Comparative Analysis Reveals Dynamic Changes in miRNAs and Their Targets and Expression during Somatic Embryogenesis in Longan (Dimocarpus longan Lour.)

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Abstract

Somatic embryogenesis (SE), which resembles zygotic embryogenesis, is an essential component of the process of plant cell differentiation and embryo development. Although microRNAs (miRNAs) are important regulators of many plant developmental processes, their roles in SE have not been thoroughly investigated. In this study, we used deep-sequencing, computational, and qPCR methods to identify, profile, and describe conserved and novel miRNAs involved in longan (Dimocarpus longan) SE. A total of 643 conserved and 29 novel miRNAs (including star strands) from more than 169 miRNA families were identified in longan embryogenic tissue using Solexa sequencing. By combining computational and degradome sequencing approaches, we were able to predict 2063 targets of 272 miRNAs and verify 862 targets of 181 miRNAs. Target annotation revealed that the putative targets were involved in a broad variety of biological processes, including plant metabolism, signal transduction, and stimulus response. Analysis of stage- and tissue-specific expressions of 20 conserved and 4 novel miRNAs indicated their possible roles in longan SE. These miRNAs were dlo-miR156 family members and dlo-miR166c* associated with early embryonic culture developmental stages; dlo-miR26, dlo-miR160a, and families dlo-miR159, dlo-miR390, and dlo-miR398b related to heart-shaped and torpedo-shaped embryo formation; dlo-miR40, dlo-miR24, dlo-miR167a, dlo-miR168a*, dlo-miR397a, dlo-miR398b.1, dlo-miR398b.2, dlo-miR808 and dlo-miR5077 involved in cotyledonary embryonic development; and dlo-miR17 and dlo-miR2089*-1 that have regulatory roles during longan SE. In addition, dlo-miR167a, dlo-miR808, and dlo-miR5077 may be required for mature embryo formation. This study is the first reported investigation of longan SE involving large-scale cloning, characterization, and expression profiling of miRNAs and their targets. The reported results contribute to our knowledge of somatic embryo miRNAs and provide insights into miRNA biogenesis and expression in plant somatic embryo development.

Introduction

Longan (Dimocarpus longan Lour.) is a tropical/subtropical fruit tree in the family Sapindaceae. In this species, embryo development status strongly influences seed size, fruit quality, fruit set, and yield. To improve longan fruit quality and yield, researchers have consequently focused much attention on mechanisms of longan embryonic development. Advances have been limited, however, primarily because of the extreme genetic heterozygosity exhibited by this species and the difficulty of sampling the early embryos. Although the longan somatic embryogenesis (SE) system has been used as a model system for investigating in vivo and in vitro regulation of embryogenesis in woody plants, most studies of longan SE have focused on a few selected aspects related to cell biology, molecular biology, and proteomics [1–5]. There are no reported studies devoted to identification and expression of microRNAs (miRNAs)—an important group of plant regulators—during longan SE.

miRNAs, a class of single-stranded noncoding RNAs that are 18–25 nt in length, negatively regulate gene expression by cleaving targeted mRNAs and repressing translation [6]. There is increasing evidence that plant miRNAs play important roles in various processes, including leaf morphogenesis and polarization, lateral root formation, floral induction, meristem boundary formation, floral organ identity, and reproduction [7–10]. SE, which resembles zygotic embryogenesis, is strongly associated with plant cell differentiation and embryo development—a process during which many specific miRNAs are undoubtedly expressed [11,12]. Several studies have accordingly shown that miRNA-mediated repression of target transcripts plays a role in embryogenesis [13,14] and that certain miRNAs may function at specific developmental stages [11,15–18]. In Arabidopsis, miRNAs enable pattern formation during embryogenesis [14] and regulate the timing of embryo maturation by repressing several key targets [13].AGO1, which is involved in miRNA function, is specifically expressed during carrot SE [19], and ptAGO9L as well as miRNAs...
such as *Pta-miR166* and *Pta-miR167* exhibit spatiotemporally variable expression in loblolly pine zygotic embryos and female gametophytes [10]. These observations suggest that a miRNA expression controlling system is required for SE in these species. The regulatory role of miRNAs during plant embryogenesis is still not well understood, however, and few relevant studies have been conducted in non-model plants. No studies have attempted to detect miRNAs in longan.

In this study, we used Solexa sequencing and an integrated bioinformatics analysis approach to clone and identify conserved and novel miRNAs in longan. We then predicted, identified, and verified longan miRNA targets using prediction analysis, degradome sequencing, and modified RNA ligase-mediated amplification of cDNA ends (RLM-RACE). Finally, we comparatively analyzed expression patterns to reveal miRNA participation in longan SE. The results of this study provide new information regarding miRNA regulatory networks and contribute to our knowledge of the role of miRNAs in longan SE.

**Results**

**Categories and Size Distribution of Small RNA Populations in *D. longan***

To identify miRNAs involved in longan SE, a pooled sRNA library was generated from embryogenic cultures and subjected to Solexa sequencing. We obtained 12,554,858 raw reads, comprising 447,851 low-quality and 12,107,007 high-quality reads. After removing low-quality reads and adapter sequences, the remaining 11,645,841 clean reads, which were 18–30 nt long, included 6,553,782 unique sequences. Only 378,143 (5.77%) of the unique sRNA sequences could be matched to the longan transcriptome (Table 1). Most of the sequences (94.23%) could not be mapped.

