African Journal of Biotechnology Vol. 10(44), pp. 8695-8702, 15 August, 2011
Available online at http://www.academicjournals.org/AJB
DOI: 10.5897/AJB11.444
ISSN 1684–5315 © 2011 Academic Journals

Full Length Research Paper

MicroRNA expression profiling during upland cotton gland forming age by microarray and quantitative reverse-transcription polymerase chain reaction (qRT-PCR)

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Accepted 20 May, 2011

Plant microRNAs (miRNAs) have important impacts on growth, development, flowering, metabolism and response to stress. Studies indicate that post-transcriptional processes are important for regulating gene expression during development. However, we still have very limited knowledge on the regulatory mechanisms associated with this process. In particular, the function of miRNAs during gland morphogenesis in cotton remains unknown. In this study, we used the Affymetrix GeneChip miRNA Array (v11.0-ther Species) and quantitative reverse transcriptase-PCR (qRT-PCR) to identify additional microRNAs during gland morphogenesis of near-isogenic lines in upland cotton. The results showed that 30 miRNAs were differentially expressed: 24 up-regulated (miR156, miR157, miR166 and miR390 families) and six down-regulated (miR149, miR169, miR289, miR705, miR1224 and miR1227 families). Some microRNAs, such as ghb-miR169a_st and ghr-miR166b_st, were confirmed by qRT-PCR assays. There was no significant difference in miRNA levels between the microarray and qRT-PCR. Analysis of the transcript data for some miRNA target genes indicated that they play an important role in the pathogenesis and development of gland morphogenesis. In summary, our results showed that some known miRNAs were expressed in the gland of upland cotton, and most of them were of low abundance. This data may be useful in future studies associated with gland control involved in the terpenoid aldehyde biosynthesis pathway, genetic engineering and molecular breeding of cotton.

Key words: MicroRNA, cotton, gland morphogenesis, microRNA microarray, quantitative real-time reverse-transcription polymerase chain reaction (qRT-PCR).

INTRODUCTION

Cotton (*Malvaceae gossypium* L.) is one of the most important crops that produces fibre and seeds, and contributes enormously to the world economy. In addition to cotton fibre, cotton seed is among the most abundant protein meals, representing 6.9% of world protein meal production (Ash and Dohlman, 2006), next only to Soybeans and rapeseed. It has been compared favorably with other traditional food sources as a source of protein in several human nutrition studies (Lusas and Jividen, 1987). However, gossypol, stored in cotton glands, is toxic to non-ruminant animals and humans, which means that large amounts of cottonseed protein that could potentially provide the annual protein requirements for half a billion people are wasted (Cai et al., 2010). *Gossypium* species are charac-terized by their lysigenous glands containing terpenoid aldehydes, important secondary phytoalexins, consisting primarily of gossypol which constitutes one of the plant's important defense systems against pests and diseases. The best approach to this

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Abbreviations: miRNAs, MicroRNAs; **qRT-PCR,** quantitative reverse transcriptase-PCR.

issue is to create glandless seeds and glanded plant cotton (Cai et al., 2010).

MicroRNA (miRNA) is a form of a small, single-stranded RNA which is 18- to 25-nucleotides long. It is transcribed from DNA and instead of being translated into protein; it regulates the functions of other genes in protein synthesis (Chen et al., 2006). Thus, miRNAs are genes that modulate other protein-coding genes. They trigger the translational inhibition of target messenger RNAs by binding to their 3'-untranslated region (Lee et al., 1993; Slack et al., 2000).

Large-scale sequencing approaches have been used to explore small RNAs at the genome level in plants (Lu et al., 2005). To date, there are 15,172 miRNA entries in the miRBase 16.0 (http://www.mirbase.org), including 40 miRNAs identified and confirmed in *Gossypium*. miR156, 157, 160, 162, 164, 393, 399, 827 (Zhang et al., 2007), 396, 414, 782 (Khan et al., 2008), 829, 836, 845 and 865 families (Ruan et al., 2009; Abdurakhmonov et al., 2008) have been predicted and identified in cotton using bioinformatics (Zhang et al., 2009) and the Solexa method. *Gossypium* miRNAs function as regulators in a wide range of processes including expression during ovule (Abdurakhmonov et al., 2008) and fibre development (Kwak et al., 2009), leaf and flower development (Pang et al., 2009; Khan et al., 2008), and transcription factors, cell division-regulating proteins and virus response genes (Kwak et al., 2009).

