

## Experimental Validation and Characterization of Sugarcane Genome-Encoded MicroRNAs and Their Targets Using PCR-Based Expressional Methodology

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### ABSTRACT

MicroRNAs (miRNAs) are typically small, endogenous, non-coding RNAs molecules that regulate gene expression at post-transcriptional level by mRNA degradation or translational repression. They are composed of 18-26 nucleotides and are conserved during evolution for the development of new miRNAs in a variety of plants. Sugarcane (*Saccharum officinarum*) is generally a valuable food and forage crop grown all over the world. Until now, different sugarcane miRNAs have been characterized for plant development and stress responses. In this research, 50 unique conserved sugarcane miRNAs from 44 different miRNA families have been predicted using a variety of genomics-based tools. The predicted sugarcane miRNAs were validated using a set of 15 randomly chosen primers and RT-PCR. Stem loop secondary structures are created using MFOLD tool. The psRNA-Target algorithm identified 7,976 various protein targets of sof-miRNAs including 55 specific GO terms. They have significant targets in biological, cellular, and molecular functions. Moreover, the sof-miR5205a regulates sulfur compound biosynthetic process and 9653a directs ubiquitin-dependent protein catabolic process. Consequently, the RNA binding and thylakoid membrane are controlled by sof-miR9657b and 2091, respectively. As a result, the outcomes of the novel sugarcane miRNAs target a variety of substantial genes that aid in controlling the environment for sugarcane to produce a higher quality crop.

**Keywords:** Biological process, miRNA, RT-PCR amplification, *Saccharum officinarum*, Web logo.

### INTRODUCTION

MicroRNAs, also known as miRNAs, are small RNAs that begin in the body endogenously and range in size from 18 to 26 nucleotides (nt). They are a subset of non-coding RNAs, and it is believed that they either control the cleavage of target mRNAs or post-transcriptionally suppress their translation (Almatroudi, 2022).

These types of small miRNAs, which are made from lengthy precursor miRNAs (pre-miRNAs), are mature miRNAs. These miRNAs are between 70 and 500 nt in length, and plants' Dicer-Like 1 (DCL1)

enzymes fold them into self-folded stem-loop secondary structures (Yusof *et al.*, 2020). Mature miRNAs regulate post-transcriptional levels of gene expression by either targeting mRNAs for degradation or preventing protein translation. Actually, the completion of both strategies depends on the miRNAs and their target mRNA sequences to couple together in a suitable complementary way (Rani and Sengar, 2022). In plants, miRNAs nearly always hybridize perfectly or almost perfectly with their targets, which directs the target mRNA breakdown (Hajieghrari *et al.*, 2022). A recent study revealed that miRNAs are

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important for a variety of developing procedures in plants, consisting of cell division, pressure response, absorption, irritation and signal transduction (Rojas et al., 2022).

After that, a growing number of miRNAs have been continuously discovered using computational and experimental techniques in animals, plants, and even viruses. So far, nearly 48860 miRNAs have been studied from 271 species of plants and animals, according to the freely accessible database miRBase (Release 22) (Kirchner, 2022).

Following this discovery, miRNAs from diverse plant species were found to have fully sequenced genomes like 738 from *Oryza sativa*, 525 from *Brachypodium distachyon*, 428 from *Arabidopsis thaliana*, 401 from *Populus trichocarpa*, 343 from *Solanum tuberosum*, 325 from *Zea mays* and 241 from *Sorghum bicolor* (Kirchner, 2022). Evidently, miRNAs with such high levels of conservation provide a useful method for profiling new miRNAs from different species. Currently, comparative genome-based approaches have been used to profile conserved miRNAs in numerous plant species. This contains switchgrass (Xie et al., 2010; Barozai et al., 2018), cherry (Baloch et al., 2018), tomato (Din et al., 2014), red alga (Barozai et al., 2018), and cowpea (Gul et al., 2017)

Sugarcane (*Saccharum officinarum*), a member from the grass family (Poaceae), is widely cultivated, providing almost 70% of the world's sugar. Sugarcane produces the greatest number of calories per unit of growth of any plant. The majority of the sugar consumed worldwide is produced from sugarcane. In addition to producing sugar and the raw materials needed to manufacture alcohol. The purpose of traditional sugar manufacturing methods is to increase the sucrose concentration and remove color by thermal and chemical processing juice, syrup and molasses (Duarte-Almeida, 2011). According to research, *S. officinarum* accounts for 70-80% of the genetic background of hybrid *Saccharum* species (Xue et al., 2017). It is feasible to assess plant improvement by

studying its genetic make-up and sowing in various locations (Achakzai et al., 2019; Fontana et al., 2021; Awaad et al., 2021; Rasheed et al., 2020).

