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IDENTIFICATION OF MICRORNA FAMILIES EXPRESSED IN SUGARCANE LEAVES SUBJECTED TO DROUGHT STRESS AND THE TARGETS THEREOF

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MicroRNA families are important regulators of plant resistance to diverse stressors, but the miRNA families expressed in response to drought, and the levels thereof, in sugarcane (an important commercial source of both sugar and biofuel) remain poorly understood. In the present study, we determined the expression profile of miRNA families synthesized by leaves of the drought-resistant cultivar ROC22 stressed by addition of PEG to the growth substrate. Totals of 23 conserved miRNA families and 34 new miRNA families were identified, and 438 putative target genes of 44 miRNA families were described. Expression analysis revealed that 11 miRNA families were differentially expressed in control and drought-exposed plants. Of these, nine families were up-regulated and two down-regulated. The potential targets of the 11 miRNA families were genes associated with plant growth and stress resistance, specifically *SPBP*, *MYB*, the *AGO1-like gene*, *NCBP*, *BCP*, *CPI,* and *LSG*. With the exceptions of *SPBP*, *NCBP* and *BCP*, the other genes were down-regulated in response to drought stress. Our preliminary identification of 11 relevant miRNA families expressed by ROC22, and their targets, suggest that these miRNA families may regulate the response to drought stress and forms a basis for planned future work. The other new miRNA families identified, along with their targets, may exert novel effects on various metabolic pathways relevant to sugarcane growth and development.
Keywords: Sugarcane, drought, microRNA, target gene, gene expression

INTRODUCTION

It has become clear that small RNAs are important regulators of eukaryotic gene expression (Reinhart *et al.*, 2000; Bartel *et al.,* 2004; Zhang *et al.*, 2006; Place *et al.*, 2008; Bazzini *et al.,* 2011; Hammell, 2011). Such novel regulation of gene expression was discovered in the 1990s (Lee *et al.*, 1993) and immediately attracted widespread interest. Small RNAs contain 20–24 nucleotides and induce cleavage of mRNAs with partially or fully complementary sequences (Reinhart *et al.*, 2002). In plants, small RNAs include microRNAs (miRNA families, mostly 21 nucleotides long) produced from hairpin-shaped precursors, and small interfering RNAs (siRNAs, 24 nucleotides long) generated from long double-stranded RNA duplexes or transcripts derived from inverted repeat regions (Chen, 2009; Voinnet, 2009).

Sugarcane is the principal source of sugar in China, yielding over 90% of all sugar produced (Zhang *et al.*, 2003). Sugarcane is a typical C4 crop and is particularly sensitive to water level and temperature. The response of sugarcane to drought was explored as far back as the 1980s; drought stress seriously affected growth, tillering, elongation, leaf expansion, and maturation (Clements, 1980; Kochler *et al.*, 1982; Inman-Bamber *et al.*, 1986). The region of southwest China in which sugarcane in grown has suffered severely from drought in recent years. In 2010, drought reduced the yield of sugar from sugarcane by 1 million tons, or 8.3% of the total annual sugar production. To date, most research effort has been focused on the cloning and expression of genes associated with the response to drought stress in sugarcane. Few works have investigated the small RNA population and the functions thereof. To systematically identify miRNA families and other small RNAs that might be involved in the sugarcane response to drought stress, we constructed small RNA libraries from control and drought-treated sugarcane ROC22 cultivars and profiled small RNA expression using Solexa sequencing technology (Genome Analyzer IIx, Illumina). Putative target genes were predicted and the functions thereof analyzed by recourse to Web-based bioinformatics data. In this study our main objective was to work out the expression pattern of miRNA families in sugarcane cultivar ROC22 exposed to drought stress, and

!The first three authors contributed equally to this paper.

tried to elucidate the complex regulatory network in sugarcane to response to drought stress.

MATERIALS AND METHODS

***Sample collection and total RNA isolation*:** The sugarcane ROC22 cultivar was studied. ROC22 seedlings were transplanted into buckets containing Hoagland's solution. Seedlings subjected to drought treatment were cultivated in solution containing PEG8000 (25%, w/v). After 16 h of treatment, leaves were collected and stored in liquid nitrogen. Total RNAs were isolated using the TRIzol reagent (TAKARA). Small RNA sequences were obtained using the Solexa sequencing technology of Illumina.

***Primary analysis of the Solexa dataset*:** Individual sequence reads, with base quality scores, were generated. Repeat sequences were eliminated. Unique reads were mapped onto the sugarcane transcript map and assembled using Saccharum EST (NCBI). Unique reads were screened for membership of a non-coding RNA database that included sequences of tRNAs, rRNAs, small nucleolar RNAs, and other ncRNAs, but not microRNAs. Unique reads that matched more than 20 genome sequences were removed prior to analysis, as were poorly expressed reads (fewer than two examples).

***Identification of conserved and novel microRNAs*:** To identify known microRNAs, perfectly matched reads were mapped onto the *Saccharum officinarum* microRNA precursor of the Sanger miRBase using Bowtie (Langmead *et al.*, 2009). The following criteria were used to define a known microRNA. First, a unique sequence had to be perfectly mapped onto the precursor. Second, alignment had to commence between +2 and –2 nt of the mature microRNA on the precursor. The presence of perfectly matched reads was sought in the mature plant microRNAs of the Sanger miRBase (Griffiths-Jones *et al.*, 2008) using Patscan (Dsouza *et al.*, 1997). Two mismatches were allowed in identification of homologs of known microRNAs.

