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Analysis of secondary structure and identification of internal repeats in miRNA precursor sequences of *Saccharum officinarum*, *Saccharum sp.* and *Sorghum bicolor*

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ABSTRACT

MicroRNAs (miRNAs) are the post-transcriptional regulators of gene expression that interact with mRNA in a sequence-specific manner. These interactions are primarily regulated by the secondary structural conformation of miRNAs. In plants, miRNAs have always been a subject to extensive research to see their explicit roles in overall development, cell to cell communications, metabolism, responses to stress and pathogen invasion. Here, we aimed to gain more understanding of the secondary structure of all possible miRNA precursor sequences (pre-miRNAs from which mature miRNAs are produced) for *Saccharum* and *Sorghum*, the two closest monocot relatives among the domesticated cultivated crops. Using computational approaches, altogether, 240 different pre-miRNAs were analyzed among which three different structural patterns were observed. The structural motifs primarily consist of stem, internal loop, bulge, and terminal loop. The pre-miRNAs of *Saccharum sp.* were found to have the most stable secondary structure with -193.05 kcal/mol free energy suggesting their resistance to nuclease in the cell. The Simple Sequence Repeats (SSRs) within the stem region of pre-miRNAs were found to be predominant with many trinucleotides, tetranucleotides and less frequent pentanucleotide repeats. AUG/AUC was the mostly observed trinucleotide in 80 percent of the studied precursors. The occurrence of these repeat sequences at varying level suggests their role in the proper functioning of miRNAs. Likewise, SSRs provide a molecular basis for the structural conformation of pre-miRNAs. All this information is substantially required for identifying miRNA targets and designing additional miRNA-based strategies to increase crop yields and enhance plant resistance to environmental stresses.

KEYWORDS: Expressed sequence tags (ESTs), miRNAs, pre-miRNAs, Secondary structure, Simple Sequence Repeats (SSRs)

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INTRODUCTION

MicroRNAs (miRNAs) are single-stranded conserved class of small (20-35 nucleotides) non-coding RNAs that preferentially binds with the target mRNAs and direct post-transcriptional silencing through a combination of translational repression and destabilization of the target mRNA (Jonas & Izaurralde, 2015). They act as a negative control device for its target mRNA. These miRNAs are generated from their precursors miRNAs (pre-miRNAs) by the enzyme RNase-III (Dicer) to release mature miRNAs and subsequently integrated into miRNA-induced silencing complexes (miRISCs) which directs the breakdown of mRNA by complementary base pairing (Iwakawa & Tomari, 2015; Huberdeau & Simard, 2019).

Plant miRNAs were first discovered in *Arabidopsis* almost 25 years ago (Reinhart *et al.*, 2002; Rhoades *et al.*, 2002; Wang *et al.*, 2004) and were shown to be important regulators of plant development (Kidner & Martienssen, 2004; Mallory & Bouche, 2008; Voinnet, 2009). In plants, they are thought to play a significant role in different cellular processes and as critical regulators of overall development, growth, and stress responses (Chen *et al.*, 2002; Yu *et al.*, 2004; Zhang *et al.*, 2022; Samynathan *et al.*, 2023). The responses of miRNAs boost the plants to endure drought, high temperature, low temperature and salinity stress (Pagano *et al.*, 2020). Pegler *et al.* (2019) performed RNA-seq in *Arabidopsis* under different stress conditions such as heat, drought, and high salt concentration. High-level fold changes were observed in the amount of total miRNA. These miRNAs bind to target mRNAs

with near-perfect complementarity. Besides, miRNAs influence nutrient availability in plants (Paul *et al.*, 2015; Islam *et al.*, 2022). Four different miRNAs such as miR379, miR398, miR408 and miR857 were thought to play crucial roles in cu-limiting conditions in plants. miR528 was associated to control cu-containing proteins in plants (Pilon, 2017). miR399 was shown to be active in the regulation of phosphate level in *Arabidopsis sp.* In rice, the genes that regulate tillering depend on miR156/miR529/SPL and miR172/AP2 modules. Though, the SPL (SQUAMOSA PROMOTER BINDING-LIKE) gene suppresses tillering, but influences the transformation of inflorescence and spikelet (Wang & Wang, 2015). Suppression of miR168 also induces early flowering and resistance against pathogen in *M. oryzae* (Wang *et al.*, 2021). Yang *et al.* (2019) reported a novel role of miR528 in the regulation of flowering time in rice. In maize, miR172 influences the determination of sex and meristem cell fate by targeting IDS1 (Indeterminate Spikelet1). Additionally, miR156a-l targets several SPL genes for the maturation of the ear, and incidentally triggers miR172 through SPLs (Lauter *et al.*, 2005; Chuck *et al.*, 2007; Salvi *et al.*, 2007). In tomatoes, miR171 is responsible for leaf morphogenesis as well as pollen ontogenesis (Kravchik *et al.*, 2019). So, all these data suggest the future utilization of miRNAs to manipulate the crops in agriculture.

