High throughput sequencing of small RNAs reveals dynamic microRNAs expression of lipid metabolism during *Camellia oleifera* and *C. meiocarpa* seed natural drying

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**Abstract**

**Background:** Camellia species are ancient oilseed plants with a history of cultivation over two thousand years. Prior to oil extraction, natural seed drying is often practiced, a process affecting fatty acid quality and quantity. MicroRNAs (miRNA) of lipid metabolism associated with camellia seed natural drying are unexplored. To obtain insight into the function of miRNAs in lipid metabolism during natural drying, Illumina sequencing of *C. oleifera* and *C. meiocarpa* small-RNA was conducted.

**Results:** A total of 274 candidate miRNAs were identified and 3733 target unigenes were annotated by performing a BLASTX. Through integrated GO and KEGG function annotation, 23 miRNA regulating 131 target genes were identified as lipid metabolism, regulating fatty acid biosynthesis, accumulation and catabolism. We observed one, two, and four miRNAs of lipid metabolism which were specially expressed in *C. Meiocarpa*, *C. oleifera*, and the two species collectively, respectively. At 30% moisture contents, *C. meiocarpa* and *C. oleifer* produced nine and eight significant differentially expressed miRNAs, respectively, with high fatty acid synthesis and accumulation activities. Across the two species, 12 significant differentially expressed miRNAs were identified at the 50% moisture content.

**Conclusions:** Sequencing of small-RNA revealed the presence of 23 miRNAs regulating lipid metabolism in camellia seed during natural drying and permitted comparative miRNA profiles between *C. Meiocarpa* and *C. oleifera*. Furthermore, this study successfully identified the best drying environment at which the quantity and quality of lipid in camellia seed are at its maximum.

**Keywords:** miRNAs, Lipid metabolism, Natural drying, *Camellia meiocarpa*, *Camellia oleifera*

**Background**

*C. oleifera* and *C. meiocarpa* belong to the camellia family, Theaceae, and are known for their high quality oilseed that is dubbed as the Eastern olive oil [1, 2]. Some of the camellia species are ancient oilseed plants with history of cultivation for over two thousand years [3]. Camellia oil is known for its edible and medicinal uses with an oleic acid content reaching above 80%, a high content of monounsaturated lipid, and the lowest level of saturated fats [4]. Camellia oil aids in cholesterol reduction, resistance to stress, oxidation reduction, reduced inflammation, and improved human immunity [5]. The drying management of camellia seed prior to oil extraction is a fundamental factor affecting its fatty acid quality and quantity [6–8].

MicroRNAs (miRNAs) are small regulatory molecules that have been shown to be involved in a wide range of biological pathways by modulating expression of specific mRNAs [9]. Sequencing small RNA libraries demonstrated the role of miRNAs in lipid metabolism in plants (safflower [10]; *Camelina sativa* [11]; soybean [12]; wheat [13]; *Jatropha* [14]; *Arabidopsis* [15]), animals [16, 17], and insects [18]). Different oil crops were differentially expressed with different seed oil content and composition.
There were 28 miRNAs regulated lipid metabolism from soybean [12], 30 miRNAs from *Camelina sativa* [11], and 13 miRNAs differentially expressed from two safflower genotypes that have difference to regulate oleic acid accumulation [10]. So there may be different miRNA expression between *C. Meiocarpa* and *C. oleifera*.

Recent research uncovered miRNAs regulation of lipid metabolism, which related to diverse pathways, such as fatty acid synthesis, accumulation, and catabolism. For example, miR159b, miR5026, and miR2911 were identified to encode Δ12-desaturase (FAD2) [10, 15, 19, 20], miR408a and miR159b for Fatty acid elongase (FAE1) [11, 15, 44], which related to fatty acid synthesis. Additionally, miR319a, miR001 and miR007 were identified to encode 1-acylglycerol-3-phosphocholine acyltransferase (LPCAT) [11, 14], which involved in fatty acid accumulation and miR166a, miR2910, miR824, miR414, and miR5206 were identified to encode fatty acid oxidase [11, 13, 14, 21], which regulate fatty acid catabolism. As above, the same fatty acid protein can be regulated by different miRNA, and the same miRNA can encode different fatty acid protein. In *C. Meiocarpa* and *C. oleifera*, the presence of different miRNAs expression pattern at different environments and, in particular, in dry seed suggest that miRNAs may play critical roles in lipid metabolism during natural seed drying [22–27].

In order to explore the potential role of miRNAs in lipid metabolism during *C. Meiocarpa* and *C. oleifera* natural drying, miRNA expression profiles of seed samples at different moisture content (10, 20, 30, 40, and 50%) were investigated using high throughput next generation small RNA sequencing technology, so the differentially expressed miRNAs are unraveled to help understand their involvement in lipid metabolism.

**Methods**

**Plant material**

In 2012, mature fruits of *C. meiocarpa* and *C. oleifera* were collected from the four cardinal directions of 3 superior trees’ crowns per species. The trees are growing at the Minhou Tongkou State Forest Farm (26°05′ N, 119°17′ E), Fujian Province, China. The collected fruits were placed in a ventilated room until they naturally cracked and seeds were extracted by manual shell cutting. The seed moisture content at the time of extraction was closed to 50%. Seeds were naturally dried and their moisture content was determined daily. Over time, seed samples were collected at moisture content of 50, 40, 30, 20, and 10% and were sequentially identified as S01 to S05 and S06 and S10 for *C. meiocarpa* and *C. oleifera*, respectively, and sampled three times, then were flash frozen in liquid nitrogen and stored at −80 °C until RNA extraction.

