Research Paper

Identification and Characterization of Novel Maize Mirnas Involved in Different Genetic Background

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Abstract

MicroRNAs (miRNAs) are a class of small, non-coding regulatory RNAs that regulate gene expression by guiding target mRNA cleavage or translational inhibition in plants and animals. At present there is relatively little information regarding the role of miRNAs in the response to drought stress in maize. In this study, two small RNA libraries were sequenced, and a total of 11,973,711 and 14,326,010 raw sequences were generated from growing leaves of drought-tolerant and drought-sensitive maize seedlings, respectively. Further analysis identified 192 mature miRNAs, which include 124 known maize (zma) miRNAs and 68 potential novel miRNA candidates. Additionally, 167 target genes (259 transcripts) of known and novel miRNAs were predicted to be differentially expressed between two maize inbred lines. Of these, three novel miRNAs were up-regulated and two were down-regulated under drought stress. The expression of these five miRNAs and nine target genes was confirmed using quantitative reverse transcription PCR. The expression of three of the miRNAs and their putative target genes exhibited an inverse correlation, and expression analysis suggested that all five may play important roles in maize leaves. Finally, GO annotations of the target genes indicated a potential role in photosynthesis, may therefore contribute to the drought stress response. This study describes the identification and characterization of novel miRNAs that are the differentially expressed in drought-tolerant and drought-sensitive inbred maize lines. This provides the foundation for further investigation into the mechanism of miRNA function in response to drought stress in maize.

Key words: Maize; Drought stress; MicroRNA; Target genes; High-throughput sequencing; qRT-PCR

Introduction

MicroRNAs (miRNAs) are small, endogenous RNAs that regulate gene expression in plants and animals [1-5] at the post-transcriptional level by translational repression or target degradation and gene silencing. Plant miRNAs function in diverse processes associated with growth and development such as developmental transitions, organ polarity, auxin signaling, boundary formation or organ separation, leaf and stem growth, floral organ identity and reproductive development [6]. Several miRNAs are involved in the regulation of root development in *Arabidopsis*, including miR167 that targets auxin response factors (ARFs) in order to regulate the emergence of shoot-borne roots [7-9], and miR164 that directly cleaves transcription factor NAC1 which inhibits lateral root development. Other miRNAs such as miR156 and miR172 affect flowering time when over-expressed in *Arabidopsis* [9-12], and miR172 exhibits a similar temporal expression pattern in maize, where it targets Glossy15, a gene required for the expression of juvenile epidermal traits [13]. Moreover, miR395 regulates sulfur assimilation and translocation by adjusting the mRNA levels of ATP sulfurylase and a low affinity sulfur transporter [14, 15], while miR399 regulates phosphate homeostasis in *Arabidopsis* by suppressing the ubiquitin-conjugating E2 en-

zyme, PHO2 [16, 17]. In addition, accumulating data demonstrate that miRNAs play major roles in response to biotic and abiotic stress [18, 19, 20], such as low nitrate condition [21, 22], low phosphorus stress [23], salt stress [24] and so on.

Bioinformatics approaches have been successful for the discovery of conserved miRNAs in numerous plant species including cotton [25], maize [26], caster [27], sorghum [28], and soybean [29]. At the time of writing, there are 18,226 mature miRNAs deposited in miRBase [30]. As an important model system for basic biological research, maize has contributed significantly to our understanding of plant development and evolution, and this knowledge has been employed to elucidate the regulatory functions of miRNA genes [26]. The total number of miRNAs in an individual organism has been estimated to represent approximately 1% of the total number of coding genes [31, 32]. To date, only 150 genes within 26 miRNA families have been identified in maize [26], and many more remain to be discovered.

Maize is one of the most important food crops worldwide, and is also used for animal feed, silage and in industrial products. Drought stress is often the limiting factor for plant growth and agricultural productivity, and this is especially true for maize. Plant microRNAs have been shown to play important functions in plant growth and development. However, role of miRNAs in the response to drought stress is poorly understood in maize. In recent years, high-throughput sequencing technologies and bioinformatics approaches have together identified many novel miRNAs [33, 34, 47]. In the present work, drought-tolerant (Hz4) and drought-sensitive (3189) inbred maize lines [35] were investigated using Illumina sequencing to identify novel drought-responsive miRNAs. Expression of the identified miRNAs was confirmed using qRT-PCR and target genes were elucidated. Probing of two small RNA libraries from young leaves led to the identification of 124 known maize (*zma*) miRNAs, and a further 68 novel maize miRNAs were discovered. Of these, five novel miRNAs that were differentially expressed in the two inbred lines were characterized, and the results suggest they may play an important role in response to drought stress.

Materials and methods

Plant materials and drought stress treatment

Drought-tolerant (Hz4) and drought-sensitive (3189) maize inbred lines were germinated in a greenhouse and grown under standard conditions until seedlings had developed three leaves. Seedlings were then subjected to drought conditions. Specifi-

cally, the soil water content was reduced from 90% to 70%, and approximately 5 g of leaves was collected from each seedling and immediately frozen in liquid nitrogen.

Construction of small RNA libraries for high-throughput sequencing

To construct small RNA libraries, total RNA was extracted from leaves using Trizol reagent (Invitrogen, USA) according to the manufacturer's protocol as modified by LC Sciences (Houston, USA). The small RNA fraction (10-40 nt) was isolated by polyacrylamide gel electrophoresis and ligated with proprietary adaptors (Illumina). Short RNAs were then reverse-transcribed into cDNA by RT-PCR. Small RNA libraries were constructed for each maize line and sequenced using a Genome Analyzer GA-IIX (Illumina) following the manufacturer's instructions. Small RNA sequencing data was processed with the in-house program ACGT101-miR (LC Sciences, USA). Program parameters were set as previously described [36] with modifications to adapt the program for plant miRNA prediction.

Sequencing data processing

To begin with, we compared the filtered small RNA sequences with known miRNAs in miRBase 20.0 [http://www.mirbase.org [37] and a 0-2 nucleotide mismatch was considered. Meanwhile, the filtered small RNA sequences were compared with *ZmB73*_4a.53 (*ZmB73*_AGPv1) [38] using BLASTn and no mismatches were permitted. Then ESTs that contained small RNA sequences were selected and their secondary structures were predicted using MFOLD (http://mfold.rna.albany.edu/?q=mfold/RNA-Folding-Form) for identifying potential novel miRNAs. At last, small RNAs with corresponding star (miRNA*) sequences were counted to be novel miRNAs according to the criteria for miRNA definition [39].

Prediction and validation of miRNA target genes

The target genes of miRNAs were predicted using the method of target prediction proposed by Allen et al. (2005) [40] and Schwab et al. (2005) [41]. The plant small RNA target analysis server psRNATarget [42] was employed for prediction through searching against the maize full-length cDNA sequences. In addition, the rules of target prediction were referred to universal criteria previously suggested [40, 41] and the parameters of psRNATarget server were the default.

Validations of 18 novel miRNAs which low abundance and more than 24 nt in length were carried out by stem-loop reverse transcription-PCR (RT-PCR).