To study the function of miRNAs it is of utmost importance to understand the structural conformation of miRNAs. The miRNA-mRNA interactions are primarily regulated by the secondary structural conformation of miRNAs and thus can explain the different degrees of genetic regulation of mRNAs in a cell. The miRNAs consist typical hairpin structure that is identified and altered by miRNA biogenesis elements. The initial 2-7 bases at the 5′ end of the miRNA are crucial to begin mRNA binding. However, the motifs of plant miRNA precursors are much consistently maintained throughout organisms (Heikkinen *et al.*, 2008) and are needed for precise miRNA biogenesis (Narjala *et al.*, 2020). Likewise, considering the huge amount of miRNA precursors produced by eukaryotes, it is difficult to recognize new miRNAs experimentally as the cloning of miRNAs that are inadequate is literally impossible. Proper uses of sequencing technology and miRNA research methods are highly recommended here. As a result, designing and employing computational approaches for miRNA analysis have become essential subjects.

Sugarcane (*Saccharum officinarum* L.) is the global sugar-producing crop and Sorghum (*Sorghum bicolor* L.) is the fifth most important cereal crop after wheat, rice, maize, and barley (Rooney & Saldivar, 2003). They are genetically closely related members of the Andropogonaceae tribe of Poaceae (Ming *et al.*, 1998). Here, our objective was to generate the secondary structures and identify the internal repeat sequences in all possible miRNA precursors of *Sorghum* and *Saccharum*. Analysis of these secondary structures will eventually help us identify potential target sites and manipulate these plants for our benefit. We hope that, in the near future, with the help of various in silico bioinformatic approaches, the regulatory pathways of these miRNAs will be revealed which will eventually provide a significant academic basis for manipulating overall development in these crops and can then be applied for agricultural benefits.

MATERIAL AND METHODOLOGY

miRNA Sequence Library Construction

The miRbase (www.mirbase.org) database is a searchable database of published miRNA sequences and annotations. Each entry in the miRBase Sequence database represents a predicted hairpin portion of a miRNA transcript, with information on the location and sequence of the mature miRNA sequence (termed miR). All miRNAs precursor sequences of *Sorghum bicolor* (205 precursors) [Sorghum_bicolor_NCBIv3], *Saccharum officinarum* (16 precursors), *Saccharum* sp. (19 precursors), were downloaded from the miRBASE database version 22.1, October 2018 in FASTA format (ftp://ftp.sanger.ac.uk/pub/mirbase/19.0/).

Sequence Submission for Generation of Secondary Structure

All the miRNA precursor sequences were individually submitted to the MFold server (http://unafold.rna.albany.edu/q=mfold/RNA Folding-Form) for the secondary structure generation. The potentially stable secondary structures from the precursors were generated by the RNAfold algorithm. The parameters were used for the secondary structure prediction using RNAfold - minimum free energy and partition function; dangling energy on both sides of the helix in any case; rescale energy parameters at a given temperature of 37 °C; interactive RNA secondary structure plot; RNA secondary structure plots with reliability annotation. Finally, these structures of precursors were manually curated using the rules of Zhang *et al.* (2005).

Identification of Internal Repeats and Mapping on Secondary Structure

The FASTA sequences of all the precursors were submitted individually to the FAIR server (http://bioserver1.physics.iisc.ernet.in/fair/) for analysis and identification of internal repeats in the sequences. Internal repeats may serve as target RNA binding sites as well as protein binding sites. To find out a probable conserved evolutionary feature that in the future can be explored for functional conservation as well. The internal repeats on the secondary structures that have been generated using MFold were mapped on the secondary structure and were analyzed for their positions, number of repeats as well as nature of repeats.

Heat Map Generation using R Studio Software

A heat map of the two-way hierarchical clustering of miRNA secondary structure data of all three species was obtained from the FAIR server. Cluster analysis classified the samples into groups based on the number of stems, bulges, internal loops, and terminal loops. Red color represents the highest number and blue color represents the lowest (absence). Heat Map was

generated using R Studio (https://posit.co/download/rstudio-desktop/).

