African Journal of Biotechnology Vol. 9(30), pp. 4741-4753, 26 July, 2010
Available online at http://www.academicjournals.org/AJB
DOI: 10.5897/AJB10.462
ISSN 1684–5315 © 2010 Academic Journals

Full Length Research Paper

Identification of 21 microRNAs in maize and their differential expression under drought stress

Chen Xu, Ruo-Fei Yang, Wan-Chen Li and Feng-Ling Fu*

Maize Research Institute, Sichuan Agricultural University, Ya'an, Sichuan, 625014, P.R. China.

Accepted 29 June, 2010

MicroRNAs (miRNAs) are a class of small, non-coding regulatory RNAs that regulate gene expression by guiding target mRNA cleavage or translational inhibition. The negative regulation of miRNAs has been found in response to stress in *Arabidopsis*, rice and other plants. The sequence of the maize genome has been completed very recently; however, only a few maize miRNAs have been identified and deposited in the miRNA registry database, and the details of their regulation of target genes remain to be elucidated. We identified 21 maize miRNAs using bioinformatics computation and small RNA cloning. They are designated as zma-miR161, zma-miR397, zma-miR446, zma-miR479, zma-miR530, zma-miR776, zma-miR782, zma-miR815a, zma-miR818a, zma-miR820, zma-miR828, zma-miR834, zma-miR1, zma-miR2, zma-miR3, zma-miR4, zma-miR5, zma-miR6, zma-miR7, zma-miR8 and zma-miR9. The characterization of these miRNAs reveals a length of 19–24 nt and the typical secondary structure. Using reverse transcriptional polymerase chain reaction (RT-PCR), 13 of these miRNAs were amplified and validated from total small RNA samples of maize. zma-miR1, zma-miR3, zma-miR6, zma-miR479, zma-miR782, zma-miR815a and zma-miR820 were found to be expressed specifically in drought-tolerant inbred line 87-1 under simulated drought stress, whereas the other six were amplified in line 87-1 and in the sensitive inbred line 200B under drought stress and in a well-watered control. The seven differentially expressed miRNAs are suggested to be associated with the regulation of gene expression under drought stress. Using the plant miRNA target finder software miRU and miRU2, 14 of the 21 miRNAs were identified to target genes encoding 21 proteins, most of which are metabolic enzymes. Seven of them are involved in signal transduction, transcription regulation, and bio- or abio-stress responses. The identification and characterization of 21 miRNAs enrich the maize miRNA database and provide valuable information for functional studies of miRNAs.

Key words: MicroRNA, maize, bioinformatics, gene cloning, differential expression, drought stress.

INTRODUCTION

MicroRNAs (miRNAs) are an abundant class of non-coding small RNAs (16-29 nt) that regulate post-transcriptional gene expression in plants and animals (Bartel, 2004; Jones-Rhoades et al., 2006; Jung et al., 2009; Zhang et al., 2009). miRNAs are encoded by MIR genes that reside in distinct genomic regions. In plants, the primary transcripts of 70 - 90 nt are processed into self-complementary foldback structures by Dicer-like enzymes (Jones-Rhoades et al., 2006). miRNAs regulate gene expression by binding to transcripts of their target genes, resulting in mRNA degradation, translational repression or RNA-directed DNA methylation. Target genes of plant miRNAs are often transcription factors (Jones-Rhoades and Bartel, 2004; Rhoades et al., 2002; Zhang et al., 2006). The regulation of expression mediated by miRNAs is related to multiple processes of plant growth and development (Jones-Rhoades et al., 2006). Several plant miRNAs are known to regulate the response to environ-

*Corresponding author. E-mail: ffl@sicau.edu.cn; ffl621010@126.com. Tel: +86-835-8569711. Fax: +86-835-2882154.

Abbreviations: miRNAs, MicroRNAs; **PCR,** polymerase chain reaction; **GSS,** genomic survey sequence; **EST,** expressed sequence tags; **NCBI,** National Center for Biotechnology Information; **BLAST,** Basic Local Alignment Search Tool; **SDS-PAGE,** sodium dodecyl sulfate-polyacrylaminde gel electrophoresis; **MFEs,** minimal free energies; **MFEIs,** minimal free energy indices.

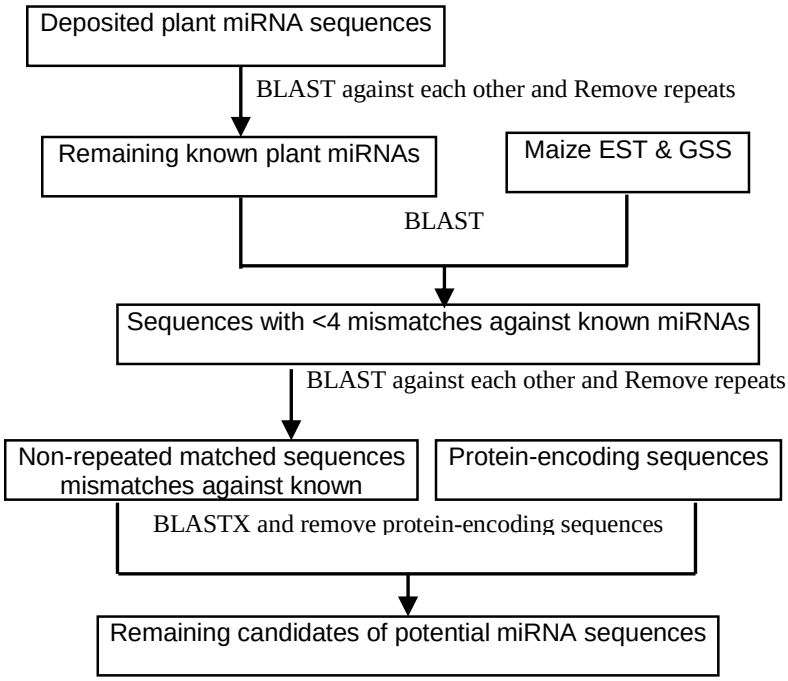


Figure 1. Bioinformatic prediction of potential miRNA sequences.

mental stresses, including drought, salinity and nutrient deficiency (Jones-Rhoades and Bartel, 2004; Sunkar and Zhu, 2004; Lu et al., 2005; Bari et al., 2006; Chiou et al., 2006; Sunkar et al., 2006; Chiou, 2007; Zhao et al., 2007; Liu et al., 2008; Ding et al., 2009).

Although, direct cloning together with genetic approaches has enabled the identification of many non-conserved miRNAs, it is difficult to clone low abundance miRNAs while avoiding invalid sequencing (Lai et al., 2003; Adai et al., 2005; Ghosh et al., 2007). Most of the known mature miRNAs are evolutionarily conserved from species to species within the plant kingdom, which makes it possible to perform a bioinformatics search for new miRNA homologues or orthologues in other plant species (Floyd and Bowman, 2004; Wang et al., 2004; Wang et al., 2004; Zhang et al., 2006). Bioinformatics computational methods used to identify new miRNAs on the basis of the genomic survey sequence (GSS), expressed sequence tags (EST) and mRNA sequences have been developed to address this disadvantage (Lai et al., 2003; Jones-Rhoades and Bartel, 2004; Adai et al., 2005; Zhang et al., 2005; Zhang et al., 2006). Most plant miRNA sequences bind to their target genes with a perfect or near-perfect complementarity and this characteristic has been exploited to predict the target genes for miRNA sequences using a computational approach (Rhoades et al., 2002; Jones-Rhoades and Bartel, 2004; Zhang et al., 2006; Cissell and Deo, 2009; Li and Ruan, 2009). Bioinformatics prediction generates a set of probable candidates for miRNAs, and might save substantial initial cloning and sequencing of fragments, and thereby help to facilitate the discovery of new miRNAs (Ghosh et al., 2007).