Only 16 mature miRNAs are reported in sugarcane from the *Poaceae* family in the miRBase (<http://www.mirbase.org/>, Release 22: January 2019), a database of miRNAs. Additionally, our research will contribute to understand and profile new sugarcane miRNAs in a more comprehensive way. However, it is essential to profile more conserved miRNAs that will help these important grain crops. In this study, a precise comparative genome-based homolog search has been employed to profile fresh sugarcane miRNAs and their targets.

## MATERIALS AND METHODS

### Finding Reference miRNA Sequences

With the aid of miRBase, a database of miRNAs (<http://www.mirbase.org/>, Release 22: January 2019), total number of attained plant precursor and mature miRNA sequences were 10523 (Kirchner, 2022). These reference miRNAs were obtained from 17 plant species like *Arabidopsis lyrata* (aly), *Arabidopsis thaliana* (ath), *Brachypodium distachyon* (bdi), *Cucumis melo* (cme), *Carica papaya* (cpa), *Gossypium hirsutum* (ghr), *Glycine max* (gma), *Gossypium raimondii* (gra), *Hordeum vulgare* (hvu), *Medicago truncatula* (mtr), *Nicotiana tabacum* (nta), *Oryza sativa* (osa), *Populus trichocarpa* (ptc), *Sorghum bicolor* (sbi), *Solanum tuberosum* (stu), *Triticum aestivum* (tae), and *Zea mays* (zma). In order to anticipate new well-maintained miRNAs from the sugarcane Expressed Sequences Tags (ESTs), the 10523 miRNAs were employed as the source for miRNAs.

### Retrieval of Candidate miRNAs

Considering the unique conserved sugarcane miRNAs via comparative

homology-based search, approximately 20,703 sugarcane ESTs were obtained from the EST-database (dbEST), (11 December 2019) available at [https://www.ncbi.nlm.nih.gov/genbank/dbest/dbest\\_summary](https://www.ncbi.nlm.nih.gov/genbank/dbest/dbest_summary). Now, for profiling of possible conserved miRNAs, the reference miRNAs and sugarcane ESTs have been exposed to BLASTn and BLASTx algorithms by removing the protein coding and repetitive sequences (Altschul *et al.*, 1990). In doing so, the putative candidate sugarcane miRNAs in FASTA format that had non-coding characteristics and up to four mismatches with the reference miRNAs were separated out, kept, and forwarded for further examination.

### Sugarcane miRNAs Stem-Loop Structures

In order to profile and describe novel conserved miRNAs in sugarcane, the key phenomenon used is the drawing of stem-loop secondary structures of preliminary probable candidate sequences, MFOLD (version 3.6) (Zuker, 2003; Rani *et al.*, 2022).

### Physical Examination

It is a key step that eliminates all the false positive miRNAs from the candidate miRNAs. It is also important to note that each newly analyzed sugarcane miRNA has an EST that identifies the organ of expression for that miRNA.

### RT-PCR Validation

In the light of the recently profiled sugarcane miRNAs, fifteen miRNAs were randomly chosen and subjected to expression analysis by RT-PCR (Reverse Transcription) (Paolacci *et al.*, 2009). Considering this, Primer-3 algorithm (<http://bioinfo.ut.ee/primer3-0.4.0>) were

employed to generate stem-loop primers from the ESTs of fifteen subjectively chosen miRNAs (Table 1). With the use of Trizol reagent (Cat No: AM9738, Thermo Scientific), total RNA was successfully extracted from sugarcane leaves. Following that, cDNA was made utilizing the RevertAid™ First Strand cDNA synthesis Kit (Cat No: K1622, Thermo Scientific), in accordance with the supplier's protocol. In order to run the PCR machine, 60 µL cDNA was used as template. Further adjustment of PCR should be like: preheat (activation) at 95°C for 5 minutes, denaturation at 95°C for 45 seconds for 35 cycles, annealing at 60°C for 45 seconds, extension at 72°C for 1 minute, and post cycling extension step at 72°C for 5 minutes. Finally, 1.5 percent (w/v) agarose gel with a 100 base pair DNA ladder was used to obtain the results for the separation of PCR products.

### Phylogenetic and Conservation Analyses

In this study, miR-399 phylogenetic analysis was started by comparing it to other monocotyledonous and dicotyledonous plant precursors associated to *Saccharum officinarum*, *Hordeum vulgare*, *Citrus sinensis*, *Brachypodium distachyon*, *Nicotiana tabacum*, and *Solanum lycopersicum* via a tool easily accessible at (<https://www.ebi.ac.uk/Tools/msa/clustalo/>). It has been accomplished in accordance with the method explained by Baloch *et al.* (2015). However, for conservation analysis, a tool termed “web logo”, which can be accessed through the link (<http://weblogo.berkeley.edu/logo.cgi>, version 2.8) was used to conduct studies on the sequence logo generator for conservation analysis of numerous plant precursors like *Hordeum vulgare*, *Brachypodium distachyon*, and *Citrus sinensis*. A similar process was utilized for logo generation, as reported by Baloch *et al.* (2018).