RESULTS

The secondary structures of all possible pre-miRNAs for *Saccharum sp* (19 miRNA precursors), *S. officinarum* (16 miRNA precursors) and *S. bicolor* (205 miRNA precursors) were obtained from miRBASE database and analyzed further. In all the precursor miRNAs the structural motifs primarily consist of stem, internal loop, bulge, and terminal loop. The detail structures were then acquired from Mfold and tabulated in Supplementary Tables 1, 2 and 3. Sample miRNA sequences are shown in Figure 1. Practically, fifty percent of miRNA precursors were found to reveal a structure ending with 1 terminal loop and the rest fifty percent with 2 terminal loops, and very few showed a different pattern. In *Saccharum sp*. the one terminal loop with one stem pattern was found to be vital for the recognition purpose during the miRNA processing. The free energy ranges from -33.47 to -193.05 kcal/mol in *Saccharum sp*., -67.50 to -113.28 kcal/mol in *S. officinarum* and -38.50 to -108.23 kcal/mol in *S. bicolor* respectively. The percentage of pentanucleotide and tetranucleotide obtained in *Saccharum sp*. is 18.79 and 34.07 respectively; while in *S. officinarum* the percentage of pentanucleotide is 16.4 and tetranucleotide is 32.08. Similarly, in *S. bicolor*, the percentage of pentanucleotide and tetranucleotide is 10.61 and 27.9 respectively (Table 1). In addition to that, mononucleotides mostly poly (U) and poly (A) were found to exist more frequently than poly (C/G) repeats. The comparison of the secondary structures obtained from the FAIR server showed the presence of the internal repeat sequences mostly in the stack region followed by the internal loop and terminal loop (Figures 2 & 3). In the heat map, each row represents one miRNA. The miRNA clusters are represented as branch connections on the left. However, among all the precursors studied, one miRNA precursor of *S. bicolor* is found to have more internal repeats in the internal loop reasonably in the stack region.

DISCUSSION

Loops and hairpin structures are pivotal components of plant miRNAs, profoundly influencing their biogenesis, stability, and regulatory functions. Plant miRNAs typically originate from longer primary transcripts, known as pri-miRNAs, which undergo several processing steps to generate mature, functional miRNAs. Central to this process is the formation of hairpin structures within pri-miRNAs (Wong & Millar, 2023). These hairpin structures, characterized by a stem-loop configuration, serve as recognition sites for the Dicer-like (DCL) endonuclease, which cleaves pri-miRNAs into pre-miRNAs. The presence of such secondary structures ensures precise processing, as DCL enzymes recognize and cleave at specific sites within the hairpin, yielding mature miRNAs with defined 5' and 3' ends. Moreover, the stability of pre-miRNAs is bolstered by the hairpin structure, shielding them from degradation

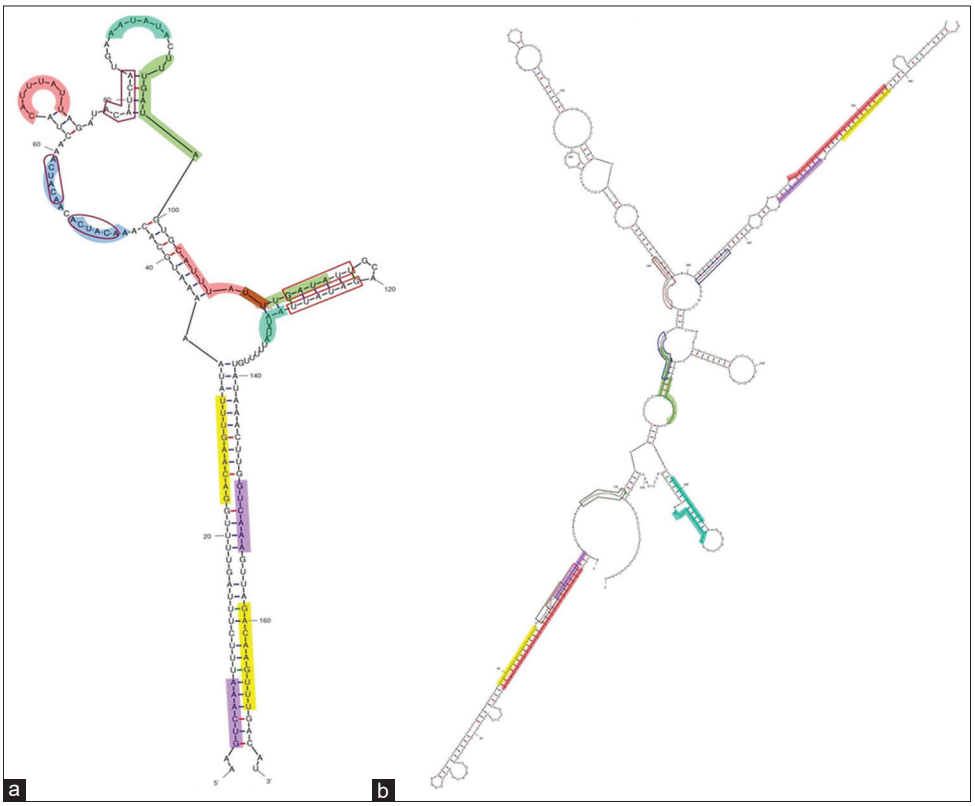

Figure 1: Representative image of repeat mapping on secondary structure of microRNA precursor sequences in a) *Saccharum* and b) *Sorghum* (In the studied miRNA precursors, the presence of Simple sequence repeats was found to be predominant with many trinucleotides, tetranucleotides and less frequent pentanucleotide repeats. AUG/AUC is the mostly observed trinucleotide in the 80percent of the studied precursors.)

