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Identification and characterization of microRNAs in the developing maize endosperm

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MicroRNAs (miRNAs) are non-coding RNAs that are approximately 20–22 nucleotides long. miRNAs have been shown to be important regulators that control a large variety of biological functions in eukaryotic cells. To investigate the roles of miRNAs in maize endosperm development, two small RNA libraries of maize endosperm at two developmental stages were sequenced. A total of 17,773,394 and 18,586,523 small RNA raw reads were obtained, respectively. Further analysis identified and characterized 95 known miRNAs belonging to 20 miRNA families. In addition, 18 novel miRNAs were identified and grouped into 11 families. Potential targets for 5 of the novel miRNA families were successfully predicted. We had also identified 12 corresponding miRNAs* of these novel miRNAs. In summary, we investigated expression patterns of miRNA in maize endosperm at key developmental stages and identified miRNAs that are likely to playing an important role in endosperm development.

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1. Introduction

MicroRNAs (miRNAs) are important regulators that control various types of biological functions in eukaryotic cells, including development, viral defense, metabolism and apoptosis [1]. In both animals and plants, miRNAs are transcribed by RNA polymerase II into primary transcripts (pri-miRNAs) [1,2]. In animals, the pri-miRNA is processed to the miRNA precursor (pre-miRNA) by an RNase III-like enzyme called Drosha [3] and then the pre-miRNA is processed by Dicer to produce the mature miRNAs [4,5]. In plants, there are no Drosha homologs. The pri-miRNA is processed by a Dicer-like protein, DCL1, to the miRNA/miRNA* duplex [6–8], which is then methylated by HEN1 [7,9–11]. HASTY, the plant homolog of exportin 5, exports the miRNA/miRNA* duplex to the cytoplasm [12]. Then, the miRNA strand is incorporated into the RNA-induced silencing complex (RISC), which guides the sequence-specific post-transcriptional repression of the target mRNA by degradation or translational repression [1,13,14]. So far researchers have developed a variety of computational approaches to predict targets and identify known/novel miRNAs. The secondary structure, the Dicer cleavage site and the minimum free energy of the unannotated small RNA tags which could be mapped to genome were mainly used to predict novel miRNA. Parameters of mismatches between miRNA & target, mismatch position, and minimum free energy (MFE) of the miRNA/target duplex were mainly used to predict target genes.

Changing miRNA expression levels and disruption of the complementarity between the miRNA and its target mRNA can result in developmental defects or phenotypic changes [15,16]. Furthermore, the targets of several miRNAs have been shown to play important roles in many regulatory pathways containing endosperm development. For example, miR164 negatively regulates ORE1, a NAC (NAM, ATAF, and CUC) transcription factor that positively regulates aging-induced cell death in Arabidopsis leaves [17]; miR156 targets SPLs that play critical roles in cell size [18], fertility [19], and embryonic development [20]; miR396 attenuates cell proliferation in developing leaves, through the repression of the transcription factors for the growth-regulating factor (GRF) [21]; and miRNA159 mediates cleavage of the *MYB101* and *MYB33* transcripts that function as positive regulators of the ABA response to desensitize hormone signaling [22]. Many ABA signal transduction proteins are involved in seed development and germination [23].

A genome-wide analysis and characterization of the microRNAs in maize has helped accelerate our understanding of the regulatory roles of miRNAs in critical biological processes [24]. Maize endosperm development involves a range of biological processes many of which may be regulated, at least in part, by miRNAs. We sequenced two small RNA (sRNA) libraries from developing maize endosperm using high-throughput sequencing technology to investigate expression difference of know miRNAs in the two libraries and to identify

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novel miRNAs in maize endosperm development. From these data sets, we characterized the expression levels of 95 known miRNA in the two sRNA libraries and identified 11 novel miRNA families. These data sets will provide insights into the known miRNA involved in endosperm development and significantly enrich the repertoire of maize miRNAs.

