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Full Length Article

Comparative Transcriptome Profiling Analysis to Unravel the Potential Mechanism of Seed Abortion in *Lumnitzera littorea*

Jingwen Zhang[1,2], Yong Yang[2] and Ying Zhang[1*]
[1]*Life Science and Technology School, Lingnan Normal University, Zhanjiang 524048, P.R. China*
[2]*Ministry of Education Key Laboratory for Ecology of Tropical Islands, College of Life Sciences, Hainan Normal University, Haikou 571158, P.R. China*
For correspondence: zhangyingred@yahoo.com
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Abstract

Lumnitzera littorea (Jack) Voigt. is a species of mangrove plant belonging to the family Combretaceae. Natural reproduction of *L. littorea* is extremely difficult due to its seed abortion in China. To reveal the molecular mechanism of seed abortion, we performed transcriptome to analyze the seeds of *L. littorea*-T (normal seeds) and *L. littorea*-S (abortive seeds). After analysis of the raw data, 64,868 transcripts (mean length = 658 bp) were assembled. Among these transcripts, 39,779 were functionally annotated. Then, differentially expressed genes (DEGs) were screened, and 23,513 transcripts were identified that were likely involved in seed abortion. About 207 DEGs assigned to Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways, and approximately 12.1% fell into reproduction categories. Genes involved in response to sucrose and starch metabolism, and phytohormone biosynthesis showed significant different expression levels between normal and abortive seeds of *L. littorea*. Further expressions patterns of key genes contribute to seed development were analyzed using quantitative real-time PCR, and the results were consistent with high-throughput sequencing data. The information obtained in this study will aid in the research of the mechanism of seed abortion in further molecular studies of *L. littorea*. © 2021 Friends Science Publishers

Keywords: Endangered mangrove; *Lumnitzera littorea*; Transcriptome; Seed abortion

Introduction

Mangroves are a group of woody plants that grow in tropical and sub-tropical intertidal zones. As the most productive and diverse wetlands in the coasts, mangroves provide important ecological services for costal ecosystems (Tomlinson 1986). In recent years, due to human activities and environmental changes, mangrove areas have been decreasing sharply, facing the biodiversity loss on ecosystems (Lovelock *et al.* 2015).

Lumnitzera littorea (Jack) Voigt. is a thermophilic mangrove species distributed in tropical Asia and Australia and listed as an endangered species in "International Convention on Wetlands" (Polidoro *et al*. 2010). Because of its specific living environment requirements, such as average temperatures of 21–25°C, 0.5–2.7% salinity, and light, *L. littorea* is only distributed in Sanya of Hainan Province and the number of individuals has decreased rapidly in the past decade, from 359 in 2006 (Fan and Chen 2006) to nine with one population in 2017 (Zhang *et al*. 2017). Currently, the extremely long dormancy periods and high abortion ratio of its seeds were seriously restricted sexual reproduction of *L. littorea* (Zhang *et al.* 2017). Generally, each flower contains 3–5 ovules. However, the rate of aborted seeds is up to 76.54 ± 0.50%, and only any well-developed seed could be found in *L. littorea* fruit in China (Zhang *et al.* 2013). Owing to its rigorous ecotope demand and low fecundity, *L. littorea* cannot realize the natural reproduction and is critically endangered in China, while there are no reports about the natural reproduction in other countries (Su *et al.* 2007).

In order to find the reason of seed abortion, several studies have carried out in *L. littorea* (Zhang *et al.* 2016, 2017). *L. littorea* is outcrossing with partial self-pollination in China. Due to the rare population, *L. littorea* is forced to self-pollination leading to ovule browning, pistil abortion, embryogenesis arrest and other phenomena. Second, the pollens vitality was lower than 10%. Third, the embryo has been eaten by a small grub that originates from eggs laid by the parent insect early in development.

Seed abortion is common in plants and has been widely studied. Many studies focus on gene regulation to illustrate seed abortion of model plant, while there are few reports for non-model woody plants, especially for endangered tree species. Based on the *de novo* sequencing technology, large-scale transcriptome data were being used

to establish the unigene library for non-model species. Therefore, it is possible to widely discover the genes in different species that do not contain any reference genome information (Xu *et al.* 2016). For example，seed abortion has caused the dove tree (*Davidia involucrata* Baill.) to become an endangered species. To investigate the mechanism by which species become endangered, *de novo* sequencing was performed. As a result, WRKY and MYB transcription factors, laccase and receptor kinase are present that play important functions in seed abortion (Li *et al.* 2016). As the study showed that GA application could change the antioxidant enzyme activities to effect on redox homeostasis by regulating the transcript levels of various genes believed to be involved in seed development (Cheng *et al.* 2013, 2015).