The unique sRNAs were then compared against all plant miRNA precursors and mature miRNAs listed in miRBase (miRBase 17: 18,299 (0.28%) of the unique sequences were found to be similar to known miRNAs. By performing a BLASTN search against the Rfam database, we then identified small RNAs corresponding to rRNA (0.49%), small nuclear RNA (snRNA; 0.03%), small nucleolar RNA (snoRNA; 0.03%), and tRNA (0.09%) in our unique sRNA dataset (Table 1). Most sRNA unique sequences (6,351,648; 96.92%) could not be annotated, however, which is consistent with results obtained in other plant studies [20–23]. The high percentage of unannotated sequences may be due to the limited number of species-specific genomes or ESTs in these databases, such that many potential miRNAs were missing; alternatively, plant sRNAs have not yet been adequately surveyed, leading to incomplete data. The distribution of sRNAs detected in longan is given in Table 1.

The size distribution pattern of unique sRNA sequences generated by Solexa sequencing is summarized in Fig. 1. Distributions were uneven. Unique sRNAs were 18–25 nt long, with 24 nt lengths predominating (55.02%), followed by 22 nt (10.67%) and 20 nt (9.90%). The percentage of 24-nt sRNAs was much higher than that of 21 nt sRNAs. This result is consistent with that observed for most angiosperms [21,23,24] other than tomato [25], wheat [26], and *Populus* [27], in which the 21-nt length is more prevalent. The 21-nt to 24-nt length ratio is highly variable among plants, indicating the existence of significant differences in sRNA biogenesis pathways among different species [28].

**Identification of Conserved miRNAs in *D. longan***

To identify conserved miRNAs in *D. longan*, unique sRNAs from longan were compared against mature plant miRNAs listed in miRBase 17. As a result, 643 miRNAs belonging to more than 169 miRNA families were identified. These miRNAs varied in length from 18 nt to 25 nt. Most miRNAs were 24-nt long (34.07%), with 21 nt (20.2%), 20 nt (12.36%), and 19 nt (10.62%) lengths also heavily represented. A few were 25 nt long, such as dlo-miR2095 and dlo-miR3436*. After miRNAs with expression levels too low to be analyzed were removed, most miRNA families were represented by only one member. Some miRNA families were represented by multiple sRNAs, including miR156 (17 members), miR166 (12 members), miR159 (8 members), miR171 (9 members), miR399 (5 members), miR441 (6 members), and miR1520 (6 members); these required further validation, however. Information about known miRNA families detected in longan, including number of members, sizes, and expression, is included in Table S1 in Supporting Information S1.

In addition to mature miRNAs, some miRNAs* with high expression were sequenced, including dlo-miR406* (3727 reads), dlo-miR946a* (1114 reads), dlo-miR390a* (723 reads), dlo-miR3441* (433 reads), dlo-miR168a* (404 reads), dlo-miR166* (346 reads), and dlo-miR1162* (300 reads) (Table S1 in Supporting Information S1). Compared with mature miRNAs, however, lower expression levels were observed during longan SE for most of the miRNAs*. This asymmetrical accumulation of transcripts may be due to preferential loading of the miRNA strand into the silencing complex, where it is protected from degradation; this is in contrast to the miRNA* strand, which is preferentially excluded from the silencing complex and subject to degradation [29].

Most of the miRNA families identified in longan are highly conserved across many plant species. For example, families dlo-miR156 and dlo-miR157 were a perfect match to families in *Arabidopsis thaliana*, *Zea mays*, *Vitis vinifera*, and *Populus*, and families dlo-miR156–dlo-miR172 and dlo-miR393–dlo-miR399 were highly homologous to families from other species. Dicot-specific miRNA families, including miR136, miR161, miR163, and miR173 [30,31], were also detected in longan. In addition to conserved miRNAs, 21 non-conserved miRNA families were present in low abundance (2–123 reads) (Table S1 in Supporting Information S1); a few, including dlo-miR535a (9232 reads), dlo-miR894 (6039 reads), dlo-miR529 (881 reads) and dlo-miR826 (753 reads), were expressed at high levels. Interestingly, several miRNA families considered to be monocot-specific–miR437, miR443, miR444, miR528, miR1122, and miR1858–were also found in our library (2–173 reads), suggesting that they also exist in dicots.

### Table 1. Distribution of small RNAs among different categories in *Dimocarpus longan*.

| category | Unique sRNA | Percent(%) | Total sRNA | Percent(%) |
|----------|-------------|------------|------------|------------|
| miRNA    | 18,299      | 0.28       | 1,208,973  | 10.38      |
| rRNA     | 32,362      | 0.49       | 342,850    | 2.94       |
| siRNA    | –           | –          | –          | –          |
| snoRNA   | 1,759       | 0.03       | 6,220      | 0.05       |
| snRNA    | 1,872       | 0.03       | 6,820      | 0.06       |
| tRNA     | 5,633       | 0.09       | 158,587    | 1.36       |
| unannotated | 6,493,857 | 99.09      | 9,922,391  | 85.20      |
| Total    | 6,553,782   | 100        | 11,645,841 | 100        |

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Prediction of Potentially Novel Longan miRNAs via Longan Transcriptome and Populus Genome Analysis

In addition to conserved miRNAs, 29 potentially novel longan miRNAs were predicted from the remaining unannotated sRNA unique sequences–22 based on the longan transcriptome and 7 based on a Populus trichocarpa genome (Table 2). With the exception of dlo-miR4 and dlo-miR27, which were represented by 2 genomic loci each, each novel longan miRNA was associated with a single genomic locus. The lengths of these miRNAs varied from 21 nt to 23 nt; 48.28% were 21 nt long. Based on Mfold calculations, their precursors had negative folding free energies ranging from $-18.20 \text{ kcal/mol}$ to $-65.24 \text{ kcal/mol}$; the average was $-40.03 \text{ kcal/mol}$, which was lower than the folding free energies reported for rice ($-71.0 \text{ kcal/mol}$) and Arabidopsis ($-59.5 \text{ kcal/mol}$) [26]. The number of reads obtained for these novel longan miRNAs varied from 5 to 8964; for example, dlo-miR7 (8964 reads), dlo-miR17 (1309), dlo-miR9 (1144 reads), and dlo-miR14 (609 reads) were highly expressed, while dlo-miR1 (5 reads) and dlo-miR18 (6 reads) were expressed at low levels. When nucleotide bias was followed by A (20.69%), C (10.34%), and G (13.79%).