**Oil content analysis**

To obtain the camellia seed oil content, the Soxhlet method was used by a fatty acid instrument (SZF-06A, Shanghai Hongji Instrument Equipment company) [28]. Camellia seeds at different moisture content were cut into thin slices, dried by silica gel and liquid nitrogen, milled into a powder by a pulverizer (FW100, Tianjin Taisite Instrument company). About 2 g of powder were weighed out (M1), packed in a folded filter paper bag and bound with a skim cotton thread, placed into the Soxhlet extraction thimble with 15 mL of petroleum ether for 1 h. Soxhlet extraction bottle was weight (M2) into which extraction thimble was placed, extracted for 5 h using 70 ml petroleum ether (75 °C). After extraction, the solvent was evaporated in Soxhlet extraction bottle and dried at 75 °C drying oven. Then Soxhlet extraction bottle was weighed again (M3).

Seed oil content = \( (M3−M2)/M1 \times 100\% \)

All experiments were carried out at least in triplicate and data were analyzed using the SPSS statistics 17.0 software.

**RNA isolation**

Total RNA was extracted from the seed samples of the two camellia species using RNA kit (RNA simply total RNA kit, Tiangen, Beijing, China) according to the manufacturer’s instructions. The purity and quality of the RNA were determined by assessing the absorbance ratio OD260/280 using NanoDrop ND1000 Spectrophotometer (NanoDrop, Wilmington, DE). The RNA was quantified with a Qubit 2.0 Fluorometer (Invitrogen Corporation, Carlsbad, CA, USA) in accordance with the manufacturer’s instructions. The integrity of the RNA samples was verified using an Agilent 2100 Bioanalyzer (Agilent, Santa Clara, CA, USA).

**Small RNA library construction and sequencing**

Equal amount of total RNA from the three samples at each moisture content of the two camellia species, was mixed to construct a transcriptome library using an Illumina TruSeq RNA Sample PrepKit following the manufacturer’s instructions. Small RNAs of 18–30 nt in length were separated and purified by denaturing polyacrylamide gel electrophoresis. After dephosphorylation and ligation of a pair of Solexa adaptors to their 5’ and 3’ ends, the products were reverse-transcribed and amplified by RT-PCR and gel purification. After the library was constructed, the Qubit 2.0 Fluorometer (Invitrogen Corporation, Carlsbad, CA, USA) were used to calculate the molar concentration and confirm the insert size. The cDNA libraries were sequenced using the Illumina HiSeq2500 Genome analyzer (Illumina Inc., San Diego, CA, USA).
miRNA-Seq data analysis

After sequencing, the raw reads (FASTQ files) were processed into clean reads, then the adaptor sequences, poy(A) tails and the inserted tag were removed, followed by filtering the low-quantity reads (ambiguous bases \(N \geq 10\%\) and more than 20% with Quality Score < 30 bases), the clean 18–30 nt sRNAs were mapped to GenBank (http://www.ncbi.nlm.nih.gov/), Rfam (version 10.1) database (http://rfam.sanger.ac.uk), tRNAdb [29], SILVA rRNA [30] and Repbase (http://www.girinst.org/), and rRNA, tRNA, snRNA, and snoRNA were discarded from the sRNA reads using bowtie2 software with perfect matches (0 mismatches) used for further analysis [31]. The unannotated sequences were then analyzed by miRDeep-P software package to predict miRNAs, which was developed by modifying miRDeep2 [32]. All mature sequences, star sequences and precursor sequences cored by miRDeep2 were retained and regarded as putative miRNAs and used for further analysis to identify known and novel miRNAs [33, 34]. Known miRNAs were annotated by identifying their homologous imRNAs in miBase database (http://www.mirbase.org/) using the following criteria: 1) seed region, nucleotides 2–7 must be identical; and 2) the remainder of the sequence alignment must contain no more than two mismatches [35, 36]. The putative miRNAs produced by miRDeep2 analysis were regarded as conservative miRNAs when it met the above criteria. Those miRNAs produced by miRDeep2 analysis that did not meet the above criteria were considered as novel miRNAs. In order to predict novel miRNAs with high confidence, only those with a miRDeep-P score higher than \(>0\) were retained as true novel miRNAs [34, 37].

Screening of differentially expressed miRNAs

Differentially expressed miRNAs were identified using the TPM [38] and IDEG6 software [39]. TPM (Tags Per Million reads) is a standardized method for calculating miRNA expression levels. TPM values were calculated using the following equation: TPM = number of mapped miRNA reads/number of clean sample reads \(\times 10^6\). In order to calculate the levels of differential expressed miRNAs, normally the value was set as 0.01 by default when the sequencing read is 0 (no reads) [40]. We calibrated miRNA expression levels using multiple hypothesis tests with a false discovery rate (FDR) \(\leq 0.01\), performed generalized chi-square tests for differential miRNA expression using the IDEG6 software (http://telethon.bio.unipd.it/bioinfo/IDEG6/), and screened the miRNAs for those with \(P\)-values less than 0.01 or TPM ratios between samples that were greater than 1 (fold change \(\geq 1\)) or FDR \(\leq 0.01\). The miRNAs that met these criteria were identified as being differentially expressed [41].

miRNA target prediction

The putative target sites of miRNAs were identified by aligning mature miRNA sequences with a draft genome sequence using TargetFinder, (http://carringtonlab.org/resources/targetfinder). miRNA targets were computationally predicted essentially as described [38, 42, 43]. Briefly, potential targets from FASTA searches were scored using a position-dependent, mispair penalty system. Penalties were assessed for mismatches, bulges, and gaps (+1 per position) and G:U pairs (+0.5 per position). Penalties were doubled if the mismatch, bulge, gap, or G:U pair occurred at positions 2 to 13 relative to the 59 end of the miRNAs. Only one single-nucleotide bulge or single-nucleotide gap was allowed, and targets with penalty scores of four or less were considered to be putative miRNA targets [42, 44].