**Table 1.** The fifteen randomly chosen sugarcane forward and reverse primers.

Sugarcane miRNAs	Accession	Primer (Forward and Reverse)	Amplicon size	Tm	GC%	Bases
sof-miR165a	CN607727	F- GAGATGAGAAGATGAGAGGG R- AGAACAACCAGGAATCTCAC	304	54.06 54.98	50.00 45.00	20 20
sof-miR530	CA257041	F- TATGCAAATGAAGACGTGTC R- TCCACCACGAGAGCTTAC	305	54.05 55.95	40.00 55.56	20 18
sof-miR823	CA103350	F- TAGGGCGTATATGGTCTGG R- AACATCACCGTCAACCAG	331	55.35 54.85	52.63 50.00	19 18
sof-miR858	CA225244	F- AGGTGCGAGTTCAGTAG R- GAAGAAGGGGAGGTGGACC	334	55.94 59.01	55.56 63.16	18 19
sof-miR1439	CA198902	F- ACGTATCTTTTGTTATGCACT R- TGCAACTAAATGACAATGAGG	335	53.56 54.47	33.33 38.10	21 21
sof-miR2907b	CA104808	F- CAAGTTGCCGGTCACCAG R- CTCCCGCTGCTTCCTCAT	330	58.66 59.09	61.11 61.11	18 18
sof-miR5049	CN608955	F- CTTGGAAGTAAAGCCTTGC R- CCGAATCTTTTGAGCCTAGT	331	55.16 55.16	45.00 45.00	20 20
sof-miR5077	CA109931	F- TTCATGACCTGCCTTGTG R- CCCGACGATAAGCATGGC	196	54.80 58.36	50.00 61.11	18 18
sof-miR5496	CA254292	F- TGGTTCCTGGGTTTGTTCAG R- ACAACTAAGTCTCATTCCGCG	194	56.07 55.91	45.00 45.00	20 20
sof-miR5566	CA222783	F- GGTTAGAGGTATGCAAATCTT R- TGTCTAATAGGTGAGGATAGG	413	53.29 54.87	38.10 40.91	21 21
sof-miR6181	CA235019	F- CTTGATCGATCTTGCATTG R- TCGATGTATTTTACTGCGGG	301	54.99 55.66	45.00 45.00	20 20
sof-miR6196	CA212234	F- CGCAGCAAGAACGTATATTT R- GCTCATAAAGTTCTCCATCG	414	54.52 53.92	40.00 45.00	20 20
sof-miR9482	DN192807	F- CTTCACTGCAGTACTTCTCG R- GATTCTGCTCTCCGAGA	413	55.93 55.36	50.00 55.56	20 18
sof-miR9653a	DN195467	F- GATTGCTCCCCTCCTTTC R- TGAGGTTATCTTCTGTTTCCA	335	55.55 54.18	52.63 38.10	19 21
sof-miR9657b	CA201285	F- CGAGCTGAGCAGGGAAGG R- CTCAGAGCAGATGTAGAAGC	337	59.81 55.38	66.67 50.00	18 20

### Targets Prediction

To predict the possible targets of the recently identified sugarcane miRNAs, psRNATarget: A Plant Small RNA Target Analysis Server (2017 Update) zhaolab.org, available at (<http://www.zhaolab.org/psRNATarget/>) (Dai and Zhao, 2011) was utilized. The sugarcane library [*Saccharum officinarum* (sugarcane), unigene, DFCI Gene Index (SOGI), version 3, released on 09-04-2010] was utilized as the preferred target library with the revised 2017 restructured parameters of psRNA Target. Moreover, agriGo's Gene Ontology functional and

enrichment studies were used to analyze the newly predicted sugarcane miRNA targets (Achakzai *et al.*, 2018).

## RESULTS

### Sugarcane New Potential miRNAs

In this research, 50 new conserved miRNAs were made from sugarcane ESTs using comparative genomics-based homology search (S1 Table). The 50 novel conserved miRNAs are related to 44 miRNA families. They include sof-miR165a, 165b, 399e, 399f, 477, 482a, 530, 531, 823, 854, 858, 1130b, 1439, 1853, 2091, 2094a, 2611,



2907b, 5049, 5077, 5205a, 5290, 5384, 5496, 5564a, 5565a, 5565b, 5565g, 5566, 5809a, 5809b, 5819, 6144a, 6144b, 6181a, 6181b, 6196a, 6196b, 6214b, 6230, 6437a, 7491, 7698, 7710, 8039, 8632, 9482, 9653a, 9657b, and 11337 (S1 Table).

Furthermore, it is confirmed that these novel 50 miRNAs of sugarcane have been reported for the first time and have not been mentioned earlier. Accordingly, these 50 novel miRNAs have been created by the assistance of reference miRNAs of *A. lyrata* (4%), *A. thaliana* (2%), *B. distachyon* (4%), *C. melo* (4%), *C. papaya* (2%), *G. hirsutum* (2%), *G. max* (2%), *G. raimondii* (2%), *H. vulgare* (12%), *M. truncatula* (8%), *N. tabacum* (4%), *O. sativa* (22%), *P. trichocarpa* (2%), *S. bicolor* (12%), *S. tuberosum* (2%), *T. aestivum* (14%), and *Z. mays* (2%).