To determine whether these novel miRNAs are conserved across other plant species, their sequences were compared with miRNA sequences of other organisms present in miRBase across other plant species, their sequences were compared with miRBase database. Although very similar to miR156d*, miR159a, miR162a*, miR171e*, miR390a*, and zma-miR390a*, they were not identical, which raises the question as to whether or not these miRNAs should be classified as known miRNAs. This problem is not unique: Pantaleo et al. [33] found that miRC9 (a new miRNA) was very similar to ape-miR477e, differing by only 2 mismatches. They classified miRC9 as a new miRNA not belonging to the miR477 family, however, because there were 6 mismatches between miRC9 and svi-miR477 and 7 mismatches between miRC9 and ptc-miR477. In our study, the 11 potentially novel miRNAs in longan showed high homology to known conserved miRNAs, and were consequently classified as known miRNAs rather than truly novel ones. As miRNAs are sequenced in an increasing number of species, this situation is expected to arise more frequently, leading to difficulties in miRNA classification.

Furthermore, miRNAs dlo-miR22 through dlo-miR27, which were predicted based on the P. trichocarpa genome, cannot be accurately classified as longan-specific miRNAs because they may also be expressed in P. trichocarpa. If possible, species-specific miRNA prediction for non-model plants should rely on ESTs or GSSs originating from the studied species. When reference sequences originate from closely-related species, any predicted “novel” miRNAs will not be species-unique, but will instead be conserved across the selected species.

Prediction and Classification of Targets of Conserved and Non-conserved miRNAs in D. longan

To better understand the biological functions of longan miRNAs identified in our study, 2040 targets of 260 conserved miRNAs and 23 targets of 12 novel miRNA families were predicted using previously-described methods (Table S2 in Supporting Information S1). Homologs of known miRNA targets were predicted for several conserved longan miRNAs, including SPL10 as a target of dlo-miR156, MIB33 for dlo-miR139/319, NAC for dlo-miR164, ARF5 for dlo-miR167, SCL6 for dlo-miR171, AP2 for dlo-miR172, TIR1 for dlo-miR393, F-box/NF-YC11 for dlo-miR394, and laccase for dlo-miR397/408. In addition, some miRNAs regulated more than one target. For example, while 23.85% (62) of the conserved miRNAs regulated only one target, 103 (39.62%) regulated 2–5 targets, and 19, including miR2628 (169 targets), miR2673 (118 targets), miR554 (119 targets), miR780 (51 targets), and miR1442 (48 targets), had at least 20 targets; this suggests that these miRNAs are probably involved in a variety of biological processes during longan SE. Finally, some targets were regulated by multiple miRNAs. Examples include Unigene 62142 (ubiquitin-associated/TS-N domain-containing protein) targeted by both miR396 and miR1520, and Unigene 68246 (GRAS family transcription factor) regulated by miR171, miR932, miR1081, and miR5078. The above-predicted targets still required further experimental validation, however.

Based on Gene Ontology (GO) annotations, these potential miRNA targets appear to be involved in a broad variety of
### Table 2. Longan-specific miRNAs identified from *Dimocarpus longan* transcriptome and populus genome.