The stem-loop RT-PCR was using the Applied Bio systems 7300 Real-Time PCR System (Applied Bio systems, Foster City, CA) and all reactions were run in triplicate. 18S rRNA was used as the internal control for stem-loop RT-PCR. In addition, quantitative real-time RT-PCR (qRT-PCR) was performed to validate the target genes of five novel miRNAs that were differentially expressed between the two inbred lines using three biological replicates. Total RNA from the leaves of the two maize inbred lines was extracted using Trizol reagent (Invitrogen, USA) according to the manufacturer's protocol as modified by LC Sciences (Houston, USA). Samples were collected at the same time as those for miRNAs sequencing. The RNase-free DNase (Promega, USA) was used to remove contaminating DNA from total RNA and first strand cDNA was synthesized according to the manufacturer's instructions (Takara, Japan). The PCR system contained 2 μL cDNA, 12.5 μL Platinum SYBR Green qPCR SuperMix-UDG (Takara, Japan), 2 μL 10 μM primer mixture and 8.5 μL RNase-free water. Sample cycle threshold (Ct) values were standardized for each template based on a GAPDH control reaction and the comparative Ct method (2-ΔΔCt) was used to determine the relative transcript abundance of each gene [43]. Gene accession and primer sequences are listed in Additional File 3: Table S4.

Gene ontology (GO) analysis

Target genes were subjected to Gene Ontology (GO) analysis to uncover the miRNA-gene regulatory network on the basis of biological process and molecular function. GO annotations were extracted using AgriGO (http://bioinfo.cau.edu.cn/agriGO/) and included alignments of individual transcripts representing diverse functions, processes or components. A graphical representation of the GO ontology distribution of the biological processes, cellular component and molecular function categories can be found in Online Resource 8.

Results

Overview of sequencing results

Two small RNA libraries constructed from the leaves of drought-tolerant and drought-sensitive maize seedlings were sequenced using high-throughput methods, and a total of 11,973,711 and 14,326,010 raw sequences were generated from the drought-tolerant and drought-sensitive libraries, respectively. After removing low quality reads and clipping adaptor sequences, 8,983,222 and 7,948,375 clean reads were remained with lengths ranging from 17 to 44 nt (Table 1). The reads of 34 nt in length were the most abundant among total reads of each small RNA library (Figure 1A). In addition, a length of 33 nt was the most abundant among unique reads of SL_HZ4 and a length of 24 nt was the most abundant among unique reads of SL_3189 (Figure 1B). To identify known miRNAs, small RNA sequences were used with BLASTn to search the miRBase database (release 20.0, June 2013). A total of 124 known maize (*zma*) miRNAs were identified, of which 76 were detected in Hz4 and 124 were detected in 3189. These miRNAs belong to different miRNA families such as the miR156, miR166, and miR167 families (Figure 2). The miR156 family was the most represented, indicating a higher level of expression in maize seedlings, as was previously reported [26]. Uridine (U) was the most common nucleotide at the 5' end (>65%), whereas this nucleotide was comparatively rare at positions 2-4 (Figure 3).

Table 1: Summary of small RNA sequencing.

Category	type	Total	% of Total	uniq	% of uniq	Total	% of Total	uniq	% of uniq
Raw reads	NA	14326010	100	817844	100	11973711	100	576650	100
3ADT&length filter	Sequence type	67178	0.47	184566	22.57	89273	0.75	142047	24.63
Junk reads	Sequence type	9707	0.07	4933	0.6	7494	0.06	3474	0.6
Rfam	RNA class	5976056	41.71	59780	7.31	2742169	22.9	45778	7.94
mRNA	RNA class	585726	4.09	146304	17.89	297523	2.48	83734	14.52
Repeats	RNA class	1280	0.01	951	0.12	320	0	237	0.04
rRNA	RNA class	457557	3.19	34204	0.24	596924	4.99	30120	0.25
tRNA	RNA class	5491075	38.33	19683	0.14	2131079	17.8	11951	0.1
snoRNA	RNA class	2407	0.02	937	0.01	2061	0.02	775	0.01
snRNA	RNA class	11645	0.08	1663	0.01	5958	0.05	1202	0.01
other Rfam RNA	RNA class	13372	0.09	3293	0.02	6147	0.05	1730	0.01
Clean reads	Sequence type	7948375	55.48	424202	51.87	8983222	75.02	303613	52.65

Overview of reads from raw data to cleaned sequences.

3ADT&length filter: reads removed due to 3ADT not found and length with <17 nt and >25 nt were removed（for plants); length with<16 and >29 were remove(for animals)

Junk reads:Junk: >=2N, >=7A, >=8C, >=6G, >=7T, >=10Dimer, >=6Trimer, or >=5Tetramer

Rfam:Collection of many common non-coding RNA families except micro RNA; http://rfam.janelia.org

Repeats:Prototypic sequences representing repetitive DNA from different eukaryotic species; http://www.girinst.org/repbase.

Notes:There is overlap in mapping of reads with mRNA, rRNA, tRNA, snRNA, snoRNA and repeats.
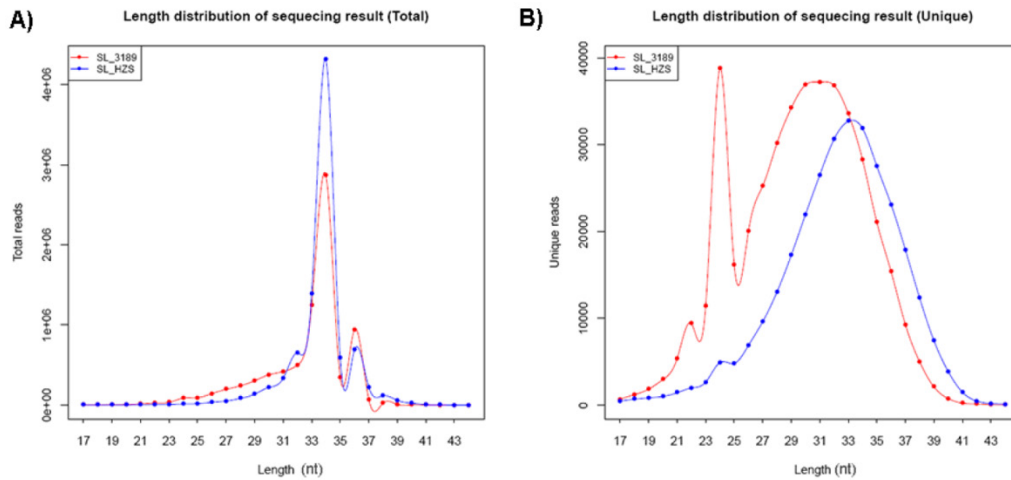
Figure 1: Length distribution of small RNAs from sequencing of the two inbred maize libraries. (A) Size distribution of total sequences. (B) Size distribution of unique sequences.
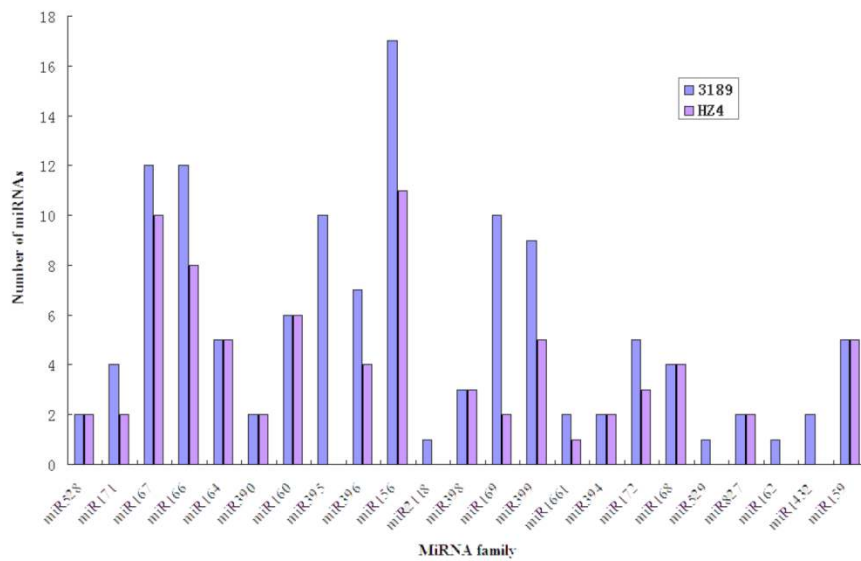

