Full Length Research Paper

Additional insights into the adaptation of cotton plants under abiotic stresses by in silico analysis of conserved miRNAs in cotton expressed sequence tag database (dbEST)

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Abiotic stress is the primary cause of crop losses worldwide. In addition to protein coding genes, microRNAs (miRNAs) have emerged as important players in plant stress responses. Though miRNAs are key in regulating many aspects of plant developmental plasticity under abiotic stresses, very few information are available in cotton. Hence, this study was conducted to identify the phylogenetically conserved miRNAs in cotton, using computational approaches. In this paper, we reported a set of miRNAs such as miR159, miR165, miR170, miR319, miR529, miR828, miR869, miR1030, miR1884, and miR2118 that are likely to be involved in abiotic stress response. Although, few of them have been described in literature for their specific role in fiber development, literature survey have shown that they may also be involved in abiotic stress response. Interestingly, miRNAs reported in this study were found to have several targets that are involved in abiotic stress resistance. Considering all together, it was concluded that these newly identified conserved microRNAs in cotton have great potential in future efforts to improve abiotic stress tolerance in cotton.

Key words: miRNA, cotton, abiotic stress resistance, *in silico* analysis.

INTRODUCTION

miRNAs, also called 'killer RNAs', are a newly identified and extensive class of endogenous non-coding small RNAs, which negatively regulate almost all biological and metabolic processes at the post-transcriptional level

(Sun, 2011). Since *lin-4* was recognized as a first miRNA in 2001, thousands of miRNAs have been identified from various organisms including plants. In plants, many miRNA targets transcription factors, which in turn regulate specific subsets of genes and hence also is designated as "regulator of regulators" (Ambros, 2004). Identification of comprehensive sets of miRNAs and other small RNAs in different plant species is a critical step to facilitate our understanding of regulatory mechanisms or networks for target genes and cell development. Both experimental methods and computational approaches have been adopted to identify miRNAs in plants. Recently, as deep sequencing technologies have become more available and affordable, more and more studies have been employing deep sequencing to discover and functionally analyse miRNAs in plants. Sun (2011) summarized the major advantages and

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Abbreviations: GSSs, Genome survey sequences; HTGSs, high throughput genomics sequences; NRs, non-redundant nucleotides; BLAST, Basic Local Alignment Search Tool; dbEST, expressed sequence tag database; NCBI, National Center for Biotechnology Information; ABA, abscisic acid; GA, gibberellic acid; AREs, anaerobic responsive elements; ABREs, abscisic acid response elements; HSEs, heat-stress-responsive elements; LTRs, low-temperature-responsive elements (LTRs).

disadvantages of each method and explained that the simplest and rapid method of identification of miRNAs is relied on *in silico* analysis.

In silico identification of miRNA is possible because plant miRNAs generally exhibit a high degree of conservation of nucleotides (Sunkar and Jagadeeswaran, 2008). Many plant miRNAs are evolutionarily conserved from angiosperms to mosses (Sunkar et al., 2008). Using publicly available nucleotide databases, numerous miRNAs were identified in diverse plant species with the aid of computational approach (Sunkar et al., 2008). Though computational approaches are successful, they require knowledge of the complete genome sequence, which is unavailable for most plant species. However, large genomic data in the form of genome survey sequences (GSSs), high throughput genomics sequences (HTGSs) and non-redundant nucleotides (NRs), are available for several plant species and can be used for identification of conserved miRNAs. On the other hand, sequence similarity cannot guarantee that the obtained sequences are real miRNAs due to the fact that miRNAs are only about 20 to 22 nt in length, and the high identity percentage in pairwise alignment may be caused by a stochastic match rather than by sequence homology. To better identify miRNAs and reduce the false positives, homolog searches always need to be combined with the major characteristics of miRNAs, particularly the stem loop hairpin structure (Ambros, 2004). It has also been noticed that the number of plant miRNAs appears not to be saturated, and many other functional miRNAs in plant species remain to be investigated. Compared to annotated miRNAs from Arabidopsis and rice (Sun, 2011), very few miRNAs from cotton plants have been identified which is suggesting that miRNA research is still limited to a few plant species, with a majority of them being model species.