| miRNA     | Location             | Sequence (5′-3′)                      | Length (nt) | count | $\text{Mfe}_9$ (kcal/mol) | $5′/3′$ |
|-----------|----------------------|--------------------------------------|-------------|-------|---------------------------|--------|
| dlo-miR1  | Contig325306_Longan-RNA:27:90:+ | TATGTTATAATATGTGATGAT                | 21          | 5     | −18.2                     | 3      |
| dlo-miR2  | Contig401867_Longan-RNA:25:90:-- | TTGGCTTGGATGTCGGGTCGG               | 22          | 9     | −25.5                     | 5      |
| dlo-miR3  | Contig24091_Longan-RNA:14:90:-- | AAGATCTTTAGTTGTCTG                   | 20          | 15    | −31.6                     | 3      |
| dlo-miR4a | Contig569989_Longan-RNA:1:81:-- | ACGGGTTCAAAGGTTGACAGA               | 21          | 11    | −27.2                     | 3      |
| dlo-miR4b | Unigene4793_Longan-RNA:488:584:+ | ACGGGTCTCAAAGGTTGACAGA              | 21          | 13    | −32.3                     | 3      |
| dlo-miR5  | Contig629412_Longan-RNA:1:95:-- | TGGAGAGTTGGAAGGTCGACAGA             | 21          | 17    | −19.75                    | 3      |
| dlo-miR6  | Scaffold39496_Longan-RNA:47:139:-- | TCAAGGGGTAGAAGTTTTAACT              | 22          | 213   | −21.3                     | 3      |
| dlo-miR7  | Unigene10300_Longan-RNA:116:231:-- | TACATCGAACACAGCGCGCATGC             | 22          | 8964  | −47.5                     | 3      |
| dlo-miR8  | Unigene12720_Longan-RNA:16:197:+ | TTGATCAATGGTCAAGGTCCTCA             | 22          | 611   | −61.5                     | 5      |
| dlo-miR9  | Unigene13717_Longan-RNA:777:907:-- | ATGTAATGATCGCGGAGACAAAT             | 23          | 1144  | −42.61                    | 3      |
| dlo-miR10 | Unigene14204_Longan-RNA:50:162:-- | TTGCAGGCGAAATTAAATTAGGA             | 21          | 12    | −51.5                     | 3      |
| dlo-miR11 | Unigene19708_Longan-RNA:14:99:-- | TGATGCCTGATATGATTGCGA               | 21          | 187   | −20.7                     | 5      |
| dlo-miR12 | Unigene3012_Longan-RNA:21:128:+ | TGAATATGAACTAAGTGGTGC               | 22          | 120   | −35.4                     | 3      |
| dlo-miR13 | Unigene30135_Longan-RNA:22:153:-- | AGAAGGTGTGATCTTCGTAAGACAGA          | 23          | 164   | −54                       | 5      |
| dlo-miR14 | Unigene31710_Longan-RNA:49:145:-- | GGAGGCAGCTGGATGACATGCAG             | 21          | 609   | −49.8                     | 5      |
| dlo-miR15 | Unigene40670_Longan-RNA:84:181:+ | TGGCTTTAATGAAGACCTGGG              | 21          | 10    | −37.89                    | 3      |
| dlo-miR16 | Unigene47226_Longan-RNA:9:101:-- | GTATGGAAAGATTGGTGGCA                | 21          | 57    | −35.2                     | 5      |
| dlo-miR17 | Unigene54607_Longan-RNA:85:298:+ | TGCAGCAGAAATACGCTGGC               | 22          | 1309  | −77.6                     | 3      |
| dlo-miR18 | Unigene56582_Longan-RNA:219:296:+ | TGGGAATTTGATGACTTACAT              | 22          | 6     | −21.2                     | 3      |
| dlo-miR19 | Unigene60375_Longan-RNA:59:202:-- | CGCTATTTCTAGTCGACATT                | 21          | 490   | −55.81                    | 5      |
| dlo-miR20 | Unigene67206_Longan-RNA:105:270:-- | TGGAGGAATGACGCAAGAAT                | 21          | 21    | −65.24                    | 3      |
| dlo-miR21 | Unigene8816_Longan-RNA:7:111:-- | GAGGATTGATGTAGACTCTAG                | 22          | 243   | −22.8                     | 3      |
| dlo-miR22 | LG_130129469:30129680:+ | AAGTTAAAGAGGGGATTGTAAGA             | 22          | 10    | −41.5                     | 5      |
| dlo-miR23 | LG_14:1008063:1401082:+ | GCTGGAGATCTGACCTGGT                | 21          | 130   | −44.8                     | 5      |
| dlo-miR24 | LG_15:15276189:15276277:+ | TGAATGATTCGACGCTGGC                | 21          | 122   | −40.8                     | 3      |
| dlo-miR25 | LG_10:10042573:1004266:+ | CCGCTTTAGTCGAGTTGGCAGA             | 23          | 72    | −48.18                    | 3      |
| dlo-miR26 | LG_11:5872450:5872561:+ | TTGAGATGACGCGGAGGATAA              | 21          | 14    | −40                       | 3      |
| dlo-miR27a | LG_2:21395857:21395930:-- | TGGGGCTGCGCGAGAATGTTATC            | 22          | 10    | −28.6                     | 5      |
| dlo-miR27b | LG_8:8501904:8501977:+ | TGGGGCTGCGCGAGAATGTTATC            | 22          | 10    | −32                       | 5      |

Identification of miRNA Cleavage Targets in *D.longan* Using Degradome Analysis

Bioinformatics analysis alone could not be used to determine whether the predicted miRNA targets were real or false positives. To verify predicted targets of known and novel miRNAs in the longan transcriptome, a high-throughput experimental approach—degradome sequencing—was therefore employed. More than 30 million raw reads were produced by Solexa sequencing. After processing, 72.14% of the resulting clean reads were 20- or 21-nt long; 58.81% (1,198,119) of the unique reads mapped perfectly to the longan transcriptome, indicating the high quality of the degradome library. The reads that mapped to the longan transcriptome were subjected to further analysis.

A total of 843 targets of 178 conserved miRNAs and 19 targets of 3 novel miRNAs (dlo-miR1, dlo-miR2, and dlo-miR5) were identified (Table S3 in Supporting Information S1). Similar to predictions based on sRNA library sequencing, degradome analysis indicated that most of the known miRNAs were capable of regulating multiple targets. These miRNAs included dlo-miR054 (90 targets), dlo-miR2673 (37 targets), dlo-miR1046 (21 targets), dlo-miR780 (13 targets), dlo-miR1442 (10 targets), and dlo-miR2628 (9 targets).
The number of targets identified through degradome sequencing was less than that obtained from bioinformatics prediction methods, perhaps because the latter approach could not distinguish genuine targets from false ones. In contrast to known miRNAs, novel miRNAs (i.e., dlo-miR1, dlo-miR2, and dlo-miR3) appeared to have only a limited number of targets.

In longan, cleavage sites for most miRNAs were usually located in the CDS of their targets. Most targeted miRNAs harbored multiple cleavage sites, a phenomenon also observed in Arabidopsis and rice [34,35]. For example, the C2H2 (Unigene 11990) mRNA (targeted by miR157) was cleaved at nucleotide positions 211, 330, 331, 387, 404, and 1143. Only the site at position 211 was mediated by miR157, however. The remaining cleavage sites were split by other sRNAs or by unknown miRNAs, suggesting a complex degradation mechanism for these targets in plants. The cleavage sites in a given transcript may be targeted either by different miRNAs or the same one; the latter is the case with Unigene 67649 (similar to At4g28230), which has 7 cleavage sites (between positions 1411 and 1417) that are all split by dlo-miR2673. This example demonstrates that cleavage does not always take place between nucleotides 10 and 11 of the target transcript, thus providing a new reference for miRNA target prediction.