2. Results and discussion

2.1. The developing maize endosperm has a complex small RNA population

To study the role of miRNA during maize endosperm development, we sequenced sRNAs from two sets of endosperm tissues, one collected at 4–6 DAP and the second collected from 7 to 23 DAP (see Material and methods), to determine the expression levels of the known miRNAs and to identify novel miRNAs. A total of 17,773,394 and 18,586,523 small RNA raw reads were obtained from the 4–6 DAP and 7–23 DAP tissues, respectively. After removing the low quality sequences and adapter sequences, 16,672,051 and 17,481,949 clean reads from 18 to 30 nucleotides (nt) long including 7,244,120 and 5,104,699 unique sequences were obtained (Table 1 and Supplementary Table 1). In the 4–6 DAP and 7–23 DAP tissues, 93.46% and 89.62% of the sRNAs, respectively, were between 20 and 24 nt in length (Fig. 1); the majority of which were between 21 and 24 nt which may be related to by-products of cleavage by Dicer-like enzymes [26]. The most abundant sRNAs were 24 nt long and accounted for 62.47% and 40.76% of the total reads in the 4–6 DAP and 7–23 DAP tissues, respectively (Fig. 1). This result is consistent with recent studies in *Zea mays* [27–29], *Oryza sativa* [30], *Phaseolus vulgaris* [31], and *Arabidopsis thaliana* [32]. The next most abundant sRNAs were the 21, 22, 23 and 20 nt long sequences in that order (Fig. 1). All the clean reads were mapped to the maize genome (B73 RefGen_v2, release 5a.57). The results indicated that 11,942,344 (71.63%) and 13,734,753 (78.57%) reads from the 4–6 DAP and 7–23 DAP tissues, respectively, were perfectly matched to the maize genome. Small RNAs (sRNA) from Solexa deep sequencing cover almost every kind of RNA, including miRNA, siRNA, piRNA, rRNA, tRNA, snRNA, snoRNA and so on. Around 3.73% and 5.59% of the unique reads in the 4–6 DAP and 7–23 DAP tissues, respectively, matched other non-coding RNAs that made up 11.44% and 23.44% of the total number of clean reads (Table 1).

2.2. Identification of known maize miRNAs

The 172 maize miRNAs registered at miRBase Release 20 can be grouped into 28 miRNA families which were defined by grouping miRNAs that share a common conserved seed region and have different precursors in different genome positions. BLASTN searches against the

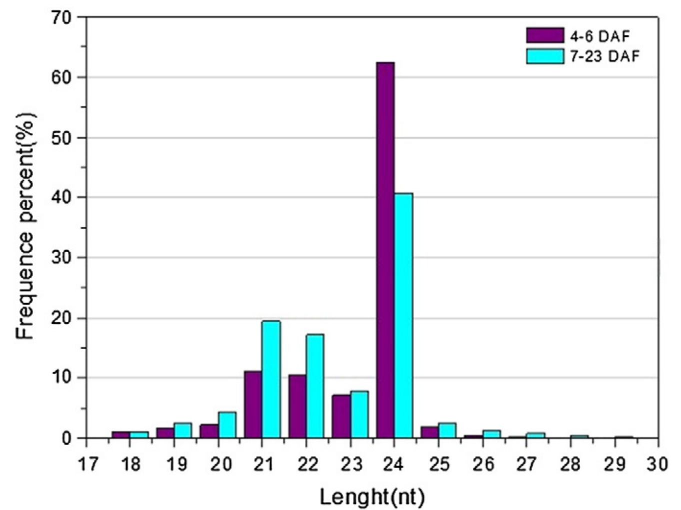

Fig. 1. Total reads of 18–30 nt small RNAs. The most abundant sRNAs were 24 nt long and accounted for 62.47% and 40.76% of the total reads in the 4–6 DAP and 7–23 DAP tissues, respectively.