Thousands of miRNAs have been found in recent years, but only a very small number of plant miRNAs have been discovered and deposited in the miRNA registry database (http://www.mirbase.org), mainly in thale cress (*Arabidopsis* thaliana) and rice (*Oryza sativa*) (Reinhart et al., 2002; Bartel and Bartel, 2003; Bonnet et al., 2004; Sunkar et al., 2005; Griffiths-Jones et al., 2006). Their function in the regulation of target genes is not known. The complete maize (*Zea mays*) genomic survey sequence has just been made available (http://www.maizesequence.org). Only 111 miRNAs have been identified for maize by bioinformatics approaches and deposited in the miRNA registry database. Very little has been reported about the direct cloning of miRNAs from maize. To provide more information for understanding the regulation network mediated by miRNAs in maize gene expression, especially under drought stress, we identified miRNAs and their target genes by bioinformatics prediction and direct cloning. Their differential expression under drought stress was investigated by reverse transcription polymerase chain reaction (PCR).

MATERIALS AND METHODS

Bioinformatics prediction of potential miRNA sequences

Sequences of plant miRNAs were downloaded from the miRNA registry database according to the procedures shown in Figure 1. After comparing against each other and removing redundant

Table 1. Specific primers for twenty-one miRNAs.

miRNA	Specific primer sequences
zma-miR1	TGCTGTTTCTAGGACGTCC
zma-miR2	GTACCTAGGACGTCCGATCA
zma-miR3	CGGACGTCCAGCTGCTCA
zma-miR4	ACTGCGTGGACGTCTCAGAA
zma-miR5	TCAGGACGTCCACGCAGC
zma-miR6	CATGCGTGTACGTCCTAGAAA
zma-miR7	ACTGAAAGAGGAGGCC
zma-miR8	CAGGTGCGTCACAGCAATGCA
zma-miR9	CGCCCCTGCAGGATCGATGT
zma-miR161	TCAATGCACTGATAGTGAA
zma-miR397	TTGAGCGCAGCGTTGATGAG
zma-miR446	ATCAATATAAATATGGAAAATG
zma-miR479	GATATTGGTTCGGCTCACA
zma-miR530	TGCAGATGCAGATGCAGG
zma-miR776	TTGAAGTCTTCTAGTGATGTT
zma-miR782	TGAAACACCTTCCATG
zma-miR815a	GAGGGGATTGAGGGGATT
zma-miR818a	ATGCCTTATATTATGGGACGG
zma-miR820	GGGCCTGGTGGATGCA
zma-miR828	GTCTTGCTTAATTGAGTATT
zma-miR834	GGTAGCAGTAGCTGTGGTTA

repeats, the remaining sequences were used as query sequences to compare against maize EST and GSS databases, downloaded from the National Center for Biotechnology Information (NCBI) GenBank nucleotide databases. A maximum of three mismatch base pairs was allowed. The sequences obtained were compared against each other to remove the repeats, and compared against the mRNA sequences deposited in the NCBI database to remove protein-encoding sequences, using Basic Local Alignment Search Tool X (BLASTX) software (http://www.ncbi.nlm.nih.gov/BLASTX/).

Cloning of potential miRNA sequences

To prevent possible deviation from differential expression of miRNA genes, a mixed leaf sample of inbred line 87 - 1 was collected from different fields with different soil, irrigation and fertilizer, and used to isolate total small RNAs (≤ 200 nt) with the mirVana^{TMP} miRNA isolation kit (Ambion, Austin, USA) according to the manufacturer's protocol. After concentration and purity assay with a UV spectrophotometer (DU 800, BECKMAN COULTER, Fullerton, USA), and integrality detection by sodium dodecyl sulfate-polyacrylaminde gel electrophoresis (SDS-PAGE) (15% polyacrylamide gel), the total small RNA samples were ligated to a 3' adapter: AAAGATCCTGCA GGTGCGTCA and a 5' adapter: CATCGATCCTGCAGGCTAGA GAC absorbed to MAGNOTEX-SA magnetic beads, and reverse transcribed into cDNA fragments, using a small RNA magnetic bead absorption cloning kit of (TaKaRa, Dalian, China) according to the manufacturer's protocol. The cDNA fragments were fractionated by SDS-PAGE (15% polyacrylamide gel), recovered and cloned into the pMD18-T vector (TaKaRa, Dalian, China). The recombinant vectors were used to transform *Escherichia coli* strain DH5α. The positive clones were detected by bacterial PCR and sequenced by two directions at Sangon (Shanghai, China). After removing the 5' adapter, the 3' adapter and any repeat cloned sequences, the sequenced results were compared against maize GSSs in the NCBI nucleotide database.

Prediction of secondary structure of potential miRNA sequences

The secondary structure and stability of the potential miRNA sequences, predicted by the bioinformatics approach or cloned directly, were analysed by online software Mfold 3.2 (http://mfold.bioinfo.rpi.edu/cgi-bin/rna-form1.cgi), and further screened with the following criteria proposed by Ambros et al. (2003), Bonnet et al. (2004) and Mathews et al. (1999) : (1) Mature miRNAs have sequences with no more than three nucleotide mismatches compared with all previously known mature plant miRNAs; (2) sequences of miRNA precursors can fold into an appropriate hairpin secondary structure that contains the 22 nt mature miRNA sequence within one arm of the hairpin structure; (3) the secondary structures of miRNA precursors have higher negative minimal free energies (MFEs) and minimal free energy indices (MFEIs) than other types of RNAs; (4) miRNA has an A+U content of 30–70%; (5) miRNA has fewer than six mismatches with the opposite miRNA sequence in the other arm; (6) no loop or break in miRNA sequence is allowed.

Reverse transcription PCR amplification of miRNAs under drought stress

In an earlier study, we identified the drought-tolerant inbred line 87 - 1 and the drought-sensitive inbred line 200B from 57 parent lines of commercial maize hybrids under strict drought stress (Fu et al., 2008). In this study, these two inbred lines were planted in eight pots filled with 0.03 m^3 of sandy loam soil. Three identical plants were established in each pot. At the seventh leaf stage, four of the eight pots for each inbred line were treated with 18% polyethylene glycol 6000 (PEG-6000) to simulate a drought stress of –0.5 MPa osmotic potential (Michel and Kaufmann 1973), while the other four pots were watered with the same volume of fresh water and used as a well-watered control. After 12 hours of drought treatment, the first fully expanded leaf was sampled from each pot. Total small RNAs were isolated, analysed for concentration, purity and integrality as described above, and polyadenylated using the poly (A) polymerase kit (NEB, Beijing, China). The products were reverse transcribed into cDNA fragments using the GeneRacer^{TM} Advanced RACE kit (Invitrogen, Carlsbad, USA) with a mixture of three poly (T) adapters: Primer A: [GCTGTCAACGATACGCTACG TAACGGCATGACAGTG$(T)_{20}$A]; Primer G: [GCTGTCAACGATAC GCTACGTAACGGCATGACAGTG$(T)_{20}$G]; Primer C: [GCTGTCAA CGATACGCTACGTAACGGCATGACAGTG$(T)_{20}$C]. These cDNA samples were used for differential expression quantification of the newly identified miRNAs by PCR amplification using a set of primers composed of the universal primer corresponding to the 5' end of the poly (T)-adapter 5'-GCTGTCAACGATACGCTACG TAACG-3' and a primer specific for the miRNA sequence (Table 1). The temperature cycle was: 94°C for 2 min; 40 cycles of 94°C for 30 s, 61°C for 30 s and 72°C for 40 s; 72°C for 3 min. The products were separated by electrophoresis in a 4% (w/v) agarose gel.