Previous studies primarily focused on miRNA expression in ovules and fibre development. We performed microarray-based miRNA expression profiling and real-time reverse transcriptase-PCR (RT-PCR) to detect differences in miRNA expression profiles before and during cotton gland formation. Furthermore, we investigated the functional role of these miRNAs and the target genes of the selected miRNAs predicted by miRU. Studies on the relationship between miRNAs and the upland cotton gland at the molecular level would be an approach towards ultimate genetically engineering of the regulation of gland and gossypol metabolism.

MATERIALS AND METHODS

The near-isogenic line 3 (glandless seed) and line 11 (glanded) were obtained from the Cotton Institute, the Chinese Academy of Agricultural Sciences (Anyang, China). The seeds were sterilized in a solution containing 70% ethanol and 15% H_2O_2 and dipped in sterilized water. Sterilized seeds were allowed to germinate and bud on plates containing sterilized filter paper and water (25°C). Then, the pigment glands began to develop. The seed germination and formation of glands was observed by a dissecting microscope. The germinating seeds of near-isogenic line 11 were at stage I at 35 h (after germination, before the formation of glands) and stage II at 50 h (after germination, new glands begin to form) (Cai et al., 2003). The germinating seeds of line 3 were collected at the same age as stage II of line 11 seeds. The newly germinating hypocotyls of line 3 variety (about 2 cm, glandless seed and plant, control group) (3-1, 3-2, 3-3), and then the one with a little small red or black glands of line 11 variety (after germination, new glands begin to form, experimental group) (11-4, 11-5, 11-6) were used as the material

RNA preparation

Total RNA was isolated from each sample using the mirVana™ RNA Isolation Kit (Ambion, lot no. AM1560) following the manufacturer's instruction. The quality of the RNA was evaluated using an Agilent 2100 Bioanalyzer.

Affymetrix GeneChip miRNA Array analysis

In this study, we used the Affymetrix GeneChip microRNA Array (Affymetrix, Santa Clara, Ca.) to identify additional microRNAs involved in the *Gossypium hirsutum* during morphogenesis of gland. This array contains 7815 mature microRNAs from 71 organisms (miRBase v. 11.0), 4 replicate features per miRNA. The RNA was labeled using the FlashTag™ Biotin RNA Labeling Kit (Genisphere, Hatfield, PA) according to the manufacturer's instructions. FlashTag labeling is fast, simple, accurate, highly sensitive and reproducible. Starting with approximately 1 µg of total RNA (or LMW RNA enriched from 1 µg of the total), the process began with a brief tailing reaction followed by ligation of the biotinylated signal molecule to the target RNA sample. The high sensitivity of FlashTag is due to Genisphere's proprietary 3DNA dendrimer signal amplification technology. The 3DNA dendrimer is a branched structure of single and double stranded DNA conjugated with numerous labels 7-8. Whereas, other labeling strategies typically target a single biotin to the sample, and FlashTag's 3DNA molecule delivers approximately 15 biotins to the sample. For Affymetrix GeneChip miRNA assay, RNA samples were cleaned with RNase-Free DNase Set(50) (Qiagen, France, lot no. 79254). The GeneChip® Scanner 3000 scan and use the free miRNA QC Tool software for data summarization, normalization and quality control. Analysis of microarray data was carried out by SAM (significance analysis of microarrays) software. Screening condition was defined as FDR controlled at 5%, fold change of ProbeSet greater than 2.0 (fold change of gossypium probe more than 1.5).

miRNA target predictions

The miRNA target prediction was using software miRU (http://bioinfo3.noble.org/miRNA/miRU.htm). The mature miRNA sequences from the refined miRNA set were reverse complemented and matched against the TIGR Cotton Gene Index 6 database, with the parameters relaxed to allow up to three mismatches and score allowed = 3, G:U wobble pairs allowed = 6, other indels allowed = 1. A maximum of three mismatches (excluding mismatch at position 1) was allowed across the length of the mature miRNA.

Reverse transcriptase-PCR and data analyses

For the first strand cDNA synthesis experiment, 1 µg/conc. of total RNA from each sample was converted to cDNA in a 20 µl reaction containing 1 µl miScript Reverse Transcriptase Mix (Qiagen, France), and 4 µl 5 × miScript RT Buffer. PCR reaction tube was placed in the instrument at 37°C for 60 min, after the reaction at 95°C for 5 min to inactivate reverse transcriptase, and was then placed on ice.

qRT-PCR of miRNAs was performed using SYBR Green Real-time Quantitative PCR methods following the manufacturer's instruction. One microliter of the reaction mixture was added to 20 µl of PCR mixture containing 10 µl of SYBR Green Realtime PCR Master Mix (TaKaRa), a 0.4 µl 10 µM universal primer and 0.4 µl 10 µM primer assay, and 0.4 µl 50 × ROX Reference Dye II and 1 µl template. Real-time PCR was carried out in 7500 FAST (ABI) using the following thermal cycling profile: Initial step 95°C for 10 s, followed by 40 cycles of amplification (95°C for 5 s, 60°C for 34 s),

final step termination reaction at 4°C. All samples were run in triplicates.