known maize mature miRNAs and their precursors identified 95 known miRNAs belonging to 20 families in our data sets (Supplementary Table 2). We found that the expression levels between different miRNA family members varied drastically in the 4–6 DAP and 7–23 DAP data sets, suggesting that there was functional divergence within the families at the stage of endosperm development. For example, there were 125,858 clean reads for miR156d and only 164 clean reads for miR156k in the 7–23 DAP endosperm data set (Supplementary Table 2). Eight of the known miRNA families, miR162, miR395, miR399, miR482, miR529, miR1432, miR2118 and miR2275 were not detected in our data sets, suggesting that these miRNAs may be tissue-specific. In our sequencing data, miR398a/b were detected only in the 7–23 DAP endosperm. We found that a few miRNA* reads were more abundant. For example, in the 7–23 DAP data set, there were 81 miR396a/b reads and 657 miR396a*/b* reads, and 36 miR408b reads and 100 miR408b* reads. In the 4–6 DAP data set, there were 13 miR396a/b reads and 57 miR396a*/b* reads, and one miR408b read and six miR408b* reads (Fig. 2). This result is consistent with a recent study in maize [29]. It is possible that these miRNAs* can either down-regulate the expression of its target gene consistent with miRNA in function or be actually a real miRNA. The two miRNAs* may play an important role in endosperm development.

Table 1
Summary of small RNA sequencing.

	4–6		7–23	
	Unique reads	Total reads	Unique reads	Total reads
Non-coding RNAs				
rRNA	57,231 (0.79%)	577,283 (3.46%)	105,712 (2.07%)	1,787,673 (10.23%)
tRNA	10,605 (0.15%)	354,300 (2.13%)	20,373 (0.4%)	1,182,563 (6.76%)
siRNA	196,790 (2.72%)	942,099 (5.65%)	155,553 (3.05%)	1,115,097 (6.38%)
snRNA	3679 (0.05%)	25,189 (0.15%)	2121 (0.04%)	6390 (0.04%)
snoRNA	1456 (0.02%)	8211 (0.05%)	1316 (0.03%)	5201 (0.03%)
Protein-coding RNAs				
Exon	336,054 (4.63%)	943,772 (5.66%)	269,746 (5.28%)	852,225 (4.87%)
Intron	835,842 (11.54%)	2,234,447 (13.40%)	584,988 (11.46%)	2,438,814 (13.95%)
Known miRNAs				
miRNA	599 (0.01%)	1,005,693 (6.03%)	610 (0.01%)	1,651,662 (9.45%)
Other sRNAs	5,801,864 (80.09%)	10,581,057 (63.47%)	3,964,280 (77.66%)	8,442,324 (48.29%)
Total	7,244,120 (100%)	16,672,051 (100%)	5,104,699 (100%)	17,481,949 (100%)

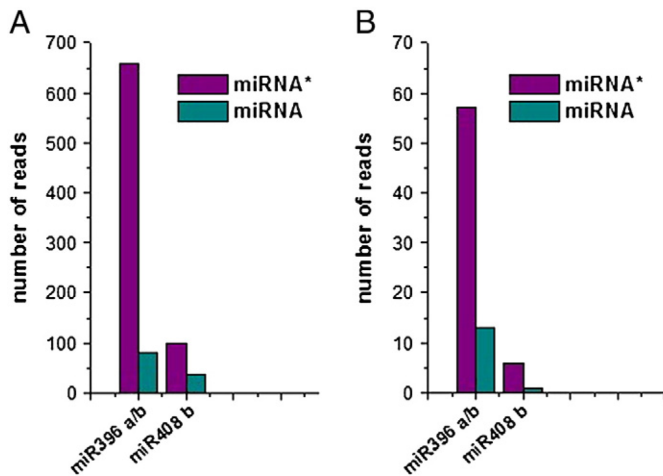

Fig. 2. A few miRNA* reads were more abundant than miRNA. (A) miRNA* reads of miR396a/b and miR408 were more abundant than corresponding miRNA in the 7–23 DAP data set. (B) miRNA* reads of miR396a/b and miR408 were more abundant than the corresponding miRNA in the 4–6 DAP data set. The two miRNAs* may play an important role in endosperm development.