Bioinformatics identification of miRNA target genes

The newly identified miRNAs were used as query sequences to blast online using plant miRNA target finder software miRU against maize mRNA transcripts in TIGR Maize Gene Index 15 and miRU2 against maize mRNA transcripts in *Zea mays* (maize) DFCI Gene Index (ZMGI) Release 17 (http://bioinfo3.noble.org/miRNA/miRU.htm). The maize mRNA sequences obtained were complementary to the newly identified miRNAs with fewer than four mismatches. The maximum expectation, the maximum circles, the maximum

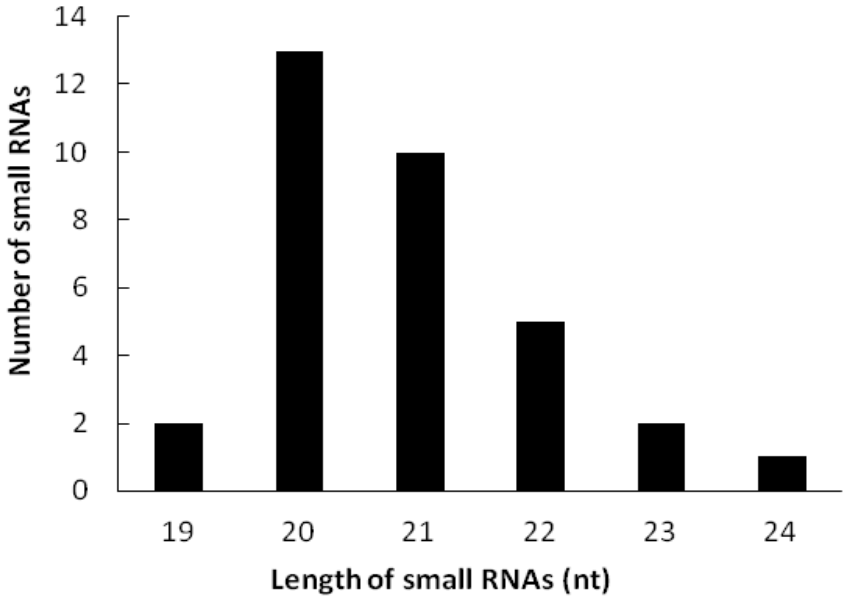


Figure 2. Size distribution of 33 cloned small RNAs. Their length varies from 19 to 24 nt, which is the typical size for mature miRNAs.

number of indels, the maximum number of G - U wobble pairs and the maximum number of other mismatches was three, three, one, six and three, respectively. The miRNA–target pairs, which were reduced to a conservative set, have minimum free energies for the miRNA-target duplexes that are less than –28.2 kcal/mol (Ambros et al., 2003). For the mRNA transcripts in TIGR Maize Gene Index 15 that were not explained, the remaining candidates identified by miRU from this database were used as query sequences to search in the maize protein database of NCBI by BLASTX software to predict the functions of their encoded proteins.

RESULTS

Potential miRNA sequences in maize predicted by a bioinformatics approach

To identify miRNA sequences in maize, 1279 sequences of plant miRNAs were downloaded from the miRNA registry database (Reinhart et al., 2002). After four rounds of BLAST and BLASTX screening, 246 potential miRNA sequences were obtained for maize; of these, 67 were predicted from EST and 179 from GSS. Each of these sequences has less than four mismatch base pairs in comparison with deposited miRNA sequences from other plant species.

Potential miRNA sequences cloned from maize

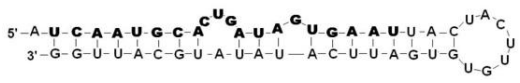
The small RNAs isolated with the mirVana^{TMP} miRNA isolation kit (Ambion, Austin, USA) should be shorter than 200 nt. After ligation to 3' and 5' adapters, and reverse transcription, they should be ≤ 240 nt. Because mature miRNAs are as long as 16 - 29 nt, the ligated products of miRNAs and the adapters should be ~ 65 nt long. Therefore, the cDNAs of miRNAs should be recovered from an electrophoresis gel between 60 and 80 bp.

After recovering and cloning, 2919 recombinant clones were obtained, and 1202 of them were identified as positive by bacterial PCR. The fragments amplified by bacterial PCR were 60-80 bp long and sequence analysis showed that some of these 1202 clones contained the same repeat sequences. Only 60 fragments were composed of a 5' adapter, a 3' adapter and a unique cloned sequence. Of these, 33 were found to be homologous to maize GSSs in the NCBI GenBank nucleotide databases (http://www.ncbi.nlm.nih.gov). They were 19-24 nt long, which is the typical size range of mature miRNAs (Figure 2).

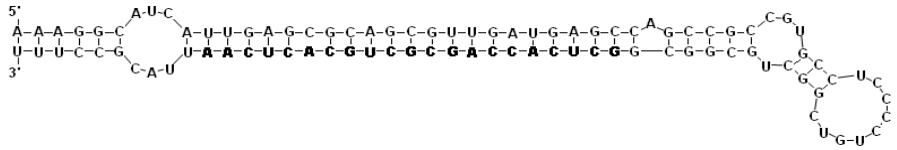
Secondary structure of newly indentified miRNAs

According to the criteria proposed by Ambros et al. (2003), Bonnet et al. (2004) and Mathews et al. (1999), 12 precursors from the 246 potential miRNA sequences predicted by the bioinformatics approach, and nine precursors from the 33 potential miRNA sequences cloned directly were able to fold into the secondary structure typical of the miRNA family, although they were variable in both length and structure (Figure 3).

