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IN SILICO PROFILING AND CHARACTERIZATION OF CONSERVED *microRNAs* IN BIOFUEL PLANT SORGHUM

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Abstract

MicroRNAs (miRNAs) are a class of small RNAs, noncoding and transcribed endogenously that directly involved in regulating gene expression at the post-transcriptional level. Majority of the miRNAs are highly conserved in plants that provide the rationality for identification of new conserved miRNAs in other species of plants through comparative genomics. In this research project, the reported known plant miRNAs were blasted against the Expressed Sequence Tags (ESTs) database of Sorghum, and following the series of stringent criteria of miRNAs profiling and characterization via homology search. Hence, a total of 25 miRNAs were identified in biofuel plant Sorghum. For them 4247 potential target genes were also predicted which belongs to the 142 Gene Ontology (GO) enrichment terms of biological processes, molecular functions and cellular components. These GO enrichment terms appear to be involved in transcription factors, growth and development, metabolism, stress related, disease related and other physiological processes. The findings of this research will enable the researchers for better devising of Sorghum plant for biofuel qualities and traits.

Key words: BLAST; Express sequence tags; Identification; MicroRNAs; Sorghum.

Introduction

MicroRNAs (miRNAs) are small, endogenous, noncoding and evolutionary-conserved RNAs with ~18-26 nucleotides in length (Bartel, 2004). They are regulated negatively their targeted genes at the post-transcriptional level via messenger RNAs (mRNAs) degradation or suppression, depending upon the complementarity between miRNA and mRNAs (Jones-Rhoades, 2006). Recent studies showed that miRNAs mediated the process of many number of genes involved in plant biotic and abiotic stress responses, signal transduction, growth and development (Dugas & Bartel, 2004). Likely mRNAs, miRNAs are also transcribed from their own genes (Chen, 2005). Biogenesis of mature miRNA is a multi-step processes. Firstly, miRNA genes are transcribed as a long primary transcripts called primary miRNAs (pri-miRNAs) by RNA polymerase II (Cui *et al*., 2009) and later, the pri-miRNAs form a stem-loop like secondary structures, which are then processed into precursor miRNAs (pre-miRNAs). The pre-miRNA is further chopped into a duplexes of short ds RNA consist of mature miRNA and its passanger strand (noted as miRNA*) by Dicer-like1 enzyme (DCL1) (Reinhart *et al*., 2002; Bartel, 2004) and afterward pass on to the cytoplasm through an enzyme termed as HASTY (Park *et al*., 2005). Finally, the duplexes are unwound and integrate into argonaute (AGO) proteins to produce a complex known as RNA-induced silencing complex (RISC) where the regulation of targeted genes expression occur (Bartel, 2004; Voinnet, 2009). Several methodologies have been established for identifying miRNAs in various plant species but exploration of miRNAs via computational approaches in plants is getting striking attention due to their conserved nature (Patanun *et al*., 2013). A large number of miRNAs have been discovered in a various plant species by the analysis of express sequence tags (ESTs) using computational tools, for example; *Arabidopsis thaliana* (Adai *et al*., 2005), maize (Zhang *et al*., 2006), cotton (Zhang *et al*., 2007; Barozai *et al*., 2008), tomato (Yin *et al*., 2008; Din & Barozai, 2014), apricot (Baloch *et al*., 2015a), and radish (Barozai *et al*., 2015), citrus (Song *et al*., 2009), potato (Xie *et al*., 2011; Din *et al*., 2014), *Brassica rapa* L. (Dhandapani *et al*., 2011), *Vigna unguiculata* (Lu & Yang, 2010; Gul *et al*., 2017), Coffea (Bibi *et al*., 2017), chilli (Din *et al*., 2016), rose (Baloch *et al*. 2015b) and switchgrass (Barozai *et al.,* 2018).

Sorghum ranks fifth among important cereal crops of the world, and is widely used as staple food for humans besides its usage as fodder for animals. Sweet sorghum is an emerging, potential candidate to serve for biofuel production due to its vast adaptability, easy cultivation, and high yield potential. Intensive exploitation of its diverse germplasm in various breeding programs has led to improved syrup, grain and forage yield. Both stalk and grain of this crop can contribute in energy sector such as 60 Mg ha^{-1} of fresh biomass can produce 5000 litres ha^{-1} of ethanol (Monk *et al.*, 1984). Despite the economically importance of sorghum, the molecular genetics information especially regarding miRNAs of this plant remains largely unknown. Only a few miRNAs in sorghum were reported. Identification of comprehensive sets of miRNAs in sorghum is a critical step to facilitate our understanding of regulatory mechanisms or networks. Based on computational prediction, therefore, we aimed to extend sorghum miRNA study by using conserved miRNAs reported in *Oryza sativa* deposited in miRNA database (miRBase). A total of 25 sorghum miRNAs (belonged to 24 miRNA families) were identified and their characteristics were also investigated.

Materials and Methods

Identification of potential candidate miRNA sequences: A similar methodology (Zhang *et al*., 2006) with a little modification as described by (Barozai *et al*.,

2008) was applied to profile the potential miRNAs from sorghum expressed sequence tags (ESTs). As reference miRNAs, all known *Oryza sativa* miRNA sequences, both precursors and matures, were downloaded from the miRNA registry database (Version Rfam 21.0 released June, 2014) (Griffiths-Jones, 2004), and subjected to basic local alignment search tool (BLAST) for alignment against publicly available 209,835 ESTs of sorghum from the dbEST (database of EST), release 130101 at http://blast.ncbi.nlm.nih.gov/Blast.cgi, using BLASTn program (Altschul *et al*., 1990). Briefly, the sorghum mature and precursor miRNA sequences were subjected as queries through BLASTn program. The parameters were adjusted as, Database; express sequence tag (EST), organism; *Sorghum bicolor* (taxid: 4558) and Program Selection; Somewhat similar sequences (blastn). The mRNA sequences showing ≥4 mismatches were selected for further inspection.