In the present study, two materials, normal and abortive seeds of *L. littorea* from two different locations, Thailand and China, were used to study the mechanism of seed abortion. The transcriptomes from developed seeds of the normal and abortive materials were performed. And the differentially expressed genes (DEGs) were identified between normal and abortive seeds. The objectives of this study were to identify functional genes involved in seed abortion and highlight the molecular mechanisms related to *L. littorea* seed abortion in China.

Materials and Methods

Sample collection

Two types of seeds, normal and abortive seeds at the same developmental stage, were collected from two different locations. The normal seeds of *L. littorea* were collected from different trees of the naturally distributed *L. littorea* population in Lam Ri-ngun, Chanthaburi, east coast, Thailand (N:12°23′/ E:102°16′). The abortive seeds were collected in the Hainan SanYaTieLu By National Nature Reserve Administration Bureau, Hainan Province, China (P.R.). The seeds were collected approximately one month after pollination. The seeds were dissected immediately from the fruits to distinguish normal or abortive seeds after collection. The normal seeds and abortive seeds were separated and kept in RNALOCKER (TIANDZ, China).

RNA extraction and sequencing

Total RNA was extracted from the two types of young seeds (200 seeds/type). We used TRIzol reagent (Invitrogen, U.S.A.) for RNA isolation. Each digital gene expression (DGE) library contained two biological replicates. After extraction of total RNAs of each sample, qualified RNA samples were used to construct complementary DNA (cDNA) libraries with NEBNext® Ultra™ RNA Library Prep Kit (NEB, U.S.A.). The raw data, including four libraries, were uploaded to NCBI SRA (accession number: SRP115695).

Transcriptome *de novo* assembly

The two transcriptome libraries were sequenced. After sequencing, the raw image data were converted into raw sequence data by base calling. Then, the raw data were treated with following steps: (1) Adaptor sequences fragments, reads with unknown sequences 'N' were greater than 10%, and low-quality sequences (the percentage of quality value $\leq$ 5 was greater than 50% in a read) were removed. (2) These filtered reads were carried out by Trinity software with min_kmer_cov set to 2 by default and all other parameters with default values (Grabherr *et al.* 2011). (3) After assembling the data, the contigs were mixed together for combined analysis. The combined assembled sequences were finally used as reference sequencing data for the following gene expression analysis.

Functional annotation of transcripts

All unigenes were searched against the following public databases, Nr (non-redundant, http://www.ncbi.nlm.nih.gov/), Swiss-Prot (http://www.expasy.ch/sprot/), KEGG (Kyoto Encyclopedia of Genes and Genomes, http://www.genome.jp/kegg/) and COG (Clusters of Orthologous Groups of proteins, http://www.ncbi.nlm.nih.gov/cog/) using the BLASTX algorithm (cut-off value of E<1e-5). To classify the unigenes, the Blast2GO program was used to get the GO annotations (Conesa *et al.* 2005). WEGO software was used to perform GO functional classification for all transcripts (Ye *et al.* 2006).

Identification of DEGs

To identify the DEGs, the gene expression value was calculated based on the FPKM method (Mortazavi *et al.* 2008). The gene expression value was calculated by using the numbers of reads that were mapped to the reference assembled sequence data. After calculating the gene expression level, the DEGs were screened. We performed differential gene expression analysis using the R package DESeq (1.10.1) (Anders and Huber 2010). In this study, DEGs were adjusted based on both genes *with P* value < 0.01 and |log2 fold change (Abortive/Normal Seeds)| $\geq$ 1.

qPCR analysis

Total RNA was extracted and reverse transcription was performed to examine the gene expression value with quantitative real-time reverse transcription PCR (qRT-PCR). qPCR reaction was performed using the SYBR premix Ex Taq kit (TaKaRa, Japan) on an ABI 7500 Real-Time System. A *L. littorea* gene, *LiActin* (unigene13201), was used as endogenous reference for data normalization. The relative expression of target genes was calculated using $2^{-\Delta\Delta CT}$ method (Quail *et al.* 2008). All experimental samples

were repeated in triplicate. All primer pairs used for qRT-PCRs were listed in Additional file 1: Table S1.