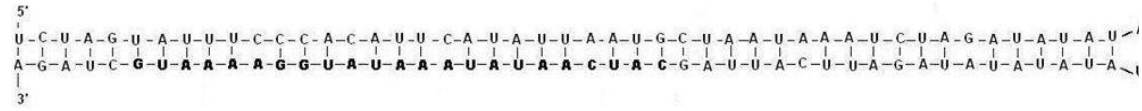
The precursors of these 21 miRNAs had an A+U content of 26.15 - 77.55%. The high negative minimal fold free energy varied between 11. 30 and 84.00 kcal/mol and the minimal fold energy index ranged from 0.48 to 1.48. These precursors were 51-166 nt long, showing greater diversity in length and structure than those of



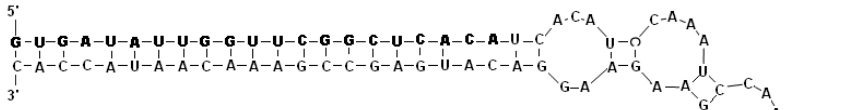
zma-miR397



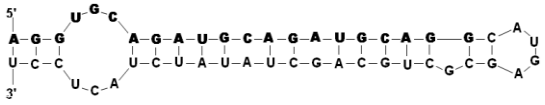
zma-miR446



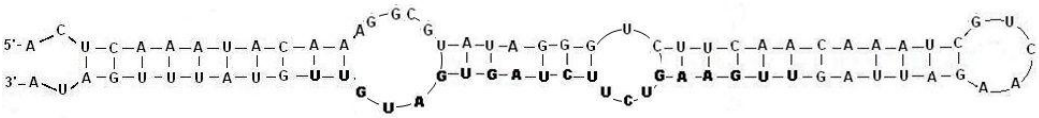
zma-miR479



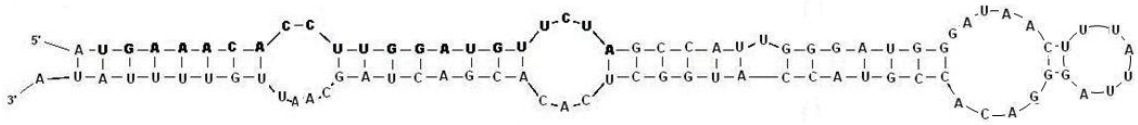
miRNA530



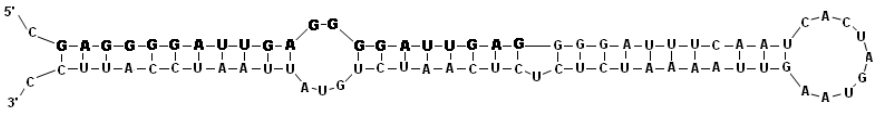
miRNA776



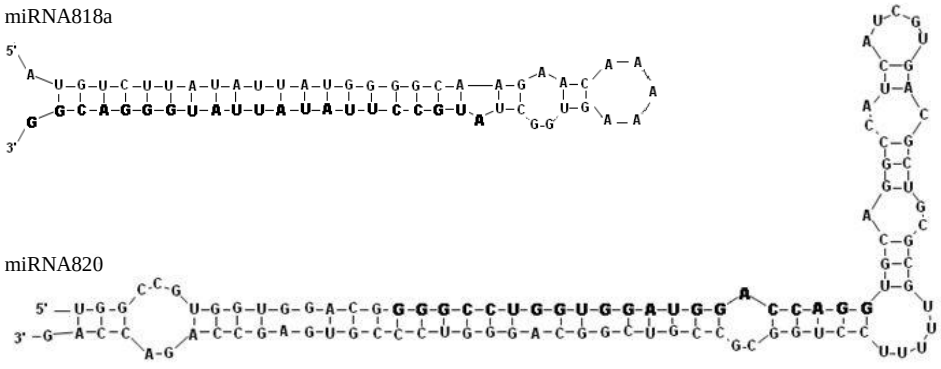
miRNA782



zma-miR815a



miRNA818a



miRNA820

zma-miR828

Figure 3. Secondary structure of twenty-one miRNAs in maize. The bold letters show mature miRNA sequences.

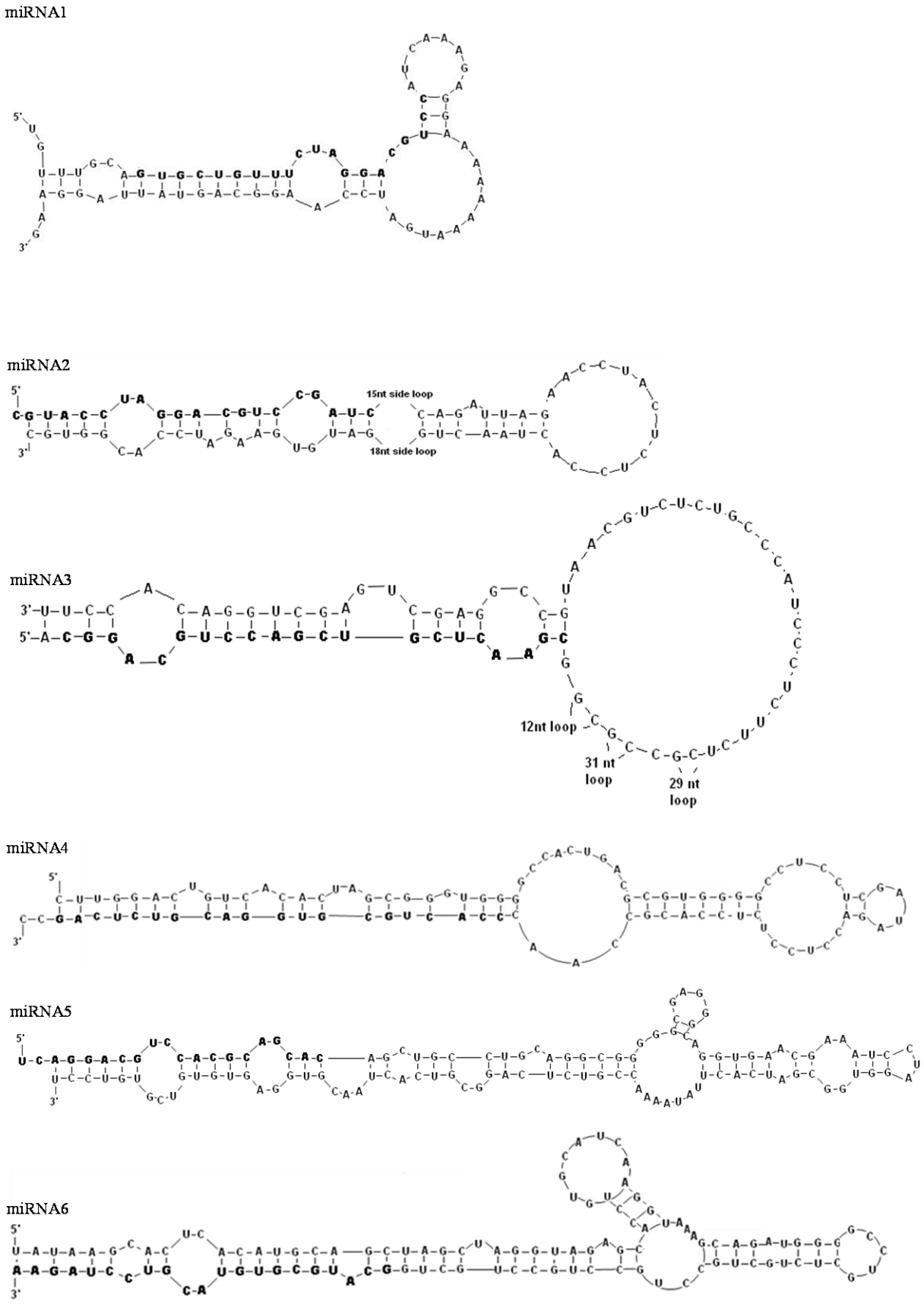


Figure 3. Continued.