Figure 2: Abundance of conserved miRNA families in the two inbred maize libraries.
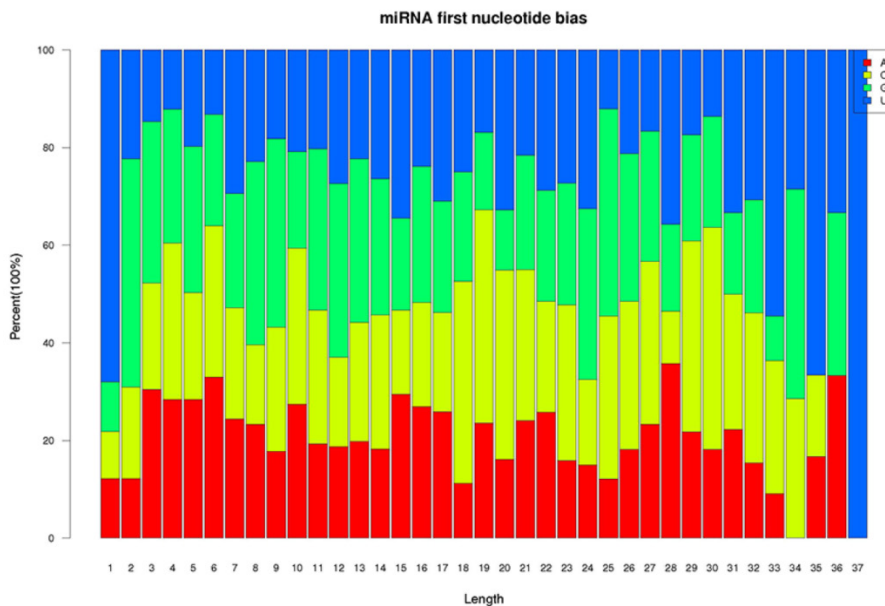

Figure 3: Relative nucleotide bias at each position of the known maize miRNAs.

Identification of novel miRNAs in the maize genome

A computational prediction method was employed to identify novel miRNAs in the maize genome. After excluding sRNAs that matched known miRNAs, rRNAs, tRNAs, snRNAs, and snoRNAs, as well as those overlapping protein-coding genes, remaining sRNAs exhibiting a perfect match to maize genomic sequences were used to predict potential novel miRNAs. The selection of novel miRNAs was based on the ability of flanking sequences to fold back in a hairpin structure [44]. Predicted secondary structures of the flanking sequences were generated using Mfold. These putative miRNA precursors were then used to extract miRNA*s, which are considered strong evidence for DICER-LIKE-1 (DCL1)-derived products [30]. A total of 68 regions satisfied these criteria and were designated as novel miRNA gene candidates (Table 2). The lengths of novel miRNAs varied from 20 to 37 nt, with 24 nt the most abundant length (18 out of 68), which is typical of DCL digestion products. Of these, 24 were found to be expressed in both maize inbred lines, with 10 weakly expressed, as indicated by a normalized sequencing read of less than 1 in both samples. In addition, the sequence frequency of most novel miRNA candidates was extremely low. It has been reported that miRNAs may be expressed at very low levels and may be present only in particular cell types and/or under particular circumstances [45]. None of the novel miRNAs were previously reported in other species and are therefore likely to be maize-specific. Then 18 novel miRNAs which low abundance and more than 24 nt in length were selected for validation by stem-loop real-time PCR (Additional File 1: Table S1). According to our RT-PCR analysis, 4 novel miRNAs (PC-3p-201205, PC-3p-359801, PC-5p-864621, zma-MIR166b-p5) had no expression quantity in both the leaves of two maize inbred lines. In addition, 5 novel miRNAs (PC-3p-201205, PC-5p-1164336, zma-MIR164e-p5, zma-MIR166b-p5, zma-MIR393a-p3) had no expression quantity in the leaves of Hz4 and 1 novel miRNA (PC-5p-864621) had no expression quantity in the leaves of 3189, which was consistent with the results of the high-throughput sequencing (Additional File 1: Table S1). Expression levels of a few members—PC-3p-129630, PC-3p-420698, PC-3p-552502, PC-3p-564523, PC-3p-793235, zma-MIR164e-p3, zma-MIR166n-p3, zma-MIR169i-p3 and zma-MIR393c-p3—were slightly different (Figure 4A). This difference may be due to variations in Hz4 and 3189 sampling times, or differences in sensitivity and specificity of the two technologies. Expression patterns of the remaining novel miRNAs (PC-3p-1072330, PC-5p-1134476, PC-5p-1164336, zma-MIR164e-p5, zma-MIR393a-p3) obtained by stem-loop real-time RT-PCR were in accordance with the sequencing data (Figure 4B).
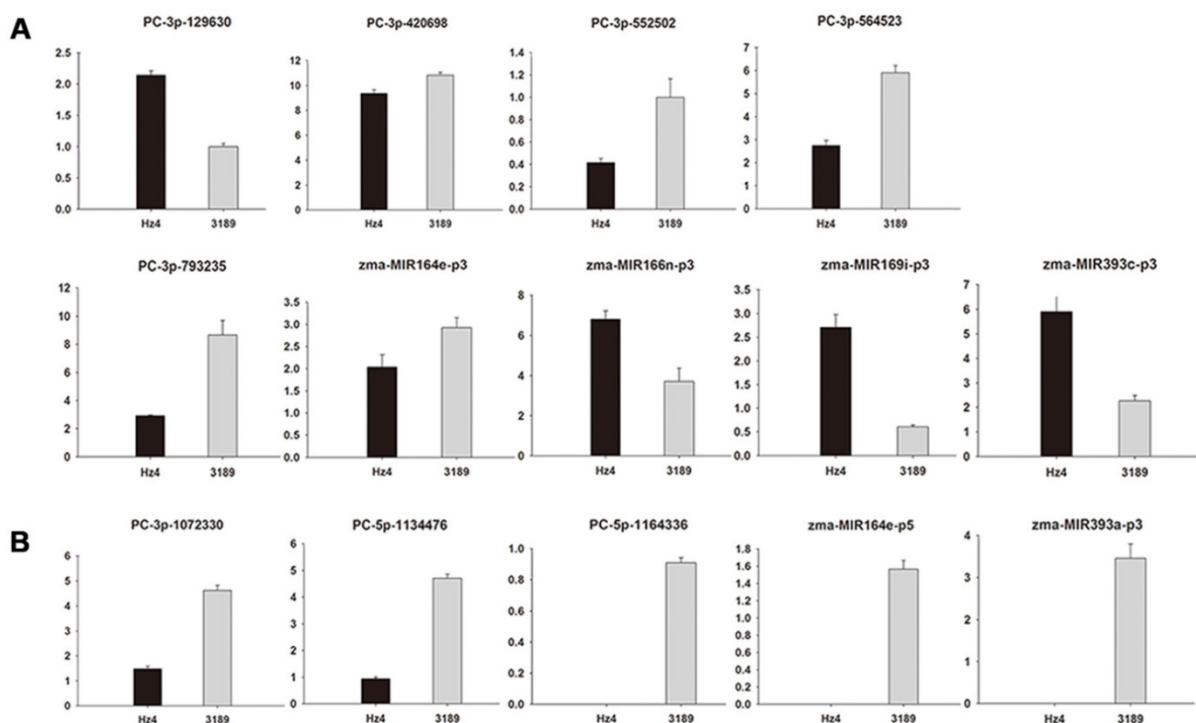