Cotton (*Gossypium* spp.,) is a very important natural textile fiber source, and cotton seed is a significant food source for human and livestock. India has the largest area dedicated to cotton production worldwide; however, the cotton production is limited by several constraints, including abiotic stresses such as drought, salinity and high temperature. Genetic improvement of cotton with abiotic stress resistance will be a viable and cost effective option to mitigate the abiotic stresses. Though significant progress has been documented before, the knowledge on stress resistance mechanism at molecular level is not yet fully identified. Despite the progress that has been made in unravelling the complex stress response mechanisms, particularly in the identification of stress responsive protein-coding genes, the key factors such as miRNAs that regulate the expression of these stress responsive genes is not yet completely documented (Wang et al., 2011). Hitherto, several miRNA family and their targets in cotton ovule development and other developmental process had been identified by computational methods based on the conserved characterization of miRNAs (Qiu et al., 2007; Zhang et al.,

2007; Abdurakhmonov et al., 2008; Barozai et al., 2008; Sunkar and Jadadeeswaran, 2008; Ruan et al., 2009). Deep sequencing studies had also reported several validated miRNAs (Kwak et al., 2009; Zhang and Pan, 2009) involved in cotton growth and development. Currently, there are 89 miRNAs deposited under *Gossypium* at Plant Micro RNA database (PMRD). Interestingly, both highly conserved and several less conserved miRNAs families specific to cotton tissues were detected. However, cotton miRNAs and its role in abiotic stress resistance have been investigated in only one report (Yin et al., 2011), even though it has been expressively established in Arabidopsis, rice and other crops (Sunkar et al., 2008). Identification of miRNAs in cotton, in response to abiotic stresses, may have important implications for gene regulation under abiotic stresses, and also contribute significantly to the goal of having a complete profile of miRNAs in cotton. In addition, knowledge on miRNA-guided stress regulatory networks should provide new tools for the genetic improvement of plant stress tolerance (Sunkar et al., 2008).

Hence, in view of the importance of miRNAs in regulation of abiotic stress resistance in crop plants, this study was conducted with the following objective: to identify novel microRNAs in cotton which are regulated by abiotic stresses using computational approach. Using this strategy, we analysed all the miRNAs that are reported in other plants but not yet reported in cotton. Using Basic Local Alignment Search Tool (BLAST) analysis, we found that 36 of these miRNAs belonging to 11 families are found in cotton expressed sequence tag database (dbEST). Critical analyses of these miRNAs and their target sequences have helped us to frame the hypothesis, that these miRNAs may play key role in controlling abiotic stress resistance in cotton.

MATERIALS AND METHODS

Identification of conserved plant microRNA using cotton dbEST BLAST analysis

All the available miRNA sequences from all plants deposited at miRBase (release 18; http://microrna.sanger.ac.uk/) and Plant microRNA database (PMRD) (http://bioinformatics.cau.edu.cn/PMRD) were retrieved. Matured miRNA sequences from all the plants with exactly the same size and nucleotide composition to the previously reported for cotton were removed, and only unique miRNA sequences from the database were utilized in this study. Those unique plant miRNAs were aligned against *Gossypium* (Taxid: 3633) ESTs available at the National Center for Biotechnology Information (NCBI), using BLASTN (2.2.25 release). All other parameters were set as default. The cotton EST sequences which closely matched with the miRNA sequences of other plants were manually chosen and reported as newly found putative miRNAs in cotton. Those cotton EST sequences which has query coverage of 95 to 100%, and identity of < 40 to maximum of 50% were considered to report as new putative miRNA in cotton. Further, CLUSTALW multiple alignment tool (www.ebi.ac.uk/Tools/msa/clustalw2) was used to find any base

pair substitutions in cotton miRNA with that of same miRNAs family of other plants. The nucleotide variations were reported when they have query coverage and identity of the above said values.

RNA secondary structure and target prediction analysis

The secondary structures were predicted for the newly identified miRNAs using RNA folding tool available at Mfold 3.2 (http://mfold.rna.albany.edu/?q=mfold/RNA-Folding-Form). Available cotton EST sequence containing the mature miRNA sequence was given in this tool in FASTA format. All the parameters were set as default, and the potential miRNA structures were selected. In order to predict the target genes, newly identified miRNA sequences from cotton dbEST were submitted to PSRNA target tool (http://plantgrn.noble.org/psRNATarget/) by specifying "search on *Gossypium* DFGI gene index release 11" (dated 15th November, 2011) using the default parameters.