Functional annotation of predicted target genes was assigned using Nr (non-redundant protein database, NCBI), Nt (non-redundant nucleotide database, NCBI), Swiss-Prot, GO (gene ontology, http://www.geneontology.org/) and COG (clusters of orthologous groups) databases. BLASTX was employed to identify related sequences in the protein databases based on an Evalue of less than \(10^{-5}\) [45]. Gene ontology (GO) and Kyoto encyclopedia of genes and genomes (KEGG) enrichment analysis were performed with package GO stats (http://www.geneontology.org/) of \(P\) value \(<0.05\) was set as the cut-off to select out significantly enriched terms [46].

Quantitative real-time PCR analysis of miRNAs

qRT-PCR was used to validate the miRNAs identified using deep sequencing and to analyze their expression patterns. Total RNA of C. meiocarpa and C. oleifera seeds samples, was extracted using TRzol Universal Reagent according to the manufacturer’s protocol. They were then reverse-transcribed into cDNA using the microRNA cDNA First Strand cDNA Synthesis Kit (CWBO, Beijing, China) according to the manufacturer’s instructions. The cDNA was quantified by microRNA Real-Time PCR Assay Kit (CWBO, Beijing, China) using a 20 \(\mu\)l reaction mixture, which consisted of 1 \(\mu\)l of diluted cDNA, 0.25 \(\mu\)l forward and reverse primer, and 10 \(\mu\l of 2 \times SYBR Green PCR Master Mix (CWBO, Beijing, China). All reactions were performed under the following conditions: 95 °C for 5 min, 40 cycles of 95 °C for 15 S, 62 °C for 45 S. Melting curve analysis was performed to verify specific amplification (from 72 to cycles at 60 °C for 15 S). Each sample was processed in triplicate, and 5.8S rRNA was used as an internal control [47, 48]. The equation ratio 2 \(^{\Delta\Delta CT}\) was applied to calculate the relative expression level of miRNAs. The qRT-PCR primers are listed in file (Additional file 1: Table S1) and \(Ct\) value of 5.8S (Additional file 1: Table S2).
Results
Oil content of two camellia species during natural seed drying
Camellia oil content were determined by Soxhlet extraction method during natural seed drying (Table 1). The two species showed increased in oil content at 50 to 30% moisture contents followed by a slow decline at 30 to 10% moisture content, with 30% moisture content producing the highest oil content. Oil content accumulation was largest when the seed moisture content dropped from 50 to 40% and 40 to 30% for C. meiocarpa and C. oleifera, respectively. At 30% moisture content, C. meiocarpa and C. oleifera showed increase in their seed oil content of 8.80 and 10.23%, respectively, indicating that the effect of appropriate seed natural drying on oil accumulation can be great. While the percentages of oil content increase seem different between the two species, the relative increase amounted to 4.40%, indicating that natural drying can promote oil accumulation in both camellia species.

Sequence analysis of small RNAs
To obtain a comprehensive profile of the sRNAs involved in natural drying, camellia seed from both species were subjected to Solexa high-throughput sequencing, with 5 libraries for each species corresponding to the sampled moisture contents. Average total of 30,641,435 and 31,012,304 reads were generated from C. meiocarpa and C. oleifera, respectively (Table 2). After filtering the low quality reads, such as 3’ insert null, poly(A), length < 18 nt or length > 30 nt, and other artifacts, the majority of the small RNAs were 21 to 24 nt in length. A total of 24,070,601 and 21,653,584 clean reads of 18–30 nt were obtained for C. meiocarpa and C. oleifera, respectively (Table 2). GC content of clean reads were more than the small RNAs were 21 to 24 nt, and other artifacts, the majority of the five moisture content levels, C. meiocarpa was largest when the seed moisture content dropped and least (2,121,984) mapped reads, respectively. Similarly, on average, C. oleifera produced 2,396,805 (14.37%) successful mapped reads, of which 50 and 20% moisture content levels (S06 and S09) produced the highest 24-nt RNA peak while 10% moisture level (sample S05) produced the highest 21-nt and 22-nt RNA peaks among the studied moisture content levels (Additional file 1: Table S3), conversely, 50% moisture (sample S06) produced the highest 24-nt, 21-nt, and 22-nt RNA peaks in C. oleifera (Additional file 1: Table S4).

Identification of miRNAs during natural drying
We used the software miRDeep2 to map the retained sequence reads to identify candidate miRNAs. Across the five moisture content levels, C. meiocarpa produced successful 2,355,539 reads (11.51%) that were mapped to the plant genomes, of which the 10 and 50% moisture content levels (S05 and S01) produced the most (2,724,598) and least (1,902,986) mapped reads, respectively. Similarly, on average, C. oleifera produced 2,396,805 (14.37%) successful mapped reads, of which 50 and 20% moisture content levels (S06 and S09) produced the most (2,803,519) and least (2,121,984) mapped reads. In total, 2,288,508 (12.80%) mapped reads were successfully annotated (Table 5).

A total of 274 candidate miRNAs, 248 and 246 unique sequences were assigned to C. meiocarpa and C. oleifera, respectively (Additional file 1: Table S5). Out of the identified candidate miRNA sequences, 110 were identified to 64 families, 57 families belonging to each of C. meiocarpa (99 out of 248 candidate miRNA) and C. oleifera (98 out of 246 candidate miRNA) (Additional file 1: Tables S5, S6 and S7). The diversity of miRNA families could be determined from their members’ number. As shown, MIR482 families were the largest with 10 members, followed by MIR159 and MIR535 with 5 members, and MIR160, MIR169_2 and MIR5272 with 4 members (Additional file 1: Table S6). Most of the conserved miRNA families had one member (65.63%) (Additional file 1: Table S6). The miRNAs sequences ranged in length from 18 to 24–26 nt and 21–25 nt for C. meiocarpa and C. oleifera, respectively.