Table 1: Summary of repetitive elements identified in precursor sequences

Organism name	Total Number of repeats identified	Number of Pentanucleotide repeats	Number of Tetranucleotide repeats	Percentage of Pentanucleotide repeats present in total repeats	Percentage of Tetranucleotide repeats present in total repeats
Saccharum officinarum	1546	254	496	16.4	32.08
Saccharum sp.	1309	246	446	18.79	34.07
Sorghum bicolor	1196	127	334	10.61	27.9

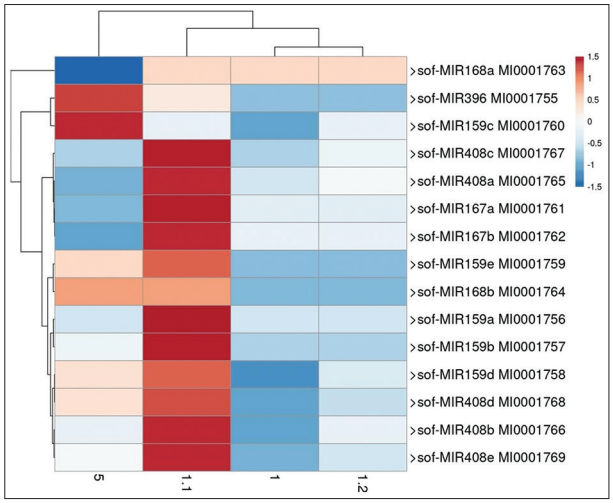

Figure 2: Heat map of two-way hierarchical clustering of microRNA (miRNA) secondary structure data of *Saccharum officinarum* obtained from Fair server (Sample miRNA sequences are shown at the right. Each row represents one miRNA. The miRNA clusters are represented as branch connections on the left. Cluster analysis classified the samples into groups based on number of stems, bulges, number of internal loops and terminal loops. Red colors represent the highest number and blue represent the lowest (absence).)

by cellular nucleases. Importantly, the loops within miRNA hairpins can harbor critical sequence motifs that influence miRNA processing and function (Xu & Chen, 2023). For instance, variations in loop size and sequence can impact the efficiency of miRNA processing by modulating the accessibility of DCL enzymes. Additionally, loop sequences may facilitate interactions with RNA-binding proteins involved in miRNA biogenesis, further fine-tuning the processing kinetics. Beyond biogenesis, loops can also mediate target specificity by affecting miRNA-mRNA interactions. Thus, loops and hairpin structures in plant miRNAs play multifaceted roles in orchestrating gene regulatory networks, underscoring their significance in plant development, stress responses, and adaptation (Bajczyk *et al.*, 2023). Internal repeat sequences within miRNA precursors of monocot plants represent integral components of miRNA biogenesis, target recognition, and evolutionary adaptation. Understanding the role of these repeat sequences in pre-miRNAs has implication for agricultural biotechnology and crop improvement. Targeting the repeat-mediated miRNA biogenesis and interactions with the target mRNAs could facilitate the development of novel strategies for manipulating stress tolerance, yield, and nutritional quality in monocot crops. Their functional significance extends beyond structural stability to encompass diverse regulatory mechanisms that shape gene expression networks in response to developmental and environmental cues (Chakraborty *et al.*, 2020). Gupta *et al.* (2022) reports showed the correlation between the different secondary structural elements and the presence of repetitive sequences in microRNA precursors of *Vriesea charlatan* and *Triticum aestivum* (Wheat), thus unraveling the roles of repeat sequences in monocot miRNA biology provides valuable insights into the regulatory complexity of these agriculturally important plants and offers opportunities for enhancing crop productivity and resilience. These minisatellites identified in the miRNA precursors may also serve as important targets for marker gene identification strategies involving expressed sequence tags (ESTs) (Vieira *et al.*, 2016).