To identify novel microRNAs, pre-mature microRNA transcript loci were first extracted from sequencing data to permit secondary structure analysis. The "Einverted of Emboss" software (Rice *et al.*, 2000) was used to predict the hairpin structures of candidate microRNAs using the parameters suggested by Jones-Rhoades and Bartel (2004). Candidate microRNAs were filtered using RNAfold (Hofacker *et al.*, 1994) and MirCheck (Jones-Rhoades and Bartel, 2004).

***miRNA target gene prediction*:** MicroRNA target genes were next sought using an approach previously described for use with the Sanger miRbase. All newly identified microRNA sequences were used to query the Sugarcane transcript (assembled using Saccharum EST of NCBI1) for potential target sequences. Patscan was employed to this end; the default parameters were three mismatches, zero insertions, and zero deletions. Only hits devoid of mismatches in positions 10 and 11 of mature miRNA families were considered to be valid target sequences.

***Differentially expressed microRNA detection*:** IDEG6 (Romualdi *et al.*, 2003) was employed to identify microRNAs that differed, with statistical significance, in terms of relative abundance (as reflected by the counts of individual sequence reads). The technique was similar to the credibility interval approaches developed for analysis of SAGE data (Vencio *et al.*, 2003), The Chi-squared method was used to analyze the significance of observed differences. A difference was considered to be significant if the expression level of a microRNA varied between the test and control samples with a P value ≤0.01 and a -fold change ≥2.

***Target gene expression levels measured using quantitative RT-PCR (qRT-PCR)*:** The expression levels of the *SPBP* (CA091338), *MYB* (CA068132), *AGO1-like* (CA099906), *NCBP* (CA092355), *BCP* (CA130641), *CPI* (CA094946), and *LSG* (BQ534061) genes were measured using quantitative RT-PCR. Total RNAs were isolated using RNeasy Plant Mini Kits (Qiagen) and the levels quantified spectrophotometrically (NanoDrop). The RNAs were

Table 1. The primers used for qRT-PCR analysis.

No.	GenBank No.	Name	Primer sequence (5'-3'direction)
1	CA091338	*SPBP*-For	CATTCCACCACCAACGCAA
		SPBP-Rev	GGGATTCACGGTCTACGGTT
2	CA068132	*MYB*-For	TCATTGCTAATCCAGAGGCT
		MYB-Rev	ATCCCTGCTTGGTCTACCT
3	CA099906	*AGO1-like gene*- For	CATCAGTTTCCTGCGATTCT
		AGO1-like gene- Rev	ATTGGTGAAGGTCTCTGTCA
4	CA092355	*NCBP*-For	CCGCAAAGAAATGGCATCAGA
		NCBP-Rev	ATTACCGCCCACAACACCA
5	CA130641	*BCP*-For	AGCACTACTTCATCTGCGG
		BCP-Rev	TAGCCGTATGGAGTTCACG
6	CA094946	*CPI*-For	GTAGTAAGTATGTCTGGTC
		CPI-Rev	TTAGGATGGAGGGAGT
7	BQ534061	*LSG*-For	CAGTGCCAAAGGGAAA
		LSG-Rev	AGCCGAGGGACTAAAT

converted into cDNAs by reverse transcription using ExScript RT Kits (Takara). For real-time PCR, 2-µl volumes of diluted cDNA products were used as templates. Quantitative RT-PCR reactions were performed using an Applied Biosystems-Step One platform and SYBR Green I Kits.

Primers were designed using dedicated software and are listed in Table 1. The reactions were performed at 95°C for 1 min, followed by 40 cycles of 95°C for 10 s and 60°C for 30 s within the linear range of reaction. The relative levels of target gene expression in each sample were calculated using the $2^{-\triangle\triangle CT}$ method.

RESULTS AND DISCUSSION

***The microRNA families of sugarcane cultivar ROC22*:** In recent years, China has suffered from widespread drought and diminishing water resources, causing serious losses in agricultural production. Sugarcane is the principal source of sugar in China, and an understanding of how miRNA families regulate the drought response of *S. officinarum* will facilitate preparation of *S. officinarum* cultivars exhibiting increased drought resistance. In the present work, miRNA expression profiles were examined in control and drought-exposed plants of *S. officinarum* cultivar ROC22. This cultivar ROC22 exhibits high-level drought resistance and, in recent years, has been planted in more than one-third of the cane-growing region of China (Pan *et al.*, 2002; Wei, 2008; Gui *et al.*, 2009). To investigate the small RNA expression profile of ROC22 leaves in response to drought stress, RNAs from control and drought-exposed plants were sequenced using Solexa technology. Many reads of 18–32 nucleotides were obtained from the two libraries (Table 2).

The distributions of different miRNA families in these reads are shown in Figure 1.