| Table 1 Seed oil content during camellia seed natural drying |
|-----------------------------|-----------------------------|
| Moisture content (%)        | Seed oil content (%)         |
|                            | C. meiocarpa                | C. oleifera                |
| 50                         | 33.41 ± 0.001d              | 37.66 ± 0.117e             |
| 40                         | 39.91 ± 0.002b              | 38.72 ± 0.116d             |
| 30                         | 42.21 ± 0.001a              | 47.89 ± 0.001a             |
| 20                         | 40.35 ± 0.002b              | 44.25 ± 0.001b             |
| 10                         | 37.83 ± 0.001c              | 42.09 ± 0.001c             |

Different letters indicate significant at P < 0.05
25 nt, with a peak of 24 nt (Additional file 1: Figure S1). The miRNA first nucleotide preference distributions are show in Fig. 2a. miRNAs of 24 nt tended to start with 5′-A while the 21 nt tended with 5′-U (Fig. 2a). Tall miRNAs tended to start with 5′-U and not 5′-G (Fig. 2b). During the seed natural drying process, the number of miRNAs across moisture content levels showed a decreasing trend which was followed by increase with a peak at 40% moisture content (S02) for *C. meiocarpa* (Table 6). *C. oleifera*, on the other hand, showed a trend of reduction, followed by increase, followed by reduction in the number of miRNAs across the studied moisture content levels with a pronounced peak at 50% moisture content (S06) (Table 6).

Next we conducted sequence homology search of these candidate miRNAs against known mature miRNAs in miRBase. Any miRNA that met the sequence homology criteria of Yang et al. [35] and Jain et al. [36] was considered a conserved miRNA gene. Through this analysis, we identified a total of 151 conserved miRNAs which belonging to 47 miRNA families in both camellia species (Additional file 1: Table S6). miRDeep2 score above 1.0 resulted in 98 pre-miRNAs (64.90%) of which 35 with a read count ranging between 10 and 100 (23.19%), 41 with read count above 100 (27.15%), and 75 with read count below 10 (49.67%) (Additional file 1: Table S6).

Those miRNA sequences, which met the threshold of miRDeep2 analysis but did not have any known homologous miRNA gene families in miRBase, were further analyzed to predict novel miRNAs in the two camellia species. The remaining miRNAs, which met the total score of >0, were considered to be true novel miRNAs. A total 123 novel mature miRNAs sequences were discovered and belonged to 36 miRNA families in both camellia species (Additional file 1: Table S7). miRDeep2 score above 1.0 had 87 pre-miRNAs (70.73%). The majority of pre-miRNA (69) read count ranged from 10 to 100 (56.10%), followed by 35 precursors in above 100 (28.45%) and 19 precursors below 10 (15.45%) (Additional file 1: Table S7).

### Prediction and annotation of miRNAs target genes
To better understand the functions of the identified miRNAs, putative target genes were predicted using TargetFinder software [42]. A total of 6250 target genes were identified (Table 6). Only 3733 target unigenes (59.73%) were annotated by performing a BLASTX search against diverse protein databases, revealing that 1368 (21.89%), 2190 (35.04%), 901 (14.42%), 2160 (34.56%), 2730 (43.68%), 2735 (43.76%), and 3718 (59.49%) unigenes have significant matches with sequences in COG, GO, KEGG, KOG, Pfam, Swissprot, and nr protein databases.

### Table 2 Statistics relating to *C. meiocarpa* and *C. oleifera* sRNA sequences produced by Solexa sequencing

| Category       | Samples | Raw reads | <18 nt reads | >30 nt reads | Clean reads | GC(%) | Q20(%) | CycleQ20(%) | Q30(%) |
|----------------|---------|-----------|--------------|--------------|-------------|-------|--------|-------------|--------|
| *C. meiocarpa* | S01     | 29,906,668| 1,658,148    | 7,025,688    | 20,417,236  | 49.32 | 93.1   | 100         | 86.98  |
|                | S02     | 32,844,767| 1,179,743    | 4,859,317    | 27,795,553  | 47.75 | 91.9   | 100         | 86.17  |
|                | S03     | 28,888,019| 1,676,660    | 3,899,605    | 23,290,661  | 48.54 | 91.4   | 100         | 85.47  |
|                | S04     | 28,599,822| 1,902,807    | 3,697,488    | 2,297,876   | 48.32 | 91.2   | 100         | 85.29  |
|                | S05     | 32,967,901| 2,333,282    | 4,738,442    | 2,581,287   | 48.67 | 91.2   | 100         | 85.36  |
|                | Average | 30,641,435| 1,750,128    | 4,641,231    | 24,070,601  | 48.52 | 91.7   | 100         | 85.86  |
| *C. oleifera*  | S06     | 34,169,238| 4,386,045    | 4,606,664    | 25,151,946  | 49.13 | 91.2   | 100         | 85.25  |
|                | S07     | 31,865,014| 1,751,974    | 9,877,436    | 19,405,898  | 49.28 | 93.2   | 100         | 87.02  |
|                | S08     | 30,501,297| 2,833,009    | 4,644,107    | 23,001,131  | 48.99 | 91.3   | 100         | 85.43  |
|                | S09     | 31,087,840| 3,519,595    | 5,720,360    | 21,016,629  | 49.7  | 93.0   | 100         | 86.74  |
|                | S10     | 27,438,132| 3,369,136    | 3,659,237    | 19,692,318  | 49.55 | 93.0   | 100         | 86.86  |
|                | Average | 31,012,304| 3,171,952    | 5,701,561    | 21,653,584  | 49.33 | 92.3   | 100         | 86.26  |
respectively (Table 7). A total of 2743 (73.48%) annotated target genes had the length of \( \geq 1000 \) (Table 7).