Results were exported to calculate mean Ct, which was then used to calculate ΔCt value for each miRNA target based on the formula: ΔCt = Ct (target miRNA)-Ct (5S rRNA). ΔΔCt for each miRNA target was calculated using the formula ΔΔCt = ΔCt (experimental group) -ΔCt (control group). Relative Expression Quantity = $2^{-\Delta\Delta Ct}$.

RESULTS AND DISCUSSION

To identify novel miRNAs during gland morphogenesis, we performed microarray-based miRNA expression profiling and real-time RT-PCR, which are powerful tools for analyzing expression patterns.

Total RNA quality

Total RNA was extracted from samples of near-isogenic lines 3 (glandless seed) and 11 (glanded). RNA quality was evaluated using an Agilent 2100 Bioanalyzer. The ratios (OD_{260}/OD_{280}) of total RNA were 2.0, indicating that samples were suitable for subsequent experiments and analysis.

Microarray analysis of miRNA expression

We employed Affymetrix GeneChip miRNA microarray technique to profile global miRNA expression during morphogenesis of gland. Every sample was repeated three times to improve GeneChip accuracy. GeneChip array image with upland cotton gland miRNAs showed that the result of test is more stable. Correlation of all signal points intensity was more than 95% after normalizes.

The signal values of reference group were linearity. The average value of hybrid background signal was less than 100. Microarray data were no abnormal data, reproducible, signal uniformity and normal distribution. miRNAs were isolated from before and after the gland formation, and the profiles of the miRNAs expression changes were gained based on the data of the microarrary.

In comparison with before the formation of glands, there were 24 differentially up-regulated miRNAs and 6 differentially down-regulated miRNAs (Table 1) during upland cotton gland forming stages. These data indicated that upland cotton gland development involved a series of expression of sequential classes of miRNAs. The regulation of these miRNAs indicated that they might play important roles during pigment gland development. The results suggested that the miRNA expression profiles can be used as markers for developmental stages.

Real-time quantitative detection of miRNAs using the SYBR Green assay

Real-time quantitative PCR (qRT-PCR) has been used for independent validation of microarray data for the relative expression of miRNAs in the cotton pigment gland. The method has been demonstrated to be quantitative and sensitive, and is sufficiently precise to discriminate single nucleotide differences between miRNAs. Moreover, it requires as little as 50 to 100 ng of total RNA (Lu et al., 2005). qRT-PCR was performed using standard protocols of Applied Biosystems 7500 Fast Real-Time PCR System. The method relies on the primer extension conversion of RNA to cDNA by reverse transcription followed by quantitative, real-time PCR (Raymond et al., 2005). An aliquot of cDNA previously used to establish the sensitivity of end-point PCRs was amplified in real-time using 40 cycles of the SYBR Green I assay. The thermal denaturation protocol was performed at the end of the PCR to determine the number of products present in the reaction (Thomas et al., 2008). All reactions were run in triplicate and included no template and no reverse transcription controls for each gene. Three ΔCt values of the control and experimental groups were obtained for each miRNA gene and were considered significant when $P < 0.05$.

The expression levels of two glands associated with miRNAs correlated with the microarray data (Figure 1) were performed and repeated three times. The melting curve of ghb-miR169a_st, ghr-miR166b_st and reference genes (5S rRNA) consisted of a single peak, showing that the specificity of the PCR amplification was good. Data from the three experimental replicates provided better reproducibility. However, the expression of two miRNA families (miRNA156 and 157) that were identified on the array could not be confirmed by qRT-PCR. This may be due to the methylation of plant miRNA, which requires the use of another quantitative analysis procedure.