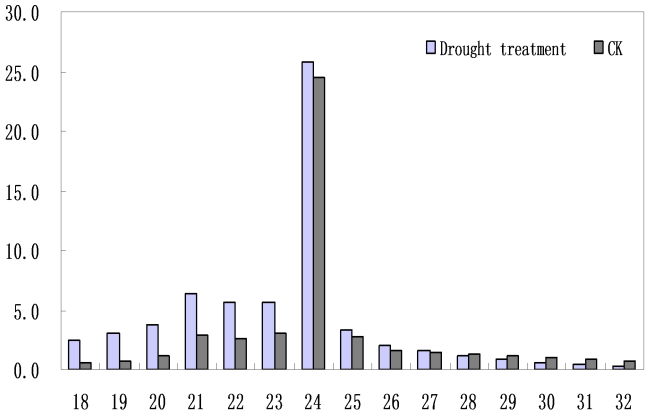

Figure 1. The size distributions of small RNAs in leaves of ROC22.

The gray bar was the size distributions of small RNAs in leaves of ROC22 subjected to drought treatment. The black bar was the size distributions of small RNAs in leaves of ROC22 under normal irrigation. The numbers in x-axis represented the number of bases and the size of small RNAs. The y-axis represented the proportion of different small RNAs in total.

After removal of adaptor sequences and low-quality reads, 2,273,348 and 2,703,934 non-redundant sequences (Table 2) were obtained from control and drought-exposed libraries, respectively. The most abundant small RNAs were 24 nucleotides long in either library, and the second-largest populations were 21-nucleotide RNAs. The size distributions of small RNAs were similar to other crops (Rajagopalan *et al.*, 2006; Chapman and Carrington, 2007; Fahlgren *et al.*,

Table 2. Sequenced reads obtained.

Class	Total reads	High-quality reads	Total matched reads	Total distinct reads	Perfect matched transcript
CK	17,597,870	13,084,908	4,506,300	2,273,348	493,778
Drought treatment	19,808,703	14,525,779	6,752,290	2,703,934	501,644

Table 3. Expression of known sugarcane miRNA families.

MiRNA families	Sequence	Length	Reads		Normalization of reads		Ratio	P-Value	Mark
			CK	DT	CK	DT			
sof-miR156	TGACAGAAGAGAGTGAGCAC	20	38	187	8.43	27.69	3.28	0.00000	Up
sof-miR159a	TTTGGATTGAAGGGAGCTCTG	21	3003	13260	666.40	1963.78	2.95	0.00000	Up
sof-miR159b	TTTGGATTGAAGGGAGCTCTG	21	3003	13260	666.40	1963.78	2.95	0.00000	Up
sof-miR159d	TTTGGATTGAAGGGAGCTCTG	21	3003	13260	666.40	1963.78	2.95	0.00000	Up
sof-miR159c	CTTGGATTGAAGGGAGCTCCT	21	1	14	0.22	2.07	9.34	0.00836	Up
sof-miR167a	TGAAGCTGCCAGCATGATCTG	21	2162	4150	479.77	614.61	1.28	0.00000	
sof-miR167b	TGAAGCTGCCAGCATGATCTG	21	2162	4150	479.77	614.61	1.28	0.00000	
sof-miR168a	TCGCTTGGTGCAGATCGGGAC	21	7166	23855	1590.22	3532.88	2.22	0.00000	Up
sof-miR396	TTCCACAGCTTTCTTGAACTG	21	18	91	3.99	13.48	3.37	0.00000	Up
sof-miR408a	CTGCACTGCCTCTTCCCTGGC	21	133	597	29.51	88.41	3.00	0.00000	Up
sof-miR408b	CTGCACTGCCTCTTCCCTGGC	21	133	597	29.51	88.41	3.00	0.00000	Up
sof-miR408c	CTGCACTGCCTCTTCCCTGGC	21	133	597	29.51	88.41	3.00	0.00000	Up
sof-miR408d	CTGCACTGCCTCTTCCCTGGC	21	133	597	29.51	88.41	3.00	0.00000	Up

Table 4. Expression of conserved sugarcane miRNA candidates.

MiRNA families	Sequence	Length	Expression		Normalization of reads		P-Value	Ratio	Mark
			CK	DT	CK	DT			
miR-156	TTGACAGAAGAGAGTGAGCAC	21	287	1409	63.69	208.67	0.00000	3.28	Up
miR-166	TCTCGGACCAGGCTTCATTCC	21	10614	30522	2355.37	4520.24	0.00000	1.92	
miR-166*	GGAATGTTGTCTGGTTCAAGG	21	18	22	3.99	3.26	0.52078	0.82	
miR-444a	TGCAGTTGTTGCCTCAAGCTT	21	521	612	115.62	90.64	0.00004	0.78	
miR-444a*	ATGAGGCAGGAACTGCATTACT	22	21	42	4.66	6.22	0.27831	1.33	
miR-444b	TGCAGTTGTTGTCTCAAGCTT	21	261	175	57.92	25.92	0.00000	0.45	Down
miR-444b*	GCTAGAGGCAGCAACTGCATA	21	5	7	1.11	1.04	0.90762	0.93	
miR-5564	TGGGGAAGCAATTCGTCGAAC	21	386	955	85.66	141.43	0.00000	1.65	
miR-5564*	TTGGCGACTTGCTTCGCCCATG	22	0	2	0.00	0.30	0.24796	0.00	
miR-6220	TCCATTCCAAATTATAAGAC	20	0	3	0.00	0.44	0.15708	0.00	

Table 5. Expression of novel sugarcane miRNA candidates.