2.3. miRNA expression changes during endosperm development

High-throughput sequencing methods now provide a rapid way to identify and profile of small RNA populations in different plants and tissues, and at different stages of development [33]. The expressions of the known miRNAs in the two data sets were compared to identify changes in miRNA expression during endosperm development. Except for the miR398 family, which was only expressed in the 7–23 DAP data set, 45 of the 95 known miRNAs were more highly expressed in the 7–23 DAP endosperm than in the 4–6 DAP endosperm (Fig. 3 and Supplementary Table 2); the highest fold change (log2 4–6/7–23) of −5.64 was observed for miR164e, while miR397b, miR408b, miR408, and miR169o all had fold changes (log2 4–6/7–23) which were more than 4. Another 23 of the known miRNAs were more highly expressed in the 4–6 DAP endosperm than in the 7–23 DAP endosperm; the highest fold change (log2 4–6/7–23) of 3.99 was observed for miR169b (Fig. 3 and Supplementary Table 2). The remaining 25 known miRNAs were expressed in approximately equal numbers in the two data sets. Differential expressions of nine selected miRNAs, comprising miR156a, miR160a, miR164a, miR164e, miR167d, miR168, miR169a, miR393a and miR528a,were validated by poly(A)-tailed RT-PCR. The results indicated that the expression patterns of all nine miRNAs are consistent with high-throughput sequencing (Fig. 4). miRNAs that showed greatest differential expression during endosperm development may play an important role in maize endosperm development. miR168a was the most abundant miRNA in the two endosperm data sets, accounting for 6.1% of all the known miRNA reads, in agreement with previous high-throughput sequencing studies in rice [30,34]. The miR168s target Argonaute-1 (AGO1), a major component of RISC, and their interaction has been reported to maintain homeostasis [35]. Recent research has demonstrated that the transcription factor ORE1 positively regulates aging-induced cell death in Arabidopsis leaves. ORE1 expression was reported to be up-regulated by EIN2 concurrently with leaf aging but negatively regulated by miR164 [17]. Kang et al. reported that miR319a/b/c/d were detected only in seeds but not in the leaves of maize [29]. Again in maize, miR319 was predicted to target several transcription factors including the MYB and TCP domain proteins [24]. In Arabidopsis, miR319 was shown to target several TCP transcription factors and to play an important role in leaf development [36,37]. MiR156 was found to target members of the squamosa promoter binding protein like (SPL) plant-specific transcription factors [38] and the SPLs in turn can affect a broad range of developmental processes in Arabidopsis.