Formation of single tone EST: The repeated ESTs from the same gene were eliminated and a single tone EST per miRNA was produced by using BLASTn program against the sorghum EST database with default parameters (Altschul *et al*., 1990).

Removal of coding sequences: The initial potential miRNA sequences of sorghum, predicted by the mature source miRNAs, were checked for protein coding. The FASTA format of initial potential sequences were subjected against protein database at NCBI using BLASTx with default parameter (Altschul *et al*., 1997) and the protein coding sequences were removed.

Creation of stem-loop structures: The initial potential candidate sorghum miRNA sequences, confirming as non-protein coding nature, having 0-4 mismatches with the reference miRNAs and representing single tone gene were subjected to generate stem-loop or secondary structures. Publicly available Zuker folding algorithm http://www.bioinfo.rpi.edu/applications/mfold/rna/form1.cgi, known as MFOLD (version 3.6) (Zuker, 2003) was used to predict the secondary structures. The MFOLD parameters were adjusted same as published by various researchers for the identification of miRNAs in various plants and animals species (Bai *et al*., 2012; Barozai, 2012a, b, c, d). For physical scrutinizing, the stem-loop structures either showing the lowest free energy ≤−18 $Kcalmol^{-1}$ or ≤ the lowest free energy of the reference miRNAs were preferred. Threshold values of Ambros *et al*., (2003) were applied as reference to finalize the potential miRNAs in sorghum. The stem regions of the stem-loop structures were checked and confirmed for the mature sequences with either at least 16 or equal to the reference miRNAs base pairing involved in Watson-Crick or G/U base pairing between the mature miRNA and the passenger strand (miRNA*).

Convergence and phylogenetic studies: The convergence and phylogenetic studies were carried out for the one of conserved sorghum miRNA (sbi-mir1436). Simply, the sbi-mir1436, for its conserved behaviour in different plant species was checked for convergence and phylogenetic investigation. The sbi-mir1436 alignment was created with *Orysa sativa* (osa) and *Hordeum vulgare* (hvu) by the publicly accessible webLogo: a sequence logo generator (http://weblogo.berkeley.edu/logo.cgi) and ClustalW2 (https://www.ebi.ac.uk/Tools/msa/clustalw2/) to produce cladogram tree using neighbor joining clustering method respectively. The results were saved.

Prediction of miRNAs targets: The psRNATarget, a plant small RNA target analysis server available at http:// plantgrn.noble.org/psRNATarget/ (Dai & Zhao, 2011) was used, to identify putative potential targets of the newly predicted sorghum miRNAs. The sorghum library (*Sorghum bicolor*, transcript, JGI genomic project, Phytozome 12, 313_v3.1) was used as selected target library with the modified 2017-updated parameters of psRNATarget as Max Expectation cutoff: 5, HSP length for scoring: 19, Penalty for GU pair: 0.5, Penalty for other mismatch: 1.0, Allowing bulge on target: Yes, Penalty for opening gap: 2.0, Penalty for extending gap: 0.5, Weight for seed region: 1.5, Seed region: 2-13, # of mismatches allowed in seed region: 2 and Calculating UPE: No. The predicted putative sorghum miRNA targets were subjected to find their *Arabidopsis* homologues and these homologues were analysed for the Gene Ontology functional and enrichment analyses through agriGO (Tian *et al*., 2017).

Results and Discussion

Detection of sorghum miRNAs: In order to identify and characterize the potential miRNAs in sorghum, a comparative genomic approach was applied using bioinformatics tools. This is in agreement with the previous reports (Barozai *et al*., 2013a, b; Silva *et al*., 2016; Bibi *et al*., 2017; Gul *et al*., 2017; Zhang *et al*., 2017) that the homology based search by applying comparative genomics is a valid and logical approach to find interesting findings in plants at genomic level. The current study resulted a total of 25 new conserved miRNAs from the analyses of 209,835 sorghum ESTs using bioinformatics tools (Table 1). The 25 potential sorghum miRNAs belonged to 24 families; (sbi-miR; 414a, 414b, 415, 417, 418, 435, 815, 1436, 1439, 1848, 1850, 1860, 1875, 1881, 2106, 2907, 2925, 2927, 5075, 5077, 5145, 5161, 5486, 5503, 5505). Available miRNAs literature revealed that all these 25 miRNAs are profiled for the first time in sorghum. In the light of the empirical formula for biogenesis and expression of the miRNAs suggested by Ambros *et al*., (2003), these miRNAs are considered as a valid candidate after justifying the criteria B, C and D. According to Ambros *et al*., (2003) only the criterion D is enough for homologous sequences to validate as potential miRNAs in other species. The present study is in agreement with the other research groups (Barozai & Husnain, 2011; Barozai *et al*., 2012; Barozai *et al*., 2013a, b; Din *et al*., 2016) where similarity based search by applying comparative genomics has produced novel and interesting findings in plants genomics.