To evaluate the potential functions of these miRNA target genes, we next applied gene ontology (GO) and KEGG pathway analyses to categorize the miRNA targets. The miRNA target genes were categorized according to biological process, cellular component and molecular function by GO analysis (Fig. 3). A total of 1860 target genes were categorized into 19 biological process. Based on molecular function, 1722 target genes were classified to 14 categories. A total of 1212 target genes were categorized as cellular components. Target genes related to lipid metabolism were found among 51 GO terms, in which 18 GO terms are related to biological process and 33 related to molecular function. There were 31 miRNAs involved in lipid metabolism and targeted 148 unigenes (Additional file 1: Table S8). The KEGG enrichment analysis revealed 12 pathways related to lipid metabolism, involved in 15 miRNA targeted 93 unigenes. There were 19 target genes in Glycolysis/Gluconeogenesis pathway, 12 in Fatty acid biosynthesis pathway, and 7 in biosynthesis of unsaturated fatty acids pathway (Additional file 1: Table S9). Integrated GO and KEGG function annotation, identified 23 miRNA regulating 131 target genes that were annotated as lipid metabolism (Additional file 1: Table S10). These miRNAs regulated the changes of seed oil content at different moisture content levels. There were high correlations between transcript abundance with added value of oil content, for example MIR482 (Additional file 1: Table S11). Finally, miRNA of lipid metabolism only expressed were identified in *C. Meiocarpa* (Group1_Unigene_BMK.30485_635795) and *C. oleifera* (Group1_Unigene_BMK.37364_696840 and Group1_Unigene_BMK.38037_703962) (Table 8).

**Differentially expressed miRNAs of lipid metabolism during natural drying**

The differences between the miRNA profiles of the two camellia species are possibly related to differences in their responses to natural drying and were investigated using the IDEG6 software. For *C. Meiocarpa*, miRNA abundance pairwise analysis between the different moisture content libraries, indicated that 40, 46, 63, and 38 significantly differentially expressed miRNAs were identified between 40 and 50% (S01 vs. S02), 30–40% (S02 vs. S03), 20–30% (S03 vs. S04), and 10–20% (S04 vs. S05) moisture content respectively (Table 7). A total of 2743 (73.48%) annotated target genes had the length of \( \geq 1000 \) (Table 7).

![Fig. 1 Length distributions of small RNAs in two camellia species. Data of *C. meiocarpa* and *C. oleifera* were averaged across five level moisture content (50, 40, 30, 20 and 10%) for each species separately.](image-url)
contents, respectively. The highest number of significantly differentially expressed miRNAs was observed between 20 and 30% (S03 vs. S04) moisture contents, with the lowest up- and highest down-regulated numbers of 10 and 53, respectively (Table 9), with 8 significantly different miRNAs involved in lipid metabolism (Tables 10 and Additional file 1: Table S12).

C. oleifera produced similar results with 71, 54, 36, and 56 significantly differentially expressed miRNAs observed between 40 and 50% (S06 vs. S07), 30–40% (S07 vs. S08), 20–30% (S08 vs. S09), and 10–20% (S09 vs. S10) moisture contents, respectively.

Comparing across the two species, the average number of miRNAs in C. oleifera seeds was higher than that of C. Mehicarpa (Table 9). Pairwise analysis of miRNA abundance between the two species for the same moisture level libraries indicated that there were 78, 51, 44, 58, and 61 significant differentially expressed miRNAs for the 50, 40, 30, 20, and 10% moisture contents, respectively. There were three differentially expressed miRNAs of lipid metabolism during the seed natural drying process of the studied two camellia species (Group1_Unigene_BMK.37987_703484, Group1_Unigene_BMK.25259_1025465, and Group2_Unigene_BMK.25259_1025498) (Tables 12 and S14). The highest up- (69) and lowest down-regulated number (9) of significant differentially expressed miRNAs were detected for 50% moisture contents (S06 vs. S01) (Table 9). This indicated that the greatest difference between the two species was observed at the 50% moisture content, with 12 significantly differentially expressed miRNAs regulating lipid metabolism during seed natural drying (Tables 12 and S14).

Validation of the expression patterns of differentially expressed miRNAs related lipid metabolism by RT-qPCR

To validate the data obtained from the high-throughput sequencing, four significantly differentially expressed miRNAs

![Fig. 2](image-url)
of the two camellia species were regulated by miRNA during the seed natural drying process.

**Discussion**

**miRNAs related to the lipid metabolism of camellia species**

Recently, sequencing of oil crops produced a large amount of miRNA associated with lipid metabolism-related genes (e.g., 97, 30, 10, and 4 miRNAs targeting 89, 133, 21, and 4 lipid biosynthesis genes were reported for soybean [12], *Camelina sativa* [11], castor bean [49], and peanut [19], respectively). In the present study, we detected 23 pre-miRNAs targeting 131 candidate genes regulating lipid metabolism functions during camellia seed natural drying (Additional file 1: Table S10). Of these pre-miRNAs, 5, 5, and 11 regulated fatty acid biosynthesis, accumulation, and catabolism, respectively, and additional 2 (Group1_ Unigene_BMK.30485_635795 and Group1_ Unigene_BMK.45675_802511) regulated not only fatty acid biosynthesis and accumulation, but also fatty acid catabolism (Additional file 1: Table S10). Group1_ Unigene_BMK.30485_635795 and Group1_ Unigene_BMK.45675_802511 encode 56 and 41 target genes involved in fatty acid biosynthesis, accumulation, and catabolism, respectively (Additional file 1: Table S10).