Because prediction methods cannot determine the authenticity of miRNA targets, experimental confirmation is required. To verify the accuracy of miRNA target prediction in our study, we compared the results of prediction analysis and degradome sequencing. Surprisingly, most of the predicted targets could not be verified by degradome sequencing. This was the case with several highly conserved longan miRNAs (miR156, miR161, miR164, miR171, miR172, miR393, miR394, and miR408) that were predicted to regulate homologs of known miRNA targets. Degradome sequencing failed to identify these targets, and also failed to identify other predicted targets for the above miRNAs. In addition, targets could not be predicted or verified for some highly conserved miRNAs using either prediction or degradome sequencing methods, although additional non-conserved targets were identified for them instead. This is consistent with the results of a previous study [36] and indicates that some conserved miRNAs had additional “novel” targets. Further analysis revealed that among 2063 predicted targets of 272 longan miRNAs, only 27 predicted targets of 18 miRNAs were verified by the degradome method. For example, 5 predicted targets of dlo-miR2673, including Unigene 49256 (expressed protein), Unigene 54104 (GL1), Unigene 60784 (similar to At1g73290), Unigene 67649 (similar to At1g28230), and Unigene 67737 (MTB), as well as several targets of dlo-miR1780, dlo-miR2099, dlo-miR5021, and dlo-miR5057, were verified by degradation analysis (Table S4 in Supporting Information S1). There are several possible explanations for the observed discrepancies. For example, it is possible that miRNAs (e.g., miR161, miR393, and miR394) or targets were not detected because of their low expression levels during longan SE or because they were specific to tissues that had not been analyzed. Another possibility is that the degradome or transcriptome data was incomplete for target analysis. Alternatively, some miRNAs, such as miR156 and miR172, inhibit expression of their target genes (SBP and AP2), primarily through translational arrest. Our results suggest that a combined prediction and degradome analysis approach is the most effective way to identify miRNA targets in non-model plants.

Validation of Longan miR398 Targets Using Modified RLM-RACE

Recent studies have demonstrated that miR398 plays a key role in plant SE [11,15,16]. Although miR398 influences plant response to oxidative stress by down-regulating Cu/Zn-superoxide dismutase (CSD) expression [37], it is not known whether this miRNA’s regulatory role in plant SE also involves CSD down-regulation. To verify the nature of predicted miR398 targets and to investigate possible regulation of CSD by miR398 during longan SE, we performed a psRNATarget analysis and a modified RLM-RACE experiment.

The psRNA Target analysis indicated that dlo-miR398 can target DlCSD1a and DlCSD1b. This result is consistent with previous studies in Arabidopsis and rice [30,38,39], indicating that these miRNA binding sites are highly conserved among different plant species. A modified RLM-RACE experiment was then conducted, which verified DlCSD2a as a target of dlo-miR398 (Fig. 2). Tags derived from cleavage of DlCSD2a by dlo-miR398 were predominant, suggesting that dlo-miR398 plays a major role in DlCSD2a regulation during longan SE. On the other hand, the conserved target DlCSD1a was not detected, implying that DlCSD1a expression is not regulated by dlo-miR398 during longan SE. These results indicate that miR398 regulates longan SE primarily through mediation of the target DlCSD2a.

Expression Patterns of Conserved and Novel miRNAs During Longan SE

Expression patterns of miRNAs were closely related to their functions. To better understand the role of miRNAs during longan SE, expression patterns of 24 miRNAs (20 conserved and 4 novel), U6 snRNA, and 5S rRNA were analyzed using quantitative real-time PCR (qPCR). The qPCR analysis revealed that all selected miRNAs were expressed at varying levels in different embryogenic tissues, with some preferentially expressed in specific tissues. The expression patterns of these miRNAs could be divided into roughly 5 groups (Fig. 3 and Fig. 4).

The first group, consisting of U6 snRNA and miRNAs, dlo-miR139, dlo-miR4a, dlo-miR808, and dlo-miR5077, displayed ubiquitous but varied expression levels during middle to late stages of longan SE. Different members of the dlo-miR139 family had contrasting expression patterns, suggesting that each member of a family may have its own function. Expression of dlo-miR139a.1 was high in heart- and torpedo-shaped embryos, but low in other tissues tested, especially in friable-embryogenic calli. dlo-miR139a.2 was strongly expressed in heart-shaped and mature embryos. dlo-miR139a.2 and dlo-miR139e had similar expression patterns during longan SE, being expressed weakly during early stages but abundantly in middle and late stages. dlo-miR4 showed strong expression in torpedo-shaped and mature embryos, moderate expression in globular and heart-shaped embryos, and weak expression elsewhere. dlo-miR5077 was highly expressed during late stages of longan SE. In addition, dlo-miR808 and U6 snRNA also exhibited a similar expression pattern: unlike the other miRNAs, they were highly expressed during both early and late developmental stages, suggesting a regulatory role in morphogenesis during longan early and late SE.