Table 1. The newly identified *Sorghum bicolor* miRNAs characterization. *Sorghum bicolor* miRNAs were characterized in terms of precursor miRNA length (PL), minimum free energy (MFE), mature sequence (MS), number of mismatches (NM), mature sequence length (ML), mature sequence arm (MSA), GC content percentage (GC%), organ of expression (OE) and source EST (SE)

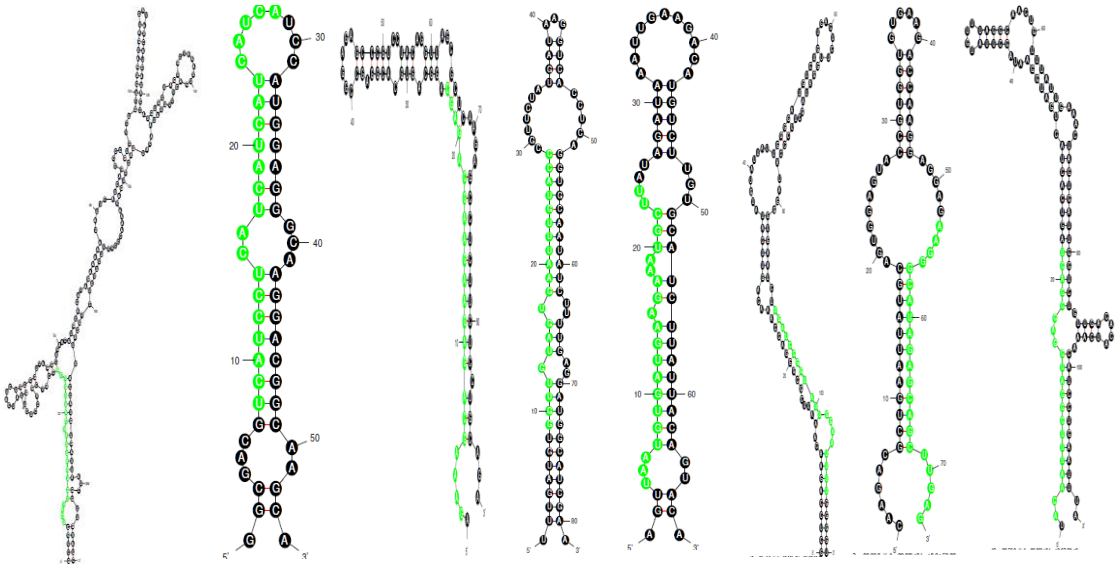
S. bicolor miRNAs	Ref- miRNA	PL	MFE	MS	NM	ML	MSA	GC%	OE	SE
sbi-miR414a	osa-miR414	210	-37.81	TCATCCTGATCATCATCGTCC	1	21	5’	47.61	Leaf	CF772139.1
sbi-miR414b	osa-miR414	54	-11.70	TCATCCTCATCATCATCATCA	2	21	5’	38.09	Mix plant	BE594086.1
sbi-miR415	osa-miR415	89	-28.30	AAGCAGAGAAGAAGCAGAGCAG	3	22	5’	50.00	Leaf & Root	CN137771.1
sbi-miR417	osa-miR417	81	-18.70	GGTTGTAGTGAATTTGTACCC	4	21	5’	42.85	Pollen	CF482712.1
sbi-miR418	osa-miR418	68	-13.20	TAATGTGATGAAGAAATGCTT	4	21	5’	28.57	Seedling	CD210389.1
sbi-miR435	osa-miR435	114	-29.40	TGATCCGGTATTGGAATTTT	4	20	3’	35.00	Root	CN128354.1
sbi-miR815	osa-miR815a	73	-20.00	AAGGGCATAGAGCAGCTTGAG	4	21	3’	52.38	Mix plant	BE593567.1
sbi-miR1436	osa-miR1436	111	-29.50	ACAATTTGGGATGGAGGGAGT	3	21	5’	47.61	Seedling	CF426836.1
sbi-miR1439	osa-miR1439	120	-21.59	ATTTGGAACGGAGTGATTACT	3	21	3’	38.09	Seedling	AW283444.2
sbi-miR1848	osa-miR1848	222	-132.40	CCGCGCCGGCGCGCGGGCA	2	21	5’	38.09	Seedling	CD430396.1
sbi-miR1850	osa-miR1850	313	-74.80	TTGTTTAGTTCACAAAAATTTT	4	22	3’	18.18	Leaf	CF771761.1
sbi-miR1860	osa-miR1860	57	-11.90	CCCAAACCAGCTTCCAGATCT	3	21	5’	52.38	Leaf	CF430189.1
sbi-miR1875	osa-miR1875	255	-113.30	ACAATGGAGTGAGGTGCAAC-GCA	3	24	3’	50.00	Leaf	CN131432.1
sbi-miR1881	osa-miR1881	107	-32.50	AATGTTATTGTAGCGGTGGTGGTTG	3	25	5’	44.00	Root	CN127844.1
sbi-miR2106	osa-miR2106	137	-37.20	AAGAAGTTTTCTGGATACTTT	4	21	3’	28.57	Root	CN130171.1
sbi-miR2907	osa-miR2907a	73	-28.40	GGCAGACGCGCGAGGGCCTCGT	3	22	3’	77.27	Callus	CD228564.1
sbi-miR2925	osa-miR2925	65	-34.50	CCGCGGCCGCGGGCCTCGT	3	19	5’	89.47	Seedling	CD205788.1
sbi-miR2927	osa-miR2927	65	-22.00	TGTCGTCGTTGATGGAGCCCATG	1	23	3’	56.52	Ovary	BF317972.1
sbi-miR5075	osa-miR5075	140	-68.30	TTCTCCGTCGCCTCCGTCCGG	2	21	3’	71.42	Leaf	CN148999.1
sbi-miR5077	osa-miR5077	85	-34.00	GGTGGCGTCGGGTTCACCA	2	19	3’	68.42	Seedling	CD424926.1
sbi-miR5145	osa-miR5145	338	-84.18	GTCTGTCTGGATTCTTGAGGACTA	4	24	5’	45.83	Pollen	CF480043.1
sbi-miR5161	osa-miR5161	182	-40.00	TTTGGAACAGAGGGAGTAATTA	4	22	5’	36.36	Pollen	CF484339.1
sbi-miR5486	osa-miR5486	59	-22.40	AGGGGCTTGCAAATTCTAGCT	4	21	3’	47.61	Seedling	EB724694.1
sbi-miR5503	osa-miR5503	99	-18.03	TTCGGATCTTTCTAGAGGCATT	0	22	3’	40.90	Callus	CD226742.1
sbi-miR5505	osa-miR5505	385	-105.42	GAGGATTTGGTATTGATCGAGC	4	22	3’	45.45	Root hairs	EH411757.1