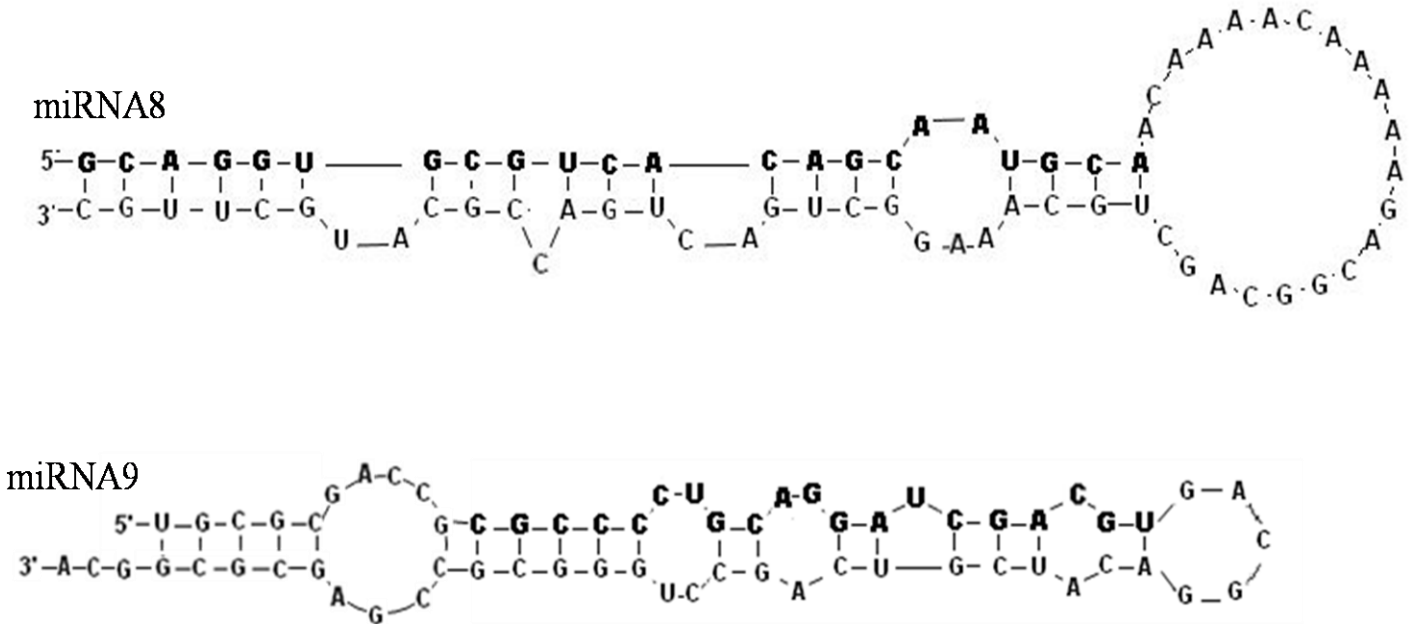


Figure 3. Continued.

animals (70–80 nt) (Griffiths-Jones et al., 2006). These features correspond to plant miRNAs deposited in the miRNA database (Zhang et al., 2006). Six of the mature miRNAs were processed from the 3' end of their primary transcripts, while the remaining 15 were processed from the 5' end (Tables 2 and 3).

According to the denomination method suggested by Ambros et al. (2003), the 12 miRNAs identified by the bioinformatics approach were designated as zma-miR161, zma-miR397, zma-miR446, zma-miR479, zma-miR530, zma-miR776, zma-miR782, zma-miR815a, zma-miR818a, zma-miR820, zma-miR828 and zma-miR834, referring to their homo-logous conserved miRNA sequences in other plant species (Table 2). For the nine miRNAs cloned directly, however, no miRNA cloned directly from maize is registered at the miRNA database, and no homologous conserved miRNA sequence from other plant species is referred to. Therefore, they were designnated as zma-miR1, zma-miR2, zma-miR3, zma-miR4, zma-miR5, zma-miR6, zma-miR7, zma-miR8 and zma-miR9 (Table 3).

Differential expression of 13 miRNAs under drought stress

Of the 21 miRNAs identified, 13 were reverse transcribed from the total small RNA samples. The remaining eight miRNAs, identified by the bioinformatics approach, need to be validated by another experimental method. Their expression abundance might be too low to be amplified by reverse transcriptase (RT)-PCR. Of the 13 reverse transcribed miRNAs, zma-miR1, zma-miR3, zma-miR6, zma-miR479, zma-miR782, zma-miR815a and zma-miR820 were strictly expressed in drought-tolerant inbred line 87 - 1 under drought stress, whereas the other six were amplified in both the drought-tolerant inbred line 87-1 and the sensitive inbred line 200B under drought stress and in the well-watered control (Figure 4).

miRNA target genes and their functions

Of the 21 miRNAs identified by plant miRNA target finder software miRU and miRU2, 14 were identified to target genes encoding 21 proteins, most of which are metabolic enzymes (Figure 5). Seven of them are involved in signal transduction, transcription regulation, biotic or abiotic stress responses (Table 4). This result reflects the wide range of roles that miRNAs have in the regulation of gene expression.

DISCUSSION

miRNAs are usually identified at the experimental level by cloning and sequencing size-selected RNAs. The outcome of that direct approach is particularly challenging because of the small size of miRNAs. Low abundance miRNAs will be under-represented and might escape detection. The results of this study provide another example of how bioinformatics can identify important biological information within existing databases. In some studies on bioinformatics for the identification of miRNAs (Jones-Rhoades and Bartel, 2004; Adai et al., 2005; Archak and Nagaraju, 2007), only ESTs were used as source

Table 2. Twelve miRNAs predicted by bioinformatic approach from maize.

miRNA	Gene Source	GenBank accession no.	Location	Number of mismatch (nt)	Length of mature miRNA (nt)	Length of percursor (nt)	A+U (%)	Minimal folding free energy (kcal/mol)	Minimal folding free energy index
zma-miR161	EST	DV493503 DT946046 DT938610 AI737398	5’	3	20	51	66.67	15.00	0.76
zma-miR397	GSS	CG106196	3’	2	21	94	37.00	57.60	0.98
zma-miR446	EST	CD447412	3’	3	23	98	77.55	36.40	1.35
zma-miR479	GSS	CG370944 CC612375	5’	1	21	82	56.10	22.50	0.62
zma-miR530	GSS	CG238117	5’	2	21	49	45.10	18.50	0.74
zma-miR776	GSS	CL253460	3’	2	21	79	65.82	26.20	0.79
zma-miR782	GSS	CL987999	5’	3	21	91	58.24	20.31	0.70
zma-miR815a	GSS	CG175870	5’	3	21	77	53.95	26.30	0.75
zma-miR818a	GSS	BZ722724	3’	1	21	57	60.34	22.30	0.97
zma-miR820	EST	DW468558 DV519308 DV171697 DR959994	5’	2	21	105	31.43	41.80	0.73
zma-miR828	GSS	BZ632191	5’	2	19	62	74.58	11.30	0.70
zma-miR834	GSS	BZ644116 BZ639894	5’	2	21	71	57.75	14.30	0.48

Table 3. Nine miRNAs directly cloned from maize.