Repeat sequences within miRNA precursors encompass duplicated or near-identical sequences, often forming stem-loop structures. These repeats can occur within the miRNA hairpin or its flanking regions. Initially rejected as genomic noise, repeat sequences are now recognized as integral components of miRNA precursors, influencing various aspects of miRNA biology. These have been reported to influence miRNA biogenesis at multiple levels. These repeats facilitate the formation of stable hairpin structures within pre-miRNAs, thereby promoting recognition and cleavage by the microprocessor complex. Additionally, repeat-containing pre-miRNAs exhibit altered processing kinetics by DCL, leading to differential production of mature miRNAs. This differential processing contributes to miRNA isoform diversity and functional specialization (De la Rosa *et al.*, 2020). They also play a crucial role in miRNA target recognition and specificity. Repeats within the miRNA seed region expand the target repertoire by enabling the recognition of multiple mRNA sequences with similar motifs. Moreover, repeat-mediated structural variations within the miRNA duplex influence target affinity and accessibility, fine-tuning miRNA-mediated gene regulation in a context-dependent manner. The evolutionary conservation of repeat sequences in miRNA precursors underscores their functional significance. Comparative genomics analyses reveal selective pressure to maintain both miRNA sequences and their flanking repeats across species, highlighting their indispensable roles in gene regulation (Attri *et al.*, 2022). Furthermore, the emergence of lineage-specific miRNA families with distinct repeat architectures suggests evolutionary innovation driven by genomic rearrangements and duplication events. The dysregulation of miRNAs and their repeat-containing precursors has been implicated in various diseases, including cancer, neurodegenerative disorders, and cardiovascular diseases. Aberrant expression or processing of miRNAs with repetitive elements can disrupt gene regulatory networks, contributing

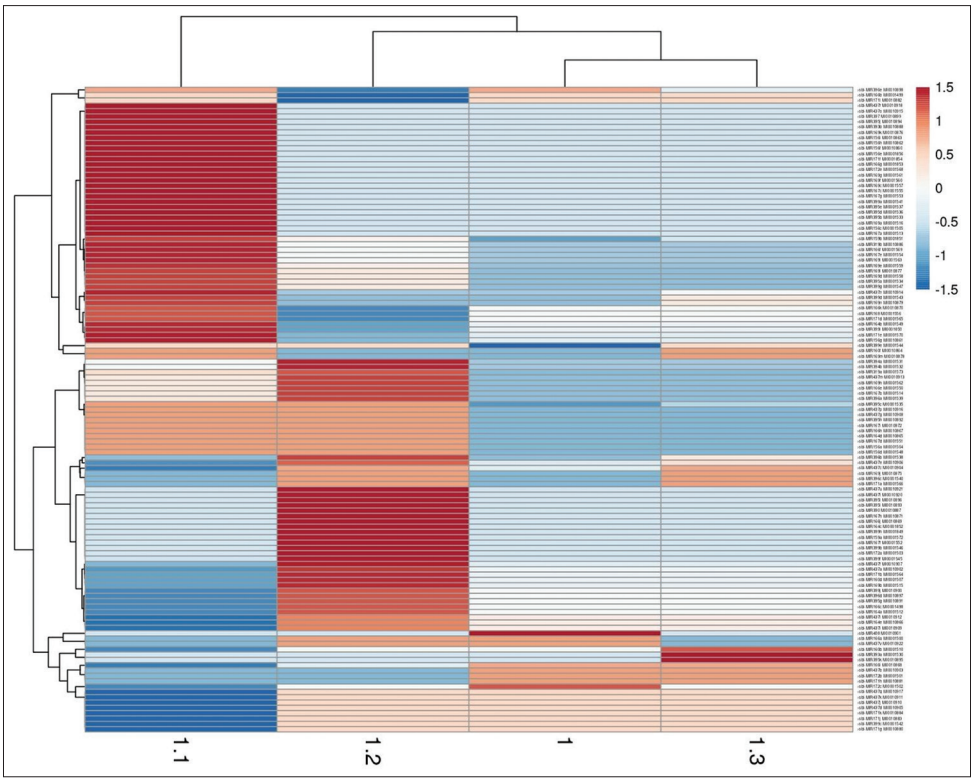

Figure 3: Heat map of two-way hierarchical clustering of microRNA (miRNA) secondary structure data of *Sorghum bicolor* obtained from Fair server (Sample miRNA sequences are shown at the right. Each row represents one miRNA. The miRNA clusters are represented as branch connections on the left. Cluster analysis classified the samples into groups based on number of stems, bulge, number of internal loops and terminal loops. Red colors represent the highest number and blue represent the lowest (absence).)

to disease pathogenesis (Kaur *et al.*, 2024). Targeting repeat-mediated mechanisms of miRNA biogenesis and function holds promise for therapeutic interventions aimed at restoring normal gene expression patterns in disease states. Their functional significance extends beyond structural stability to encompass diverse regulatory mechanisms that shape gene expression networks. There are few reports on the miRNA function of *Saccharum sp.* as well as in *Sorghum sp.* where they were supposed to regulate stress conditions (Thiebaut *et al.*, 2012). However, a detailed analysis of secondary structure is constantly needed to speculate the diversified functions of these miRNAs in plants. Our findings will provide a basis for further analysis of the functional aspects of these miRNAs and will eventually help in generating novel strategies on the agricultural aspects.