Figure 4: Validation of 18 novel miRNAs which low abundance and more than 24 nt in length using stem-loop real-time PCR. (A) different expression levels of miRNAs compared with the sequencing data. (B) similar expression levels of miRNAs compared with the sequencing data. Ordinates indicate relative expression levels.

Table 2: Novel maize miRNAs identified in this study.

PC-3p-687149_1	AGATGAGAAATGAAGGCACCAGAT	24	tag_chr3	+	3'	-169.7	49.6	1.3	0	2
PC-5p-691043_1	TTAGGCTCGGGGACTACGGT	20	tag_chr3	+	5'	-97.3	59.3	0.9	0.5	0
PC-3p-711926_1	CCGTGGCTCCTGCTCCTGAT	20	tag_chr3	+	3'	-97.3	59.3	0.9	0	0.5
PC-3p-687149_1	AGATGAGAAATGAAGGCACCAGAT	24	tag_chr3	-	3'	-154.7	49.1	1.2	0	2
PC-5p-1134476_1	TCTTACTTTTGGCATTTGTGACATTGACTT	30	tag_chr3	-	5'	-58.2	31.3	0.8	0	2
PC-3p-1072330_1	ATCGCCCTGATCGATGCCTAATCGCG	26	tag_chr4	-	3'	-33.5	57.6	1	0	2
PC-5p-431452_1	TCGTGTTTTTTCCTCAGCTGTGCC	24	tag_chr4	-	5'	-91.1	40.7	1.5	0	1
PC-3p-1160150_1	AGGACACAGCTGAGTAAAAACAC	24	tag_chr4	-	3'	-91.1	40.7	1.5	0	1
PC-5p-366433_1	TGCCTTTAGGGCTGATTTGGTGC	23	tag_chr5	+	5'	-118.2	49.7	1.6	0	1
PC-3p-564523_1	TTCTCCCCCATGGATCCCTTTGGGA	25	tag_chr5	+	3'	-118.2	49.7	1.6	0	1
PC-5p-12469_78	ATAGTTTTTTCTACCACACTTTAGATTCTT	30	tag_chr5	+	5'	-69.6	29	0.9	68.5	184.5
PC-3p-359801_1	AGAATAGACTAGAATAGATTATAGTAAAAG	30	tag_chr5	+	3'	-69.6	29	0.9	1	3
PC-3p-1033669_1	ATAGATGAGCACACTACCAAAACT	24	tag_chr6	-	3'	-123.9	45.5	1.8	0	2.5
PC-3p-201205_3	AGAAAAGATTGAGCCGAATTGAATTA	26	tag_chr6	-	3'	-33.2	30	1.8	0	6.5
PC-5p-1123590_1	TCGCAGTCGGCCGTGTCCTCGGAG	24	tag_chr6	-	5'	-62.7	58.8	0.5	0	2
PC-3p-237604_2	AATATGGAAACGGGACGGAAACGG	24	tag_chr7	-	3'	-32.7	37.9	1	0	2
PC-5p-691043_1	TTAGGCTCGGGGACTACGGT	20	tag_chr8	+	5'	-99	53.3	1	0.5	0
PC-3p-711926_1	CCGTGGCTCCTGCTCCTGAT	20	tag_chr8	+	3'	-99	53.3	1	0	0.5

Clustering of miRNAs

Clusters of miRNAs are often present in the genome where multiple miRNAs are aligned in the same orientation and transcribed as a polycistronic structure to facilitate cooperative action [46]. A total of 69 of the miRNAs identified in this research were located within 22 miRNA clusters, with chromosomes 1, 2, 3, 4, 5, 6, 7, 8 and 10 containing 5, 1, 5, 1, 4, 2, 1, 2 and 1 clusters, respectively (Table 3). The largest miRNA cluster contained 7 miRNAs, all of which belonged to the same miRNA family (miRNA395). Some miRNAs in the same cluster may be transcribed by the same primary miRNA, although their expression levels can differ, as was observed with PC-3p-1105743/ PC-3p-897448, PC-3p-552502/PC-5p-62968 and PC-5p-1164336/PC-3p-104764. This is presumably due to these miRNAs undergoing different transcriptional regulation during maturation.

Differentially expressed miRNAs

To identify miRNAs involved in the response to drought stress, differential expression in the two libraries was estimated from the read counts of the high-throughput sequencing. In the two libraries, miRNAs exhibiting a log2 (Hz4/3189) fold change higher than 1 were designated up-regulated, while a log2 (Hz4/3189) fold change less than -1 was designated as down-regulated. In total, 29 miRNAs were differentially expressed between the two inbred lines, with 18 down-regulated and 11 up-regulated (Table 4). Among these differentially expressed miRNAs, five novel miRNAs were selected for expression analysis using qRT-PCR. These were PC-3p-190, PC-3p-104764, PC-3p-129630, PC-3p-552502 and PC-5p-139812. Of these, two were up-regulated and three were down-regulated. Secondary structures were predicted to be of a typical hairpin shape, which

is different from siRNAs and piRNAs (Figure 5). The expression patterns of these five miRNAs, obtained by qRT-PCR experiments, were consistent with the results of the high-throughput sequencing reads (Figure 6). However the fold changes obtained from the qRT-PCR data were much lower than those estimated from the high-throughput sequencing data, presumably due to differences in sensitivity and specificity between the experimental approaches [47].