Characterization of sorghum miRNAs: Characterization of newly identified candidate miRNAs is a set crucial step for their validation, as reported earlier (Frazier *et al*., 2010; Wang *et al*., 2012). The pre-miRNA length of the profiled sorghum miRNAs ranges from 54 to 385 nt with an average of 140 nt. The pre-miRNAs were further illustrated on the basis of their length (nt) as 50-100= 12 (48.00%), 101-150= 06 (24%), 151-200= 02 (08.00%), 201-250= 02(08.00%), 251-300= 01 (04.00%), 301-350= 02 (08.00%), 351-400= 01 (04.00%). The minimum folding free energy (MFE) of pre-miRNA is a vital and valid term of characterization. The newly identified potential sorghum pre-miRNAs have shown MFEs in range from −11.70 to −132.40 $Kcalmol^{-1}$ with an average of −42.78 $Kcalmol^{-1}$ described as -10 to -30= 13 (52%), -31 to -60= 6 (24%), -61 to -90= 3 (12%), -91 to -120= 2 (8.%), -121 to -150= 1 (4%). The numbers of mismatches of mature sequences with their reference sequences were observed in a range of 0-4 with an average of three mismatches categorized as 0= 1 (4%), 1= 2 (8%), 2= 4 (16%), 3= 8 (32%), 4= 10 (40%). These values are matched with the previously reported values in different plants, (Wang *et al*., 2012; Din *et al*., 2016; Ghani *et al*., 2013). Mature miRNA sequences length were observed from 19 nt to 25 nt with an average of 21.52 nt categorised as 19= 2 (8%), 20= 1 (4%), 21= 12 (48%), 22= 6 (24%), 23= 1 (4%), 24= 2 (8%), 25= 1 (4%). These findings of mature sequences length are in agreement to prior published data in other plant species (Frazier *et al*., 2010; Xie *et al*., 2010; Barozai *et al*., 2012). The 48% of sorghum miRNAs sequences were found at 5′ arm, while 52% were at 3′ arm (Fig. 1). The GC content ranged from 18% to 89% with an average of 47% as further categorised as 10 – 40= 9 (36%), 41 – 70= 13 (52%), 71 – 100= 3 (12%). The identified conserved sorghum miRNAs were also characterized on the basis of their organ of expression as shown in Table-1, as Callus = 2 (8%), Leaf =5 (20%), Leaf & Root =1 (4%), Mix plant =2 (8%), Ovary =1 (4%), Pollen =3 (12%), Root =3 (12%), Root hairs =1 (4%), Seedling =7 (28%). These findings are similar with the earlier reports (Wang *et al*., 2012; Bibi *et al*., 2017) and suggesting organ dependent expression pattern of miRNAs in sorghum. The miRNA organ specific expression would be utilized to manage the organogenesis in sorghum. The secondary structures of the sorghum pre-miRNAs are observed with at least 16 nt engaged in Watson-Crick or G/U base pairing between the mature miRNA and the opposite arms (miRNAs*) in the stem region. Except few where the reference miRNAs have also less base pairing and these precursors do not contain large internal loops or bulges. The mature miRNA sequences are observed in the double stranded stem region of the pre-miRNA secondary structures, as shown in (Fig. 1). Almost similar findings for various plant and animal species were reported by many researchers (Barozai, 2012a, b, c, d; Gul *et al*., 2017; Bibi *et al*., 2017; Din *et al*., 2016; Chen *et al*., 2012). Furthermore, the newly identified sorghum miRNAs were also confirmed as non-protein coding nature by showing no significant similarity with known proteins. This validation strengthens the expressed nature for computationally identified miRNAs as non-coding RNAs. Similar results were observed in various research papers by many groups (Barozai *et al*., 2012; Ji *et al*., 2012; Din *et al*., 2016).

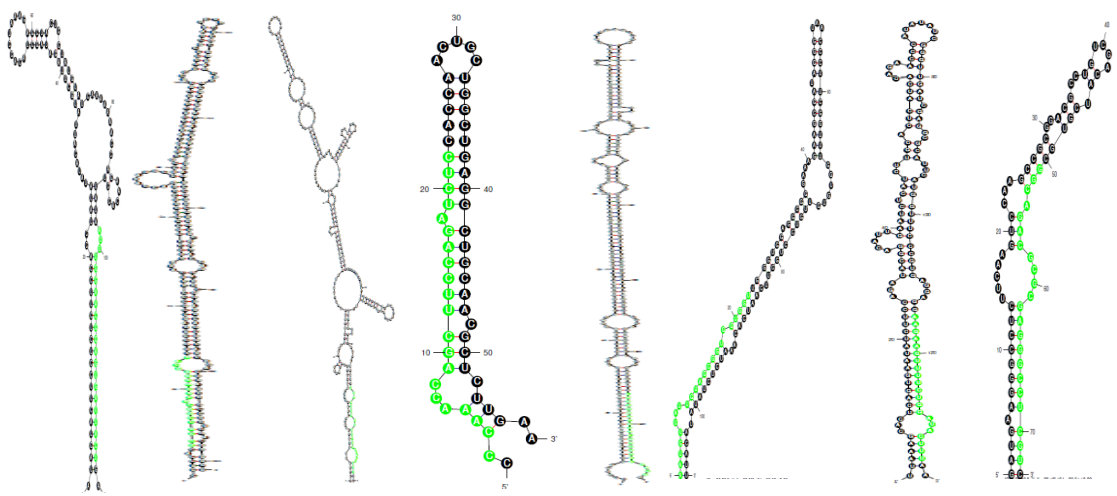
Convergence analysis: The newly characterized sorghum miRNA; sbi-mir1436, due to its conserved nature, was investigated for convergence. Simply, the sorghum miRNA sbi-mir1436 alignment was created with *Oryza sativa* (osa) and *Hordeum vulgare* (hvu) by the publicly available WebLogo, a sequence logo generator (Crooks *et al*., 2004). The sorghum miRNA sbi-mir1436 is observed in convergence with *Oryza sativa* (osa) and *Hordeum vulgare* (hvu) as shown in (Fig. 2). Zeng *et al*., (2009) have also reported conserved nature in Euphorbiaceous plants.