Results

De novo assembly

Two cDNA libraries of normal and abortive seeds of *L. littorea* were generated and sequenced. In total, 485,698,877 raw sequencing reads were generated from the 200 bp insert library. After filtering, 102,164,440 clean data were obtained. Then, the clean data were used to assembly analysis. After clustering the contigs, 71,390 and 63,066 transcripts were obtained. Then, the unigene information of the two libraries was used for comparative analysis. As a result, 64,868 transcripts were obtained, and the average length was 714 bp with an N50 of 1,180 bp. For the length distribution analysis, approximately 41.95% of the transcripts had lengths greater than 500 bp (Table 1).

Annotation of all nonredundant transcripts

To further validate and annotate the assembled transcripts, all assembled transcripts were searched against the Nr and SwissProt databases by BLAST 2.2.28+ program (E-value < 1E−5, Fig. 1a). For all 64,868 transcripts, 39,779 transcripts (61.32%) had at least one significant match to an existing gene. Using the NR database, 37,523 (57.85%) transcripts matched sequences annotated in NR (Table S2). Among them, 8,466 (22.6%) shared more than 80% similarity with an established sequence (Fig. 1b). For the species similarity analysis, all of the transcripts could be mapped to approximately 200 species; a high percentage of *L. littorea* sequences (24.7%) were homologous to *Vitis vinifera* genes, followed by *Amygdalus persica* genes (15.4%) and *Ricinus communis* genes (14.8%) (Fig. 1c). Using the NT database, 30,671 (47.28%) transcripts matched sequences annotated in NT (Table S2). A total of 23,964 transcripts (36.94%) were mapped to SwissProt compared with the NR database (Table S2). In total, 15,082 unigenes were hit the Nr and SwissProt protein databases, indicating that this study produced a substantial fraction of the fertility-related genes in *L. littorea*.

Functional classification by GO and COG

Based on the NR annotation, a Gene Ontology (GO) analysis was conducted. In total, 29,094 transcripts were assigned to GO classes with 55 functional terms. As shown in Fig. 2, the primary category was biological process (14,142, 48.61%), followed by cellular component (10,907, 37.49%), and molecular function (4,044, 13.90%). In the biological process category, cellular process (18,349, 15.85%) and metabolic process (17,488, 15.10%) was prominent (Fig. S2). The results indicated that important cell activities and metabolic processes occurred in the embryos formation and development stages of *L. littorea*. For cellular component, cell and cell part, organelles, and membrane and membrane components accounted for about 50, 26.03 and 14.04%, respectively. In the molecular function category, the major subcategories were catalytic activity (14,216, 42.93%) and binding (13,671, 41.29%). The following categories contained 3,562 transcripts, representing only 10.76%. Among the 37,523 transcripts with significant similarity to the NR proteins, 13,998 transcripts could be matched to COG database (Fig. S1). In total, 25 COG categories were assigned, and the major subcategories were general function prediction cluster (4,384, 16.63%), followed by transcription (2,376, 9.01%), recombination, replication, and repair (2,158, 8.19%).

Functional classification by KEGG pathway

In addition to COG category analysis, KEGG pathway was used to analyze all 64,868 transcripts in *L. littorea*. The results indicated that 42.26% (27,415) of transcripts were positively matched with the database and could be divided into 5 main categories in 128 KEGG pathways. Of which, metabolism represented the largest proportion (17,363, 63.33%), followed by general information (5,713, 20.84%), organismal systems (1,631, 5.95%), environmental information processing (1,539, 5.61%), and cellular processes (1,169, 4.26%). These results indicated that the relative active metabolic processes occurred in the gamete formation process. As shown in Table S3, the KEGG pathway metabolism contained 11 categories, including lipid metabolism, nucleotide metabolism, energy metabolism, and carbohydrate metabolism.

Analysis of DEGs between normal and abortive seeds

According to screening criteria, 23,513 DEGs were detected between the normal seeds from Thailand and abortive seeds from Sanya (Table S4). A total of 13,043 transcripts were upregulated and 10,470 transcripts were downregulated in *L. littorea*-S compared with *L. littorea*-T. Among the differentially expressed genes, 1,920 DEGs had no homologs in the NCBI database, 10,459 transcripts were annotated with GO terms (Fig. 2a), and 8,127 transcripts were identified in the KEGG pathway annotation (Fig. 2b). Among them, approximately 5.94% transcripts were related to plant hormone signal transduction, nitrogen metabolism, and starch and sucrose metabolism, indicating that these pathways might respond to seed formation processes.