Identification and confirmation of miRNA targets using qRT-PCR

In most cases, the identified miRNAs were predicted to cleave two or more different targets. With a specific value of 0.85 and a signal-to-noise value of 6.28, a total of 167 genes were predicted to be potential targets for 23 of the 29 differentially expressed miRNAs. Among these, nine genes were predicted to be targets of the five novel miRNAs (Table 5). Interestingly, PC-3p-104764, PC-3p-129630, PC-3p-552502 and PC-5p-139812 shared a common target (GRMZM2G448344), and PC-3p-104764 and PC-3p-129630 shared three common targets (GRMZM2G360821, GRMZM2G385635, GRMZM2G308907), suggesting differnet miRNAs may function in combination in gene regulatory networks.

To confirm whether the miRNAs regulated their potential targets under drought stress conditions, the expression patterns of putative target genes were analyzed using qRT-PCR. It is known that miRNAs regulate gene expression at the post-transcriptional level by translational repression or target degradation and gene silencing. As expected, an inverse relation was observed between the expression of three miRNAs (PC-3p-190, PC-3p-552502 and PC-5p-139812) and their putative target genes, suggesting miRNA-mediated regulation of their potential targets (Figure 6A). The other two novel miRNAs

(PC-3p-104764 and PC-3p-129630) did not exhibit this inverse relationship with their putative target genes (Figure 6B). On account of the regulatory mechanism between miRNAs and their target genes is so complicated that one target gene may be correspond to multiple miRNAs. Therefore, we speculated that these two novel miRNAs had little effect on their target genes and the expression of the target genes might be affected by other regulators.

GO functional and transcriptomics analysis of target genes

The results of GO analysis demonstrated that most of the target genes were involved in cellular or metabolic processes such as response to stimuli or biological regulation among other categories (Table 5). Of the nine target genes characterized, seven could be classified into 21 biological processes including carbon fixation, photosynthesis and proteolysis, while the remaining two were of unknown function. For example, PC-3p-190 targets a gene involved in RNA-binding and PC-3p-129630 targets a gene involved in carbon fixation. PC-3p-552502 targets a gene involved in the light reaction of photosynthesis and the target genes of PC-3p-104764 are ATP-dependent

peptidase and serine-type endopeptidase. Interestingly, the common target (GRMZM2G448344) of four of the five novel miRNAs, and the common targets (GRMZM2G360821 and GRMZM2G308907) of PC-3p-104764 and PC-3p-129630 are all involved in photosynthesis.

To gain more insight into the expression patterns of the target genes of the five novel miRNAs, a comprehensive expression analysis was performed using the publicly available maize transcriptome data. A total of 18 tissues from five organs have been analyzed using RNA sequencing (RNA-Seq) [48]. Distinct expression profiles were identified for nine target genes from the transcriptome data (Additional File 2: Table S2). Transcriptome data were imported into R and Bioconductor (http://www.bioconductor.org/) for expression analysis to generate a heat map (Figure 7). Based on the heat map, all nine target genes were appeared to be expressed only in the leaves. GRMZM2G360821 and GRMZM2G308907 exhibited the highest expression levels, suggesting important roles for these target genes. These results indicated that these genes may participate in photosynthesis, which was consistent with the GO analysis.

Table 3: Genome location clusters of pre-miRNAs.

Pre-miRNA Cluster ID	Chromosome	Start	End	Strand	miRNA Name
1	tag_chr1	6415393	6415814	+	bdi-MIR528-p3_1ss21GT
1	tag_chr1	6415393	6415814	+	zma-miR528a-5p_R+1
1	tag_chr1	6415592	6415714	+	zma-miR528a-3p
1	tag_chr1	6415592	6415714	+	zma-miR528a-5p
2	tag_chr1	19971673	19972096	-	PC-3p-1105743_1
2	tag_chr1	19971742	19972165	+	PC-3p-897448_1
3	tag_chr1	114311615	114312042	+	PC-3p-46539_19
3	tag_chr1	114311615	114312042	+	PC-5p-62968_15
3	tag_chr1	114311584	114312011	-	PC-3p-552502_1
3	tag_chr1	114311584	114312011	-	PC-5p-97360_9
4	tag_chr1	203925757	203926180	-	PC-3p-270024_3
4	tag_chr1	203925757	203926180	-	PC-5p-1164336_1
4	tag_chr1	203925786	203926210	+	PC-3p-104764_7
5	tag_chr1	274716509	274716929	-	osa-miR166m
5	tag_chr1	274716709	274716784	-	zma-miR166a-3p
5	tag_chr1	274716709	274716784	-	zma-miR166h-5p_L-1R+4
6	tag_chr10	144744354	144744457	+	zma-miR395a-3p_L-1R-1
6	tag_chr10	144744531	144744772	+	zma-miR395a-3p_L-1R-1
6	tag_chr10	144744844	144744970	+	zma-miR395a-3p_L-1R-1
7	tag_chr2	6321644	6321709	-	zma-miR395a-3p_L-1R-1
7	tag_chr2	6322300	6322369	-	zma-miR395a-3p_L+11R-1
7	tag_chr2	6331941	6332012	-	zma-miR395a-3p_L+11R-1
7	tag_chr2	6331941	6332012	-	zma-miR395i-5p_R+11
7	tag_chr2	6332583	6332667	-	zma-miR395a-3p_L-1R-1
7	tag_chr2	6332742	6332855	-	zma-miR395a-3p_L+11R-1
7	tag_chr2	6333397	6333545	-	zma-miR395a-3p_L-1R-1
8	tag_chr3	7774272	7774395	-	zma-miR156a-5p_L+1
8	tag_chr3	7774558	7774696	-	zma-miR156a-5p
9	tag_chr3	25490777	25491197	-	ath-miR159a
9	tag_chr3	25490976	25491190	-	zma-MIR159f-p5
9	tag_chr3	25490976	25491190	-	zma-miR159a-3p_R-1
10	tag_chr3	27314699	27315122	-	PC-3p-687149_1
10	tag_chr3	27314819	27315242	+	PC-3p-687149_1
11	tag_chr3	37610192	37610611	+	PC-3p-711926_1