Name	Sequence	Length	Reads		Normalization of reads		P-Value	Ratio	Mark
			CK	DT	CK	DT			
Novel-1	TATTAAGCCTAATTAATCCAT	21	1	11	0.22	1.63	0.02504	7.34	
Novel-2	GAGAGATATATAAGATTTGTGAAC	24	3	8	0.67	1.18	0.38798	1.78	
Novel-3	TTCAATGCATACACTGAGGGG	21	4	7	0.89	1.04	0.80422	1.17	
Novel-4	TTCCAAATTATAAGTCGCTTT	21	5	6	1.11	0.89	0.71323	0.80	
Novel-5	AGCGACTTATAATTTGGAACAGAG	24	3	8	0.67	1.18	0.38798	1.78	
Novel-6	CTGCGGATCAAGCTGCAACTGCGGCT	26	2	8	0.44	1.18	0.19618	2.67	
Novel-7	CGAGACAAATTTTTTAAGCCT	21	2	8	0.44	1.18	0.19618	2.67	
Novel-8	TAAGTTGGTTGTCATCAATT	20	5	5	1.11	0.74	0.51971	0.67	
Novel-9	AAAGCGACTTATAATTTGGAA	21	4	4	0.89	0.59	0.56473	0.67	
Novel-10	GCCGAGTGCCAAGATTCTGACACT	24	2	6	0.44	0.89	0.38572	2.00	
Novel-11	TTTTTTGGTACATCTATTTTG	21	6	2	1.33	0.30	0.04348	0.22	
Novel-12a	TCAAACTTAAGATGCTTTGACT	22	18	112	3.99	16.59	0.00000	4.15	Up
Novel-12a*	TCAAAGGATCTCAAGTTTGACC	22	18	21	3.99	3.11	0.43472	0.78	
Novel-12b	TCAAACTTGAGATCCTTTGAC	21	12	38	2.66	5.63	0.02073	2.11	
Novel-12b*	AGAGTCAAAGCATCTTAAGTTTGA	24	3	1	0.67	0.15	0.15338	0.22	
Novel-13	GTTCTTTCTACCACACTTTAGATTCT	26	1	5	0.22	0.74	0.24288	3.34	
Novel-14	TTTTGGTAATTGATGACAACC	21	4	2	0.89	0.30	0.18288	0.33	
Novel-15	ATACTCCCTCTGTCCCTAAATGTT	24	5	1	1.11	0.15	0.03037	0.13	
Novel-16	CGGCCTCCGTGGCCGGCCGACGAC	24	1	4	0.22	0.59	0.36075	2.67	
Novel-16*	GCTGGTTGTTGGCTGGCCATGGCT	24	1	3	0.22	0.44	0.53964	2.00	
Novel-17	TAATGATGGATTAATTAGGCT	21	1	4	0.22	0.59	0.36075	2.67	
Novel-18	AAGCCTAATTAATCTGTCATTAGC	24	3	2	0.67	0.30	0.36197	0.44	
Novel-19	GACGTGATCGAGAGCGGCGCTGGC	24	2	3	0.44	0.44	0.99907	1.00	
Novel-20	AAATTATAAGTCGCTTTGACT	21	14	38	3.11	5.63	0.05380	1.81	
Novel-20*	TCAAAGCGACTTATAATTTG	20	2	0	0.44	0.00	0.08342	0.00	
Novel-21	ATATTATATGTTCACTGTGTA	21	6	42	1.33	6.22	0.00010	4.67	Up
Novel-21*	TACACAGTGAACATATAATAT	21	1	4	0.22	0.59	0.36075	2.67	
Novel-22	CGAGACGAATCTATTAAGCCT	21	186	297	41.28	43.99	0.49644	1.07	
Novel-23	CTGTACCCGAAACCGACAC	19	4	30	0.89	4.44	0.00077	5.01	Up
Novel-24a	AATTAGGCTTAAAAGATTCGTCTC	24	163	121	36.17	17.92	0.00000	0.50	Down
Novel-24b	AATTAGGCTTAATAGATTCGTCTC	24	129	104	28.63	15.40	0.00000	0.54	
Novel-25	AGTCCCGGTCGGTGCCACGGACCG	24	8	7	1.78	1.04	0.29281	0.58	
Novel-26	GTTATATGGGCTCAAGTGCGAGAA	24	8	6	1.78	0.89	0.19113	0.50	
Novel-27	AAATTATAAGACGTTTTGGCT	21	90	135	19.97	19.99	0.99378	1.00	

2007; Martinez *et al.*, 2011). Analysis of genomic location showed that 16.11% of all nonredundant small RNAs were perfectly matched to regions encoding reported *S. officinarum* miRNA families or sequences homologous to miRNA genes of other species. After removal (by filtering) of rRNA, tRNA, and snoRNA sequences, and other poorly expressed small RNAs, the remaining reads accounted for 28.54% of perfectly matched miRNA families.