Expression levels of selected miRNAs in association with gland development from qRT-PCR analyses confirm data obtained by miRNA microarrays. The results of the two methods (array and qRT-PCR) are consistent in fold change. We concluded that these differences were primarily due to the limitations associated with using microarray technology. In short, the miRNA expression profiling by the Affymetrix GeneChip miRNA microarray and qRT-PCR provided some valuable information (Figure 2). However, the additional effort required to validate the data makes this approach less attractive for quantifying miRNA expression effects in target genes.

miRNA target gene predictions

The computational miRU method effectively predicts plant miRNA targets. We used this method to predict conserved mRNA targets of gland-associated miRNAs (Table 2). Mature miRNA sequences from the refined miRNA set were reverse complemented and matched against the TIGR Cotton Gene Index 6 database. The

Table 1. Differential expression miRNAs during gland forming age in cotton.

Parameter	Gene ID	Species scientific name	Fold change	q-value (%)	Localfdr (%)
Positive genes (24)	ath-miR157a_st	*Arabidopsis thaliana*	8.45	0.000	0.00
	ath-miR157b_st	*Arabidopsis thaliana*	6.37	0.000	0.03
	ath-miR157c_st	*Arabidopsis thaliana*	6.70	0.000	0.08
	ath-miR157d_st	*Arabidopsis thaliana*	6.66	0.000	0.00
	bna-miR156b_st	*Brassica napus*	8.03	0.000	0.01
	bna-miR156c_st	*Brassica napus*	7.86	0.000	0.00
	ghr-miR156c_st	*Gossypium hirsutum*	1.69	0.051	0.01
	ghr-miR166b_st	*Gossypium hirsutum*	1.83	0.000	0.80
	ghr-miR390a_st	*Gossypium hirsutum*	1.80	1.306	0.01
	ghr-miR390b_st	*Gossypium hirsutum*	1.71	0.052	0.01
	ghr-miR390c_st	*Gossypium hirsutum*	1.63	2.306	0.06
	gma-miR156c_st	*Glycine max*	7.76	0.000	0.05
	gma-miR156d_st	*Glycine max*	6.07	0.000	0.12
	gma-miR156e_st	*Glycine max*	8.12	0.000	0.03
	gra-miR157a_st	*Gossypium rammindii*	4.27	0.000	0.15
	gra-miR157b_st	*Gossypium rammindii*	3.61	0.000	0.19
	ptc-miR156g_st	*Populus trichocarpa*	6.59	0.000	0.05
	ptc-miR156h_st	*Populus trichocarpa*	7.95	0.000	0.00
	ptc-miR156i_st	*Populus trichocarpa*	7.15	0.000	0.11
	ptc-miR156j_st	*Populus trichocarpa*	8.64	0.000	0.02
	smo-miR156b_st	*Selaginella moellendorffii*	6.72	0.000	0.12
	vvi-miR156f_st	*Vitis vinifera*	6.27	0.000	0.11
	vvi-miR156g_st	*Vitis vinifera*	7.12	0.000	0.06
	vvi-miR156i_st	*Vitis vinifera*	6.80	0.000	0.00
Negative genes (6)	dme-miR-289_st	*Drosophila melanogaster*	0.44	0.000	0.01
	ghb-miR169a_st	*Gossypium herbecium*	0.51	0.000	0.56
	hsa-miR-149-star_st	*Homo sapiens*	0.33	0.076	0.02
	mml-miR-1227_st	*Macaca mulatta*	0.28	0.000	0.01
	mmu-miR-705_st	*Mus musculus*	0.29	1.921	0.02
	ptr-miR-1224-5p_st	*Pan troglodytes*	0.33	0.000	0.02

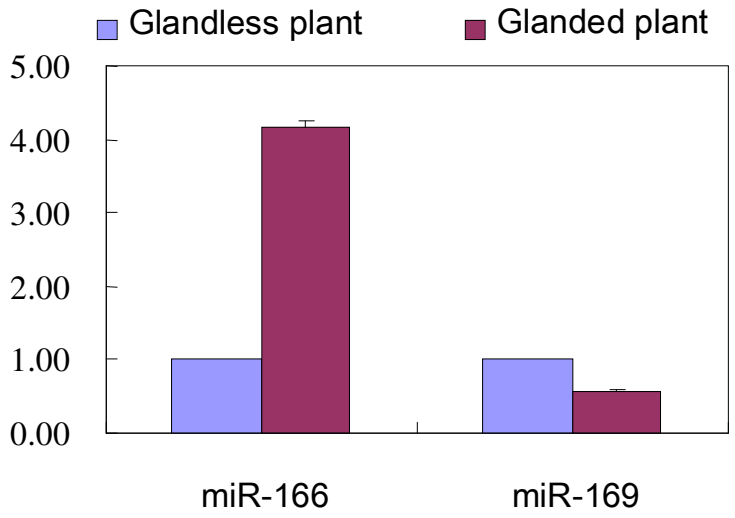

Figure 1. The results of miRNA quantitative real-time analyses of two gland-associated miRNAs (ghb-miR169a_st and ghr-miR166b_st) in glandless plant and glanded plant