11	tag_chr3	37610192	37610611	+	PC-5p-691043_1
11	tag_chr3	37610280	37610409	+	zma-MIR169a-p5_1ss22TC
12	tag_chr3	119175685	119175874	+	zma-miR167a-5p
12	tag_chr3	119177648	119177890	+	zma-miR167e-5p_R+1
13	tag_chr4	173295127	173295263	+	zma-miR396a-3p_R-1
13	tag_chr4	173295127	173295263	+	zma-miR396a-5p
13	tag_chr4	173300108	173300273	-	zma-miR396e-5p
14	tag_chr5	21933496	21933916	-	cme-miR166i_L+2R-1
14	tag_chr5	21933694	21933797	-	zma-miR166a-3p
15	tag_chr5	146894716	146894809	+	zma-miR399a-3p
15	tag_chr5	146903662	146903752	+	zma-miR399e-3p
16	tag_chr5	210001500	210001929	+	PC-3p-359801_1
16	tag_chr5	210001500	210001929	+	PC-5p-12469_78
16	tag_chr5	210008725	210008868	-	bdi-MIR5056-p3
17	tag_chr5	210632198	210632365	-	zma-miR166j-3p
17	tag_chr5	210632469	210632624	-	zma-miR166l-3p
17	tag_chr5	210632469	210632624	-	zma-miR166m-5p
18	tag_chr6	84226293	84226713	-	osa-miR166m
18	tag_chr6	84226489	84226701	-	zma-miR166a-3p
19	tag_chr6	159686859	159686963	-	zma-miR399f-3p_L+10R-1
19	tag_chr6	159686859	159686963	-	zma-miR399f-5p_R+11
19	tag_chr6	159694634	159694840	+	zma-miR399a-3p
19	tag_chr6	159694634	159694840	+	zma-miR399c-5p
20	tag_chr7	9830090	9830510	-	cpa-miR167c
20	tag_chr7	9830212	9830330	-	zma-miR167e-5p_R+1
21	tag_chr8	4791774	4792193	+	PC-3p-711926_1
21	tag_chr8	4791774	4792193	+	PC-5p-691043_1
21	tag_chr8	4791975	4792130	+	zma-miR169a-3p_L+8R-1
21	tag_chr8	4791975	4792130	+	zma-miR169a-5p_R+14
22	tag_chr8	10528867	10529066	+	zma-MIR159h-p3
22	tag_chr8	10532710	10532961	+	zma-MIR159i-p3
22	tag_chr8	10544675	10544926	+	zma-miR159a-3p_R-1
22	tag_chr8	10585048	10585247	+	zma-miR159a-3p_R-1
22	tag_chr8	10588904	10589324	+	ath-miR159a
22	tag_chr8	10588923	10589143	+	zma-miR159a-3p_R-1

Table 4: miRNAs differentially expressed in the two maize inbred lines.

miR name	miR seq	3189(norm)	HZS(norm)	Fisher exact test	Chis quare	Log2(Hz4/3189)
zma-miR167e-5p_R+1	TGAAGCTGCCAGCATGATCTGA	0.850384	3.271683	0.629352	0.94767	down
zma-miR390a-5p	AAGCTCAGGAGGGATAGCGCC	0.831897	5.708753	0.823974	0.56767	down
zma-miR160a-5p	TGCCTGGCTCCCTGTATGCCA	0.277299	1.101689	1	0.95794	down
zma-miR396c_L-1	TCCACAGGCTTTCTTGAACTG	11.64656	43.5668	0.591401	0.87501	down
zma-miR160f-5p_1ss21GA	TGCCTGGCTCCCTGTATGCCA	0.277299	1.101689	1	0.95794	down
zma-miR399a-3p	TGCCAAAGGAGAATTGCCCTG	0.554598	2.203378	0.524953	0.94054	down
zma-miR168a-5p	TCGCTTGGTGCAGATCGGGAC	7.487077	17.72718	0.30102	0.34827	down
zma-miR168b-3p_R+1_1ss12TC	CCCGCCTTGCACCAAGTGAAT	7.487077	15.02304	0.191204	0.19522	down
zma-miR168a-5p	TCGCTTGGTGCAGATCGGGAC	7.487077	17.72718	0.30102	0.34827	down
zma-miR827-5p_L+1	TTTTGTTGGTGGTCATTTAACC	11.64656	25.83962	0.110772	0.17769	down
zma-miR827-3p	TTAGATGACCATCAGCAAACA	38.26728	141.8175	0.638664	0.81446	down
zma-miR164a-5p	TGGAGAAGCAGGGCACGTGCA	0.332759	1.201843	1	0.99321	down
zma-miR167h-3p_L+1R+1	AGATCATGTTGCAGCTTCACT	1.663795	10.81659	0.814904	0.46092	down
zma-miR408a	CTGCACTGCCTCTTCCCTGGC	4.991384	35.45436	0.957678	0.13853	down
zma-miR408b-5p	CAGGGACGAGGCAGAGCATGG	4.991384	0.600921	0.002503	0.000121	up
zma-miR398a-3p_L+9R-1	GATCTTGCATGTGTTCTCAGGTCGCCCCC	0.831897	3.004607	0.629352	0.98926	up
zma-miR398a-3p_L+7R-1	TGCTGCATGTGTTCTCAGGTCGCCCCC	64.0561	3.004607	9.53E-39	3.33E-48	up
zma-miR172a_R+1	AGAATCTTGATGATGCTGCAT	1.663795	0.400614	0.048259	0.041885	up
zma-miR396f-3p_L+3	GAAGGTCAAGAAAGCTGTGGGAAG	1.663795	0.600921	0.123582	0.061209	up
zma-MIR397b-p3	TCACCAGCGCTGCACTCAATT	1.663795	0.600921	0.123582	0.061209	up
zma-miR399f-3p_L+10R-1	GTGCCACTGCTGCCAAAGGAAATTTGCCCC	6.655179	0.600921	0.000159	5.64E-06	up
zma-miR169a-5p_R+14	CAGCCAAGGATGACTTGCCGATCTATCGTCGATCA	3.32759	1.201843	0.035379	0.008106	up
zma-miR159a-3p_R-1	TTTGGATTGAAGGGAGCTCT	3.535564	1.547373	0.023761	0.009544	up
PC-5p-139812_4	GAAGGGTAGAAAAAGTTATTAGATAGCGA	48.25005	3.605528	1.89E-27	2.99E-35	up
PC-3p-793235_1	TCCAATGCTATCTAGTAATTTTTCTACCTACA	1.663795	0.600921	0.123582	0.061209	up
PC-3p-552502_1	ACTAGAATGAACAATGCTGTAGCAATAAATGCGAGAA	8.318974	3.004607	0.000464	2.82E-05	up
PC-3p-129630_5	TTAGAAAGATTGAGCCGAATTGAATTA	1.663795	5.107832	0.476801	0.87006	down
PC-3p-104764_7	AGAAAAGATTGAGCCGAATTGAATT	1.663795	17.42672	0.943169	0.16161	down
PC-3p-190_11180	CCAACAGGATATTGGGTATTTCTT	1435.855	8141.283	1	0	down

Table 5: Gene Ontology (GO) analysis of potential targets of the five novel miRNAs.