RESULTS

Identifying new plant microRNAs

After obtaining the miRNAs from all the plants, we analysed cotton EST database in order to find the new putative miRNAs which have not yet been reported in cotton at miRBase and PMRD databases. Our strategy includes the following steps. First, the miRNAs from all plants other than cotton miRNAs were retrieved. Then we searched the collected plant miRNA sequences against cotton EST databases using BLASTN 2.2.25. Sequences with 0 mismatch miRNA nucleotides were manually chosen (Table 1) and hence all the reported miRNA in Table 1 showed 100% sequence identity. Totally, 11 conserved candidate miRNA families that were representing 36 members in different plants were identified in this study. An attempt had also been made to document if there was any nucleotide change in the reported cotton miRNA with the same miRNA identified in other plants. They were blasted using BLASTN 2.2.25 against cotton EST database as before. The cotton EST hits which has 0 to 3 mismatch were taken into consideration. Fascinatingly, we were able to notice several number of deposited cotton miRNAs having one to three nucleotide change(s) in the matured sequences when compared with the same miRNA family of other plants (because of the huge size of the table, the data was not shown here). Most of the reported miRNAs had one mismatch, and the similarity percentage was between 40 to 50%. Interestingly, some of the miRNA sequences in other plants had two nucleotides mismatches with the same miRNA family reported in cotton but their similarity percentages was high (40 to 50%).

Secondary structure prediction

To be annotated as a miRNA, it is essential to establish

that sequences representing both miRNA and miRNA* should be identified in the same transcript containing miRNA candidates. MiRNA* sequences of those newly identified conserved miRNA families were analysed using Mfold tool, and all the putative miRNAs identified in this study have shown potentially folded structures. As an example, miR1884b:miR1884b* has been shown in Figure 1. Hairpin secondary structures of conserved miRNA precursors, mature miRNA positions are highlighted in red.

miRNA target analysis

Target prediction of the conserved miRNA families was performed by miRU (http://bioinfo3.noble.org/psRNATarget/) using nearly perfect sequence complementary as criteria. The identified conserved miRNA and their predicted targets are listed in Table 2 along with E-value and target accessibility. The interesting point to note in this result is, more than 90% of the identified target genes were involved in abiotic stress resistance. For example, the role of MYB related genes (Table 2) in abiotic stress resistance has been shown in several plants (Sun, 2011).

DISCUSSION

Plant growth and development is highly dependent on a variety of environmental conditions, such as temperature, light, water availability and soil conditions that strongly affect productivity worldwide. Therefore, there is an urgency to understand how plants behave when facing adverse conditions, in order to reduce productivity losses in sub-optimal environments. Plants have evolved complicated mechanisms to overcome a variety of environmental stresses, where miRNA also plays an important role (Zhang et al., 2008). About 25.8% of ESTs, containing miRNAs, were found in stress-induced plant tissues (Zhang et al., 2005) and many studies have provided supportive evidence for this hypothesis (Liu et al., 2008; Sunkar et al., 2008). Many miRNAs that are up- or down-regulated in drought condition were discovered by global miRNA expression profiling experiments, with either microarray hybridization or small RNA deep sequencing (Barrera-Figueroa et al., 2011). It has also been found that there is a cross-talk among the high salinity, drought, and cold stress signalling pathways, which extends to the current view about miRNA as ubiquitous regulators under stress conditions (Liu et al., 2008).

Several recent experimental studies have shown that abiotic stresses (drought, salinity, high and low temperature, and osmotic stress) induce differential expression of a set of miRNAs in a variety of plant species, including arabidopsis, rice, maize, populus, and

Table 1. Identification of new miRNAs in cotton.