miRNA	GenBank accession number of GSS	Location	Length of mature miRNA (nt)	Length of percursor (nt)	A+U (%)	Minimal folding free energy (kcal/mol)	Minimal folding free energy index
zma-miR1	DX848724	5’	20	69	59.42	9.3	1.48
zma-miR2	BZ806558	5’	19	101	45.00	62.1	1.15
zma-miR 3	BH256202	5’	21	166	36.36	84.0	1.04
zma-miR 4	CG115991	3’	20	96	34.38	30.5	0.96
zma-miR 5	CG309026	5’	20	122	40.98	55.2	0.77

Table 3. Nine miRNAs directly cloned from maize.

zma-miR 6	CG225043	3’	21	111	46.30	37.1	0.73
zma-miR 7	DX854351	5’	20	132	45.80	42.8	0.65
zma-miR 8	CG298167	5’	22	69	46.38	39.5	1.06
zma-miR 9	BZ818160	5’	20	65	26.15	26.6	0.90

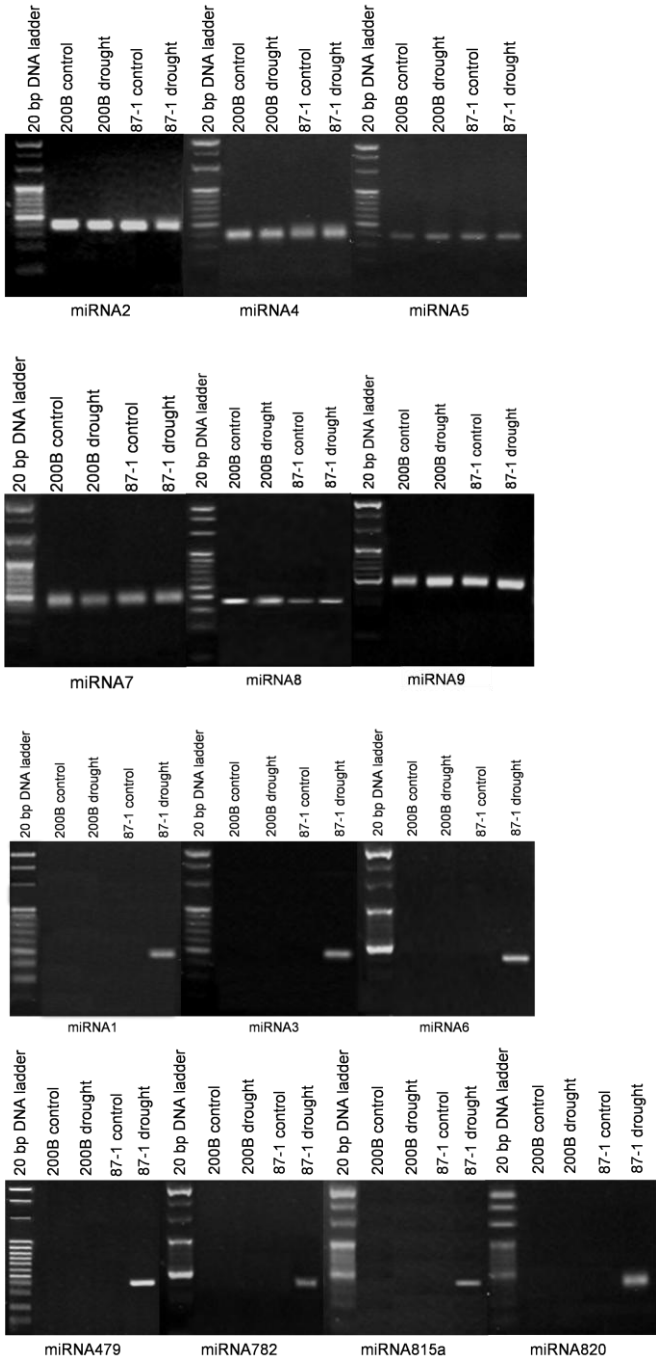


Figure 4. Reverse transcript PCR amplified products of thirteen miRNAs separated by 4% agarose gel. zma-miR1, zma-miR3, zma-miR6, zma-miR479, zma-miR782, zma-miR815a and zma-miR820 were specifically expressed in drought-tolerant inbred line “87-1” under drought stress, while the other six were amplified both in drought-tolerant inbred line “87-1” and sensitive inbred line “200B” under drought stress and well-watered control.