| miR name	Target gene	GO annotation
PC-3p-190	GRMZM2G427404	GO:0015934 large ribosomal subunit
		GO:0015935 small ribosomal subunit
		GO:0003735 structural constituent of ribosome
		GO:0016740 transferase activity
		GO:0003723 RNA binding
		GO:0006412 translation
		GO:0015934 large ribosomal subunit
		GO:0015935 small ribosomal subunit
		GO:0003735 structural constituent of ribosome
		GO:0016740 transferase activity
	GRMZM2G330095	GO:0003735 structural constituent of ribosome
		GO:0006412 translation
		GO:0005840 ribosome
PC-3p-104764	GRMZM2G360821	GO:0015977 carbon fixation
		GO:0016984 ribulose-bisphosphate carboxylase activity
	GRMZM2G448344	GO:0015977 carbon fixation
		GO:0016984 ribulose-bisphosphate carboxylase activity
	GRMZM2G308907	GO:0015977 carbon fixation
		GO:0016984 ribulose-bisphosphate carboxylase activity
	GRMZM2G385622	GO:0004176 ATP-dependent peptidase activity
		GO:0045261 proton-transporting ATP synthase complex, catalytic core F(1)
		GO:0015986 ATP synthesis coupled proton transport
		GO:0004252 serine-type endopeptidase activity
		GO:0046933 hydrogen ion transporting ATP synthase activity, rotational mechanism
		GO:0000166 nucleotide binding
		GO:0046961 proton-transporting ATPase activity, rotational mechanism
		GO:0006508 proteolysis
		GO:0008553 hydrogen-exporting ATPase activity, phosphorylative mechanism
	GRMZM2G385635	Unknown
PC-3p-129630	GRMZM2G308907	GO:0015977 carbon fixation
		GO:0016984 ribulose-bisphosphate carboxylase activity
	GRMZM2G360821	GO:0015977 carbon fixation
		GO:0016984 ribulose-bisphosphate carboxylase activity
	GRMZM2G385635	Unknown
	GRMZM2G448344	GO:0015977 carbon fixation
		GO:0016984 ribulose-bisphosphate carboxylase activity
PC-3p-552502	GRMZM2G448344	GO:0015977 carbon fixation
		GO:0016984 ribulose-bisphosphate carboxylase activity
	GRMZM2G030695	GO:0019684 photosynthesis, light reaction
		GO:0016021 integral to membrane
		GO:0009523 photosystem II
	GRMZM2G055151	Unknown
PC-5p-139812	GRMZM2G448344	GO:0015977 carbon fixation
		GO:0016984 ribulose-bisphosphate carboxylase activity
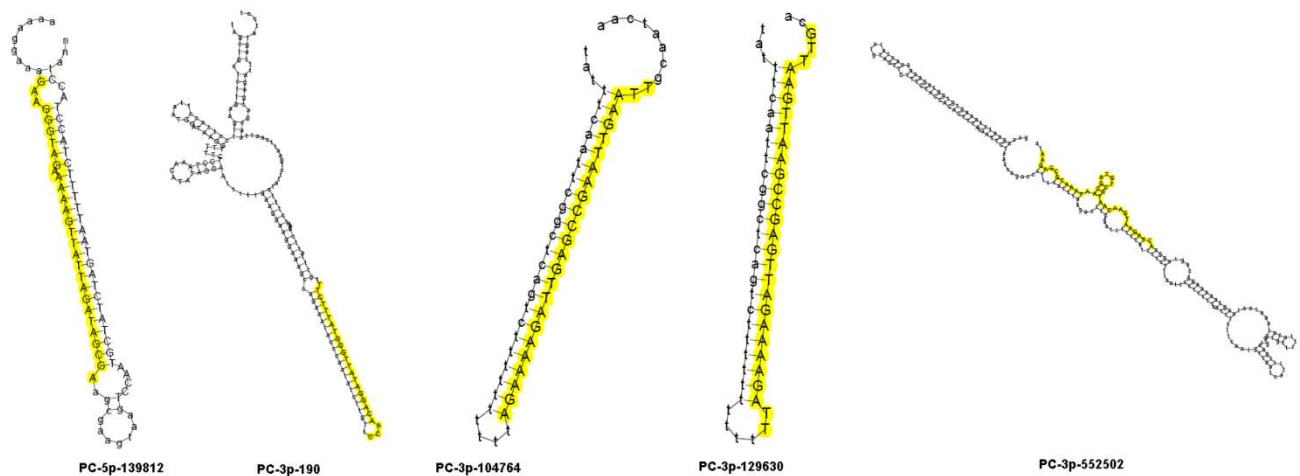
Figure 5: Secondary structure of five novel miRNA precursors. Mature miRNA sequences are shown in yellow.
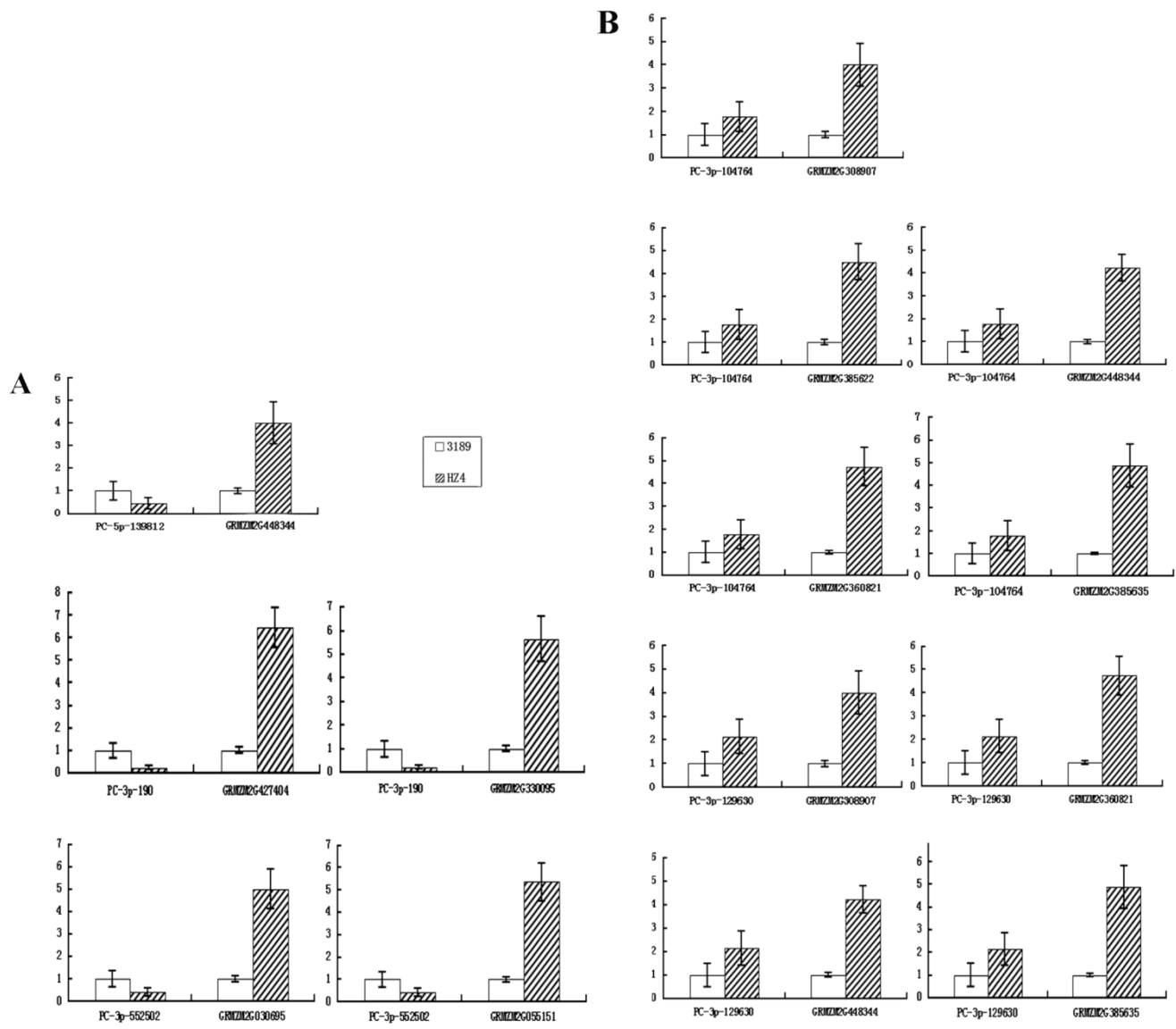
Figure 6: Quantitative real-time RT-PCR analysis of five novel miRNAs and their target genes. Expression levels of miRNAs were normalized against 18S rRNA. Fold changes in expression level were estimated using the 2−ΔΔCT method. Data are reported as mean ± SE for three independent experiments. (A) The inverse relationship between three novel miRNAs (PC-3p-190, PC-3p-552502 and PC-5p-139812) and their putative target genes. (B) The uniform relationship between two novel miRNAs (PC-3p-104764 and PC-3p-129630) and their putative target genes.