S/N	miRNA ID	Organism in which miRNA is identified	miRNA sequence	Cotton dbEST ID which contains the same miRNA	Score	E-value
1	ahy-miR159 ath-miR159a ptc-miR159b ptc-miR159c	*Arachis hypogaea* *A. thaliana* *Populus trichocarpa* *P. trichocarpa*	UUUGGAUUGAAGGGAGCUCUA	ES824206.1	34.2	0.080
	gma-miR159d	*Glycine max*	AGCUGCUUAGCUAUGGAUCCC			
2	mtr-miR165 hce-miR165	*Medicago truncatula* *Hedyotis centranthoides*	UCGGACCAGGCUUCAUUCCCC	ES803085.1	42.1 34.2	3e-04 0.080
	ath-miR165a,b	*A. thaliana*	UCGGACCAGGCUUCAUCCCCC		34.2	0.080
3	ace-miR170a,b,c,d stu-miR170 sbi-miR170 ppd-miR170 ath-miR170m ath-miR170n zma-miR170p	*Allium cepa* *Solanum tuberosum* *Sorghum bicolor* *P. trichocarpa x P. deltoides* *A. thaliana* *A. thaliana* *Zea mays*	UGAUUGAGCCGCGCCAAUAUC	ES822760.1	42.1	3e-04
	osa-miR170a osa-miR170b gma-miR170m hvv-miR170 ppe-miR170 tae-miR170b zma-miR170m	*Oryza sativa* *O. sativa* *G. max* *Hordeum vulgare* *Prunus persica* *Triticum aestivum* *Zea mays*	UGAUUGAGCCGUGCCAAUAUC		34.2	0.080
4	vvi-miR828a vvi-miR828b	*Vitis vinifera* *V. vinifera*	UCUUGCUCAAAUGAGUAUUCCA UCUUGCUCAAAUGAGUGUUCCA	ES846207.1 DR453866.1	36.2 40.1	0.024 0.002
5	gma-miR2118	*G. max*	UUGCCGAUUCCACCCAUUCCUA	CO108124.1	36.2	0.024
6	mtr-miR319b	*Medicago truncatula*	UUGGAUUGAAGGGAGCUCUA	ES824206.1	32.2	0.26
7	gso-miR2118	*G. soja*	UUGCCGAUUCCACCCAUUCCUA	CO108124.1	36.2	0.023
8	gma-miR869	*G. max*	CAUGGUUCAAUGCUGGUGUUA	DR458018.1	34.2	0.080
9	mtr-miR529	*M. truncatula*	CUCUCACCUCUCUCUUCUUCU	BQ405642.1	34.2	0.080
10	osa-miR1884b	*O. sativa*	AAUGUAUGACGCUGUUGACUUUUA	EX172380.1	46.1	3e-05
11	ppt-miR1030a ppt-miR1030b	*Physcomitrella patens*	UCUGCAUCUGCACCUGCACCA	DT560748.1	34.2	0.079

Table 2. Results of cotton target gene prediction for the newly identified cotton miRNA.

miRID	Cotton psRNA accession number	E-value	Target accessibility (UPE)	Target Discription	Inhibition
miR159	EV486340	2.5	17.3	Beta-ketoacyl-CoA synthase	Cleavage
miR165	ES797005	3.0	24.276	Glutathione S-transferase	Cleavage
	ES834023	3.0	24.803	Glutathione S-transferase	Cleavage
miR828a	CO085283	2.5	11.769	MYB-like DNA-binding domain protein	Cleavage
	TC2263	3.0	13.331	GHMYB36	Cleavage
	TC235742	1.0	14.943	MYB-like DNA-binding domain protein	Cleavage
	DV437953	1.5	18.847	MYB family transcription factor	Cleavage
	TC255078	2.0	9.303	MYB family transcription factor	Cleavage
	TC245389	2.0	12.719	MYB-like DNA-binding domain protein	Cleavage
	TC252232	2.0	12.719	MYB-like DNA-binding domain protein	Cleavage
	TC252167	2.5	11.307	BNLGHi233	Translation
	TC257339	2.5	11.787	BNLGHi233	Translation
	NP869199	2.5	15.715	Transcription factor myb109	Cleavage
	TC230487	2.5	15.715	Myb family transcription factor 109	Cleavage
	TC241019	3.0	13.331	GHMYB36	Cleavage
miR828b	CO085283	1.0	14.943	MYB-like DNA-binding domain protein	Cleavage
	TC235742	1.5	18.847	MYB-like DNA-binding domain protein	Cleavage
	DV437953	2.0	9.303	MYB family transcription factor	Cleavage
	TC255078	2.0	12.719	MYB family transcription factor	Cleavage
	TC245389	2.0	12.719	MYB-like DNA-binding domain protein	Cleavage
	TC252232	2.5	11.307	MYB-like DNA-binding domain protein	Cleavage
	TC252167	2.5	11.787	BNLGHi233	Translation
	TC257339	2.5	15.715	BNLGHi233	Translation
	NP869199	2.5	15.715	transcription factor myb109	Cleavage
	TC230487	2.5	15.715	Myb family transcription factor 109	Cleavage
	TC268719	3.0	17.302	Myb transcription factor MYB30	Cleavage
	TC185502	1.5	14.943	MYB-like DNA-binding domain protein	Cleavage
	DV437953	2.0	18.847	MYB family transcription factor	Cleavage
	TC204562	2.5	9.303	Myb family transcription factor 2	Cleavage
	TC194914	2.5	12.719	MYB-like DNA-binding domain protein	Cleavage
	TC201708	2.5	12.719	MYB-like DNA-binding domain protein	Cleavage
	TC201640	3.0	11.307	BNLGHi233	Translation
	TC206803	3.0	11.787	BNLGHi233	Translation
	TC180411	3.0	15.715	Myb family transcription factor 109	Cleavage
	TC218138	3.0	17.302	Myb transcription factor MYB30	Cleavage
	TC135442	1.5	14.943	MYB-like DNA-binding domain protein	Cleavage
	TC154488	2.5	9.303	Myb family transcription factor 2	Cleavage
	TC144838	2.5	12.719	MYB-like DNA-binding domain protein	Cleavage
	TC151641	2.5	12.719	MYB-like DNA-binding domain protein	Cleavage
	TC151574	3.0	11.307	BNLGHi233	Translation
	TC156739	3.0	11.787	BNLGHi233	Translation
	NP869199	3.0	15.715	transcription factor myb109	
	TC130346	3.0	15.715	Myb family transcription factor 109	
	TC168071	3.0	17.302	Myb transcription factor MYB30	Unpredictable
	TC85900	1.5	14.943	MYB-like DNA-binding domain protein	
	DV437953	2.0	18.847	MYB family transcription factor	
	TC104698	2.5	9.303	Myb family transcription factor 2	Cleavage