Concurring with the above, the most abundant miRNAs (osa-miR2118e: Group1_Unigene_BMK.37987_703484, Group2_Unigene_BMK.25259_1025465, and Group2_Unigene_BMK.25259_1025498 (Additional file 1: Table S10 and Table 8)) that target genes encoding a long-chain-alcohol oxidase involving fatty acid-oxidation [50]. The second abundant miRNAs (Group2_Unigene_BMK.63506_1315063) targeting diacylglycerol kinase, a lipid kinase converting diacylglycerol to phosphatidic acid, regulating balance of diacylglycerol and phosphatidic acid [51], and potentially involved in regulating lipid deposition [52]. The third, Group2_Unigene_BMK.38504_1137258 (Additional file 1: Table S10 and Table 8), targeting lipases/acylhydrolase, fatty acid hydroxylase, and carboxylesterase, which are hydrolytic enzymes involved in degradation of fatty acid [53–55], collectively suggesting that these miRNAs may play important roles.
Table 8 miRNA of lipid metabolism transcript abundance in the two camellia species during seed natural drying

| Pre-miRNA          | miRNA                | C. meiocarpa | C. oleifera |
|--------------------|----------------------|--------------|-------------|
|                    |                      | S01 S02 S03 S04 S05 Average | S06 S07 S08 S09 S10 Average |
| CL22479Contig1_234494 | Unknown              | 0 0 0 1 0 0.2 | 1 0 1 0 1 0.6 |
| CL22738Contig1_61999 | Unknown              | 0 0 1 0 0 0.2 | 0 0 0 0 1 0.2 |
| Group1_Unigene_BMK.23434_588836 | MIR5067        | 20 21 33 25 24 24.6 | 19 20 15 9 10 14.6 |
| Group1_Unigene_BMK.30485_635795 | Unknown              | 0 1 0 0 0 0.2 | 0 0 0 0 0 0.0 |
| Group1_Unigene_BMK.37364_696840 | Unknown              | 0 0 0 0 0 0.0 | 1 2 0 0 0 0.6 |
| Group1_Unigene_BMK.37987_703484 | MIR2118             | 302 563 747 807 602 604.2 | 650 280 434 325 359 409.6 |
| Group1_Unigene_BMK.38037_703962 | Unknown              | 0 0 0 0 0 0.0 | 0 0 0 0 1 0.2 |
| Group2_Unigene_BMK.25259_1025465 | MIR482             | 302 563 747 807 602 604.2 | 650 280 434 325 359 409.6 |
| Group2_Unigene_BMK.25259_1025498 | MIR482             | 302 563 747 807 602 604.2 | 650 280 434 325 359 409.6 |
| Group2_Unigene_BMK.34335_1,093,229 | MIR5067         | 20 21 33 25 24 24.6 | 19 20 15 9 10 14.6 |
| Group2_Unigene_BMK.50808_1268477 | Unknown              | 0 0 0 1 0 0.2 | 1 0 0 1 0 0.4 |
| Group2_Unigene_BMK.9543_1378570 | Unknown              | 15 22 11 26 21 19.0 | 17 10 15 11 7 12.0 |
| CL19455Contig1_54014 | Unknown              | 26 26 23 22 27 24.8 | 40 18 28 25 14 25.0 |
| CL19777Contig1_314088 | Unknown              | 23 34 30 29 26 28.4 | 35 18 31 26 20 26.0 |
| CL2440Contig1_359627 | MIR1861             | 2 6 13 5 4 6.0 | 5 1 5 3 4 3.6 |
| CL4378Contig1_191450 | Unknown              | 11 8 10 14 15 11.6 | 35 13 22 17 12 19.8 |
| CL9644Contig1_380257 | Unknown              | 26 24 21 21 18 22.0 | 20 12 18 10 24 16.8 |
| Group1_Unigene_BMK.45675_802511 | Unknown              | 4 4 8 1 10 5.4 | 9 4 4 5 3 5.0 |
| Group2_Unigene_BMK.24252_1018543 | Unknown              | 1 1 1 0 0 0.6 | 1 2 2 1 0 1.2 |
| Group2_Unigene_BMK.38504_1137258 | Unknown              | 35 31 29 40 33 33.6 | 45 25 24 13 25 26.4 |
| Group2_Unigene_BMK.38504_1137263 | Unknown              | 6 3 4 3 4 4.0 | 2 0 2 2 1 1.4 |
| Group2_Unigene_BMK.63506_1315063 | Unknown              | 58 47 89 49 53 59.2 | 96 49 34 50 20 49.8 |
| Group2_Unigene_BMK.39605_1150110 | Unknown              | 0 1 1 0 0 0.4 | 2 1 0 1 0 0.8 |
in decreasing the rate of lipid breakdown during camellia seed natural drying. This can be confirmed by the 4% increase in oil content in two camellia seed through seed nature drying (Table 1).

**miRNAs regulate lipid metabolism during camellia seed natural drying**

Several studies have shown that miRNAs are differentially regulated in response to stress [56] and that this differential regulation varied among different plant species [57]. For example, distinct responses to drought stresses were reported for miRNAs in *Arabidopsis* [58], switchgrass [59], *Populus* [60] and *Caragana intermedia* [61]. Especially, drought stress in switchgrass [59] and *Populus* [60] were regulated by miRNAs related to lipid metabolism; however, the linkage between drought responses and lipid metabolism miRNAs changes is not established.

In the present study, *C. meiocarpa* produced 9 significant differentially expressed miRNAs regulating lipid metabolism with only 4 at 20–30% moisture contents. These pre-miRNAs belonged to Group2_Unigene_BMK.34335_1,093,229, Group1_Unigene_BMK.23434_588836, CL19777_Contig1_314088, and CL2440_Contig1_359627, with the former three showing more than 100 TPM (Table 10 and Additional file 1: Table S12) and targeting 3-ketoacyl-CoA synthase III, which catalyze the initial elongation step of fatty acid biosynthetic process [62] and glycerol-3-phosphate transporter, a precursor protein for phospholipid biosynthesis [63]. It is interesting to note that these three pre-miRNAs were down-regulated resulting in a reduction in the fatty acid biosynthesis, so seeds with 30% moisture content have high fatty acid synthesis and accumulation activities (Table 1).