### Sugarcane miRNAs Characterization

The recently profiled sugarcane miRNAs was categorized and explained in respect of pre-miRNAs length, MFE of pre-miRNAs, mature miRNA sequences with mismatches, number of mismatches, mature sequence length, ESTs, strand orientation, mature sequences arm, GC percentage, and organ of expression (S1 Table). Consequently, all of the mature sequences of the newly conserved sugarcane miRNAs are noted in the stem portions of the stem-loop structures (Figure 1).

According to length, sugarcane pre-miRNAs range from 41 to 205 nt, having an average length of 88 nt. Considerably, it is found in the arrangement of pre-miRNAs lengths that 1–50 nt (6 out of 50) and formed 12% of the overall pre-miRNA, from 51–100 nt (28 out of 50= 56%), 101–150 nt (14 out of 50= 28%), 151–200 nt (1 out of 50= 2%), and 201–250 nt (1 out of 50= 2%) (Figure 2-a).

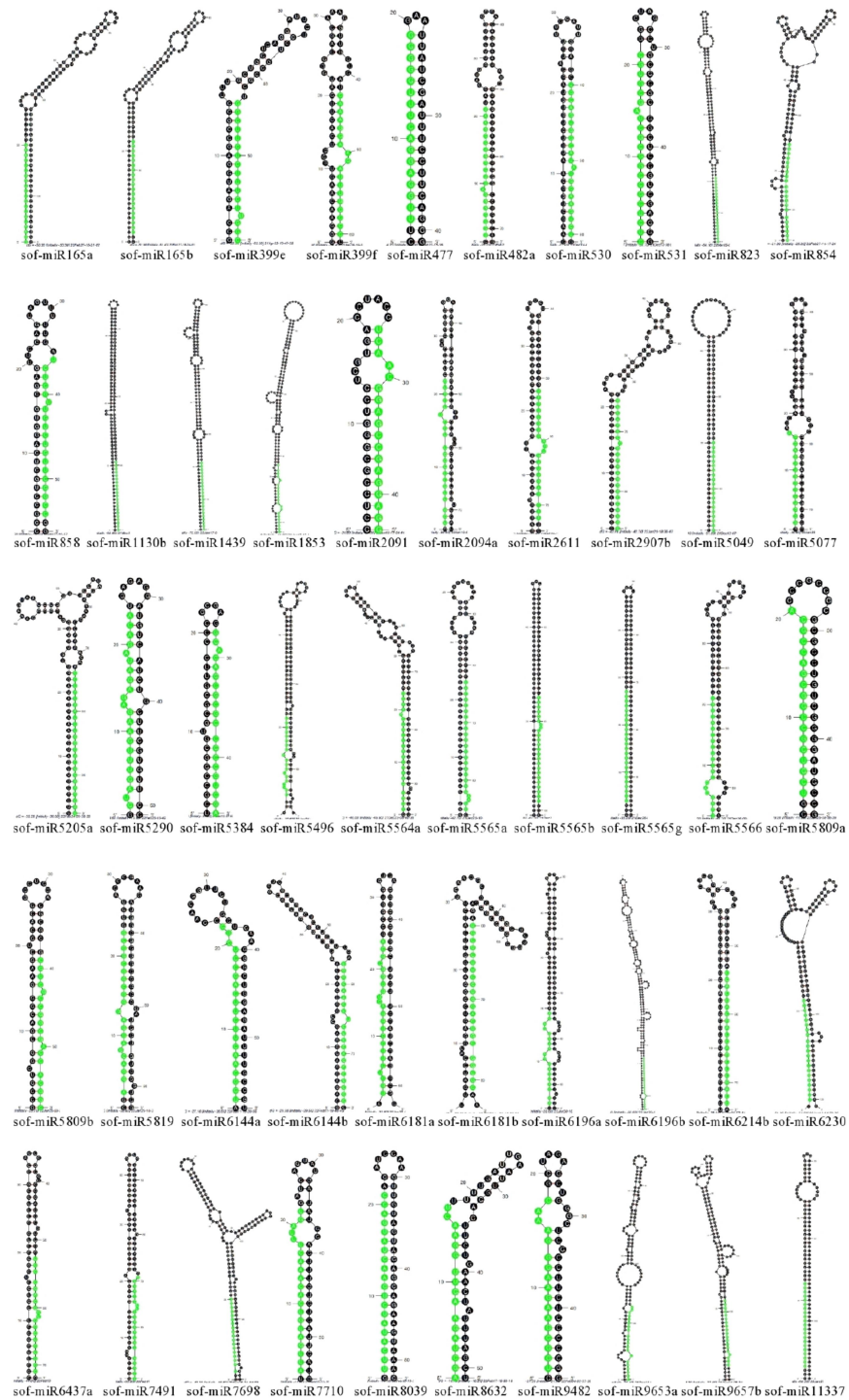
Additionally, this work has noted that the MFE of the freshly found sugarcane pre-miRNAs ranges from –74.3 to –10.1 kcal mol<sup>-1</sup>, having an average of –35.6 kcal mol<sup>-1</sup>.