Table 2. Contd.

	TC101889	2.5	12.719	MYB-like DNA-binding domain protein	Cleavage
	TC95133	2.5	12.719	MYB-like DNA-binding domain protein	Cleavage
	TC101824	3.0	11.307	BNLGHi233	Translation
	TC106913	3.0	11.787	BNLGHi233	Translation
	NP869199	3.0	15.715	transcription factor myb109	Cleavage
	TC80980	3.0	15.715	Myb family transcription factor 109	Cleavage
	TC118126	3.0	17.302	Myb transcription factor MYB30	Cleavage
miR2118	TC5598	1.5	21.967	NBS-type resistance protein	Translation
	ES807202	1.5	24.015	NBS-type resistance protein	Cleavage
miR869	TC184013	2.0	7.597	MADS-box protein MADS4	Translation
	TC216699	2.0	10.744	MADS-box protein MADS4	Translation
	TC260	2.5	19.744	Alpha-tubulin	
	TC235637	2.5	19.729	Alpha-tubulin	
	TC237554	2.5	20.582	Alpha-tubulin	
	EX172142	2.5	20.656	Alpha-tubulin	Unpredictable
	TC256259	2.5	20.656	Alpha-tubulin	
	BG440577	3.0	0.168	ACS-ike protein	
	TC249535	3.0	13.574	Heat shock protein	
	TC220509	1.5	21.441	Elongation factor 1-alpha	
	TC185391	2.5	19.729	Alpha-tubulin	
	EX172142	2.5	20.656	Alpha-tubulin	Cleavage
	TC205723	2.5	20.656	Alpha-tubulin	
	BG440577	3.0	0.168	Alpha-tubulin	
miR529	TC199026	3.0	13.81	Heat shock protein	Unpredictable
	TC135331	2.5	19.729	Alpha-tubulin	
	TC137173	2.5	20.582	Alpha-tubulin	
	EX172142	2.5	20.656	Alpha-tubulin	
	TC155654	2.5	20.656	Alpha-tubulin	Cleavage
	BG440577	3.0	0.168	Alpha-tubulin	
	TC148957	3.0	13.81	Heat sock protein	
	TC85795	2.5	19.729	Alpha-tubulin	
	TC87611	2.5	20.582	Alpha-tubulin	
	EX172142	2.5	20.656	Alpha-tubulin	Unpredictable
	TC105847	2.5	20.656	Alpha-tubulin	
	BG440577	3.0	0.168	Alpha-tubulin	
	TC99232	3.0	13.81	Heat shock protein	

tobacco (Sun, 2011). However, very little information is available in cotton (Yin et al., 2011). In this paper, we provided literature based evidences that have shown in other crops for the involvement of the newly identified putative miRNAs in the regulation of gene expression under abiotic stress in cotton. Since, almost all of the miRNAs are evolutionarily conserved, miRNA-mediated regulatory mechanisms in responding to environmental stresses in plants may also be evolutionarily conserved. To this end, cotton EST analysis plays a vital role in identification of undiscovered miRNAs by computational techniques using novel bioinformatics approach. Although, this is not a direct evidence for miRNA-involved plant response to environmental stress, this at least gives clues to the role of miRNAs in plant responses to stress. This study has identified 11 families of conserved miRNA in cotton. Though miR159, miR165, miR170, miR828 and miR319 were reported in publication, they have not been included in miRBase or PMRD. Further, all the miRNA families that have been described in this paper have