Validation of DEGs by qPCR

qRT-PCR analyses were performed to verify RNA-seq data. Eight genes with various expression patterns, related to plant hormone signal transduction, starch biosynthesis and catabolism, and cell wall invertase, were chosen for qRT-PCR analysis. As shown in Fig. 3, five genes were

Table 1: Summary of the *L. littorea* transcriptome assembly

Type	Sample	Total number	Total length (bp)	Mean length (bp)	N50 (bp)
Contig	*L. littorea*-T	174,024	44,746,514	257	350
	L. littorea-S	119,090	40,150,743	337	645
Unigene	*L. littorea*-T	71,390	37,175,316	521	919
	L. littorea-S	63,066	39,730,396	630	1,040
	All	64,868	46,291,877	714	1,180

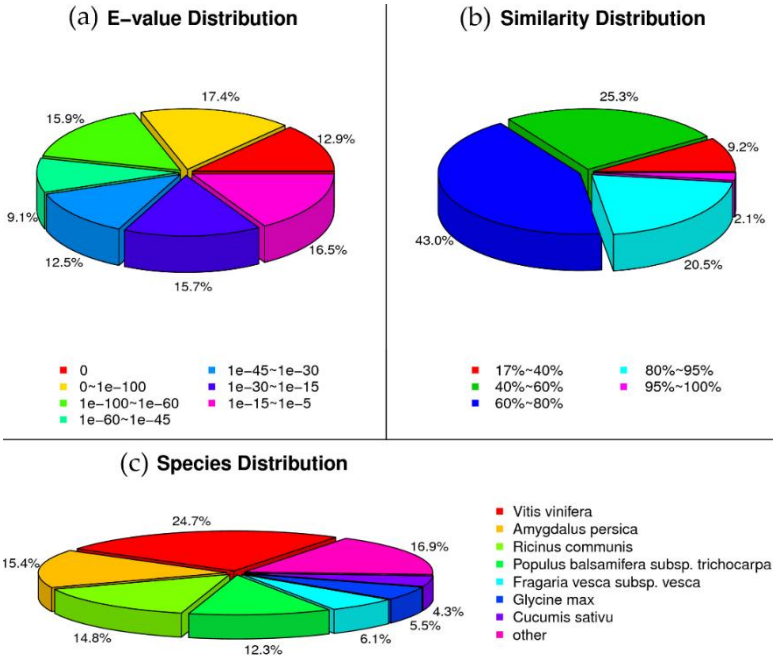

Fig. 1: BLAST results of *L. littorea* transcriptome. (**a**) E-value and (**b**) Similarity distribution of the top BLAST hits. (**c**) Species distribution of the BLAST hits for each unigene in the NR database

significantly upregulated in abortive seeds, and the other three genes were downregulated in abortive seeds. The gene expression pattern was similar between the DGE profile and qRT-PCR, indicating that the sequencing libraries were truly representative of the differentially expressed genes between the normal and abortive seeds from the two different locations. Moreover, these genes may be target genes that cause seed abortion and require further validation.

Discussion

Being an important and endangered species of mangrove plants in China, *L. littorea* is a very valuable resource that has many useful genes that could be used for cultivated plant improvement. Due to its unique living environment requirements, the distribution area of *L. littorea* is restricted, and research is limited (Zhang *et al.* 2013). Most of the research has focused on cytological and tissue culture, and few molecular biology studies have investigated the mechanism of seed abortion. In this study, we analyzed the transcriptomic changes that occurred in the normal and abortive seeds of *L. littorea* from two different locations. As expected, numerous known genes were identified, including starch and sucrose metabolism genes, hormone signal transduction genes, and some possible new genes to be candidates for studying mechanisms involved in the abortion of L. littorea seeds in Sanya Province, China (Table S4 and S5).

In angiosperms, as the key yield components, the development of seeds and fruits has been studied for decades. These processes are energy-intensive and depend greatly on an adequate import of photo assimilates sucrose, which is produced in photosynthetically active leaves to support non-photosynthetic tissues, such as seeds, fruits and tubers. In the sucrose metabolism pathway, sucrose is often hydrolyzed by cell wall invertase (CWIN) into glucose and fructose (Ruan *et al.* 2012). Combined the transcriptomic and metabolomics analysis, high CWIN activity could promote fruit set by altering cell cycle and cell wall synthesis (Ru *et al.* 2017). In tomato, the activity of a major CWIN gene, *LIN5*, is significantly increased after pollination in comparison with an unpollinated control (Shen *et al.* 2019). Interestingly, genes (Unigene11647_All) encoding cell wall invertase were downregulated in the abortive seeds in our study, suggesting