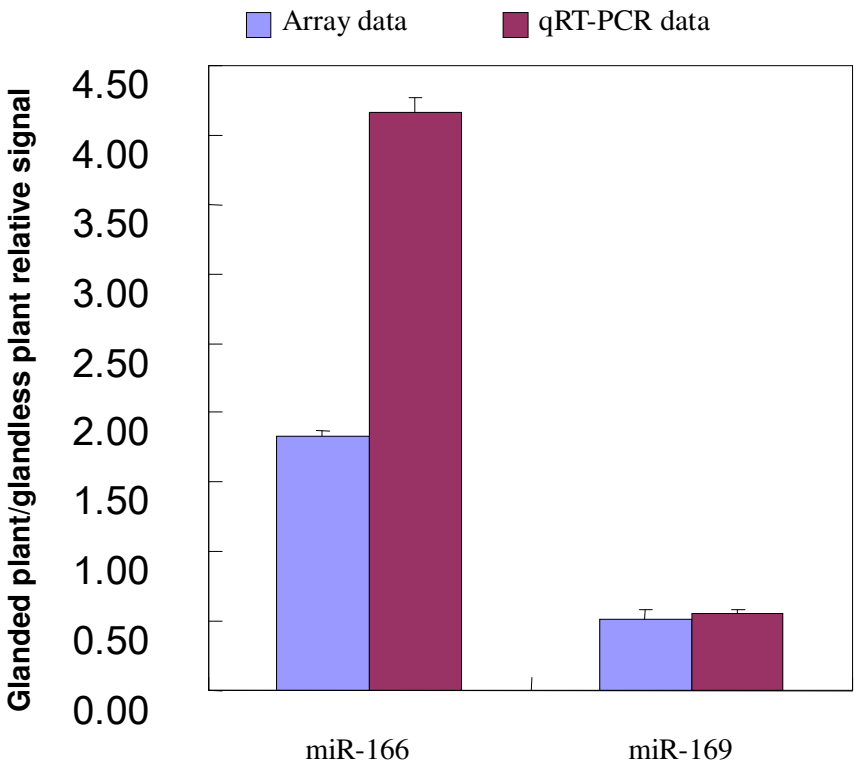

Figure 2. Comparison of expression profiles of quantitative RT-PCR (qRT-PCR) and microarray for two miRNAs.

functional annotation of the mRNA sequence of the partial target gene was not available in the database. Thus, we obtained the mRNA sequence that would serve as a probe by searching the encoded protein using BLASTX and predicted the protein function. Most target genes of miRNA are transcription factors or protein-coding genes associated with development and regulation of signal transduction proteins, such as triacyl-glycerol/steryl ester lipase-like protein, tubulin-binding cofactor C, translation initiation factor and lipid transfer protein. Analysis of the miRNA target genes revealed that they participated in the developmental and multicellular organismal process of cotton. They may also be involved in the plant hormone signaling pathway, growth of leaves, organ regeneration and formation, developmental stages of conversion and small RNA metabolism. Thus, these genes may play important roles in cell differentiation and gland formation in cotton.

Conclusions

We first used the qRT-PCR method for quantitative analysis of two microRNAs during gland morphogenesis. The method relies on the primer extension conversion of RNA to cDNA by reverse transcription, followed by real-time quantitative PCR. miRNAs were enriched from total RNA extracted from near-isogenic lines 3 (glandless) and 11 (glanded). Thirty (30) differentially expressed miRNAs were detected using GeneChip and two miRNAs were confirmed by real-time RT-PCR. Correlation of target genes involved in gland formation was predicted by programs and will be further analyzed.

The experiment detected target genes involved in multiple aspects of cell function. The squamosa promoter binding protein-like (SPL) gene family represents a group of structurally diverse genes encoding putative transcription factors found apparently only in plants. These genes show highly diverse genomic organizations and are found scattered over the genome. Some SPL genes are constitutively expressed, while transcriptional activity of others is under developmental control. SPL genes might play a role in the control of plant development. N-acylethanolamine amidohydrolase, triacylglycerol/steryl ester lipase-like protein and lipid-transfer proteins are responsible for metabolism, synthesis and transport of fat, respectively (Gomez et al., 2009). Tubulin binding cofactor C (TBCC) is a folding cofactor that participates in tubulin biogenesis. PHAVOLUTA-like HD-ZIPIII protein participates in any process that modulates the frequency, rate or extent of cellular DNA-dependent transcription. SET domains are associated with histone lysine methylation. Trehalose-6-phosphate synthase like protein and glycine-rich protein, all catalyzes reactions in sugar metabolism. In addition, target genes contain a complex series of basal translation

Table 2. The main target genes and encoded proteins of differentially expressed miRNAs.