In accordance with class boundaries –100 to –60 kcal mol<sup>-1</sup> (5) formed 10% of the overall pre-miRNA, from –61 to –20 (37) formed 74%, and from –21 to –00 kcal mol<sup>-1</sup> (8) formed 16% of all the pre-miRNAs.

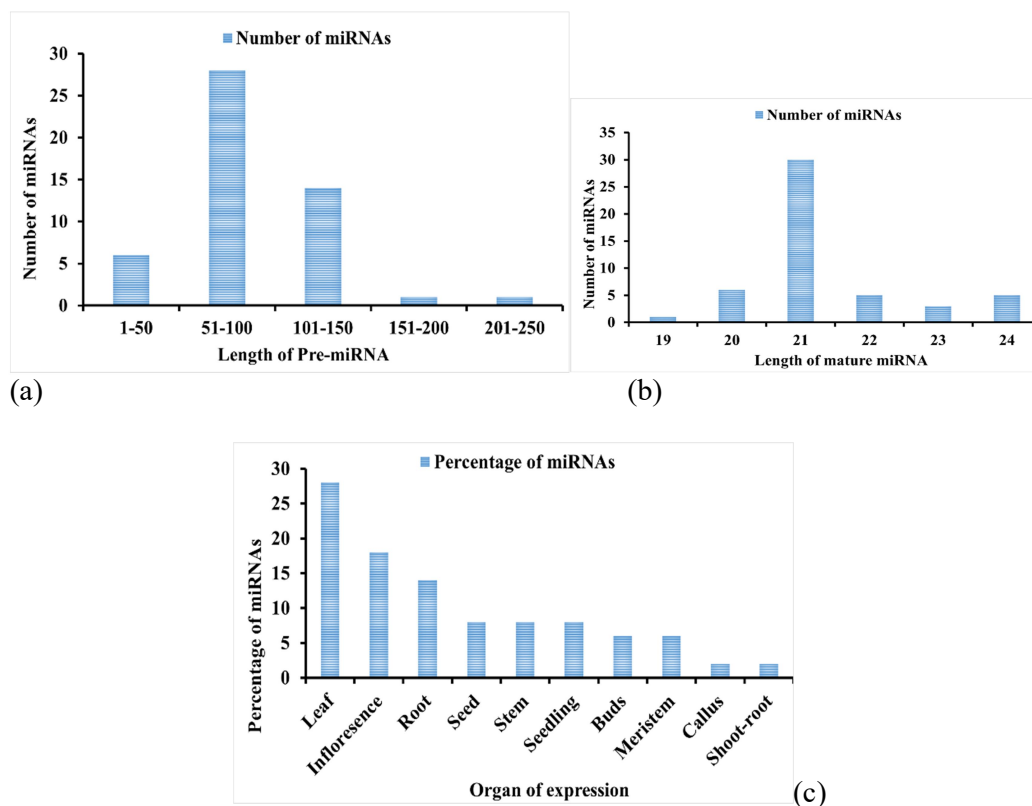
According to the aforementioned study, the crucial outcomes concerning the total mismatches noticed in the predicted sugarcane mature miRNAs as well as their source sequences vary between 1-4, with an average of 2 mismatches. Henceforth, 3 mismatches (13 miRNAs out of 50) were sought 26% of all miRNAs, 2 mismatches (9 miRNAs out of 50) with 18%, 4 mismatches (24 miRNAs out of 50) with 48%, and 1 mismatch was 8% (4 miRNAs out of 50).

Accordingly, the mature lengths of sugarcane miRNAs, which had a minimum and maximum of 19 and 24 nt, respectively, with an average of 21 nt, were found. Now, assuming the class boundaries, the lengths of mature sequences ranging from shortest to longest are; 19 nt have (1 out of 50) formed 2% of total, 20 nt (6 out of 50) 12%, 21 nt (30 out of 50) 60%, 22 nt (5 out of 50) 10%, 23 nt (3 out of 50) 6%, 24 nt (5 out of 50) 10% (Figure 2-b). This study showed that, among the 50 newly analyzed miRNAs, 31 were exhibited in the sense strand, accounting for 62% of the overall miRNAs. In contrast, 19 miRNAs out of 50 were observed to have been created in an anti-sense strand orientation that produced 38% of all the miRNAs.