Discussion

Identification of maize miRNAs by high-throughput sequencing

Identification of miRNAs in model plants using high-throughput sequencing or miRNA arrays has been previously reported. High-throughput sequencing has proven particularly successful for identifying plant miRNAs involved abiotic stress responses [49, 50, 51]. In this study libraries constructed from the leaves of two maize inbred lines were used to identify novel drought-associated miRNAs and their mechanisms of action were subsequently investigated.

Of the miRNAs identified using high-throughput sequencing, 85.48% of those already known were expressed at low levels (less than 10 raw reads; Table 1). This suggests that high-throughput sequencing is a powerful strategy for identifying poorly expressed miRNAs in plants. The miR159, miR167 and miR396 families, which are believed to target MYB transcription factors, auxin responsive factors and GRF transcription factors, respectively, were abundantly represented in both libraries. By comparing the expression levels of all members of an miRNA family, dominant members could be found, such as zma-MIR396d in the miR396 family, zma-MIR167h in the miR167 family and zma-MIR169a in the miR169 family. These dominant members may perform key

regulatory roles in response to abiotic stress. Some family members exhibited comparable expression levels, such as zma-MIR166b/c/e/f/g/h/I in the miR166 family and zma-MIR168a and zma-MIR168b in the miR168 family. This indicates that several members of a family may have a synergistic effect in the regulation network.

Potential targets of differentially expressed miRNAs

Earlier research in *Arabidopsis* demonstrated that many of the potential targets of candidate miRNAs were transcription factors [52] and the majority of miRNAs were involved in multiple diverse biological processes [49]. In this study, 29 differentially expressed miRNAs were identified using high-throughput sequencing, and potential targets of 23 of these were successfully predicted (Additional File 3: Table S3). No targets were identified for the remaining 6 miRNAs, and this may be due to differences in the spatial or temporal expression of a miRNA and its target that could result in insufficient degradation of the target [50]. As anticipated, many of the potential targets were involved in transcription regulation, including MYB, CRP, PPA, HRT, GRF, SR,

C2H2, SLS, SMH, RAV, GLH and Phytocyanin-type transcription factors. These transcription factor families are conserved in other plant species [53-56] and are known to regulate plant development.

Potential targets of drought-associated miR-NAs

This study identified five novel miRNAs that were differentially expressed in drought-resistant and drought-sensitive maize inbred lines. GO analysis was performed to infer whether further investigation into these miRNAs would be appropriate, and many of the potential target genes play important roles in diverse biological processes, but especially in photosynthesis. Transcriptomics analysis suggested that the nine target genes were expressed only in leaves, which are the primary location for photosynthesis. The chemical constituents of leaves are known to change in response to drought stress, therefore we propose that these target genes are involved in drought stress. The results presented here will guide further functional analysis of the role of these novel drought-associated miRNAs and their target genes in the response to drought stress in maize.
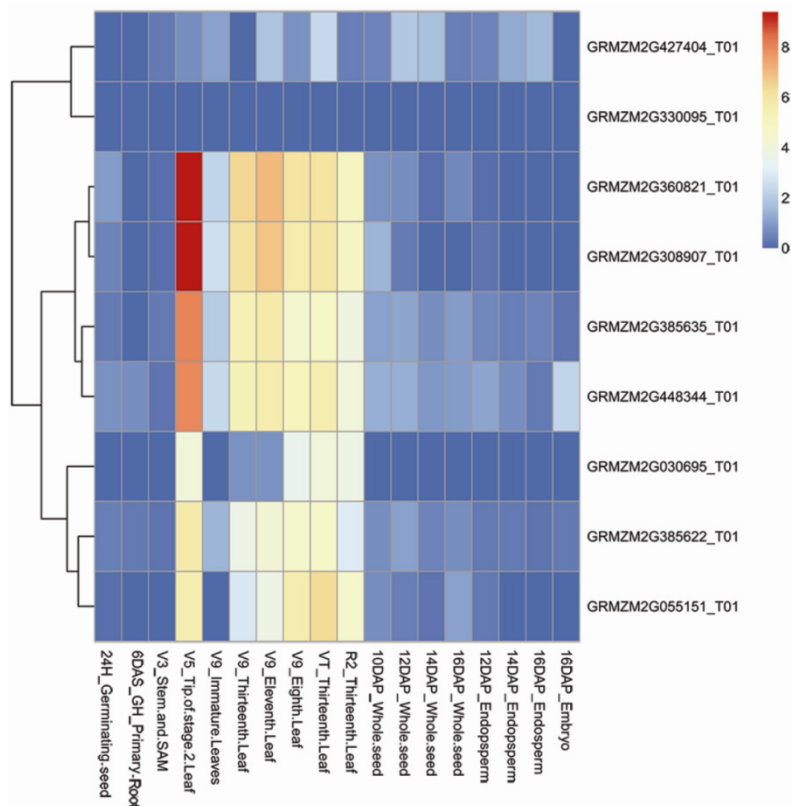

Figure 7: Expression profiles of nine target genes. A heat map was generated by hierarchical clustering using a dedicated heat map package [57]. Expression data were normalized and hierarchically clustered with average linkage. The color scale in the top right corner represents the relative gene expression level, where red, yellow and blue indicate high, medium and low levels of gene expression, respectively.

Conclusion

Differentially expressed miRNAs were detected in the leaves of two maize inbred lines, and 124 known and 68 novel miRNAs were identified using a high-throughput sequencing approach. The sRNA-seq data has been deposited into NCBI GEO database and the GEO accession number is GSE61700. The novel drought-associated miRNAs identified and their predicted target genes may play important roles in the regulation of stress responses in this important food plant. These findings have enriched the maize miRNA databases and provide the foundation for further analysis of their roles in maize growth and development.

Supplementary Material

Additional File 1:
Supplementary Table S1.
http://www.ijbs.com/v11p0781s1.xls
Additional File 2:
Supplementary Table S2.
http://www.ijbs.com/v11p0781s2.xls
Additional File 3:
Supplementary Tables S3-S4.
http://www.ijbs.com/v11p0781s3.pdf

Acknowledgements

Competing Interests

The authors have declared that no competing interest exists.

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