The potential sorghum miRNAs targeted genes: Profiling the potential sorghum miRNAs targeted genes is a vital step for validation of the computationally identified miRNAs. A total of 4247 targeted genes were predicted for the 25 potential sorghum miRNAs. These targets belong to the 142 Gene Ontology (GO) enrichment terms, where 48 are involved in the GO-biological process, 76 in GO- molecular Functions and 18 in the GO-cellular components. The detail description is mentioned in Table 2. Different sorghum miRNAs targeting same proteins and vice versa were predicted here. This showed that one miRNA target more than one mRNAs and a single mRNA targeted by many miRNAs (Bartel, 2009). GO-biological process showed that the newly identified potential targets of the sorghum miRNAs were significantly engaged in metabolic process (GO:0008152), post-translational protein modification (GO:0043687), cell surface receptor linked signaling pathway (GO:0007166), lipid metabolic process (GO:0006629), cellular catabolic process (GO:0044248), response to stimulus (GO:0050896), response to external stimulus (GO:0009605) defense response (GO:0006952), regulation of biological process (GO:0050789) multicellular organismal development (GO:0007275), signal transduction (GO:0007165) and developmental process (GO:0032502). Similar targets were reported by many researchers (Baloch *et al*., 2015a; Barozai *et al*., 2015; Song *et al*., 2009; Xie *et al*., 2011; Din *et al*., 2014).

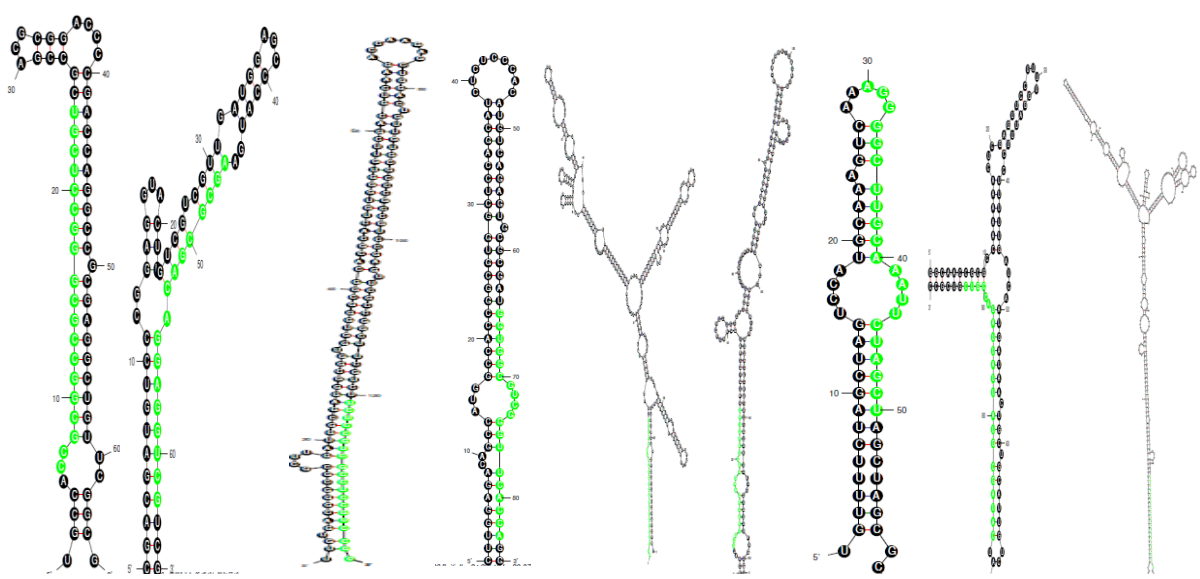
Fatty acids are essential components of all plant cells and play vital roles in cell division, growth and development. Besides this, fatty acids are also reported to have important roles in biofuel production (Rogalski & Carrer, 2011). Many newly identified sorghum miRNAs are predicted here to target the fatty acids related targets such as, fatty acid elongase activity (GO:0009922), very-long-chain fatty acid metabolic process (GO:0000038), fatty acid biosynthetic process (GO:0006633) and fatty acid beta-oxidation (GO:0006635).



sbi-miR414a, sbi-miR414b, sbi-miR415, sbi-miR417, sbi-miR418, sbi-miR435, sbi-miR815, sbi-miR1436



sbi-miR1439, sbi-miR1848, sbi-miR1850, sbi-miR1860, sbi-miR1875, sbi-miR1881, sbi-miR2106, sbi-miR2907



sbi-miR2925, sbi-miR2927, sbi-miR5075, sbi-miR5077, sbi-miR5145, sbi-miR5161, sbi-miR5486, sbi-miR5503, sbi-miR5505

Fig 1. The newly identified sorghum miRNAs' secondary structures. Sorghum pre-miRNAs secondary structures were developed through Mfold algorithm. These structures clearly showing the mature miRNAs in stem portion of the stem-loop structures (Green highlighted).