Additionally, on the 5' arm of secondary structures, there are 23 out of 50 miRNAs found, which account for 46% of all mature sequences whereas 27 out of 50 miRNAs were found to make up 54% on the 3' arm. Taking the nucleotide sequence into account, the crucial measure of characterization is the GC percentage. As a result, the GC percentage for the newly projected sugarcane miRNAs ranged from a minimum of 30% to a maximum of 86%, with an average of 55%. Now, from the class boundaries, the entire values of GC% are presented as: 10 to 40% (7 out of 50) 14%, 41 to 60% (26 out of 50) 52%, 61 to



**Figure 1.** The newly identified sugarcane miRNAs secondary structures (mature in green).



**Figure 2.** The distributions identified in sugarcane ESTs: (a) Length of precursor miRNAs, (b) Length of mature miRNAs, and (c) Organ of expression.

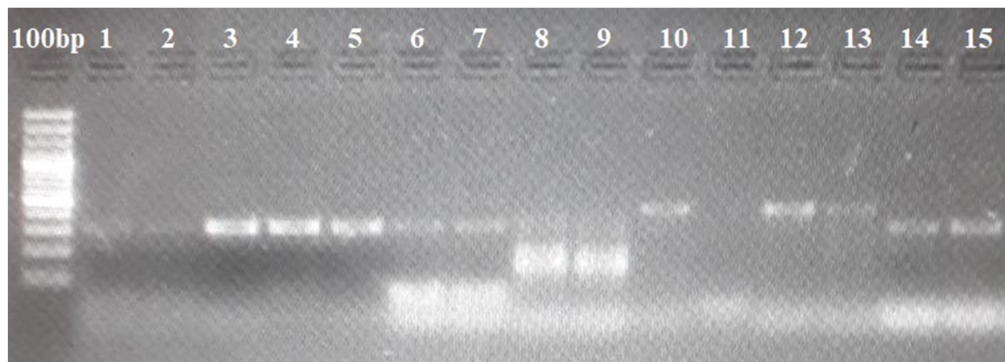
80% (14 out of 50) 28%, and 81 to 95% (3 out of 50) 6% of the total.

Likewise, the organ of expression of the newly examined sugarcane miRNAs was also calculated for their ESTs. The majority of miRNAs were in the leaf (14 out of 50), which accounted for 28% of the total, and were followed by inflorescence 18%, root 14%, seed 8%, stem 8%, seedling 8%, buds 6%, meristem 6%, callus 2%, and shoot-root 2% (Figure 2-c). The expression of sugarcane miRNAs at the organ level plays special functions in the initiation of the development and regulation of improved plant organs. The previously reported data in other plant species are consistent with the reported diverse organ-based expression of miRNAs, using comparative genomics methodologies (Din *et al.*, 2014; Barozai *et*

*al.*, 2018; Baloch *et al.*, 2015; Bibi *et al.*, 2017).

### Amplification and validation of sugarcane miRNAs

In order to experimentally validate the newly profiled sugarcane miRNAs, the substantial analysis used is the RT-PCR. The 15 sugarcane miRNAs along with the 100 base pair ladders were used for amplification (Paolacci *et al.*, 2009) in RT-PCR expression assay (Figure 3). The arrangement will be like: 1 (sof-miR165a), 2 (sof-miR530), 3 (sof-miR823), 4 (sof-miR858), 5 (sof-miR1439), 6 (sof-miR2907b), 7 (sof-miR5049), 8 (sof-miR5077), 9 (sof-miR5496), 10 (sof-miR5566), 11 (sof-miR6181), 12 (sof-miR6196), 13 (sof-miR9482), 14 (sof-



**Figure 3.** Sugarcane miRNAs RT-PCR expression validation.

miR9653a), and 15 (sof-miR9657b). Among the 15 sugarcane miRNAs, 14 miRNAs were validated through RT-PCR in an appropriate way and results are shown in Figure 3. However, RT-PCR validation of just one miRNA, 11 (sof-miR6181), was not verified. The cause could be a result of a sugarcane variety difference, environmental element, or developmental stage difference. So, an agarose gel with a 1.5% concentration and a 100 base pair DNA ladder were used to verify the 15 products. Such outcomes were used by numerous researchers studying various plant types (Din *et al.*, 2016; Zhang *et al.*, 2008).

#### Phylogenetic and Conservation Studies of Sugarcane miRNAs

The phylogenetic tree and conservation studies for sugarcane miRNAs were generated and displayed (Figures 4 and 5). Sugarcane and barley (*Hordeum vulgare*) are closely related, as seen by the red highlighted box (Figure 4). In accordance with conservation analyses of the pre-miRNA 399 (Figure 5), red highlighted frame displays the conserved areas of matures associated with other plants such as *H. vulgare*, *B. distachyon*, and *C. sinensis*.