Ferreira *et al.* (2012) identified 18 miRNA families in sugarcane cultivars RB867515. Only six putative precursors were found in the sugarcane EST databases, representing five miRNA families (Ferreira *et al.*, 2012). To date, although many miRNA families have been identified, only 16 conserved miRNA families from *S. officinarum* have been lodged in miRBase. In the present study, both plant-conserved and species-specific miRNA families were systematically identified in ROC22. A total of 13 nonredundant reads, identical in sequence to miRBase-lodged sugarcane miRNA families (Table 3), were noted, and 10 sequences (Table 4) that were highly homologous to miRBase-recorded plant miRNA families, with hairpin-shaped precursors, were also identified as miRNA families. In addition, 34 new miRNA genes (Table 5) were predicted; all had well-formed hairpin-shaped secondary structures.

***Differentially expressed miRNA families in control and drought-exposed plants*:** The expression levels of miRNA families in ROC22 were calculated by reference to Solexa read levels. Of previously identified miRNA families, the expression levels of 57 were analyzed. Of six *S. officinarum* miRNA families lodged in miRBase, five were more highly expressed in drought-exposed ROC22; these were miR156, miR396, miR159, miR168, and miR408. The conserved miRNA families expressed by ROC22 were miR-156, miR-166, miR-444, miR-5564, and miR-6220. The miR-156, miR-166, and miR-5564 families were expressed at 3.28-, 1.92-, and 1.65-fold higher levels in drought-exposed ROC22 (Table 4). The expression level of miR-444b in drought-exposed plants was 0.45-fold that in control plants (Table 4).

The new miRNA families identified in ROC22 were termed "novel" and were grouped into 27 families. Of these, the expression levels of novel-12a, novel-21, and novel-23 in drought-exposed plants were respectively 4.15-, 4.67-, and 5.01-fold that of control plants. The expression level of novel-24a in drought-exposed plants was 0.50-fold that of controls. In most studies, the reported abundances of miRNA families* (sequences complementary to miRNA families) have been far lower than those of miRNA families. In the present study, nine miRNA families* were detected; these were miR-166*, miR-444a*, miR-444b*, miR-5564*, novel-12a*, novel-12b*, novel-16*, novel-20*, and novel-21*. All occurred at lower levels than those of the corresponding miRNA families.

Ferreira *et al*. (2012) found that the expression levels of five miRNA families (ssp-miR164, ssp-miR394, ssp-miR397, ssp-miR399 and miR528) were validated by RT-qPCR. The expression of ssp-miR164, ssp-miR393, ssp-miR397, ssp-miR399 and ssp-miR528 were up-regulated in drought treatment, and the expression of ssp-miR394 was down-regulated, which were different from our results. In our work, 11 miRNA families were differentially expressed in control and drought-exposed ROC22 plants. Of these, nine families were up-regulated upon exposure to drought; these were miR156, miR159, miR168, miR396, miR408, miR-156, novel-12a, novel-21, and novel-23. Two families were down-regulated; these were miR-444b and novel-24a.

***The differential expression of target genes associated with drought*:** Recognized plant miRNA target prediction criteria were used in a search for putative targets of conserved and new *S. officinarum* miRNA families. A total of 438 putative targets were identified. The targets of most miRNA families were homologous to genes of *Saccharum* and other plants. The functions of the targets could be grouped into 34 broad categories of cellular components, molecular function, and biological processes (Fig. 2). The target genes of miRNA families that were up- or down-regulated in response to drought had functions associated with plant growth regulation and reaction to stress (Table 6). Expression of the potential target genes *SPBP* (CA091338), *MYB* (CA068132), *AGO1-like* (CA099906), *NCBP* (CA092355), *BCP* (CA130641), *CPI* (CA094946), and *LSG* (BQ534061) was detected in ROC22 leaves after 0, 2, 4, 8, 16, and 24 h of short-term PEG treatment (Fig. 3). Expression of the *MYB*, *AGO1-like*, *CPI,* and *LSG* genes was down-regulated in response to drought stress (Fig. 3).

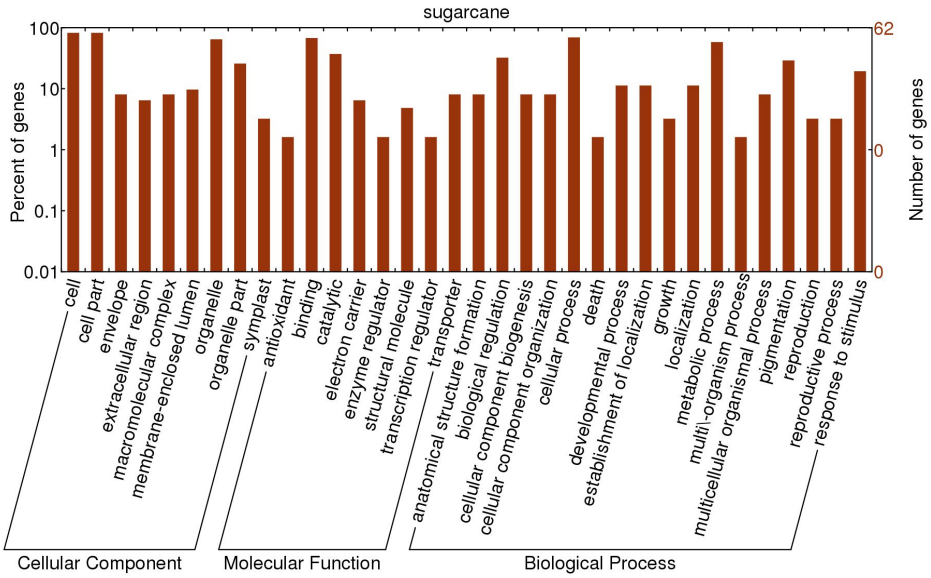

Figure 2. Putative functions of target genes for sugarcane miRNA families.