Similarly, *C. oleifera* produced 8 significant differentially expressed miRNAs during seed natural drying, with up- and down-regulated for the 40–50 and 30–40% moisture contents, respectively (Tables 11 and S13). The largest fold changes were observed for Group1_Unigene_BMK.23434_588836 and Group2_Unigene_BMK.34335_1,093,229, which target 3-ketoacyl-CoA synthase III (Additional file 1: Table S10 and Table S13). These pre-miRNAs regulate fatty acid biosynthesis with seeds at 40–50% moisture content showing low fatty acid biosynthesis activities while those at 30–40% moisture content exhibiting high activities (Table S13). This can be confirmed by the observed 1.06 and 9.17% increase in oil content at 40–50 and 30–40% moisture contents, and reaching the highest point at 30% moisture content (Table 1). So seeds with 30% moisture

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**Table 10** Significant differentially expressed miRNAs of lipid metabolism during *C. meiocarpa* seed natural drying across different moisture content levels (%)

| miRNA                         | 50 vs. 40% | 40 vs. 30% | 30 vs. 20% | 20 vs. 10% |
|-------------------------------|------------|------------|------------|------------|
| Pre-miRNA                     |            |            |            |            |
| Group2,Unigene_BMK.34335_1,093,229 | 1          | 1          | 1          | 1          |
| Group1,Unigene_BMK.23434_588836 | 0          | 0          | 1          | 0          |
| CL2440_Contig1_359627         | 0          | 0          | 1          | 0          |
| Group1,Unigene_BMK.23434_1,093,229 | 0          | 0          | 0          | 0          |
| CL19777_Contig1_314088        | 0          | 0          | 1          | 0          |
| Group2,Unigene_BMK.23434_588836 | 1          | 1          | 0          | 1          |
| Group1,Unigene_BMK.23434_802511 | 0          | 0          | 1          | 1          |
| Group2,Unigene_BMK.63506_1315063 | 0          | 1          | 1          | 0          |

1 Represents significant DEG and 0 represents non-significant DEG

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**Table 11** Significant differentially expressed miRNAs of lipid metabolism during *C. oleifera* seed natural drying across different moisture content levels (%)

| miRNA                         | 50 vs. 40% | 40 vs. 30% | 30 vs. 20% | 20 vs. 10% |
|-------------------------------|------------|------------|------------|------------|
| Pre-miRNA                     |            |            |            |            |
| Group2,Unigene_BMK.34335_1,093,229 | 1          | 1          | 0          | 1          |
| CL19455_Contig1_54014         | 0          | 0          | 0          | 1          |
| Group1,Unigene_BMK.23434_703484 | 1          | 1          | 0          | 1          |
| Group1,Unigene_BMK.23434_588836 | 1          | 1          | 0          | 0          |
| Group2,Unigene_BMK.23435_1025498 | 1          | 1          | 0          | 1          |
| Group2,Unigene_BMK.23435_1025465 | 1          | 1          | 0          | 1          |
| Group2,Unigene_BMK.38504_1137258 | 1          | 1          | 0          | 0          |
| Group2,Unigene_BMK.34335_1,093,229 | 1          | 1          | 0          | 0          |

1 Represents significant DEG and 0 represents non-significant DEG

1 significance at *P* < 0.05
content have also high fatty acid synthesis and accumulation activities.

Comparing the significant differentially expressed miRNAs of *C. meiocarpa* (9) with those of *C. oleifera* (8), indicated that the two species share 6 miRNAs involved in lipid metabolism, with unique miRNAs belonging to each species (CL19455Contig1_54014 and Group2_ Unigene_BMK.38504_1137258 in *C. oleifera*, and CL2440 Contig1_359627, CL19777Contig1_314088 and Group1_ Unigene_BMK.45675_802511 in *C. meiocarpa*) (Tables 10

Table 12 Significant differentially expressed miRNAs of lipid metabolism during the two camellia species seed natural drying across the same moisture content level (*C. oleifera* vs. *C. meiocarpa* MC%)

| Pre-miRNA                                      | 50 vs. 50% | 40 vs. 40% | 30 vs. 30% | 20 vs. 20% | 10 vs. 10% |
|------------------------------------------------|------------|------------|------------|------------|------------|
| CL19777Contig1_314088                          | 1          | 0          | 0          | 0          | 0          |
| CL19455Contig1_54014                           | 1          | 0          | 0          | 0          | 0          |
| Group2_ Unigene_BMK.25259_1025465              | 1          | 1          | 1          | 1          | 1          |
| Group2_ Unigene_BMK.9543_1378570               | 1          | 0          | 0          | 0          | 0          |
| CL9644Contig1_380257                           | 1          | 0          | 0          | 0          | 0          |
| Group2_ Unigene_BMK.25259_1025498              | 1          | 1          | 1          | 1          | 1          |
| Group2_ Unigene_BMK.38504_1137258              | 1          | 0          | 0          | 0          | 0          |
| Group2_ Unigene_BMK.34335_1,093,229            | 1          | 0          | 1          | 0          | 0          |
| Group2_ Unigene_BMK.63506_1315063              | 1          | 0          | 1          | 1          | 1          |
| Group2_ Unigene_BMK.38504_1137263              | 1          | 0          | 0          | 0          | 0          |
| Group1_ Unigene_BMK.23434_588836               | 1          | 0          | 1          | 0          | 0          |
| Group1_ Unigene_BMK.37987_703484               | 1          | 1          | 1          | 1          | 1          |

1Represents significant DEG and 0represents non-significant DEG

Fig. 4 qRT-PCR validation of miRNA in *C. meiocarpa* and *C. oleifera*. Relative expression of miRNA in 10, 20, 30, 40, and 50% moisture contents. Reference gene was 5.8 rRNA. Normalized miRNA in 50% moisture content of *C. oleifera* were arbitrarily set to 1. Error bars were calculated based on three replicates.
and 11). Group2_Unigene_BMK.38504_1137258 and CL2440Contig1_359627 regulated fatty acid catabolism, CL19455Contig1_54014 and CL19777Contig1_314088 regulated fatty acid accumulation, and Group1_Unigene_BMK.45675_802511 regulated fatty acid synthesis, accumulation, and catabolism (Additional file 1: Table S10). These indicate that oil content differences between the two camellia species are mainly due to their differential abilities miRNAs of fatty acid accumulation and catabolism.