Figure 1. Formation of typical stem loop structure when analysing the cotton dbEST ID EX172380.1 that contains miR1884b.

been found to be associated with drought stress in previous studies, and the following facts describe their involvement in detail.

Abscisic acid (ABA) and gibberellic acid (GA) are two kinds of phytohormones which are involved in plant responses to different environmental stresses (Sunkar and Zhu, 2004). Two independent studies revealed that ABA and GA treatment regulates miR159 expression (Sunkar and Zhu, 2004; Achard et al., 2004; Zhang et al., 2008). Accumulation of miR159 is induced by ABA through the seed specific ABA-dependent transcription factor ABI3 (Reyes and Chua, 2007). miR159 targets the cleavage of transcripts for the MYB transcription factors,

MYB33 and MYB101 that are positive regulators of ABA signalling. Thus, miR159 may act to attenuate ABA responses in plants. Further, miRNA 159 were regulatedby GA, cold, water and sulfate starvation stress (Zhang et al., 2005). Different concentrations of NaCl down-regulated the expression of miR159 in tobacco plants (Frazier et al., 2011). In an another study, the expression of maize miR159 was found to be induced after submergence (Zhang et al., 2008). Therefore, miR159 appears to be involved in plant responses to a variety of biotic and abiotic stresses. In a recent study, Yin et al. (2011) have shown that miR159 was down-regulated in a salt sensitive cotton cultivar under salt stress. Interestingly, when we analysed the promoters of miR159 in different plants, they have the Skn-1 motif required for endosperm expression and P-box motif for the gibberellin responsive element, GC motif which is a enhancer like element involved in anoxic specific indelibility, TATC element involved in gibberellin responsiveness besides having a TATA-box, the core element for start of transcription and anaerobic responsive elements (AREs) (Zhou et al., 2010). This enrichment of stress responsive cis-elements provide additional evidence that miR159 is very likely to be involved in the response to abiotic stress.

The class III homeodomain leucine-zipper (HD-ZIP III) genes are thought to be targets of miR165. Zhao et al. (2007) demonstrated a link between miR165 overexpression, and altered expression of genes involved in auxin signalling and vascular development. miR165 may also be involved in the developmental polarity of leaves and roots (Rubio-Somoza and Weigel, 2011). Many of these processes are involved in auxin signalling. Given the key role of auxin in lateral root development, and the fact that several miRNAs are linked to auxin signalling, it is not surprising that miRNAs play an important role in lateral root development. It is needless to emphasise the importance and involvement of well-developed roots in abiotic stress resistance mechanisms. Leaf development, shape and function are dependent on the vasculature, which not only provides mechanical support, but also conveys metabolites and signalling molecules. Elements of the miR165 and miR159 nodes have essential roles in the development of leaf vasculature (Rubio-Somoza and Weigel, 2011). Further, it has also been shown that miR165 was up regulated under cold stress and targets *Phabulosa*, homeobox genes (Zhou et al., 2010; Lv et al., 2010). miR165 may also be involved in responses to light and radiation stimuli. Zhao et al. (2007) gathered data from promoter element analysis and the expression of protein-coding genes predicted to be targeted by miRNAs, and identified *in silico* a group of potentially UV-B inducible miRNAs in Arabidopsis. Later, using a home-made array, Jia et al. (2009) identified miRNAs in black cottonwood that were differentially expressed upon 2 h of treatment with the same radiation. As previously predicted for Arabidopsis (Zhao et al., 2007), miR165 was induced in these conditions,

whereas the same pattern was not observed for miR159 (Jia et al., 2009). Also, in both Arabidopsis and *Populus tremula*, the majority of the selected miRNAs exhibited stress and light-inducible *cis*-acting elements, like GT-1 site (GGTTAA) and I-boxes (GATAAGA), upstream of their coding genes. Besides being regulated by UV radiation, miR165 also seem to be regulated by the photoperiod (Jia et al., 2009; Zhou et al., 2010).