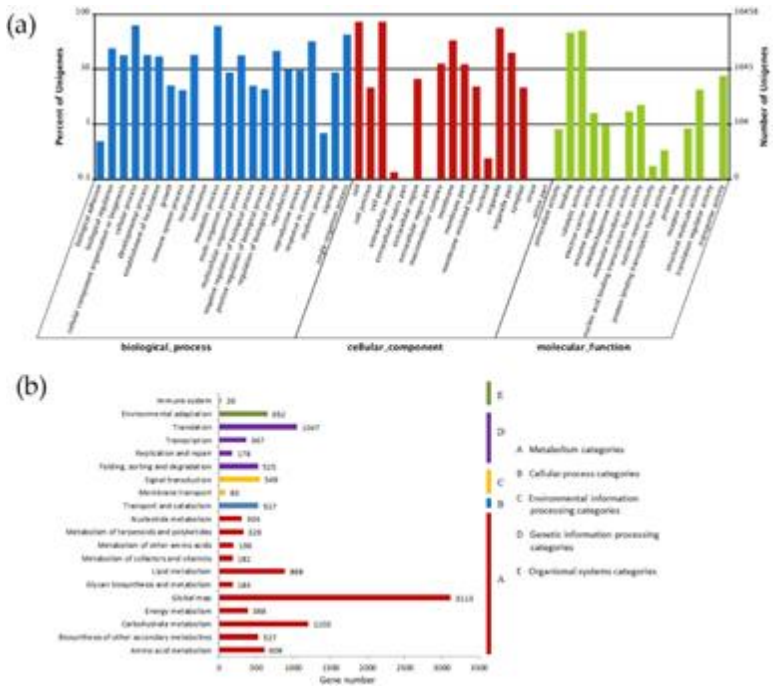

Fig. 2: Proportions of DEG transcripts by GO and KEGG. (**a**) GO classification of DEGs; (**b**) KEGG pathway of DEGs between normal and abortive seeds. The left y-axis indicates the number of transcripts in that main category. The bottom x-axis indicates the specific category of transcripts in that main category

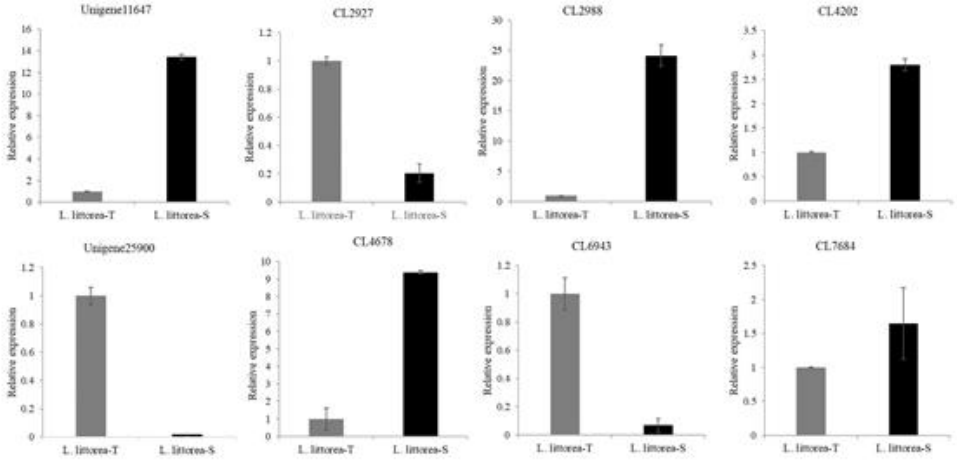

Fig. 3: Expression patterns of DEGs in *L. littorea* seeds. The *unigene13201* was used as an internal reference. Bar depict SD (n=3)

that cell wall invertase activity deficiency may cause the seed abortion in *L. littorea*.

Starch, as another energy source, can be hydrolyzed into glucose, particularly under environmental stress. The resultant glucose leads to a inhibition in programmed cell death (PCD) genes and promotion in cell division, which together lead to seed and fruit set (Ruan *et al.* 2012). Some genes involved in starch biosynthesis and catabolism, including catalase, starch branching enzyme and fructokinase, were downregulated in *L. littorea*-S, suggesting that the starch content in abortive seed is low. These findings provide insights into the roles of sucrose activation in fruit and seed set and identify new genetic targets to improve reproductive success.