Table 2. Putative sorghum targets enrichment analysis.

GO term	Ontology	Description	Number	p-value	FDR
GO:0008152	BP	metabolic process	728	1.80E-11	6.00E-08
GO:0043687	BP	post-translational protein modification	128	1.90E-10	2.20E-07
GO:0009791	BP	post-embryonic development	86	1.30E-10	2.20E-07
GO:0006464	BP	protein modification process	140	2.80E-09	2.30E-06
GO:0009987	BP	cellular process	772	3.40E-09	2.30E-06
GO:0044238	BP	primary metabolic process	607	2.90E-08	1.60E-05
GO:0007166	BP	cell surface receptor linked signaling pathway	31	8.30E-08	4.00E-05
GO:0006629	BP	lipid metabolic process	87	1.10E-07	4.70E-05
GO:0043412	BP	macromolecule modification	144	1.30E-07	4.70E-05
GO:0007167	BP	enzyme linked receptor protein signaling pathway	27	1.70E-07	5.20E-05
GO:0007169	BP	transmembrane receptor protein tyrosine kinase signaling pathway	27	1.70E-07	5.20E-05
GO:0006468	BP	protein amino acid phosphorylation	94	1.90E-07	5.40E-05
GO:0006508	BP	proteolysis	81	1.10E-06	0.00027
GO:0044248	BP	cellular catabolic process	73	7.10E-06	0.0016
GO:0016310	BP	phosphorylation	97	6.70E-06	0.0016
GO:0044237	BP	cellular metabolic process	568	8.00E-06	0.0017
GO:0006796	BP	phosphate metabolic process	102	1.60E-05	0.0031
GO:0006793	BP	phosphorus metabolic process	102	1.70E-05	0.0031
GO:0050896	BP	response to stimulus	283	4.40E-05	0.0062
GO:0007275	BP	multicellular organismal development	156	3.70E-05	0.0062
GO:0007165	BP	signal transduction	103	4.80E-05	0.0062
GO:0034641	BP	cellular nitrogen compound metabolic process	52	4.50E-05	0.0062
GO:0006511	BP	ubiquitin-dependent protein catabolic process	36	4.80E-05	0.0062
GO:0043632	BP	modification-dependent macromolecule catabolic process	36	4.80E-05	0.0062
GO:0065007	BP	biological regulation	291	4.40E-05	0.0062
GO:0019941	BP	modification-dependent protein catabolic process	36	4.80E-05	0.0062
GO:0051603	BP	proteolysis involved in cellular protein catabolic process	36	6.90E-05	0.0082
GO:0032501	BP	multicellular organismal process	159	6.80E-05	0.0082
GO:0006575	BP	cellular amino acid derivative metabolic process	36	9.80E-05	0.011
GO:0019538	BP	protein metabolic process	277	9.90E-05	0.011
GO:0044257	BP	cellular protein catabolic process	36	9.80E-05	0.011
GO:0048856	BP	anatomical structure development	134	0.00011	0.011
GO:0007018	BP	microtubule-based movement	11	0.00015	0.015
GO:0044255	BP	cellular lipid metabolic process	58	0.00016	0.016
GO:0009605	BP	response to external stimulus	44	0.00017	0.016
GO:0007017	BP	microtubule-based process	18	0.00018	0.017
GO:0006952	BP	defense response	68	0.00021	0.019
GO:0050789	BP	regulation of biological process	253	0.00035	0.029
GO:0016567	BP	protein ubiquitination	18	0.00035	0.029
GO:0006446	BP	regulation of translational initiation	5	0.00035	0.029
GO:0032502	BP	developmental process	167	0.00037	0.03
GO:0019748	BP	secondary metabolic process	47	0.00041	0.032
GO:0009404	BP	toxin metabolic process	11	0.00044	0.034
GO:0009407	BP	toxin catabolic process	11	0.00044	0.034
GO:0008610	BP	lipid biosynthetic process	43	0.00049	0.036
GO:0032446	BP	protein modification by small protein conjugation	19	0.00049	0.036
GO:0009416	BP	response to light stimulus	54	0.00058	0.041
GO:0006082	BP	organic acid metabolic process	72	0.00065	0.045
GO:0003824	MF	catalytic activity	826	4.50E-43	6.60E-40

Table 2. (Cont'd.).