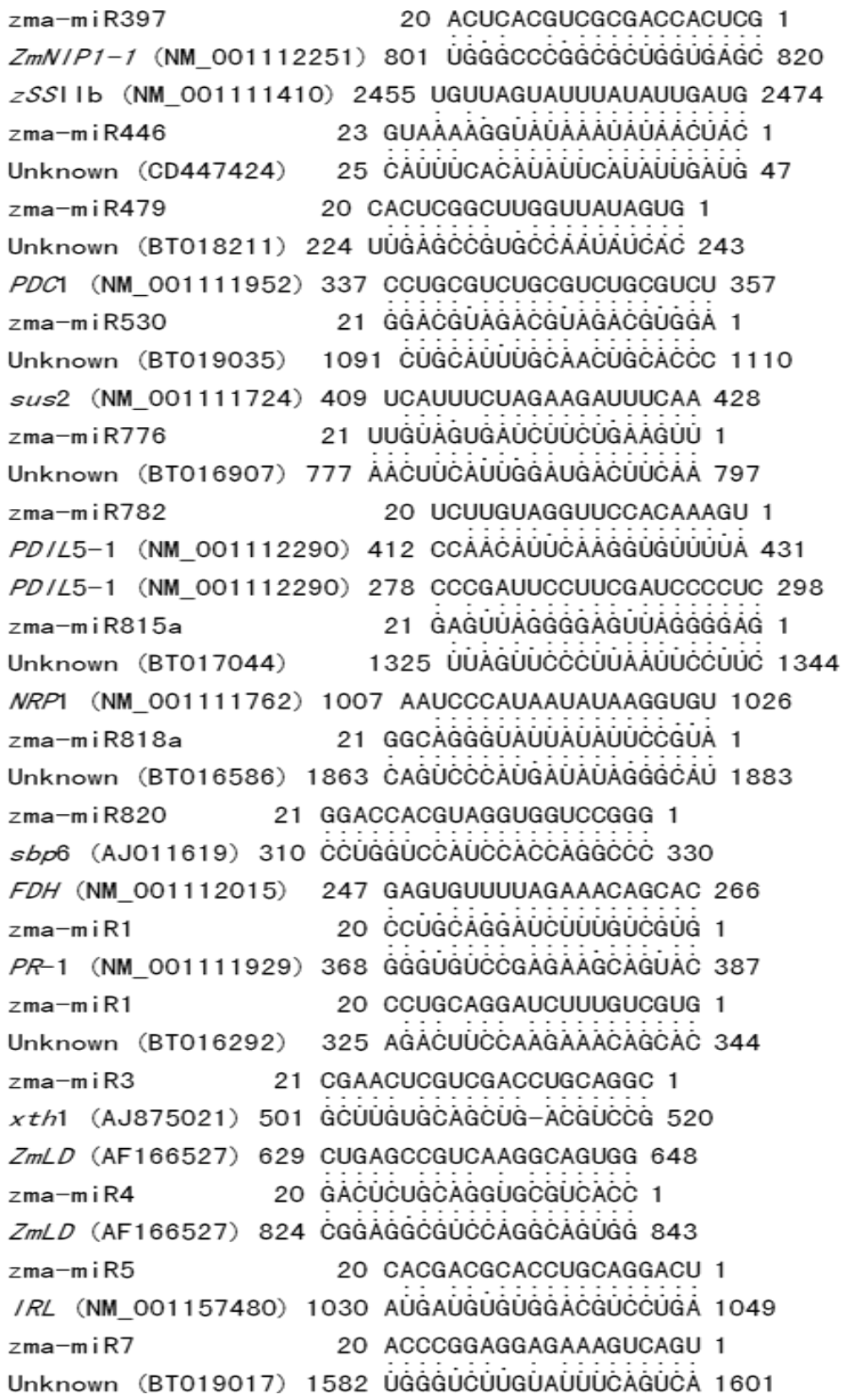


Figure 5. Alignments between miRNAs and their target genes. "꞉ " shows ordinary base pairing between adenine and thymine, and between guanine and cytosine. "·" shows wobble base pairing between guanine and uracil.

Table 4. Target genes and encoding proteins of the newly identified miRNAs.

microRNA	Target gene (Accession number)	Encoded protein	Protein function
zma-miR397	*ZmNIP1-1* (NM_001112251)	Nodulin 26-like membrane integral protein ZmNIP1-1	Transmembrane transport, abiotic stress
zma-miR446	*zSS*IIb (NM_001111410)	Starch synthase isoform zSTSII-2	Starch synthesis
	Unknown (CD447424)	Not annotated	Unknown
zma-miR479	Unknown (BT018211)	Not annotated	Unknown
zma-miR530	*PDC*1 (NM_001111952)	Pyruvate decarboxylase 1	pyruvate metabolism, abiotic stress
	Unknown (BT019035)	Not annotated	Unknown
zma-miR776	*sus*2 (NM_001111724)	sucrose synthase 2	sucrose synthesis
	Unknown (BT016907)	Not annotated	Unknown
zma-miR782	*PDIL*5-1 (NM_001112290)	Protein disulfide isomerase	disulfide bond formation
zma-miR815a	*PDIL*5-1 (NM_001112290)	Protein disulfide isomerase	disulfide bond formation
	Unknown (BT017044)	Not annotated	Unknown
zma-miR818a	*NRP*1 (NM_001111762)	no-apical-meristem -related protein 1	Transcriptional factor
	Unknown (BT016586)	Not annotated	Unknown
zma-miR820	*sbp*6 (AJ011619)	SBP-domain protein 6	Transcriptional factor
	PR-1 (NM_001111929)	Pathogenesis related protein-1	Biostress
zma-miR1	*FDH* (NM_001112015)	Alcohol dehydrogenase class 3	Methane metabolism
	Unknown (BT016292)	Not annotated	Unknown
zma-miR3	*xth*1 (AJ875021)	Xyloglucan endo-transglycosylase / hydrolase enzyme	endotransglucosylation of two xyloglucan polysaccharides
zma-miR4	*ZmLD* (AF166527)	Flowering-time protein isoform alpha	Signal transduction
zma-miR5	*IRL* (NM_001157480)	Isoflavone reductase IRL	Biostress, abiotic stress
zma-miR7	Unknown (BT019017)	Not annotated	Unknown

sequences. The advantage of that approach is that the miRNAs identified are known to be expressed, which provides an alternative solution to the difficulties associated with large-scale experimental validation of the expression of miRNAs, and facilitates further miRNA functional analysis. However, many potential miRNAs might not be found, as the identification depends upon the ESTs available. In this study, we added GSSs to the source sequences to identify miRNAs and, of the 12 miRNAs identified by the bioinformatics approach, three were identified from ESTs and nine from GSSs (Table 2). A disadvantage of this approach is that the results can include some false predictions. Although the quality of prediction was improved by the use of multiple screening criteria, the miRNAs identified from GSSs, such as zma-miR161 and zma-miR834, remain to be validated experimentally. However, the stringent screening criteria we used might have prevented us from identifying more miRNAs. Some miRNAs identified from rice do not fulfil all these criteria (Wang, et al., 2004), suggesting that miRNAs might be much more abundant in the maize genome than the number that have been deposited. No target mRNA sequence was identified from maize for zma-miR161, zma-miR834, zma-miR2, zma-miR6, zma-miR8 and zma-miR9 and they remain to be validated by the experiment. Some target mRNA sequences were identified for zma-miR446, zma-miR479, zma-miR530, zma-miR776, zma-miR815a, zma-miR818a, zma-miR1 and zma-miR7, but their encoding proteins are not known (Table 4). This result suggests that the targets of miRNAs are not limited to protein-encoding sequences. Recent evidence has substantiated an epigenetic role of miRNAs, in that miRNAs can regulate the epigenetic state of genes by targeting intergenic regions (Brodersen and Voinnet, 2006;

Royo, et al., 2006). For example, miRNAs regulate heterochronic shifts that control polarization during leaf abaxial/adaxial determination and organ ontogeny during floral development (Kidner and Martienssen, 2005). Along with rapid progress of functional genomics research, more and more mRNA sequences will be identified and annotated. This will facilitate identifying the target genes of miRNAs and explaining the detailed regulation of miRNAs in different physiological processes and pathways.

We identified 21 miRNAs with a length of 19–24 nt from maize using bioinformatics computation and small RNA cloning. They followed the typical secondary structure of plant miRNAs. Of these, 13 were amplified and validated by reverse tran-scriptional PCR from total small RNA samples of maize leaf. zma-miR1, zma-miR3, zma-miR6, zma-miR479, zma-miR782, zma-miR815a and zma-miR820 were found to be expressed speci-fically in drought-tolerant inbred line 87-1 under simulated drought stress. Of the 21 miRNAs identified, 14 were found by the use of plant miRNA target finder software miRU and miRU2 to target genes encoding 21 proteins, most of which are metabolic enzymes. Some of them are involved in signal transduction, transcription regulation, biotic stress or abiotic stress responses. The identification of the 21 miRNAs is a useful complement to the maize miRNA database. The seven differentially expressed miRNAs are sug-gested to be related to gene expression regulation under drought stress, whereas the other six probably function in other pathways.

ACKNOWLEDGEMENTS

Financial support from the Project of Development Plan of the State Key Fundamental Research (973 Project) (2009CB118400), the National Natural Science Foundation of China (30571172, 30721140554 and 30971795), and the National Key Science and Technology Special Project (2008ZX08003-004 and 2009ZX08003-012B) are sincerely appreciated. The authors are grateful to Dr Jihong Liu Clarke for pre-reviewing this manuscript. The English writing has been revised by the International Science Editing.

REFERENCES

Adai A, Johnson C, Mlotshwa S, Archer-Evans S, Manocha V, Vance V, Sundaresan V (2005). Computational prediction of miRNAs in *Arabidopsis thaliana*. Genome Res. 18: 78-91.

Ambros V, Bartel B, Bartel DP, Burge CB, Carrington JC, Chen X, Dreyfuss G, Eddy SR, Griffiths-Jones S, Marshall M, Matzke M, Ruvkun G, Tuschl T (2003). A uniform system for microRNA annotation. RNA, 9: 277-279.