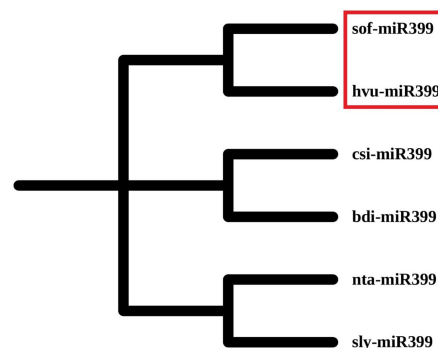
#### Estimate of Sugarcane miRNAs Significant Targets

The targets estimation is a crucial step in the explanation and characterization of the recently found sugarcane miRNAs. As a

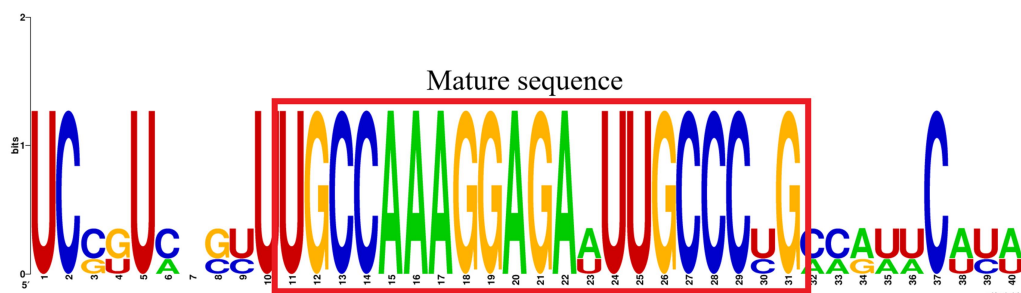
whole, almost 7976 target genes were predicted for the recently predicted 50 newly conserved sugarcane miRNAs with the use of a very complex method as described above. Additionally, taking into account the gene ontology annotation, such targets comprise 55 GO-terms (S2 Table) and are essential to important activities such as cellular component biogenesis, response to stimulus, RNA biosynthetic process, regulation of biological quality, response to stress, protein binding, molecular transducer activity, mitochondrion and insoluble fraction (Achakzai *et al.*, 2018; Tian *et al.*, 2017; Eskandarynasab *et al.*, 2020).

#### DISCUSSION

The miRNAs presented in this research include homologs of both dicots and monocots. Some of the 50 miRNAs have



**Figure 4.** Sugarcane miRNA and their phylogenetic analysis.



**Figure 5.** Conservation analysis of the miRNA in sugarcane. Mature miRNA sequences and their conserved nature are shown in the red boxed area that has been highlighted.

homologs in both dicots and monocots, whereas others are exclusive to one or the other. In addition, to find out new interesting results of several organisms, the widely used approach is the comparative genomics-based research (Wahid *et al.*, 2016; Jahan *et al.*, 2017; Ghani *et al.* 2018; Barozai *et al.*, 2017; Shah *et al.*, 2021). This assisted in the prediction of 50 novel sugarcane miRNAs. Following this, to satisfy the empirical formula, A, B and D for the synthesis and expression of the miRNAs, presented by Ambros *et al.* (2003), all of the newly identified conserved sugarcane miRNAs were presumed to be genuine candidates. Evidently, the principle D is only enough for homologous sequences in order to confirm new miRNAs in several plant species as described by Ambros *et al.* (2003).

It is demonstrated that the stem loop structures of the predicted miRNAs contain roughly 11–21 nucleotides that are involved in Watson–Crick or G/U base pairings between the mature miRNA and the opposing arms (pre-miRNAs) in the stem section. Similar to this, the ancestors to hairpins lack significant interior loops or bulges. Similar findings for the miRNAs in many plants and animals have been reported in a number of studies (Din *et al.*, 2016; Baloch *et al.*, 2015; Bibi *et al.*, 2017). The MFE (Minimal Free Energy) of the freshly noted sugarcane miRNAs range from  $-74.3$  to  $-10.1$  kcal mol $^{-1}$ , with an average of  $-35.6$  kcal mol $^{-1}$ . Earlier, several researchers in different organisms confirmed the

conclusions about the reported MFEs of pre-miRNAs that were discussed above (Rojas *et al.*, 2022; Din *et al.*, 2016; Zhang *et al.*, 2008; Gasparis *et al.*, 2017; Bibi *et al.*, 2017).

Considering the total mismatches in sugarcane, they vary between 1–4, with an average of 2 mismatches. Therefore, the results of sugarcane miRNA mismatches, which have a range of 0–4, are similar to those for other species of plants and animals that have been previously mentioned (Din *et al.*, 2016; Xie *et al.*, 2010; Baloch *et al.*, 2015; Bibi *et al.*, 2017). Moreover, the nucleotides in the mature length of sugarcane miRNAs are 19 and 24, with an average of 21. As a result, the length range of sugarcane mature sequences is observed to be consistent with the other recognized plant miRNAs (Gul *et al.*, 2017; Bibi *et al.*, 2017).