Parameter	miRNA family	Target gene	Encoded protein
Up-regulated genes	miR156	TC41322	Annotation not available
	miR157 same as miR156 family	TC41514	Annotation not available
		TC34708	Squamosa promoter binding protein-like (SPL)
		TC35884	SPL
		TC36356	SPL
		TC38424	SPL
		CO092899	SPL
		TC34910	SPL domain class transcription factor
		TC31499	N-acylethanolamine amidohydrolase
		TC34032	N-acylethanolamine amidohydrolase
		TC37796	Triacylglycerol/steryl ester lipase-like protein
		TC39015	Transcription factor (TF)
		TC35182	Cryptochrome 1
		BF270515	Annotation not available
		CO120687	Tubulin binding cofactor C
		TC32276	Translation initiation factor
	miR166	TC31648	PHAVOLUTA-like HD-ZIPIII protein
		BQ409053	Lipid transfer protein
		TC29068	Ribonucleotide reductase small subunit
		TC29418	SET domain-containing protein
	miR390	CO115013	Leucine-rich repeat transmembrane protein kinase
Down-regulated genes	miR169	TC32844	TF
		CO107474	Unknown protein
		TC29763	Nuclear transcription factor Y subunit A-3
		TC40955	TF
		TC31014	TF
		TC36082	HMILVC
		BG442852	Annotation not available
		AW726500	Similar to GP
		BM360138	Neural Wiskott-Aldrich syndrome protein
	miR149	CO077095	Light sensitive hypocotyls 3
		TC31476	Trehalose-6-phosphate synthase like protein
		TC40663	Annotation not available
		AI729425	Glycine-rich protein
	miR289	BF271974	Oleosin
		TC31535	Chloroplast RNA processing protein-like
	miR705	No hit was found	
		TC32682	BCL-2-associated athanogene 7
		TC34306	2OG-Fe(II) oxygenase
		CF932092	Zn(II)2Cys6 transcription fact
	miR1224	CO091949	Integral membrane Yip1 family protein
		TC27280	Translation elongation factor 1A-1
		TC28087	Auxin response factor-like protein
		TC29677	SINAH1 protein

Table 2. Continue.

	TC36333	Acyltransferase
	TC33703	WD-40 repeat regulatory protein tup1 homolog - *Arabidopsis thaliana*
	TC36052	Homeobox protein NK-2 homolog B
	CO094562	Glycoprotein-like protein
miR1227	No hit was found	

factor. Analysis of the miRNA target genes showed that they participated in the developmental process of gland formation, suggesting that these genes may play important roles in cell differentiation during gland formation.

The results of this study showed the benefits of using the Affymetrix GeneChip miRNA Array platform for global miRNA expression profiling in cotton. However, the quantity of the detected miRNA and target gene mRNA was limited in our study. The mechanism underlying gene silencing of gland morphogenesis in cotton via miRNA regulation is complex. We identified a number of miRNAs specifically expressed during the upland cotton gland forming age. However, the functions and cell type distributions of most of the miRNAs remain unclear. These will be studied further in detail. Future studies should investigate not only miRNAs and their effect on the expression of target genes, but also their influence on protein expression.

These results may be useful in future studies of gene expression that regulates miRNA-169 and -166. They may also provide new information for molecular marker-assisted selection, molecular enhancement and candidate gene mining associated with gland traits in cotton. It would be a major breakthrough to convert cotton into both a fibre product and a food crop; it would be a great accomplishment for the sustainable development of agriculture. Studies on the relationship between glands and their secondary inclusions at the molecular level would be one approach to genetically engineer the regulation of the glands and gossypol content (Cai et al., 2010).

ACKNOWLEDGEMENTS

This work was supported by grants from the National Natural Science Foundation of China (No. 31071461 and No. 30771311), Chongqing Municipal Commission of Education(No. KJ110506), Natural Sciences Foundation of Chongqing cstc 2007 BB1328 and BB1329, CQUPT (A2008-04) and Nan'an 2008.

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