Archak S, Nagaraju J (2007). Computational prediction of rice (*Oryza sativa*) miRNA targets. Genomics, Proteomics Bioinformatics, 5: 196-206.

Bari R, Pant BD, Stitt M, Scheible WR (2006). PHO2, microRNA399, and PHR1 define a phosphate-signaling pathway in plants. Plant Physiol. 141: 988-999.

Bartel B, Bartel DP (2003). MicroRNAs: at the root of plant development? Plant Physiol. 132: 709-717

Bartel D (2004). MicroRNAs: genomics, biogenesis, mechanism and function. Cell, 116: 281-297.

Bonnet E, Wuyts J, Rouze P, van de Peer Y (2004). Evidence that microRNA precursors, unlike other non-coding RNAs, have lower folding free energies than random sequences. Bioinformatics, 20: 2911-2917.

Bonnet E, Wuyts J, Rouze P, Van de Peer Y (2004). Detection of 91 potential conserved plant microRNAs in *Arabidopsis thaliana* and *Oryza sativa* identifies important target genes. Proc. Natl. Acad. Sci. USA. 101: 11511-11516.

Brodersen P, Voinnet O (2006). The diversity of RNA silencing pathways in plants Trend. Genetics, 22: 268-280.

Chiou TJ (2007). The role of microRNAs in sensing nutrient stress. Plant Cell Environ. 30: 323-332.

Chiou TJ, Aung K, Lin SI, Wu CC, Chiang SF, Su CL (2006). Regulation of phosphate homeostasis by microRNA in *Arabidopsis*. Plant Cell, 18: 412-421.

Cissell KA, Deo SK (2009). Trends in microRNA detection. Anal. Bioanal. Chem. 394: 1109-1116.

Ding D, Zhang L, Wang H, Liu Z, Zhang Z, Zheng Y (2009). Differential expression of miRNAs in response to salt stress in maize roots. Ann. Bot. 103: 29-38.

Floyd SK, Bowman JL (2004). Ancient microRNA target sequences in plants. Nature, 428: 485-486.

Fu F-L, Feng Z-L, Gao S-B, Zhou S-F, Li W-C (2008). Evaluation and quantitative inheritance of several drought-relative traits in maize. Agric. Sci. Chin. 7: 280-290.

Ghosh Z, Chakrabarti J, Mallick B (2007). miRNomics-the bioinformatics of microRNA genes. Biochem. Biophys. Res. Commun. 363: 6-11.

Griffiths-Jones S, Grocock RJ, van Dongen S, Bateman A, Enright AJ (2006). miRBase: microRNA sequences, targets and gene nomenclature. Nucleic Acids Res. 34: D140-D144.

Jones-Rhoades MW, Bartel DP (2004). Computational identification of plant microRNAs and their targets, including a stress-induced miRNA. Mol. Cell, 14: 787-799.

Jones-Rhoades MW, Bartel DP, Bartel B (2006). MicroRNAs and their regulatory roles in plants. Ann. Rev. Plant Biol. 57: 19-53.

Jung J-H, Seo PJ, Park C-M (2009). MicroRNA biogenesis and function in higher plants. Plant Biotech. Rep. 3: 111-126.

Kidner CA, Martienssen RA (2005). The developmental role of microRNA in plants. Curr. Opin. Plant Biol. 8: 38-44.

Lai EC, Tomancak P, Williams RW, Rubin GM (2003). Computational identification of *Drosophila* microRNA genes. Genome Biol. 4: R42.

Li W, Ruan K (2009). MicroRNA detection by microarray. Anal. Bioanal. Chem. 394: 1117-1124.

Liu HH, Tian X, Li YJ, Wu CA, Zheng CC (2008). Microarray-based analysis of stress-regulated microRNAs in *Arabidopsis thaliana*. RNA, 14: 1-8.

Lu S, Sun YH, Shi R, Clark C, Li L, Chiang VL (2005). Novel and mechanical stress-responsive microRNAs in *Populus trichocarpa* that are absent from *Arabidopsis*. Plant Cell, 17: 2186-2203.

Mathews DH, Sabina J, Zuker M, Turner DH (1999). Expanded sequence dependence of thermodynamic parameters improves prediction of RNA secondary structure. J. Mol. Biol. 288: 911-940.

Michel BE, Kaufmann MR (1973). The osmotic potential of polyethylene glycol 6000. Plant Physiol. 51: 914-916.

Reinhart BJ, Weinstein EG, Rhoades MW, Bartel B, Bartel DP (2002). MicroRNA in plants. Gene Dev. 16: 1616-1626.

Rhoades MW, Reinhart BJ, Lim LP, Burge CP, Bartel B, Bartel DP (2002). Prediction of plant microRNA targets. Cell, 110: 513-520.

Royo H, Bortolin M-L, Seitz H, Cavaille J (2006). Small non-coding RNAs and genomic imprinting. Cytogenet. Genome Res. 113: 99-108.

Sunkar R, Girke T, Jain PK, Zhu JK (2005). Cloning and characterization of microRNA from rice. Plant Cell, 17: 1397-1411.

Sunkar R, Kapoor A, Zhu JK (2006). Posttranscriptional induction of two Cu/Zn superoxide dismutase genes in *Arabidopsis* is mediated by downregulation of miR398 and important for oxidative stress tolerance. Plant Cell, 18: 2051-2065.

Sunkar R, Zhu JK (2004). Novel and stress-regulated microRNAs and

other small RNAs from *Arabidopsis*. Plant Cell, 16: 2001-2019.
Wang J, Zhou H, Chen Y, Luo Q, Qu L (2004). Identification of 20 microRNAs from *Oryza sativa*. Nucleic Acids Res. 32: 1688-1695.
Wang XJ, Reyes JL, Chua NH, Gaasterland T (2004). Prediction and identification of *Arabidopsis thaliana* microRNAs and their mRNA targets. Genome Biol. 5: 1-15.
Zhang B, Pan X, Anderson TA (2006). Identification of 188 conserved maize microRNAs and their targets. FEBS Lett. 580: 3753-3762.
Zhang B, Pan X, Cobb GP, Anderson TA (2006). Plant microRNA: a small regulatory molecule with big impact. Dev. Biol. 289: 3-16.
Zhang B, Stellwag EJ, Pan X (2009). Large-scale genome analysis reveals unique features of microRNAs. Gene, 443: 100-109.
Zhang BH, Pan XP, Cannon CH, Cobb GP, Anderson TA (2006). Conservation and divergence of plant microRNA genes. Plant J. 46: 243-259.
Zhang BH, Pan XP, Cox SB, Cobb GP, Anderson TA (2006). Evidence that miRNAs are different from other RNAs. Cell. Mol. Life Sci. 63: 246-254.
Zhang BH, Pan XP, Wang QL, Cobb GP, Anderson TA (2005). Identification and characterization of new plant microRNAs using EST analysis. Cell Res. 15: 336-360.
Zhao B, Liang R, Ge L, Li W, Xiao H, Lin H, Ruan K, Jin Y (2007). Identification of drought-induced microRNAs in rice. Biochem. Biophys. Res. Commun. 354: 585-590.