dlo-miR398 23 CGCCCCCGUCGGACUCUUUUGUAC 1
DlCSD2a 565 CGACAUUGCAGCUUGACUGGAAA–ACUGUUGACG 594

Figure 2. Cleavage site mapping of miR398 target gene. mRNA sequence of DlCSD2a is aligned with miR398. Nucleotides flanking the cleavage site are in bold. Numbers indicate the fraction of cloned PCR products terminating at different positions.
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In the second category, dlo-miR159b*, dlo-miR160a, dlo-miR167a, dlo-miR390, dlo-miR398b, and dlo-miR26 exhibited tissue-specific expression, indicating that they may have stage-specific functions during longan SE. Among these, dlo-miR159b* expression patterns were similar to those of the miR398b family (b.1, b.2, and b.3): they were all highly expressed in heart-shaped, torpedo-shaped, and cotyledonary embryos, but barely detectable in mature embryos. In an earlier study in oranges [16], miR398 also exhibited high expression in globular and cotyledonary embryos, suggesting that its function may be conserved across different species. dlo-miR167a was barely detectable during early longan SE stages; during late developmental stages, however, it was strongly expressed, suggesting a major role in SE during cotyledonary and mature embryonic stages. This is consistent with previous studies [16]. In addition, dlo-miR390a.1 and dlo-miR390a*.1, differing by only 2 mismatches, showed low expression during early and late SE stages but high...
expression during middle stages; dlo-miR390a.1 was not detected in longan globular and mature embryos, but was expressed in orange globular embryos [16]. dlo-miR160a and dlo-miR26 were highly expressed in the middle stages of SE, implying that their accumulation may be required for heart- and torpedo-shaped embryo formation and morphogenesis. On the other hand, dlo-miR160a was barely detectable during the period from the friable-embryogenic callus stage (stage 1) to the incomplete compact pro-embryogenic culture stage (stage 3), and dlo-miR26 was not detected in mature embryos; this suggests that their targets of regulation were consistently promoted.

Members of the third group, comprising dlo-miR156, dlo-miR166c*, dlo-miR168a*, dlo-miR397a, dlo-miR2089*-1, dlo-miR24, dlo-miR17, and 5S rRNA, were expressed at varied levels in different embryogenic tissues, suggesting their wide involvement in various developmental stages during longan SE. dlo-miR156a and dlo-miR156c were both highly expressed during early stages of longan SE. dlo-miR156a was strongly expressed in cotyledonary embryos, whereas dlo-miR156c expression was concentrated in embryogenic callus (stage 2). dlo-miR166c* showed high expression during friable-embryogenic callus through compact pro-embryogenic culture stages, moderate expression in globular embryos, and weak expression in cotyledonary and mature embryos; this indicates it may play a key role in early stages of longan SE. In contrast, miR166 has been found to be abundantly expressed in orange cotyledonary embryos [16] and Pinus taeda zygotic embryos [18]. dlo-miR168a* expressed relatively high levels from heart-shaped embryos to mature embryos, especially in cotyledonary embryos, and high expression in cotyledonary embryos and moderate expression elsewhere was observed for both dlo-miR397a and dlo-miR24. In addition to dlo-miR17, which was weakly expressed during late developmental stages, the remaining miRNAs dlo-miR2089*-1 and 5S rRNA showed ubiquitous expression during longan SE.

In summary, the above results indicate that different miRNA families and their individual members, as well as miRNAs*, have different regulatory roles during longan SE.

**Discussion**

In this study, 29 novel and 643 conserved miRNAs in more than 169 miRNA families were identified during longan SE by Solexa sequencing. This research represents the first large-scale cloning and characterization of plant SE-related miRNAs. Although many miRNAs remain to be identified, our results reveal that longan has a larger and more diverse miRNA (snRNA) population than other plants [11,15,16] when conserved miRNAs are compared. Bioinformatics prediction, degradome analysis, and modified RLM-RACE demonstrated that putative miRNA targets were involved in a broad variety of biological processes, including plant metabolism, signal transduction, and stimulus response. The stage- and tissue-specific expression patterns observed for 24 conserved and novel miRNAs suggest their probable roles in longan SE, and imply that different miRNA families are responsible for morphogenesis at different developmental stages.

**Putative Functions of miRNAs during Longan SE Based on Analysis of Expression Patterns**

SE is a multi-step regeneration process which begins with proembryogenic mass formation followed by somatic embryo
formation, maturation, desiccation, and plant regeneration. This complicated process involves a large number of genes with complex expression patterns. By identifying miRNAs and their targets and studying their expression patterns, we can propose possible roles for miRNAs during longan SE.

The largest miRNA family in plants—miR156—plays critical regulatory roles during juvenile-adult transitions [40] and flowering [41] by targeting SPL genes. In Arabidopsis, beginning as early as the eight-cell stage, miR156-mediated repression of zygomorphic SPL10 and SPL11 prevents premature accumulation of transcripts from genes normally induced during the embryonic maturation phase [14]. In the study, the highest concentrations of dlo-miR156a were observed in cotyledonary embryos, which is consistent with miR156 patterns in larch [15]. In contrast, dlo-miR156c expression was strongest in embryogenic calli II (stage 2), similar to results in citrus, in which miR156 levels were at a maximum during the E2 (EC induced for 2 weeks) stage [16]. These results suggest that miR156 members play regulatory roles during early stages of longan SE or cotyledonary embryogenesis. Such data is consistent with the notion that miR156a has a conserved role in developmental regulation among dicots throughout the plant kingdom [17].

miRNA

miR159 and ARF

miR159, like its Arabidopsis homolog, is conserved in all angiosperms and plays important regulatory roles during both vegetative and reproductive development in many species. miR159 promotes cell division, whereas ARF family members promote cell expansion, and together they promote cell expansion and flowering [42,43]. In citrus, the low expression of miR159 suggests it may have stage-specific functions, because it is expressed at different levels in globular-shaped embryos [16]. During plant embryonic development, cells not only increase in number, but also undergo differentiation. By inducing autonomous cell division, oxidative stress can modulate plant SE [51,52].

miR166

miR166 targets HD-ZIP III transcription factors [55] and is required for cotyledon-shaped embryogenesis in longan. Like miR168, dlo-miR390 a, b, and c also accumulated during heart- and torpedo-shaped embryonic stages. Previous studies have shown that miR390 directs the formation of ta-siRNAs, which target ARF17, ARF3, and ARF4 genes to modulate developmental timing and patterning in Arabidopsis [50], and that this miRNA is most highly expressed in citrus in globular-shaped embryos [16] and in larch in cotyledonary embryos [15]. These differing results among different species may be a consequence of different miR390 members being examined, and suggest that a strict division of labor may exist in this miRNA family.