Previous studies have shown that seed and fruit development is closely related to phytohormone regulation. The normal development of seed and fruit requires a variety of hormones, such as auxins, GAs, ethylene and brassinolides (Sun *et al.* 2010).

Research shows that postfertilization accumulation of auxin is required to initiate endosperm development, even in woody plants (Sun *et al.* 2017). Dove tree (*D. involucrata*),

as an endangered species, has several genes encoding auxin-response factors that are downregulated in abortive seeds based on comparative transcriptomics (Li *et al.* 2016). According to our data, auxin-response factors were found among the DEGs. A number of auxin-response factors were upregulated in normal seeds. Gibberellin is a key player in fruit initiation, and GA biosynthesis genes are upregulated after pollination (Serrani *et al.* 2008). There were nine genes encoding gibberellin receptors among the DEGs, of which five and four gibberellin receptors were up- and downregulated, respectively. Study showed that ethylene biosynthesis and ethylene signaling genes down-regulate after pollination (Ruan *et al.* 2012). In our data, most ethylene-responsive transcription factors were least expressed in abortive seeds. Due to the limited samples, the DEGs involved in phytohormone biosynthesis were not as obvious as reported. Therefore, more detailed samples and sequencing should be collected for analysis.

Impact of genetic diversity and population on *L. littorea*

Genetic diversity plays an important role in allowing individual species to resist climate change (Ravenscroft *et al.* 2015). In endangered *Acer yangbiense*, a high selfing rate in seedlings was found, resulting in a low level of genetic diversity (Yang *et al.* 2015, 2019). The population decline in the critically endangered *Ostrya rehderiana* has resulted in self-pollination and seed abortion, which has caused extensive homozygosity and increased genetic load (Yang *et al.* 2018a). Studies comparing genomic patterns of diversity between the endangered *Ostrya rehderiana* (IUCN Red List) and the widespread *O. chinensis* show that *O. rehderiana* accumulates more deleterious mutations than *O. chinensis* (Yang *et al.* 2018b). According to our investigation, *L. littorea* is forced to undergo selfing in China, resulting in breeding difficulties and reduced genetic diversity in Sanya Province, China (Su *et al.* 2007; Zhang *et al.* 2017). The patterns detected in *L. littorea* may be similar to those detected in *O. rehderiana*, which requires detailed genomic information.

Plant populations are often adapted to their local environments (Leimu and Fischer 2008). Rubisco catalyses a rate-limiting step in photosynthesis and have long been a target for improvement due to its slow turnover rate (Sharwood 2017). However, the overexpression of the Rubisco assembly chaperone RUBISCO ASSEMBLY FACTOR 1 (RAF1) resulted in a >30% increase in Rubisco content, which could improve the tolerance of maize to extreme growth environments (Salesse-Smith *et al.* 2018). Furthermore, Rubisco activase could regulate the activity of Rubisco and keep Rubisco in a high activation level under in vivo conditions (Bracher *et al.* 2017; Zhang *et al.* 2019). *L. littorea* grows in harsh environments and in ecosystems that are highly fragile and found in very limited areas in China (Zhang *et al.* 2017). Interestingly, three genes (*Unigene36992*, *Unigene41498*, and *Unigene17234*) encoding Rubisco activase were found among the DEGs, all of which showed dramatically increased expression in abortive seeds according to our research. This result showing no difference with the finding that the reduction in severely deleterious recessive variations may have allowed endangered *O. rehderiana* to survive at low population sizes over extended time periods (Yang *et al.* 2018a, b). Thus, further research should focus on designing an appropriate hybrid strategy to avoid inbreeding and increasing the genetic diversity rather than improving the total number of seedings through the collection of inbred seeds. A large number of mangrove plants are currently dying out, and the same strategy should be carried out to facilitate population recovery.

Conclusion

In the current study, two cDNA libraries of developing seeds from Sanya and Thailand were evaluated via high-throughput sequencing. After combined sequence assembly, 64,868 unigenes were identified, and GO, KEGG, and COG analyses were performed with these unigenes. In the DEG analysis, 23,513 DEGs were discovered, including sucrose and starch metabolism and phytohormone biosynthesis genes, which are potentially involved in seed development. Furthermore, the living environments, distribution and genetic diversity also affect the seed reproduction of *L. littorea*. Our study provides a foundation to understand and further unravel the molecular mechanism of seed abortion of *L. littorea* in China.

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Author Contributions

Ying Zhang designed the experiments and reviewed drafts of the paper. Jingwen Zhang analyzed the data, performed the experiments and wrote the paper. Yong Yang contributed materials.

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