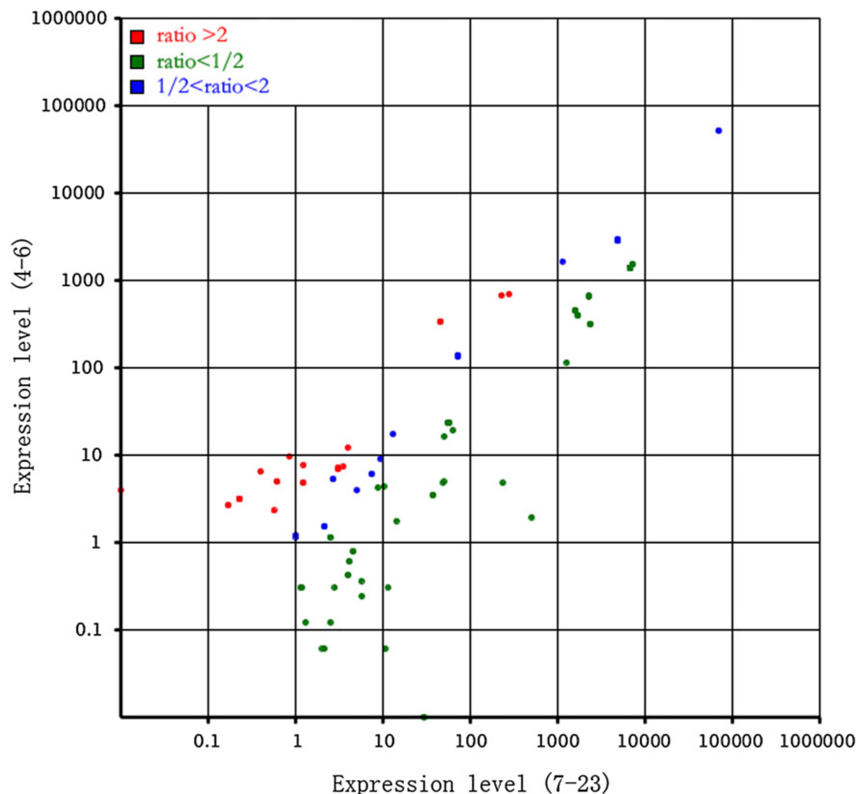

Fig. 3. Known miRNA expression changes during 4–6 DAP data set and 7–23 DAP data set. Green points represent that miRNAs were more highly expressed in the 7–23 DAP data set than in the 4–6 DAP data set. Red points represent that miRNAs were more highly expressed in the 4–6 DAP data set than in the 7–23 DAP data set. Blue points represent that miRNAs were expressed in approximately equal numbers in the two data sets.

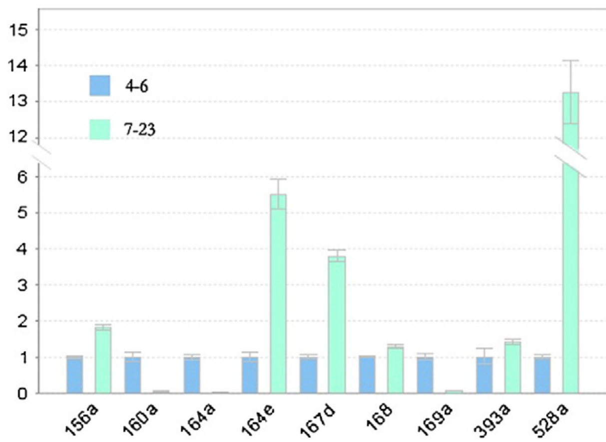

Fig. 4. Difference in expression of known miRNAs validated by poly(A)-tailed RT-PCR. Nine known miRNAs (miR156a, miR160a, miR164e, miR164a, miR167d, miR168, miR169a, miR393a, miR528a) were validated by poly(A)-tailed RT-PCR. The results indicated that the expression patterns of all nine miRNAs are consistent with high-throughput sequencing.

For example, miR156 was found to regulate the expression of miR172 via SPL9, which directly promotes the transcription of miR172b, which in turn accelerates flowering [39,40]. Also, miR156-targeted SPL9 negatively regulates anthocyanin accumulation by directly preventing expression of anthocyanin biosynthetic genes through the destabilization of the MYB-bHLH-WD40 transcriptional activation complex [16]. In addition, SPLs play critical roles in cell size [18], fertility [19], and embryonic development [20]. MiR160 and miR167 target the mRNAs of the auxin response factors (ARFs) that regulate levels of auxin [41–44]. In Arabidopsis, the mRNA of the transcription factor ARF17 is cleaved by miR160 and the over-expression of a miRNA-resistant version of ARF17 (5mARF17) resulted in increased ARF17 mRNA accumulation causing dramatic developmental defects that correlate with reduced accumulation of the GH3 family that encodes auxin-conjugating proteins [41]. In rice, miR167-targeted ARF8 positively regulated the expression of GH3-2, an IAA-conjugating enzyme [43]. Thus, it has been suggested that miRNAs that target positive regulators such as ARF6 and ARF8, and negative regulators such as ARF17, appear to fine-tune the expression of the GH3 family genes, thereby regulating the availability of free IAA [45]. In the present study, the expression level of miR160 was down-regulated and miR167 was up-regulated in the 7–23 DAP endosperm compared with the 4–6 DAP endosperm, suggesting that a higher level of free IAA may be required for endosperm development. In maize, miR393 and miR394 were predicted to target cyclin-like F-box [24]. The phytohormone auxin, an important regulator of plant growth and development, exerts its functions through F-box receptors. MiR393 down-regulates the TAAR genes that encode the transport inhibitor response 1/auxin signaling F-box 1 auxin receptor by guiding the cleavage of their mRNAs, thereby regulating auxin-related development of leaves in Arabidopsis [46]. MiR396 attenuates cell proliferation in developing leaves through the repression of the activity of the growth-regulating factor (GRF) transcription factors, resulting in a decrease in the expression of the cell cycle genes in Arabidopsis [47]. In the present study, the expression level of miR396 in the 7–23 DAP endosperm was higher than that in the 4–6 DAP endosperm. This may be because the early mitotic phase of development represented by the 4–6 DAP data set has been shown to be characterized by active cell proliferation throughout the endosperm [25]. MiR159 mediates the cleavage of the *MYB101* and *MYB33* transcripts that function as positive regulators of ABA responses, to desensitize hormone signaling [22]. Many ABA signal transduction proteins are reported to be involved in seed development and germination [23]. A mir159a/b double mutant has been shown to have pleiotropic morphological defects, including altered growth habit, curled leaves, small siliques, and small seeds [48].