The 438 putative targets were classified into 34 functions. Different functions of putative targets were further group into 3 categories. The y-axis represented the number of targets in different functions and the proportion of targets in total.

Table 6. Predicted target genes of sugarcane miRNA families.

MiRNA families	Quantity	Potential target gene	main putative function
sof-miR156	11	CA091338(1): 163-182, CA075190(1): 304-323, CA071566(1): 533-552, CA231663(1): 609-628, CA075255(1): 588-607, CA072223(1): 391-410, CA072223(2): 328-347, CA072223(3): 616-635, CA120099(1): 829-848, CA084820(1): 470-489, CA084820(2): 527-546	CA091338: Squamosa promoter binding protein (SBP)
sof-miR159	1	CA068132(1): 549-569	CA068132: MYB protein
sof-miR167	1	CA232593(4), CA292372(4), CA201181(4), CA070734(4)	CA232593: Auxin response factor
sof-miR168	1	CA099906(4)	CA099906: AGO1-like protein
sof-miR396	3	CA240723(1): 155-175, CN610810(1): 179-199, CA092355(1): 892-912	CA092355: Nucleic acid binding protein
sof-miR408	6	BQ534106(1): 21-41, BQ534106(2): 214-234, CA162377(1): 83-103, CA067690(1): 206-226, CA130641(2): 313-333, CA130641(5): 717-737	CA130641: blue copper protein
miR-156	11	CA091338(1): 163-183, CA075190(1): 304-324, CA071566(1): 533-553, CA231663(1): 609-629, CA075255(1): 588-608, CA072223(1): 391-411, CA072223(2): 328-348, CA072223(3): 616-636, CA120099(1): 829-849, CA084820(1): 470-490, CA084820(2): 527-547,	CA091338: Squamosa promoter binding protein (SBP)
miR-166*	1	CA066625: 536-556	CA066625: G-type lectin S-receptor-like serine/threonine-protein kinase
miR-444a	4	CA274433(1): 166-186, CA133153(1): 288-308, CA125236(2): 957-977, CA102008(3): 418-438	CTL-like protein DDB, putative RING/U-box superfamily protein
miR-444b	3	CA274433(1): 166-186, CA133153(1): 288-308, CA102008(3): 418-438	No homology
miR-444b*	2	CA181743: 539-559, BQ535322(3): 269-289	CA181743: sugar transporter
miR-5564	2	CA287982: 52-72, CA185455: 475-495	CA287982: Rf-1 gene for fertility restorer
Novel-12a	11	CA178353: 240-261, CA113700: 197-218, CA202476: 28-49, CA094946(1): 608-629, CA208275(1): 907-928, CA227712(1): 526-547, CA071984(1): 826-847, CA067469(1): 247-268, BQ535281(1): 819-840, CA068395(1): 484-505, BQ533665(3): 294-315	CA094946: cysteine proteinase inhibitor B
Novel-21	2	CA200000: 509-529, BQ534061(1): 382-402	BQ534061: LSG (LSG) gene, LSG-6 allele
Novel-23	1	BQ533059(2): 464-482	No homology
Novel-24a	41	DV548986: 7-30, CA218782: 70-93, CA101078: 371-394, CA086415: 43-66, CF574469: 55-78, CA267600(1): 1111-1134, CA199000(1): 440-463, CA176358(1): 345-368, CA220500(2): 866-889, CA125017(1): 880-903, CA091532(2): 752-775, CA074041(2): 570-593, BQ533191(2): 10-33, CA101090(1): 304-327, CA203894(1): 174-197, BU103176(2): 611-634, CA084181(1): 609-632, CA127474(4): 10-33, CA067099(2): 393-416, CA070907(2): 732-755, CA066863(2): 82-105, CA140768(2): 682-705, CA082646(1): 702-725, EC324620(1): 29-52, CA091242(1): 533-556, CA092719(1): 636-659, BQ534446(1): 344-367, BQ534446(2): 657-680, CA073046(2): 126-149, CA086354(3): 423-446, BQ533051(3): 294-317, CA098733(1): 237-260, CA098733(8): 35-58, CN609178): 282-305, CA066879(4): 666-689, CA066879(4): 355-378, BQ532994(1): 1031-1054, BQ534413(2): 74-97, BQ535543(5): 427-450, BQ530109(4): 655-678, BQ532969(9): 685-708	No homology