Another miRNA identified in this study, miR319, has several roles in abiotic stress resistance, among them, the repression of the onset of senescence has been considered as key activity. miR319 over expression causes plants to stay green much longer while compromised miR319-dependent regulation of one of its main targets, TCP4, results in increased expression of genes that are normally active in older leaves (Rubio-Somoza and Weigel, 2011). miR319/Jaw overexpression also leads to delayed flowering. miR319/Jaw targets a group of TCP transcription factors for which a role in flowering is currently unknown (Palatnik et al., 2003). In plants, oxidative stress usually emerges as a secondary effect of abiotic stress conditions and is highly responsible for losses in crop productivity. Several works have reported the involvement of miRNAs in counteracting this stress (Trindade et al., 2011). In an attempt to mimic oxidative stress, Li et al. (2011) treated rice seedlings with different concentrations of H_2O_2, and found that the conserved miR319 were down-regulated in this conditions. miR319 was shown to be up-regulated by both GA3 and ABA, and down-regulated by cytokinins (Liu et al., 2009), suggesting that miRNAs can be involved in processes of crosstalk between different phytohormones and between those and abiotic stress conditions. For example, miR165 and miR319, were up-regulated by both high salinity and cold in Arabidopsis (Liu et al., 2009) and miR319 and miR165 was found to be conserved in more than 40 species (Sunker and Jagadeeswaran, 2008). miR319 has been shown to be differentially expressed in maize lines under drought stress; however it has not shown any response to salt stress in maize (Kong et al., 2010). miR319 was also found to be regulated during cold stress in sugarcane (Thiebaut et al., 2011). This finding will be useful for tracing the evolution of small RNAs by examining their expression in common ancestors besides unravelling the evolutionary role of miRNAs in plants against abiotic stresses.

Interestingly, miR159, miR165 and miR319 target transcription factors are involved in further regulation of gene expression and signal transduction that probably function in stress responses (Jones-Rhoades and Bartel, 2004; Sunkar and Zhu, 2004; Liu et al., 2008). In particular, though miR159 and miR319 families share sequence identity at 17 of 21 nucleotides, yet they affect different developmental processes through distinct targets. miR159 regulates MYB mRNAs, while miR319 predominantly acts on TCP mRNAs. In the case of

miR319, MYB targeting plays at most a minor role because miR319 expression levels and domain limit its ability to affect MYB mRNAs. In contrast, in the case of miR159, the miRNA sequence prevents effective TCP targeting (Palatnik et al., 2007). Further, Liu et al. (2008), identified several known stress-responsive elements, such as the ABA-response elements (ABREs), anaerobic induction elements (AREs), MYB binding site (MBS) involved in drought inducibility, heat-stress-responsive elements (HSEs), low-temperature-responsive elements (LTRs), and defence and stress-responsive elements (TC-rich repeats) in miR159, miR165 and miR319. In plants, most ABA-responsive genes have the conserved ABREs in their promoters, which are significant cis-elements for genes responsive to abiotic stress in Arabidopsis. The presence of ABREs and AREs suggests that these miRNA might be regulated by stress conditions as protein coding genes.

This study identified miR529 as new putative cotton miRNA. MiR529 was reported as drought-associated miRNA in rice (Zhou et al., 2010) contrary to the pattern that was found in cowpea, it was down regulated under drought stress in rice (Barrera-Figueroa et al., 2011). It is not clear whether it was caused by different sampling time or tissue, or species-specific stress response mechanisms. Deep sequencing of Brachypodium small RNAs have shown that miR529 was also involved in response to cold stress (Zhang et al., 2009). Li et al. (2011) have identified H_2O_2 responsive and more abundant miR529 in the seedlings of rice. Similarly, it was found that miR2118 was up regulated under water deficit and ABA treatments (Arenas-Huertero et al., 2009) and it has also been shown to be strongly expressed under nutrient deficiency and manganese toxicity in common bean, irrespective of the stress treatments used (Lopez et al., 2010). In addition, significant level of expression of miR2118 was reported in drought tolerant cowpea genotype (Barrera-Figueroa et al., 2011). miR2118 was also up regulated in response to drought stress in *Medicago truncatula* (Wang et al., 2011). Further, it has been shown that miR2118 targets TIR-NBS-LRR domain protein, which usually expressed in response to drought, cold, salinity and ABA (Wang et al., 2011). Under drought stress, Wang et al. (2011) found that there was up-regulation of miR2118, whose targets may be proteins associated with disease resistance. Kulcheski et al. (2011) have identified mir2118 as a novel miRNA involved in biotic and abiotic stress response in soybean. In another study, miRNAs such as miR159, miR319 and miR2118 in roots of *Vigna unguiculata* grown under salt stress were identified and validated (Paul et al., 2011). Using these potential miRNA sequences, several potential target genes were predicted and all of them were identified as transcription factors that may be involved in abiotic stress responses. Thus, it is envisaged that miR2118 may enhance the ability of drought tolerance through unknown mechanisms

associated with cross adaptation in plants.