CONCLUSION

It is noteworthy to identify more miRNAs in plants and their target genes in distal organs in a non-cellular autonomous way. Using this data as reference, one can identify and characterize additional enzymes or modulators involved in overall regulation of various fundamental processes in plants. These are pretty much crucial for crop development since many miRNAs can safely modify plants at gene expression level. Additionally, one can study their involvement in host-parasite interactions. More analysis of miRNAs will eventually throw lights on the initial steps in the hidden world of small RNAs.

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SUPPLEMENTARY TABLES

Supplementary Table 1: Secondary Structural analysis of *Saccharum sp.*

S. No.	Accession Number and Sequence	Number of Stem	Number of Bulges	Number of Internal Loop	Number of Terminal Loop	Free Energy Value
1	>ssp-MIR169 MI0018180	1	2	2	2	-74.78
2	>ssp-MIR827 MI0018181	2	3	1	2	-60.5
3	>ssp-MIR437a MI0018182	1	0	2	2	-54.11
4	>ssp-MIR437b MI0018183	1	0	2	3	-33.47
5	>ssp-MIR437c MI0018184	1	0	3	2	-48.79
6	>ssp-MIR444a MI0018185	1	0	0	1	-59
7	>ssp-MIR444b MI0018186	1	0	1	1	-63.7
8	>ssp-MIR444c MI0018187	1	0	1	1	-62.8
9	>ssp-MIR528 MI0018188	2	2	1	2	-47.4
10	>ssp-MIR1128 MI0018189	1	1	4	3	-100.25
11	>ssp-MIR1432 MI0018190	1	2	1	1	-56.9
12	>ssp-MIR156 MI0018191	3	9	11	6	-193.05
13	>ssp-MIR159a MI0018192	1	2	7	2	-106.28
14	>ssp-MIR167b MI0018193	2	1	3	2	-61.88
15	>ssp-MIR168a MI0018194	1	0	1	1	-66.6
16	>ssp-MIR396 MI0018195	1	3	1	1	-48.3
17	>ssp-MIR408d MI0018196	1	4	4	2	-72.04
18	>ssp-MIR408a MI0018197	1	0	5	3	-97.18
19	>ssp-MIR166 MI0025546	1	3	2	1	-63.4

Supplementary Table 2: Secondary Structural analysis of *S. officinarum*

S. No.	Accession Number and Sequence	Number of Stem	Number of Bulges	Number of Internal Loop	Number of Terminal Loop	Free Energy Value
1	>sof-MIR156 MI0001754	1	5	1	1	-67.7
2	>sof-MIR396 MI0001755	1	3	2	1	-67.5
3	>sof-MIR159a MI0001756	2	2	7	2	-107.08
4	>sof-MIR159b MI0001757	2	3	6	2	-106.85
5	>sof-MIR159d MI0001758	2	4	5	3	-102.23
6	>sof-MIR159e MI0001759	2	4	5	2	-103.58
7	>sof-MIR159c MI0001760	2	5	3	3	-106.26
8	>sof-MIR167a MI0001761	2	1	5	2	-80.52
9	>sof-MIR167b MI0001762	2	1	4	2	-84.12
10	>sof-MIR168a MI0001763	1	0	1	1	-67.7
11	>sof-MIR168b MI0001764	1	2	2	1	-56.5
12	>sof-MIR408a MI0001765	2	1	6	3	-113.28
13	>sof-MIR408b MI0001766	2	3	5	3	-107.85
14	>sof-MIR408c MI0001767	2	2	6	3	-112.77
15	>sof-MIR408d MI0001768	1	4	6	2	-79.54
16	>sof-MIR408e MI0001769	2	4	7	3	-96.03

Supplementary Table 3: Secondary Structural analysis of *Sorghum bicolor*

S. No.	Accession Number and Sequence	Number of Stem	Number of Bulges	Number of Internal Loop	Number of Terminal Loop	Free Energy Value
1	>sbi-MIR166d MI0001497	1	1	1	1	-45.3
2	>sbi-MIR166c MI0001498	1	0	2	1	-54.2
3	>sbi-MIR166b MI0001499	1	1	0	1	-52.3
4	>sbi-MIR166a MI0001500	2	1	2	1	47.5
5	>sbi-MIR172b MI0001501	3	2	2	3	-59.85
6	>sbi-MIR172c MI0001502	3	1	2	2	-46.88
7	>sbi-MIR172a MI0001503	1	1	3	1	-48.2
8	>sbi-MIR156a MI0001504	1	2	2	1	-51.2
9	>sbi-MIR156c MI0001505	1	2	1	1	-46.6
10	>sbi-MIR156b MI0001506	1	1	1	1	-54
11	>sbi-MIR160d MI0001507	1	0	3	1	-51
12	>sbi-MIR160a MI0001508	1	1	1	1	-44.8
13	>sbi-MIR160c MI0001509	1	1	1	1	-38.5
14	>sbi-MIR160b MI0001510	1	0	1	2	-41.6
15	>sbi-MIR160e MI0001511	1	1	1	1	-53.9

(Contd...)