MiR156 is conserved in various plants and exhibits opposing functions in terms of plant shoot maturation, being highly expressed early in shoot development but decreasing in level over time in both *Arabidopsis* and maize (Wang *et al.*, 2009). Overexpression of miR156 prolonged expression of juvenile vegetative traits and delayed flowering (Gou *et al.*, 2011). A few studies have reported that miR156 expression was induced by stress. The expression level of the miR156 target CA091338 (the squamosa promoter binding protein, *SPBP*) did not significantly differ between control and test plants in the present study. In contrast, it has been reported that miR159 contains stress-related *cis*-elements including a W-box (TTGAC), a G-box (ACGTG), an ABREcore (ACGTGG/TC), a TGA-box (ACGTG), an LTRE-core (A/GCCGAC), a P-box (CCA/TA/TCC), and a GATA-box (GATATTT) (Zhou *et al.*, 2008). Transcripts accumulated in the inflorescences and floral tissues of several plants (Reyes and Chua, 2007), and expression of the miRNA was modulated by gibberellic acid (GA) during anther development (Achard *et al.*, 2004). In *Arabidopsis*, miR159 expression was induced by ABA and drought, and the miRNA regulated the expression of *AtMYB33*, *AtMYB35*, *AtMYB65,* and *AtMYB101* of the MYB family (Allen *et al.*, 2007).

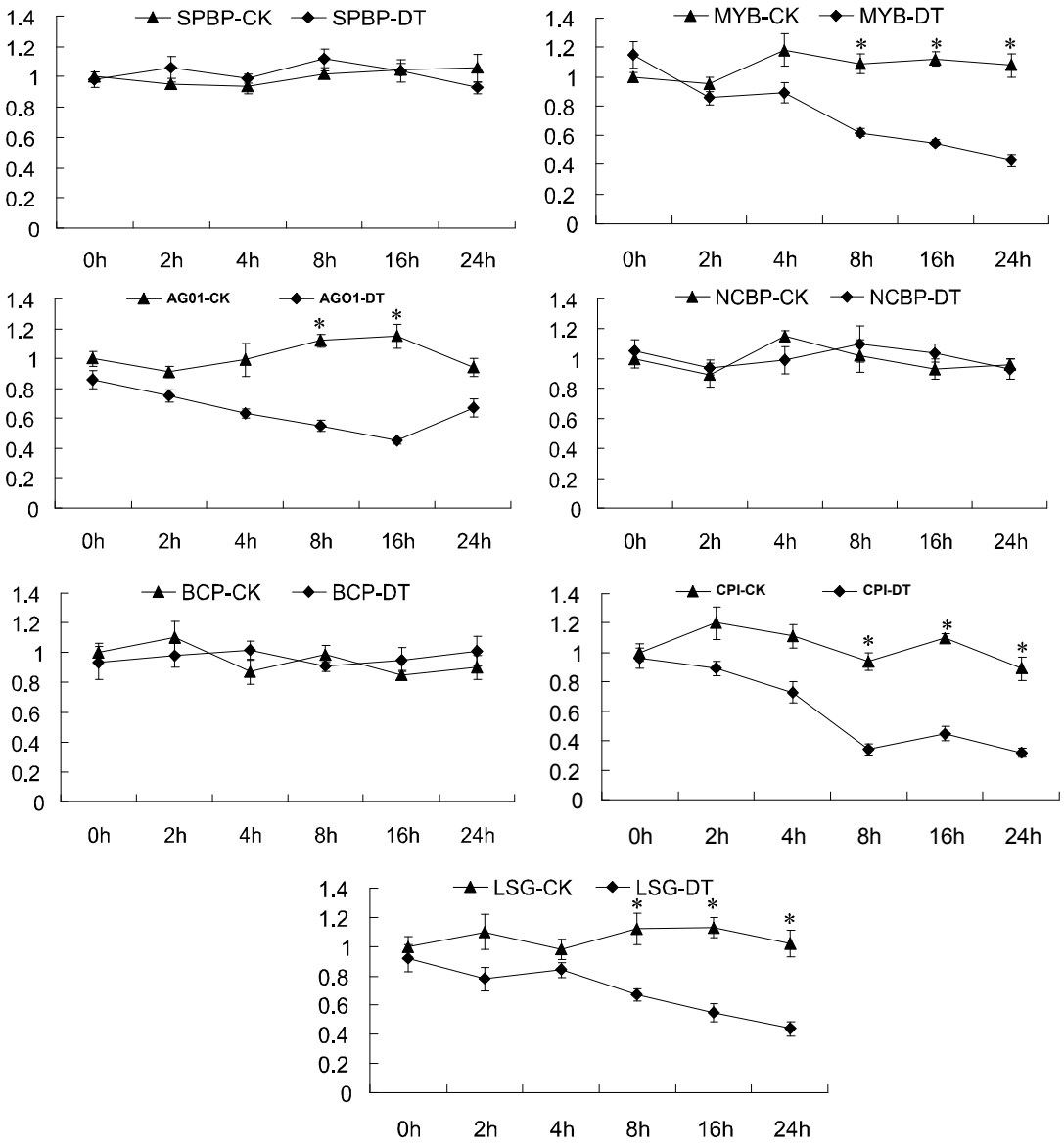

Figure 3. Expression of target genes in response to drought dress.
The *SPBP*, *MYB*, *AGO1*, *NCBP*, *BCP*, *CPI* and *LSG* were the target of miR156, miR159, miR168, miR396, miR408, novel-12a and novel-21, respectively. The CK was the control plant.
The DT was drought-exposed plants. Asterisks indicated significant differences of $P<0.05$.