GO term	Ontology	Description	Number	p-value	FDR
GO:0016740	MF	transferase activity	330	9.00E-24	6.60E-21
GO:0008194	MF	UDP-glycosyltransferase activity	45	2.50E-12	1.20E-09
GO:0019825	MF	oxygen binding	48	7.20E-12	2.60E-09
GO:0016757	MF	transferase activity, transferring glycosyl groups	67	1.00E-08	2.10E-06
GO:0035251	MF	UDP-glucosyltransferase activity	24	8.80E-09	2.10E-06
GO:0016301	MF	kinase activity	150	7.80E-09	2.10E-06
GO:0016772	MF	transferase activity, transferring phosphorus-containing groups	165	2.20E-08	4.10E-06
GO:0005488	MF	binding	739	3.00E-08	4.50E-06
GO:0016787	MF	hydrolase activity	269	3.10E-08	4.50E-06
GO:0001883	MF	purine nucleoside binding	133	8.10E-08	9.10E-06
GO:0001882	MF	nucleoside binding	133	8.10E-08	9.10E-06
GO:0030554	MF	adenyl nucleotide binding	133	8.10E-08	9.10E-06
GO:0070008	MF	serine-type exopeptidase activity	20	9.80E-08	9.60E-06
GO:0004185	MF	serine-type carboxypeptidase activity	20	9.80E-08	9.60E-06
GO:0004180	MF	carboxypeptidase activity	20	1.40E-07	1.30E-05
GO:0046914	MF	transition metal ion binding	141	2.90E-07	2.50E-05
GO:0043167	MF	ion binding	172	4.10E-07	3.20E-05
GO:0043169	MF	cation binding	172	4.10E-07	3.20E-05
GO:0008238	MF	exopeptidase activity	22	6.00E-07	4.40E-05
GO:0005515	MF	protein binding	227	6.40E-07	4.40E-05
GO:0046872	MF	metal ion binding	162	2.10E-06	0.00014
GO:0046527	MF	glucosyltransferase activity	24	3.20E-06	0.0002
GO:0017076	MF	purine nucleotide binding	141	5.00E-06	0.00031
GO:0005506	MF	iron ion binding	26	5.30E-06	0.00031
GO:0000166	MF	nucleotide binding	177	6.10E-06	0.00034
GO:0008236	MF	serine-type peptidase activity	28	7.70E-06	0.0004
GO:0017171	MF	serine hydrolase activity	28	7.70E-06	0.0004
GO:0005524	MF	ATP binding	117	8.80E-06	0.00045
GO:0032559	MF	adenyl ribonucleotide binding	117	1.10E-05	0.00053
GO:0004674	MF	protein serine/threonine kinase activity	67	1.40E-05	0.00067
GO:0016207	MF	4-coumarate-CoA ligase activity	8	2.70E-05	0.0012
GO:0048037	MF	cofactor binding	32	2.60E-05	0.0012
GO:0016765	MF	transferase activity, transferring alkyl or aryl (other than methyl) groups	24	2.90E-05	0.0013
GO:0050660	MF	FAD binding	16	3.20E-05	0.0013
GO:0016758	MF	transferase activity, transferring hexosyl groups	39	4.80E-05	0.0019
GO:0004672	MF	protein kinase activity	84	5.10E-05	0.002
GO:0016874	MF	ligase activity	56	5.50E-05	0.0021
GO:0016405	MF	CoA-ligase activity	9	5.80E-05	0.0022
GO:0008146	MF	sulfotransferase activity	8	6.60E-05	0.0024
GO:0016878	MF	acid-thiol ligase activity	9	7.40E-05	0.0026
GO:0008270	MF	zinc ion binding	106	0.0001	0.0035
GO:0016462	MF	pyrophosphatase activity	74	0.00011	0.0037
GO:0060089	MF	molecular transducer activity	44	0.00012	0.004
GO:0004871	MF	signal transducer activity	44	0.00012	0.004
GO:0016818	MF	hydrolase activity, acting on acid anhydrides, in phosphorus-containing anhydrides	74	0.00013	0.0041
GO:0016817	MF	hydrolase activity, acting on acid anhydrides	74	0.00014	0.0042
GO:0042623	MF	ATPase activity, coupled	39	0.00014	0.0042
GO:0004872	MF	receptor activity	27	0.00015	0.0044

Table 2. (Cont'd.).

GO term	Ontology	Description	Number	p-value	FDR
GO:0016881	MF	acid-amino acid ligase activity	39	0.00018	0.0051
GO:0017111	MF	nucleoside-triphosphatase activity	70	0.00019	0.0055
GO:0004311	MF	farnesyltranstransferase activity	6	0.00022	0.0061
GO:0009055	MF	electron carrier activity	33	0.00025	0.0069
GO:0032555	MF	purine ribonucleotide binding	125	0.00029	0.0076
GO:0032553	MF	ribonucleotide binding	125	0.00029	0.0076
GO:0080043	MF	quercetin 3-O-glucosyltransferase activity	8	0.00029	0.0076
GO:0016887	MF	ATPase activity	47	0.0003	0.0077
GO:0019787	MF	small conjugating protein ligase activity	36	0.00031	0.0077
GO:0016877	MF	ligase activity, forming carbon-sulfur bonds	9	0.00038	0.0093
GO:0016782	MF	transferase activity, transferring sulfur-containing groups	9	0.00038	0.0093
GO:0016773	MF	phosphotransferase activity, alcohol group as acceptor	92	0.00057	0.014
GO:0031072	MF	heat shock protein binding	18	0.00074	0.017
GO:0004364	MF	glutathione transferase activity	11	0.00075	0.017
GO:0070011	MF	peptidase activity, acting on L-amino acid peptides	52	0.00087	0.019
GO:0016491	MF	oxidoreductase activity	111	0.00085	0.019
GO:0050662	MF	coenzyme binding	23	0.00087	0.019
GO:0003774	MF	motor activity	15	0.0011	0.023
GO:0004091	MF	carboxylesterase activity	37	0.0012	0.025
GO:0004842	MF	ubiquitin-protein ligase activity	33	0.0012	0.025
GO:0004888	MF	transmembrane receptor activity	21	0.0011	0.025
GO:0016879	MF	ligase activity, forming carbon-nitrogen bonds	40	0.0014	0.028
GO:0042626	MF	ATPase activity, coupled to transmembrane movement of substances	21	0.0022	0.043
GO:0043492	MF	ATPase activity, coupled to movement of substances	21	0.0022	0.043
GO:0004650	MF	polygalacturonase activity	12	0.0023	0.045
GO:0016820	MF	hydrolase activity, acting on acid anhydrides, catalyzing transmembrane movement of substances	21	0.0024	0.047
GO:0003777	MF	microtubule motor activity	12	0.0025	0.048
GO:0044464	CC	cell part	992	2.00E-11	6.40E-09
GO:0005623	CC	cell	992	2.00E-11	6.40E-09
GO:0016020	CC	membrane	311	8.20E-09	1.80E-06
GO:0005886	CC	plasma membrane	134	3.50E-08	5.60E-06
GO:0005622	CC	intracellular	645	4.60E-08	5.90E-06
GO:0044424	CC	intracellular part	622	6.60E-08	7.00E-06
GO:0005737	CC	cytoplasm	471	1.80E-07	1.60E-05
GO:0043231	CC	intracellular membrane-bounded organelle	508	2.50E-06	0.00018
GO:0043229	CC	intracellular organelle	539	2.80E-06	0.00018
GO:0043227	CC	membrane-bounded organelle	508	2.80E-06	0.00018
GO:0043226	CC	organelle	539	3.10E-06	0.00018
GO:0044444	CC	cytoplasmic part	424	9.00E-06	0.00048
GO:0005856	CC	cytoskeleton	27	3.30E-05	0.0016
GO:0044430	CC	cytoskeletal part	23	9.00E-05	0.0041
GO:0043234	CC	protein complex	111	0.00055	0.023
GO:0015630	CC	microtubule cytoskeleton	17	0.00064	0.025
GO:0005875	CC	microtubule associated complex	9	0.0012	0.044
GO:0044459	CC	plasma membrane part	25	0.0013	0.045