Supplementary Table 3: *(Continued)*

S. No.	Accession Number and Sequence	Number of Stem	Number of Bulges	Number of Internal Loop	Number of Terminal Loop	Free Energy Value
16	>sbi-MIR164a MI0001512	2	1	3	2	-60.19
17	>sbi-MIR167a MI0001513	1	2	1	1	-49
18	>sbi-MIR167b MI0001514	2	3	4	2	-83.85
19	>sbi-MIR169b MI0001515	1	0	3	1	-52.4
20	>sbi-MIR169a MI0001516	1	2	1	1	-46.2
21	>sbi-MIR393a MI0001530	1	1	1	2	-73.75
22	>sbi-MIR394a MI0001531	1	2	4	1	-43.2
23	>sbi-MIR395b MI0001533	1	4	1	1	-49.5
24	>sbi-MIR395a MI0001534	1	3	2	1	-78.5
25	>sbi-MIR395d MI0001536	1	2	1	1	-50.2
26	>sbi-MIR395e MI0001537	1	3	1	1	-50.2
27	>sbi-MIR396b MI0001538	1	1	3	2	-61.15
28	>sbi-MIR396a MI0001539	1	2	3	1	-49.8
29	>sbi-MIR396c MI0001540	1	1	2	2	-40.66
30	>sbi-MIR399a MI0001541	1	2	1	1	-68.9
31	>sbi-MIR399c MI0001542	2	1	2	2	-59.58
32	>sbi-MIR399d MI0001543	2	4	2	3	-108.23
33	>sbi-MIR399e MI0001544	1	2	2	2	-54.04
34	>sbi-MIR399f MI0001545	1	1	3	1	-47.4
35	>sbi-MIR399b MI0001546	1	1	2	1	-59
36	>sbi-MIR399g MI0001547	1	3	2	1	-53.7
37	>sbi-MIR156d MI0001548	1	3	3	1	-64.9
38	>sbi-MIR164b MI0001549	1	3	0	1	-71.7
39	>sbi-MIR166e MI0001550	1	2	3	1	-77.6
40	>sbi-MIR167d MI0001551	1	3	3	1	-77.6
41	>sbi-MIR167f MI0001552	2	2	3	2	-63.34
42	>sbi-MIR167g MI0001553	1	2	1	1	-64.6
43	>sbi-MIR167e MI0001554	1	7	3	1	-77.8
44	>sbi-MIR167c MI0001555	1	6	1	1	-80.6
45	>sbi-MIR168 MI0001556	1	2	0	1	-65.8
46	>sbi-MIR169c MI0001557	2	3	2	2	-69.2
47	>sbi-MIR169d MI0001558	2	4	3	2	-91.4
48	>sbi-MIR169f MI0001560	1	6	1	1	-83.6
49	>sbi-MIR169g MI0001561	1	7	1	1	-79.2
50	>sbi-MIR169i MI0001563	2	5	3	2	-79.75
51	>sbi-MIR171b MI0001564	1	0	3	1	-74.7
52	>sbi-MIR171d MI0001565	2	3	1	2	-59.94
53	>sbi-MIR171a MI0001566	2	2	3	3	-71.83
54	>sbi-MIR171c MI0001567	1	1	1	1	-45.5
55	>sbi-MIR172e MI0001568	1	4	1	1	-62.2
56	>sbi-MIR166f MI0001569	1	4	2	1	-61.1
57	>sbi-MIR171e MI0001570	1	4	0	1	-53.9
58	>sbi-MIR159a MI0001572	2	2	6	2	-92.27
59	>sbi-MIR319a MI0001573	1	4	6	1	-116.7
60	>sbi-MIR399h MI0001849	1	1	3	1	-59.3
61	>sbi-MIR399i MI0001850	1	3	0	1	-57.2
62	>sbi-MIR159b MI0001851	2	6	4	3	-104.36
63	>sbi-MIR164c MI0001852	1	1	3	1	-90
64	>sbi-MIR166g MI0001853	1	4	1	1	-75.2
65	>sbi-MIR171f MI0001854	1	3	1	1	-51.7
66	>sbi-MIR395f MI0001855	1	1	1	1	-61.4
67	>sbi-MIR156e MI0001856	1	6	1	1	-71
68	>sbi-MIR156f MI0010860	2	4	2	2	-66.3
69	>sbi-MIR156g MI0010861	1	4	0	1	-59.9
70	>sbi-MIR156h MI0010862	1	3	1	1	-61.1
71	>sbi-MIR156i MI0010863	1	5	1	1	-67.3
72	>sbi-MIR160f MI0010864	1	2	1	2	-56.6
73	>sbi-MIR164d MI0010865	1	2	2	1	-79.7
74	>sbi-MIR164e MI0010866	3	1	4	3	-96.65
75	>sbi-MIR166h MI0010867	1	3	3	1	-79.8
76	>sbi-MIR166i MI0010868	2	0	1	2	-46.1
77	>sbi-MIR166j MI0010869	1	1	2	1	-48.2
78	>sbi-MIR166k MI0010870	1	2	0	1	-53.8
79	>sbi-MIR167h MI0010871	1	1	2	1	-40.5

(Contd...)