2.4. Identification and characterization of novel maize miRNAs

We used a bioinformatics pipeline to identify novel miRNAs from our sequence data sets (see Material and methods for details). First, we utilized a prediction software Mireap to predict novel miRNA. Then, the candidate miRNAs that had MFEIs more than 0.85 were selected for further analysis. Lastly, MiPred was used to predict whether or not the candidate miRNA precursors were a real microRNA precursor. Because of the stringent criteria that we used to predict the novel miRNAs, these 18 sequences are good candidates as novel miRNAs.

The 18 novel miRNAs were grouped into 11 miRNA families (Table 2, Supplementary Table 3). Except for miR8, the remaining novel miRNA were detected less than 1000 times, the least was detected only seven times. We identified 12 of the corresponding miRNAs* of the novel miRNAs in the combined data sets. Most miRNAs* show low abundance (sequencing frequency < 50) and their expression levels are much lower than their corresponding miRNAs (Supplementary Table 3). Five novel miRNAs were validated by stem–loop RT-PCR and sequencing (Fig. 5).

2.5. Target prediction of novel miRNAs

The prediction of the target genes of the newly identified miRNAs was performed to assign functions which might assist in future comprehensive studies of biological functions. TargetSearch is based on GenomeMapper, a short sequence alignment tool developed for the 1001 genomes project (http://wmd3.weigelworld.org). We used the *Z. mays* ZmB73 V5b (MGC) database for target finding (see Material and methods for details).

Of the 11 miRNA families that we identified for the novel miRNAs, 5 had at least one predicted target gene (Supplementary Table 4). A total of 22 target genes with functional annotations for the miRNA families were predicted. An examination of the functions of the predicted target genes showed that they had diverse functions, such as transcription regulation, carrier proteins, enzymes, structural molecules, and kinase. These target genes may have important regulation roles in endosperm development.

3. Material and methods

3.1. Construction of the small RNA libraries and RNA sequencing

Maize seeds undergo a complex series of developmental events leading to the production of the mature embryo and endosperm. This division separates the two major phases of endosperm development. The early mitotic phase of development is represented by 4–6 days after pollination (DAP) and is characterized by active cell proliferation throughout the endosperm. The basal transfer layer undergoes differentiation, but there is little other cytological evidence of cell specialization. Between 7 and 10 DAP, mitotic divisions become localized to the periphery, differentiation of the aleurone becomes evident, and central cells enlarge, undergo endoreduplication, and begin to accumulate proteins and starch. By 16 DAP, the maturation program is initiated, preparing the seeds for desiccation and dormancy, and by 23 DAP, the process of desiccation has begun [25]. During the summer of 2010, two sets of endosperm tissues were harvested from the maize inbred line 08-641. One set was collected at 4–6 days after pollination (DAP) and pooled. The second set was collected at 2-day intervals from 7 to 23 DAP and pooled. Total RNA was extracted and purified using TRIzol reagent (Invitrogen). The integrity of the RNA was checked by 1% agarose gel electrophoresis. The sRNAs were separated on 15% denaturing PAGE and 5′ and 3′ adaptors were added. Amplification was performed by reverse transcription PCR (RT-PCR) and the product was sequenced directly on a Solexa machine (BGI-Shenzhen, Shenzhen, China). Clean reads were generated from the raw reads after filtering out the adaptor sequences and removing the low quality reads.