Collectively, the studied two camellia species produced 12 significantly differentially expressed miRNAs to regulate lipid metabolism during seed natural drying (Tables 12 and S14). These pre-miRNAs indicated that C. meiocarpa has higher abilities to regulate the lipid metabolism and this can be confirmed by its lower oil content as compared to C. oleifera (Table 1). It should be stated that all these 12 differentially expressed miRNAs were significantly expressed in the 50% moisture content stage (Tables 12 and S14). Seeds with 50% moisture content had only significant differentially expressed pre-miRNAs (CL19777Contig1_314088, CL19455Contig1_54014, Group2_Unigene_BMK.9543_1378570, CL9644Contig1_380257, Group2_Unigene_BMK.38504_1137258 and Group2_Unigene_BMK.38504_1137263) (Tables 8 and S14). CL19777Contig1_314088 and CL19455Contig1_54014 target glycerol-3-phosphate transporter (Additional file 1: Table S10), Group2_Unigene_BMK.9543_1378570 target acetyl-CoA-carboxylase (Additional file 1: Table S10), which catalyze the ATP-dependent carboxylation of acetyl-CoA to form malonyl-CoA, the rate limiting and first committed reaction in fatty acid synthesis [64]. So these three pre-miRNA control different fatty acid synthesis and accumulation in the two camellia species. For all significantly differentially expressed miRNAs, the largest fold change was observed for Group2_Unigene_BMK.38504_1137263 (Fatty acid hydroxylase), followed by CL9644Contig1_380257 (Carboxylesterase), and Group1_Unigene_BMK.23434_588836 (3-ketoacyl-CoA synthase III) and Group2_Unigene_BMK.34335_1,093,229 (3-ketoacyl-CoA synthase III) (Additional file 1: Table S10 and Table S14). So the oil content of C. oleifera is higher than C. meiocarpa and this is attributable to four pre-miRNAs, of which Group2_Unigene_BMK.38504_1137263 and CL9644Contig1_380257 regulating fatty acid catabolism and Group1_Unigene_BMK.23434_588836 and Group2_Unigene_BMK.34335_1,093,229 regulating fatty acid synthesis.

**Conclusion**

The present study identified 274 candidate miRNAs, with 248 and 246 unique sequences to C. meiocarpa and C. oleifera, respectively. Integrated GO and KEGG function annotation, produced 23 miRNAs regulating 131 target genes, all were annotated as lipid metabolism, regulating fatty acid biosynthesis, accumulation and catabolism during seed natural drying. Lipid metabolism primarily focused on fatty acid catabolism, with five miRNAs (Group1_Unigene_BMK.37987_703484, Group2_Unigene_BMK.25259_1025465, Group2_Unigene_BMK.25259_1025498, Group2_Unigene_BMK.63506_1315063, and Group2_Unigene_BMK.38504_1137258) playing important roles in decreasing the rate of lipid breakdown and additional two miRNAs (Group2_Unigene_BMK.34335_1,093,229 and Group1_Unigene_BMK.23434_588836) regulating fatty acid synthesis. Across the two species, four pre-miRNAs were identified to regulate lipid metabolism, with Group2_Unigene_BMK.38504_1137263 and CL9644Contig1_380257, and Group1_Unigene_BMK.23434_588836 and Group2_Unigene_BMK.34335_1,093,229 regulating fatty acid catabolism and synthesis, respectively.

To our knowledge, this work provides the first small RNA expression analysis of lipid metabolism in camellia seed during natural drying as well as the first comparative miRNA profiling analysis between C. Meiocarpa and C. oleifera that exhibit significantly different fatty acid profiles.

**Additional file**

**Additional file 1: Figure S1.** Length distributions of miRNAs in two camellia species. **Table S1.** qRT-PCR primer sequences. **Table S2.** Length distributions of small RNAs in C. meiocarpa during seed natural drying. **Table S3.** Length distributions of small RNAs in C. oleifera during seed natural drying. **Table S4.** miRNAs transcript abundance in the two camellia species during seed natural drying. **Table S5.** All conserved miRNA discovered in the two camellia species during seed natural drying. **Table S6.** All novel miRNA discovered in the two camellia species during seed natural drying. **Table S7.** GO terms related with the lipid metabolism in the two camellia species during seed natural drying. **Table S8.** KEGG enrichment related with the lipid metabolism in the two camellia species during seed natural drying. **Table S9.** miRNA of lipid metabolism targets and their putative functions. **Table S10.** Differentially expressed miRNAs of lipid metabolism during C. meiocarpa seed natural drying. **Table S11.** Differentially expressed miRNAs of lipid metabolism during C. oleifera seed natural drying. **Table S12.** Differentially expressed miRNAs of lipid metabolism between two camellia species during seed natural drying. (DOCX 168 kb)

**Acknowledgments**

We thank Minhou Tongkou State Forest Farm of Fujian Province for providing access to research material. We also thank Professor Bruce C Larson, University of British Columbia, for helpful suggestions. This work was supported by grants received under the Doctoral Fund of the Ministry of Education of China (K4112020A) and Fujian Province Major Science and Technology Project (2013NZ0207).

**Availability of data and materials**

The data supporting this study are available in the Dryad Digital Repository, doi: 10.5061/dryad.1mj01

**Authors’ contributions**

J-LF, HC, YAK conceived and designed the experiment, J-LF, Z-JY performed the experiment, J-LF, Z-JY, S-P, C, collected the data collection and prepared the figures, J-LF performed the data analysis, and J-LF, YAK wrote the manuscript. All authors read and approved the final manuscript.
Ethics approval and consent to participate
Not required.

Competing interests
The authors declare that they have no competing interests.

Publisher’s Note
Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Received: 7 December 2016 Accepted: 4 July 2017
Published online: 20 July 2017

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