Dolichocarpus

During plant embryonic development, cells not only increase in number, but also undergo differentiation. By inducing autonomous cell division, oxidative stress can modulate plant SE [51,52]. miR398 is the first miRNA to be directly linked to regulation of plant oxidative stress response via down-regulation of CSD expression [37]. Recent studies have shown that miR398 is required for cotyledon-shaped embryo morphogenesis in citrus [16] and is involved in modulation of proembryogenic mass propagation and transition to single embryos in larch [15]. Surprisingly, dlo-miR398a was barely detected during longan SE in our study (data not shown). Moreover, in a previous study, pre-miR398a was only weakly expressed during early longan SE stages and was not detected at all during middle and late stages [53]. These results indicate that dlo-miR398a is not a key developmental factor in longan SE. In addition, the low expression of dlo-miR398a led to increased accumulation of its target (DICS1a), such that miRNA cleavage barely took place; this may explain why cleaved DICS1a mRNAs were not detected using modified RLM-RACE in our study. In contrast to dlo-miR398a, the dlo-miR398b family was highly expressed in heart-shaped, torpedo-shaped, and cotyledonary embryos, suggesting its involvement in these developmental stages. miR398 targets CSD and is down-regulated under stress conditions [39]. In our study, the addition of 5% sucrose to the mature embryo induction medium produced a stress-like osmotic pressure. Low expression levels of dlo-miR399b during cotyledonary embryogenesis permitted accumulation of DICS1a as a response to this stress, promoting maturation of longan cotyledonary embryos, consistent with a previous report [16].

miR390

High expression in cotyledonary embryos and moderate expression elsewhere was observed for both dlo-miR397a and dlo-miR24. miR397 is known to target laccases, which are associated with cell wall lignification and thickening during secondary cell growth [54]. In citrus, miR397 is specifically expressed at high levels in globular embryos; in larch, miR397 levels are highest in early cotyledonary embryos—consistent with our results—with low levels observed for its target mRNA. We consequently propose that dlo-miR397a helps regulate cell wall thickness during SE through cleavage of laccase mRNA.

miR166

miR166 targets HD-ZIP III transcription factors [55] and is required for cotyledon-shaped embryogenesis in longan [15,16]. In our study, similar targets, such as Unigenes 12408 and 14044 (class III HD-Zip proteins 4 and 6), were also predicted for dlo-miR166, demonstrating the evolutionarily conserved nature of these miRNA targets in different plants. In another study, miR166 was induced by gibberellic acid (GA3) [56]. Expression levels of endogenous GA3 showed a gradual downward trend during longan SE [49], which was mirrored in our study by dlo-miR166a expression levels. This suggests that changes in miR166a levels are caused by alterations in endogenous GA3 concentrations. GA3 inhibits the early stage of embryogenic cell differentiation/development leading to globular embryos [57]. dlo-miR166a in
our study exhibited a decreasing level of expression during this transition, suggesting a role for dlo-miR166* during early stages of longan SE. dlo-miR166a* expressed relatively high levels from stages heart-shaped embryos to mature embryos, especially in cotyledonal embryos, which is consistent with miR168 from larch [15]. miR168 is associated with repression of AGO1 accumulation, and AGO1 is the most important AGO protein in the miRNA pathway, which has specific expression during carrot SE [19]. Therefore, we speculated the accumulation of dlo-miR166* during cotyledonal embryos develop- ment repressed the expression of AGO1, thus leading to the development of longan embryo.

In addition, several miRNAs, such as dlo-miR80*, −2089* and −5077 were also detected in longan SE, which have rarely been studied in plant development. Among them, dlo-miR80* and −5077 both showed highly expression embryos at late developmental stages of longan somatic embryo, the former reaches an abundance peak at cotyledonal embryos; and the later, which is similar to osa-miR5077, reaches peak at mature embryos, suggesting the accumulation of the two miRNAs is required for the development of somatic embryo at late stages. Besides, dlo-miR80*, which considered to be legume-specific [30], showed ubiquitous expression levels and exerted regulatory function during longan SE. Further, the expression analysis of the 4 novel miRNAs uncovered in this study (dlo-miR4a, dlo-miR17, dlo-miR26, and dlo-miR24*), showed varied levels in different embryogenic tissues during longan SE, has laid a foundation for future functional studies of longan embryogenesis. Such a study is currently underway in our laboratory. The investigation reported here represents a small but significant step towards the elucidation of functions of miRNAs during longan embryogenesis.

**Materials and Methods**

**Plant Materials and RNA Isolation**

Synchronized embryogenic cultures at different developmental stages were obtained following previously published methods for longan [1,2,58–60]. The synchronized cultures, consisting of friable-embryogenic calli, embryogenic calli II, incomplete compact pro-embryogenic cultures, compact pro-embryogenic cultures, globular embryos, heart-shaped embryos, torpedo-shaped embryos, cotyledonal embryos, and mature embryos, were collected and stored at −80°C for later use. Total RNAs were extracted from the above-described cultures using Trizol reagent (Invitrogen, Carlsbad, CA, USA). Only RNA samples with A260/A230 ratios between 1.9 and 2.1 and A260/A280 ratios between 1.9 and 2.0 were used for further analyses.