Yet another miRNA identified in study miR170 was found to target scarecrow-like transcription factor, sodium inducible calcium-binding protein (ACP1) and those genes involved in floral development. It was also identified in response to NaCl, mannitol, and 4°C stresses (Jones-Rhoades and Bartel, 2004; Sunkar and Zhu, 2004; Zhou et al., 2010). Among the 30 miRNAs identified by Zhou et al. (2010) as significantly down- or up-regulated under the drought stress in rice, miR170 and miR1030 were down-regulated miRNAs, and were revealed for the first time to be induced by drought stress in plants, and miR170 and miR319 showed opposite expression to that observed in drought-stressed Arabidopsis. The most conserved down-regulated miRNAs were ath-miR170. This analysis of cis-elements provides molecular evidence for the possible involvement of these miRNAs in the process of drought response and/or tolerance in rice. Xu et al. (2010) have identified miR828 in maize and their differential expression under drought stress. Induced by orthophosphate (Pi) deficiency, miR828 not only targets the transcript of a MYB transcription factor (MYB113) but also the TAS4 transcript that produces clusters of phased transacting, miRNAs involved in anthocyanin accumulation (Kuo and Chiou, 2011). Because anthocyanin accumulation is a common abiotic stress response, such an auto regulation mechanism may apply to other stress conditions (Hsieh et al., 2009). Another miRNA found in this study, miR1030, was down regulated under drought stress in rice (Zhou et al., 2010) and was expressed in response to biotic and abiotic stresses in several plants (Khraiwesh et al., 2011). Though there was no much evidence for the involvement of miR869 in abiotic stress resistance, available report such as Amor et al. (2009) have shown that mirR869 was involved in Arabidopsis differentiation and abiotic stress response. It has been shown that miR1884 was down regulated under cold stress in rice. It was predicted that this gene targets *ATPase, URICASE,* and ABA stress-ripening, TMV response-related proteins (Lv et al., 2010). However, this downregulated rice miRNA was not present in Arabidopsis, implying species-specific miRNAs functions in the response to cold-stress (Lv et al., 2010). miR2118 was found in the first draft of pigeon pea genome, a hardy crop (Singh et al., 2011). However, its response to abiotic stresses and its role is yet to be demonstrated.

Considering all these facts, it could be possible to assume that the putative miRNAs reported in this paper may be involved in abiotic stress resistance in cotton. Nevertheless, if the miRNAs identified in this study are confirmed by experimental evidence, such as northern blotting, it will provide most convincing conclusion. It is also worth to note the point that ESTs are the true products of gene expression, and in some case they may be considered as the result of northern blotting. There are evidences like Bonnet et al. (2004) who used the EST

database and confirmed 91 potential *Arabidopsis* and rice miRNAs identified by a computational strategy. Thus, we conclude that these newly identified putative miRNAs by EST analysis will sure have a great deal for abiotic stress responses in cotton plants and offer new scopes for genetic improvement of cotton.

Conclusion

To gain insight into the molecular mechanisms of cotton's responses to abiotic stresses, conserved miRNAs were examined. Widespread effects of miRNAs on major plant biochemical pathways were observed, and novel strategies to enhance abiotic stress tolerance in sensitive crops such as cotton suggested. Currently, a majority of plant miRNA-related researches are purely descriptive and provide no further detailed mechanistic insight into miRNA-mediated gene regulation and other functions. Research in this direction is progressing at this laboratory. The findings reported in this study provide valuable information for further functional characterization of miRNAs in response to abiotic stress in general, and drought stress, particularly in cotton. However, due to the potential uncertainty in this experiment, it is not feasible to just estimate the exact effects of these miRNAs in plants. These data provide a starting point for future studies, and continued efforts are needed to confirm the function of miRNAs in stress response and stress adaptation.

ACKNOWLEDGEMENT

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