Table 2
Novel maize miRNAs identified in this study (Supplementary Table 3 for the detailed pre-miRNA sequences and MFEI; a/b represent miRNA form different pre-miRNA).

miRNA Id	Mature	Count	L (nt)	Arm	Mature*	Count*
zma-miR1	AAGTTAGATCTAAGGGCCAAA	5	21	3p	TGGCCCTTAGATCTGACACGG	2
zma-miR2a	ACAACTTTAGATATATACCAT	7	21	5p	GGTATATATCTAAAGTTGCAA	1
zma-miR2b	ACAACTTTAGATATATACCAT	7	21	5p	GGTATATATCTAAAGTTGTAA	1
zma-miR3	CCCTTTCTTGTTCGGATTCT	5	20	3p	ATTCCTGACTGGGAAGGGTT	4
zma-miR4	CCGTCGGGTACATATATGGTG	10	21	5p	CTATATAGTATCCGCGGGT	1
zma-miR5	GCGGCGATCATGTACTACCCT	102	21	3p		
zma-miR6	TACATATGTTGTCTGCCTCAC	25	21	5p	AGACAGACAACATATGTAGAA	26
zma-miR7	ATTTTTACCCTTAGTCGTGTT	53	21	5p	TACGACTAAGGGTAAAAATGA	5
zma-miR8a	TACGTGGGCTTTCTGTACGGCA	33,448	22	5p	CCGTAGAGAAAGCTCGCGATAAG	44
zma-miR8b	TACGTGGGCTTTCTGTACGGCA	33,448	22	5p	CCGTAGAGAAAGCTCGCGATAAG	44
zma-miR9a	TTATTAGTCGCTGGATAATGC	172	21	5p		
zma-miR9b	TTATTAGTCGCTGGATAATGC	172	21	5p		
zma-miR9c	TTATTAGTCGCTGGATAATGC	172	21	5p		
zma-miR9d	TTATTAGTCGCTGGATAATGC	170	21	5p		
zma-miR9e	TTATTAGTCGCTGGATAATGC	172	21	5p		
zma-miR10	CCATGCAGCCAAGACACAAAC	1	21	5p	TTGTGTTTTGGTTGCACGGTT	24
zma-miR11a	TTTTTTTATTTGTCGCTGGAT	33	21	5p	CAAGCGACAAATAAAAAGAAAC	2
zma-miR11b	TTTTTTTATTTGTCGCTGGAT	32	21	5p	CCGGCGACAAATAAAAAGAAA	3

3.2. Identification of known and novel miRNAs

The length distribution of the clean reads is shown in the figure. All the clean reads were mapped to the maize genome (B73 RefGen_v2, release 5a.57) using SOAP [49]. Sequences that did not match the genome were discarded. Known miRNAs were identified by BLAST searches against the known maize mature miRNAs and their precursors in miRBase database (www.mirbase.org). Sequences with perfect matches to the known sequences were regarded as known miRNAs. To discover potential novel miRNA precursor sequences in our data sets, we aligned the remaining clean sequences against the GenBank, Rfam and miRbase databases to filter out protein-coding RNAs and to identify the non-coding RNAs. We used the MIREAP software tool to predict novel miRNA (http://cloud.genomics.cn/). MIREAP works by exploring the secondary structure, the Dicer cleavage site and the minimum free energy of the remaining reads that were mapped to the genome. The parameter is shown as below: 1) Minimal miRNA sequence length (20); 2) maximal miRNA sequence length (25); 3) minimal miRNA reference sequence length (21); 4)maximal miRNA reference sequence length (24); 5) maximal free energy allowed for a miRNA precursor (−25 kcal/mol); 6) maximal space between miRNA and miRNA* (300); 7) minimal base pairs of miRNA and miRNA* (20); and 8) maximal bulge of miRNA and miRNA* (2). Next, the minimal folding free energy index (MFEI) values of the remaining unidentified reads were checked. Previous studies have found that the average MFEI of miRNA precursors in plant pre-miRNAs is 0.97, and that while more than 90% of miRNA precursors have MFEIs greater than 0.85, no other RNAs have MFEIs higher than 0.85 [28]. Thus, the MFEI of miRNA precursors can be used to distinguish miRNAs from other non-coding and coding RNAs. MFEI can be calculated as MFE/(precursor length) × 100/(G + C)%. In the present study, only reads with an MFEI value greater than 0.85 were considered to be miRNAs. Finally, Mipred was used to predict whether or not it is a real miRNA precursor [50].