**Longan sRNA cDNA Library Construction, Solexa Sequencing, and Data Analysis**

To identify potential conserved and novel miRNAs from longan SE, a pooled sRNA library was generated from the synchronized embryogenic cultures (friable-embryogenic calli, globular embryos, heart-shaped embryos, torpedo-shaped embryos, and cotyledonal embryos) and sequenced on a Solexa system (Illumina). First, embryogenic cultures (friable-embryogenic calli, globular embryos, heart-shaped embryos, torpedo-shaped embryos, cotyledonal embryos, and mature embryos, were collected and stored at −80°C for later use. Total RNAs were extracted from the above-described cultures using Trizol reagent (Invitrogen, Carlsbad, CA, USA). Only RNA samples with A260/A280 ratios between 1.9 and 2.1 and A260/A230 ratios higher than 2.0 were used for further analyses.

**Solexa Transition, suggesting a role for A280 ratios between 1.9 and 2.1 and (Invitrogen, Carlsbad, CA, USA). Only RNA samples with extracted from the above-described cultures using Trizol reagent and stored at −80°C for later use. Total RNAs were purified, cloned, and sequenced.

**Bioinformatics analyses were conducted on the resulting Solexa sequencing data.** The 33 nt sequence tags were first trimmed of adaptors, regions of low complexity, and low-quality sequences, and the length distribution of clean tags was summarized. The remaining sRNA sequences (clean reads) were mapped to both a longan embryogenic callus transcriptome (SRA050205) and a P. trichocarpa genome. To classify degradation fragments of noncoding RNA, the clean tags were then compared against non-coding RNAs in Rfam and NCBI GenBank databases. Any sRNAs having exact matches to these sequences were excluded from further analysis. To identify conserved miRNAs in longan, the unique sRNA sequences were searched using miRBase 17.0. Novel miRNAs were predicted from unamortized sRNAs using Mireap, and their secondary structures were predicted using the computational software package Mfold 3.1. At the same time, first position base bias among sRNA candidates of certain lengths was summarized to determine prediction accuracy. Finally, to understand the biological functions of miRNAs, potential longan novel and conserved miRNA targets were predicted as described in [41,61].

**Degradome cDNA Library Construction and Sequencing**

To experimentally verify both conserved and novel miRNA targets in longan, a degradome cDNA library from the above pooled total RNA samples was constructed using the following steps: ligation of polyA-enriched RNA samples to a custom RNA adaptor containing an MmeI site, followed by reverse transcription (RT), second- strand synthesis, MmeI digestion, ligation of a 3’ double-stranded DNA adaptor, gel purification, and PCR amplification. The amplified degradome tag library was sequenced on a Solexa/Illumina genome analyzer. After sequencing, 20- and 21-nt reads from the degradome library were analyzed using PairFinder to identify sRNA targets.

**psRNATarget and Modified 5’ RLM-RACE**

To verify the nature of predicted miR398 targets and to investigate possible regulation of CSD by miR398 during longan SE, a psRNATarget [62] analysis and a modified 5’ RLM-RACE experiment were performed. For the psRNATarget analysis, sequences of the newly-identified longan miR398 were matched against nucleic acid sequences of longan SOD gene families (Ca/SOD, Fe-SOD, and MnSOD). All predicted targets were scored using criteria described in [62]. Any sequences with total scores less than 5.0 were considered to be miRNA targets. To identify cleavage sites of target transcripts, a modified RLM-RACE experiment was performed using a GeneRacer kit (Invitrogen). Briefly, total RNAs from equal mixtures of friable-embryogenic calli, globular embryos, heart-shaped embryos, and torpedo- shaped embryos were ligated to a 5’ RACE RNA adapter. The resulting samples were reverse-transcribed to cDNA using a GeneRacer Oligo dT primer and Superscript III reverse transcriptase, and PCR-amplified with GeneRacer 5’ primers and gene- specific primers (Table S5 in Supporting Information S1). The resulting PCR products were gel-purified, cloned, and sequenced.

**Real-time Quantitative PCR of Longan Conserved and Novel miRNAs**

qPCR was used to validate results obtained from high-throughput sequencing of longan miRNAs. RNA samples from the 9 embryogenic cultures described above were reverse-transcribed using an NCcode VILO miRNA cDNA Synthesis kit. Expression profiles of 24 miRNAs were examined using an NCcode
Express SYBR GreenER miRNA qPCR kit (Invitrogen). All reactions were performed in triplicate in a LightCycler 480 qPCR instrument (Roche Applied Science, Switzerland), with a dissociation curve used to control for primer dimers in the reactions. Mature miRNA abundance was calculated relative to expression of reference genes dia-miR156c, dia-miR2089*-1, and 5S rRNA. miRNA names and primer sequences are provided in Table S6 in Supporting Information S1.

Supporting Information
Supporting Information S1 File containing the following supporting information tables. Table S1. Conserved and non-conserved longan miRNAs. Table S2. Longan-known miRNA targets by prediction method. Table S3. Identification targets for longan miRNAs by Degradome sequencing. Table S4. The consensus targets for longan miRNAs obtaining from prediction and degradome analysis. Table S5. Primer sequences. Table S6. The miRNA names, primer sequences.

Author Contributions
Conceived and designed the experiments: ZL YL. Performed the experiments: ZL YL. Analyzed the data: ZL YL. Contributed reagents/materials/analysis tools: ZL YL. Wrote the paper: ZL YL.

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