where, BP = Biological process, MF = Molecular function, CC = Cellular component, p-value=0.5, FDR = False discovery rates

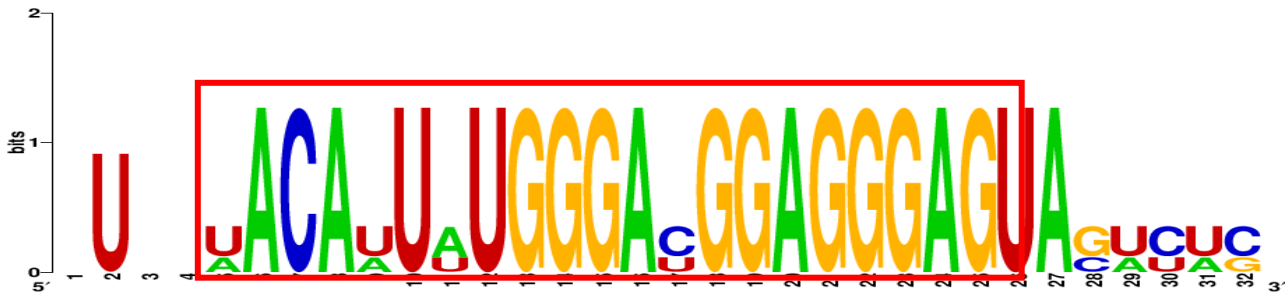


Fig. 2. Sorghum miRNA's conservation studies. Alignment of *Sorghum bicolor* (sbi) miRNA (sbi-mir1436) with *Oryza sativa* (osa) and *Hordeum vulgare* (hvu) was generated using WebLogo: a sequence logo generator, showing conserved nature mature miRNA sequences. The mature sequences highlighted in a rectangle red box.

Sweet sorghum stem juice has mostly sucrose and invert sugars such as glucose, fructose, maltose and xylose. These stem carbohydrates (sucrose and invert sugar) is a good and suitable source for ethanol production for biofuel with an easily and rapidly approaches (Almodares & Hadi, 2009). Several newly putative targets of the sorghum miRNAs are identified having roles in sucrose and invert sugar related activities as, sucrose mediated signaling (GO:0009745), regulation of carbohydrate metabolic process (GO:0006109), sucrose synthase activity (GO:0016157), sucrose biosynthetic process (GO:0005986), response to sucrose stimulus (GO:0009744), glucose-1-phosphate guanylyltransferase (GDP) activity (GO:0010474), xylan 1,4-beta-xylosidase activity (GO:0009044), xylan catabolic process (GO:0045493) and fructose-bisphosphate aldolase activity (GO:0004332). These putative targets in sorghum can serve as potential source to enhance the biofuel related production.

Biotic and abiotic stresses are the main restrictions in plant growth and production (Barozai & Whaid, 2012). miRNAs are reported to manage and help in the plant survival under various stresses (Barozai *et al*., 2018). In this study, many stress related genes are predicted as targets of newly identified sorghum miRNAs as, cold acclimation (GO:0009631), response to cold (GO:0009409), response to water deprivation (GO:0009414), response to salt stress (GO:0009651), response to heat (GO:0009408), response to oxidative stress (GO:0006979), response to fungus (GO:0009620) and response to bacterium (GO:0009617). The stress related miRNAs' targets are also predicted in many plant species ((Barozai *et al*., 2012; Ji *et al*., 2012; Din *et al*., 2016). These target gene can be used to increase sorghum resistance against various biotic and abiotic stresses.

Some significant GO cellular components based on enrichment analysis are found as plasma membrane (GO:0005886), intracellular (GO:0005622), cytoplasm (GO:0005737), intracellular organelle (GO:0043229) and cytoskeleton (GO:0005856). All these targets are reported as potential targets of miRNAs in various plant species (Barozai, 2013; Din & Barozai, 2014a,b).

Conclusion

This study resulted in the identification of 25 new miRNAs and their 142 GO-enrichment targeted genes in an important commercial plant sorghum. All these miRNAs are profiled for the first time in sorghum. The new miRNAs identified in this study should enable investigation of the complexity of miRNA-mediated genes such as growth and development, and various stress responses in sorghum. The knowledge gained from such study should be beneficial in development of sorghum variety with desired biofuel properties.

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