3.3. Comparison of the expressions of the known miRNAs in the two libraries

The expressions of the known miRNAs in the two data sets were compared to identify the miRNAs that were differentially expressed in the two libraries. The expression patterns in the two libraries were compared using a log2 ratio plot and a scatter plot. We used a two step procedure as shown: (1) the expression of the miRNAs in the two libraries were normalized in transcripts per million (TPM) for each library as normalized expression = actual miRNA count/total count of clean reads × 1,000,000; and (2) the fold-change and P-values were calculated from the normalized expression values. The log2 ratio plot and scatter plot were generated using the fold-change formula, fold-change = log2 (treatment/control).

3.4. Prediction of novel miRNA targets

To predict the target genes of the candidate novel miRNAs, we used the target search tool in the WMD3 software (http://wmd3.weigelworld.org) and the *Z. mays* ZmB73 V5b (MGC) database for target prediction. Briefly, the following criteria were applied: (1) no more than three mismatches between the sRNA and the target; (2) minimum free energy (MFE) of the miRNA/target duplex should be ≥70% of the MFE of the miRNA bound to its perfect complement; (3) the temperature for the calculation of RNA–RNA hybridization is 23 °C; and (4) RNA–RNA interaction was computed using the RNAcofold program from the ViennaRNA package. Functional annotations for the predicted targets were retrieved from the ZmB73_5a_gene_descriptors.txt in the MaizeGDB database.

3.5. Validation of known miRNA expression by poly(A)-tailed RT-PCR

Nine known maize miRNAs were selected to validate the deep-sequencing results by poly(A)-tailed RT-PCR. Real-time PCR was performed in accordance with instruction manual of TaKaRa SYBR® PrimeScript™ miRNA RT-PCR Kit (TaKaRa, Japan). 5S rRNA served as an internal control, and the 2-△△Ct method was used to calculate relative expression amounts. The PCR primers used can be found in Supplementary Table 5. Each real-time RT-PCR reaction was done in technical triplicates.

3.6. Validation of novel miRNAs by stem–loop RT-PCR and sequencing

The stem–loop RT-PCR method was adopted for novel miRNA detection [29]. Total RNA from endosperm was extracted using TRIzol

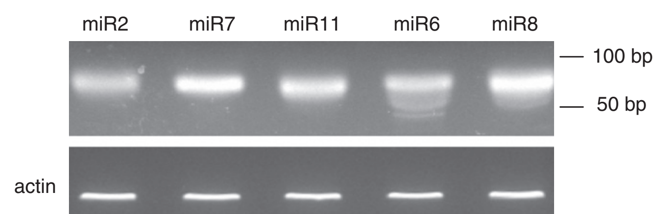

Fig. 5. Novel miRNAs validated by stem–loop RT-PCR and sequencing.

reagent (TaKaRa). Stem–loop reverse transcription reactions were used for the mature miRNAs using specific stem–loop RT primer. PCR was performed with the following procedures: 94 °C for 2 min; 30 cycles of 94 °C for 30 s and 60 °C for 1 min. The PCR products were analyzed by 3% agarose gel and purified, then ligated to pMD19-T vector (TaKaRa) and transformed to JM109. Sequencing was carried out by Invitrogen (Shanghai). All primers are listed in Supplementary Table 6.

4. Conclusions

We had studied the roles of miRNAs in maize endosperm development by small RNA sequencing. By sRNA sequencing, expression patterns of 95 known miRNAs belonging to 20 miRNA families were identified and characterized. Seventy miRNAs that had significant differences in expression may be involved in regulating maize endosperm development. These miRNAs would be exciting candidates to investigate in elucidating how they impact upon development. Furthermore, 18 novel miRNAs that take part in regulating endosperm development were identified and grouped into 11families. We had also identified 12 corresponding miRNAs* of these novel miRNAs.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.ygeno.2013.08.007.

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