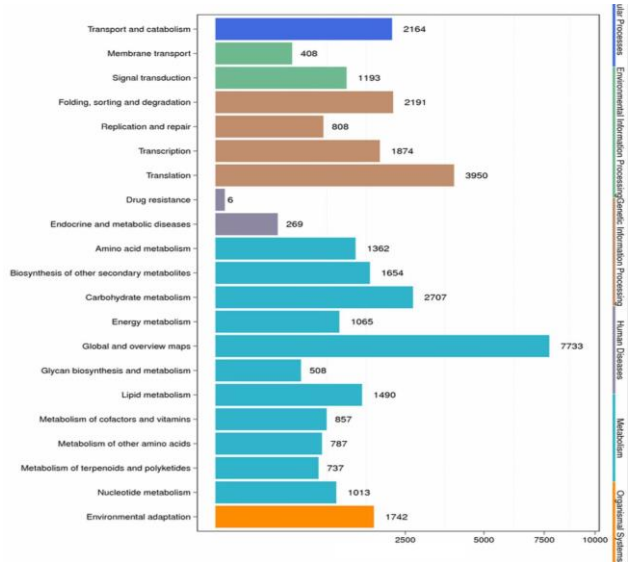

Fig. 5: Clusters of KEGG functional classification of the *P. integrifolia*

(Zhang *et al*., 2016), *A. konjac* (108,651), *A. bulbifer* (119,678) (Diao *et al*., 2014) (Table 3). More unigenes identified in this study may have been resulted from sample mixture of petiole and use of tuber. The average unigene length (831 bp) was slightly greater than that of other plants of the Araceae family, including *P. ternata* (703 bp) (Zhang *et al*., 2016), *P. ternata* (611 bp), *A. konjac* (439 bp), *A. bulbifer* (430 bp) (Diao *et al*., 2014).

Different functional annotation with transcriptome sequencing for *P. ternata*, *P. integriflolia*, and *Amorphophallus* spp. has been observed (Table 4).

Table 2: Unigene No. of *PEBP* genes in four species identified based on transcriptome

Species	*P. integrifolia*	*A. bulbifer*	*P. ternata*	*C. esculenta*
Transcriptome	--	SRX182517	SRX484200	SRX290678
Materials	Petiole and tubers	Leaves	Tuber	Leaves
Total nucleotides (Gb)	6.62	4.71	6.77	4.57
Unigene No. of *FT*	8	6	3	1
Unigene No. of *TFL1*	1	0	0	0
Unigene No. of M*FT*	1	0	1	1

Table 3: Transcriptome sequencing with different species (Araceae)

	P. integrifolia	*P. ternata* (Zhang *et al*., 2016)	*A. konjac* (Diao *et al*., 2014)	*A. bulbifer* (Diao *et al*., 2014)
Number of contigs	166,017	120,983	187,459	199,257
Total length of contigs (bp)	94,985,034	90,698,333	51,822,856	54,982,674
Mean length of contigs(bp)	572	750	276	276
Contig size N50 (bp)	972	1,112	381	372
Number of unigese	74,550	89,068	108,651	119,678
Total length of unigese (bp)	61,962,063	62,683,550	47,711,327	51,480,410
Mean length of unigenes(bp)	831	703	439	430
N50 length(bp)	1,440	1,078	534	524

Table 4: Different functional annotation with transcriptome sequencing

		P. integrifolia	*Amorphophallu* (Diao *et al*., 2014)	*P. ternata* (Zhang *et al*., 2016)
COG	maximum	General Function prediction (4,250 unigenes)	General Function prediction (6,808 unigenes)	General Function prediction (6,282 unigenes)
	minimum	Extracellular structures (5 unigenes)	Extracellular structures (13 unigenes)	Extracellular structures (7 unigenes)
GO	Biological process	12,962 unigenes	40,810 unigenes	29,454 unigenes
	Cellular component	11,419 unigenes	54,943 unigenes	35,061 unigenes
	Molecular Function	7,066 unigenes	25,109 unigenes	16,875 unigenes
KEGG	pathway	135	121	126
	maximum	Global and overview maps (7,733 unigenes)	Metabolic pathways (5,957 unigenes)	Metabolic pathways (3,612 unigenes)
	minimum	Betalain biosynthesis (2 unigenes)	Betalain biosynthesis (9 unigenes)	Betalain biosynthesis (1 unigene)

According to the COG classification, "General Function prediction" and "Extracellular structures" was the largest and the smallest category, respectively for these three species. For Go classification, unigenes were related to "Biological process", "Cellular component", and "Molecular Function". The smallest group identically was "Molecular Function" (7,066 for *P. integrifolia*, 25,109 for *Amorphophallu*, and 17,857 for *P. ternata*). In the *P. integrifolia*, the most unigenes was grouped in "Biological process" (12,962). However, the most unigenes were grouped in Cellular component: *Amorphophallu spp.* (54,943) and *P. ternata* (35,061). In the KEGG pathway analysis, 135, 121, 126 KEGG pathways have been revealed in *P. integrifolia*, *Amorphophallus* spp., and *P. ternata*, respectively. This result indicated that *P. integrifolia* possess additional secondary metabolic pathways to adopt its particular habitat.

The *PEBP* gene family could be subdivided into three subfamilies: *FT*, *TFL1* and *MFT*. The *PEBP* gene family is presented in all eukaryotic organisms. In Angiosperm, various members of the *PEBP* gene family were involved in many developmental process, such as flowering (Li *et al*., 2015b), stomatal control (Kinoshita *et al*., 2011), inflorescence meristem stabilization (Liu *et al*., 2014), tuber formation (González-Schain *et al*., 2012), bulb formation (Lee *et al*., 2013), and so on. Ten, six, four, and two unigenes of *PEBP* were identified in *P. integrifolia*, *A. bulbifer*, *P. ternata*, and *C. esculenta*, respectively. Differences of unigenes number for *PEBP* gene may be resulted from two reasons except for species identity. Firstly, different plant materials were used: petiole and tubers for *P. integrifolia*, leaves for *A. bulbifer*, tubers for *P. ternata*, and leaves for *C. esculenta*. Secondly, total bases sequenced for these four species: 6.62 Gb, 4.71 Gb, 6.77 Gb, and 4.57 Gb, respectively. *TFL* unigene identification revealed only one member in *P. integrifolia* and no unigene in other three species. Compared to these Araceae species, three members of the *TFL1* in the *Jatropha curcas* (Li *et al*., 2015a), two in *Poplar* (Igasaki *et al*., 2008) and three in grapevines (Carmona *et al*., 2007) were identified. The difference of *TFL1* members maybe resulted from differential evolutionary history for these species. Furthermore, these differences between four Araceae species and other species in *TFL1* members may result from the difference of reproductive strategies: asexual reproduction mainly for four Araceae species, sexual reproduction for other species. These differences coincide with the function of *TFL1* that mainly represses flowering (Wickland and Hanzawa, 2015).

Conclusion

The transcriptome sequencing of *P. integrifolia* has been reported for the first time. Based on the anlysis of transcriptomic data of *P. integrifolia* and other related species, the difference for members of the *PEBP* gene family that plays various roles in plant development including the formation of vegetative organs was also revealed.

Acknowledgements

The present investigation is financially supported by the National Natural Science Foundation of China (31560081).

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(Received 05 December 2017; Accepted 28 December 2017)