In the present work, the expression levels of-miR159 in drought-exposed plants were higher than in controls. Computational methods were used to identify a possible miR159 target; this is a putative *MYB* gene (GenBank No. CA068132). The *MYB* genes encode important plant transcription factors, and many such genes are involved in the responses to biotic or abiotic stresses. In soybean, expression of 43 of 156 *MYB* genes was induced by ABA and the gene products participated in the response to drought stress (Liao *et al.*, 2008). Transcription of *MYB* genes in ROC22 was down-regulated in response to short-term PEG treatment. These results indicate that *MYB* genes are potential targets of miR159 in ROC22. Future work should include study of the functions of *MYB* families in sugarcane subjected to drought stress.

The targets of other miRNA families that were up- or down-regulated in terms of expression after exposure to drought stress included genes encoding the AGO1-like protein (the

AGO1-like gene), the nucleic acid binding protein (*NCBP*), the blue copper protein (*BCP*), the cysteine proteinase inhibitor B (*CPI*), and the loading stem gene (*LSG*). The potential targets of miR396 and miR408 are (respectively) the genes encoding nucleic-acid-binding protein and blue copper protein. However, the expression levels of these genes did not differ significantly between test and control plants. The transcription levels of other targets did differ significantly. AGO1 forms part of the RNA-induced silencing complex (RISC) which, when combined with miRNA, inhibits translation of target genes (Vaucheret, 2006). The AGO family of *Arabidopsis* has 10 family members (AtAGO1–10; Vaucheret, 2008). Only AtAGO1, AtAGO4, and AtAGO7 have been shown to exert cleavage activity (Vaucheret, 2008). AtAGO1 cut the *TAS1* gene (in the *ta*-siRNA pathway) when associated with miR173, and the *TAS4* gene when associated with miR828 (Mallory and Bouche, 2008). In the present work, the gene encoding the AGO1-like protein (the *AGO1-like gene*) was a potential target of miR168 and was down-regulated in response to drought stress, indicating that the transcript level of the *AGO1-like gene* per se may be regulated by miRNA families in ROC22. However, little effort has been devoted to analysis of structure and function within the AGO or AGO-like families of sugarcane; the topic requires further research effort.

The novel-12a and novel-21 miRNA families may be key components of the drought response of ROC22. One potential target of novel-12a is the gene encoding the cysteine proteinase inhibitor (*CPI*). In plants, accumulation of *CPI* transcripts is induced by biotic or abiotic stresses including fungal infection, mechanical damage, high temperature, and sodium chloride treatment (Kouzuma *et al.*, 2000; Kuroda *et al.*, 2001; Martinez *et al.*, 2005; Lima *et al.*, 2006). However, *CPI* expression in ROC22 was down-regulated during short-term PEG stress. A target of novel-21 may be a putative *loading stem gene* (*LSG*, BQ534061), which has nine alleles in sugarcane and is preferentially expressed in the sucrose-loading zone of the sugarcane stem (Moyle *et al.*, 2013). The gene encoding BQ534061 is similar to the *LSG-6* allele and is down-regulated in ROC22 during short-term PEG stress.

In sugarcane cultivar RB867515, the targets of the differentially expressed miRNA were predicted using an *in silico* approach and validated by RT-qPCR (Ferreira *et al.*, 2012). NAC transcription factor, Auxin-responsive factor TIR1-like protein, Glyceraldehyde 3-Phosphate Dehydrogenase, Lacasse 23-Like, Inorganic pyrophosphatase 2-like, UBX Domain Containing Protein and B-ZIP transcription factor may be the targets of miR164, miR393, miR394, miR397, miR399, miR528 and miR1432, respectively. These targets had the functions of transcription factors, kinases, phosphatases and chaperones. In our study, the targets of miR156, miR159, miR168, miR396, miR408, novel-12a and novel-21 were associated with stress resistance and growth. Four targets were up-regulated, i.e. *MYB*, *AGO1*, *CPI* and *LSG*. The expression of *SPBP*, *NCBP* and *BCP* had no change. Interestingly, the corresponding miRNA families of these targets were up-regulated. These results indicated that these targets had a certain correlation with the corresponding miRNA families, which may be sometimes affected by other factors, such as miRNA methylation and 3′-end uridylation activity (Chen, 2009; Voinnet, 2009). Therefore, the expression of the miRNA families and their targets did not directly display clear correlations with the differences in drought tolerance observed in different sugarcane cultivars (Ferreira *et al.*, 2012). However, our work showed clearly the expression pattern of miRNA families in sugarcane cultivar ROC22.

In summary, the short-term PEG stress induces differential expression of miRNA families and targets thereof in ROC22, and these may form part of the response to drought stress. As the full sequence of the sugarcane genome is not available, we may have missed some important functional small RNAs. In future, various sugarcane cultivars (including drought-resistant and drought-sensitive cultivars, and wild species) will be exposed to different drought conditions to identify new functional miRNA families or small RNAs and their associated targets, and to investigate the expression patterns of these materials. In addition, the key functions of miRNA families and target genes will be addressed. We seek to define the miRNA-mediated metabolic network triggered by drought stress in *S. officinarum*.

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