Supplementary Table 3: *(Continued)*

S. No.	Accession Number and Sequence	Number of Stem	Number of Bulges	Number of Internal Loop	Number of Terminal Loop	Free Energy Value
80	>sbi-MIR167i MI0010872	1	3	3	1	-59.1
81	>sbi-MIR169e MI0001559	1	8	4	1	-121.6
82	>sbi-MIR169h MI0001562	2	3	4	2	-69.03
83	>sbi-MIR169j MI0010875	1	1	2	2	-63.48
84	>sbi-MIR169k MI0010876	1	2	1	1	-45.3
85	>sbi-MIR169l MI0010877	1	3	2	1	-49
86	>sbi-MIR169m MI0010878	1	2	1	2	-40.03
87	>sbi-MIR169n MI0010879	1	3	1	2	-42.65
88	>sbi-MIR171g MI0010880	1	0	1	1	-36.6
89	>sbi-MIR171h MI0010881	1	0	0	1	-51.5
90	>sbi-MIR171i MI0010882	1	1	0	1	-42.3
91	>sbi-MIR171j MI0010883	2	1	2	2	-43.4
92	>sbi-MIR171k MI0010884	1	0	1	1	-47.5
93	>sbi-MIR172d MI0001571	1	1	1	1	-49.4
94	>sbi-MIR319b MI0010886	1	4	2	1	-92
95	>sbi-MIR390 MI0010887	1	1	5	1	-93
96	>sbi-MIR393b MI0010888	1	4	1	1	-48.5
97	>sbi-MIR394b MI0001532	1	2	4	1	-48.5
98	>sbi-MIR395c MI0001535	1	5	5	2	-74.14
99	>sbi-MIR395g MI0010891	1	0	2	1	-35.4
100	>sbi-MIR395h MI0010892	1	2	2	1	-37.5
101	>sbi-MIR395i MI0010893	1	1	2	1	-45.3
102	>sbi-MIR395j MI0010894	1	2	1	1	-43
103	>sbi-MIR395k MI0010895	1	1	1	2	-31.14
104	>sbi-MIR395l MI0010896	1	1	3	1	-39.5
105	>sbi-MIR396d MI0010897	1	0	2	1	-42.9
106	>sbi-MIR396e MI0010898	4	4	2	3	-86.93
107	>sbi-MIR397 MI0010899	1	2	1	1	-67.6
108	>sbi-MIR399j MI0010900	1	0	2	1	-45.2
109	>sbi-MIR408 MI0010901	4	3	3	3	-87.83
110	>sbi-MIR437a MI0010902	2	0	5	2	-49.49
111	>sbi-MIR437b MI0010903	2	1	1	2	-54.92
112	>sbi-MIR437c MI0010904	2	1	3	3	-29.84
113	>sbi-MIR437d MI0010905	2	1	2	2	-35.72
114	>sbi-MIR437e MI0010906	1	0	3	2	-16.9
115	>sbi-MIR437f MI0010907	1	0	4	1	-57.8
116	>sbi-MIR437g MI0010908	1	2	2	1	-67
117	>sbi-MIR437i MI0010909	2	0	3	2	-60.94
118	>sbi-MIR437j MI0010910	2	1	2	2	-61.68
119	>sbi-MIR437k MI0010911	2	0	2	2	-55.57
120	>sbi-MIR437l MI0010912	2	0	3	2	-34.39
121	>sbi-MIR437m MI0010913	1	2	3	1	-41.2
122	>sbi-MIR437n MI0010914	1	4	1	2	-49.37
123	>sbi-MIR437o MI0010915	2	4	2	2	-36.8
124	>sbi-MIR437p MI0010916	1	3	3	1	-66.5
125	>sbi-MIR437q MI0010917	2	0	2	2	-42.02
126	>sbi-MIR437r MI0010918	1	3	1	1	-54.6
127	>sbi-MIR437s MI0010919	2	2	2	2	-57.33
128	>sbi-MIR437t MI0010920	1	1	3	1	-62
129	>sbi-MIR437u MI0010921	2	2	3	2	-34.4
130	>sbi-MIR437v MI0010922	3	2	3	2	-25.54