According to phylogenetic and conservation analyses of sugarcane miRNAs, the sof-miRNA399 is more closely related to *H. vulgare* (hvu) than to *C. sinensis* (csi), *B. distachyon* (bdi), *N. tabacum* (nta) and *S. lycopersicum* (sly). Similar findings have already been reported by experts from several professions (Achakzai *et al.*, 2019; Din *et al.*, 2018).

GO-biological method exposed that the assumed targets of the recently identified sugarcane miRNAs are prominently contained of multi-organism process (GO:0051704), response to abiotic stimulus (GO:0009628), regulation of biosynthetic





process (GO:0009889), RNA metabolic process (GO:0016070), regulation of biological process (GO:0050789), biological regulation (GO:0065007), ubiquitin-dependent protein catabolic process (GO:0006511), protein transport (GO:0015031), defense response (GO:0006952) and sulfur compound biosynthetic process (GO:0044272) (S2 Table, Figure 6) (Achakzai *et al.*, 2018; Eskandarynasab *et al.*, 2020). These putative targets are regulated and annotated by the novel identified sugarcane miRNAs like sof-miR8039, sof-miR7698, sof-miR5566, sof-miR399e, sof-miR5809a, sof-miR2091, sof-miR9653a, sof-miR165b, sof-miR6196b, and sof-miR5205a. Thus, these recently discovered sugarcane miRNAs contribute to the better crop management by controlling the environment for sugarcane.

In the light of this, the GO cellular component is the next significant target of

sugarcane (Achakzai *et al.*, 2018; Eskandarynasab *et al.*, 2020). This contains the key targets in the membrane-bounded organelle (GO:0043227), organelle (GO:0043226), cytoplasmic part (GO:0044444), intracellular part (CA129594), cell (GO:0005623), nucleus (GO:0005634), cytosol (GO:0005829), plastid (GO:0009536), membrane (GO:0016020), and nucleolus (GO:0005730) which are plainly displayed (S2 Table, Figure 7). These essential tasks are carried out by the sugarcane miRNAs like sof-miR5077, sof-miR2611, sof-miR1439, sof-miR1130b, sof-miR5565b, sof-miR11337, sof-miR530, sof-miR6230 and sof-miR5565a.

Additionally, a large number of genes have key roles in a variety of activities, most notably in the GO molecular function (Achakzai *et al.*, 2018; Eskandarynasab *et*

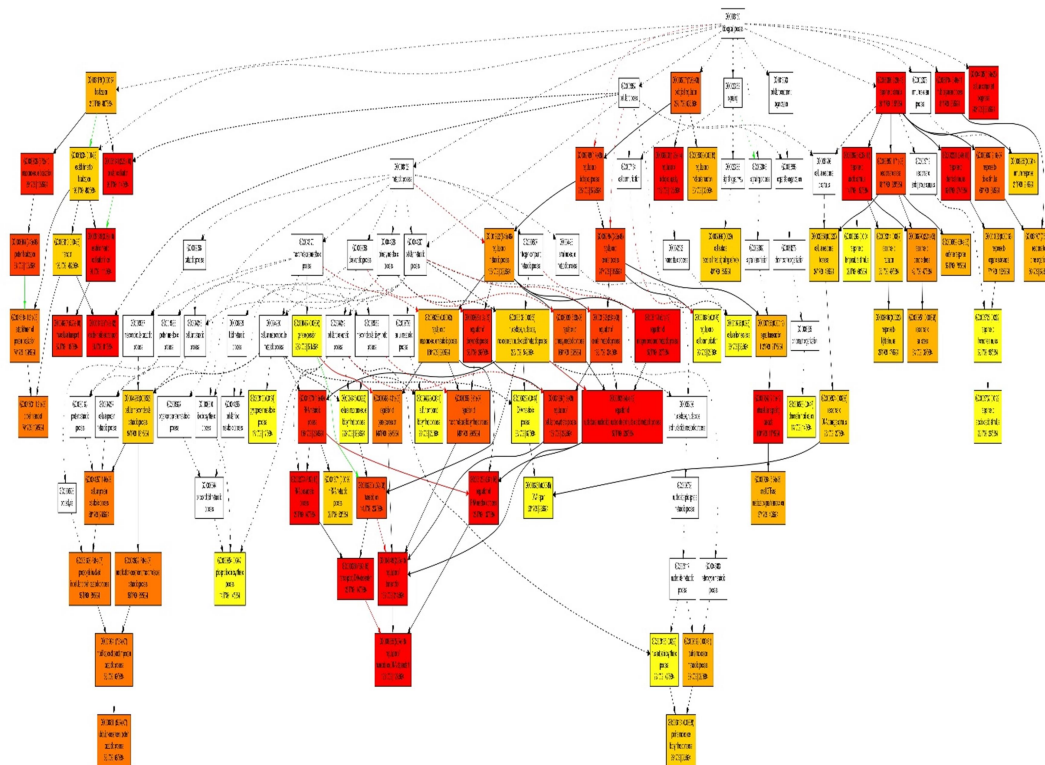


Figure 6. GO-biological processes.

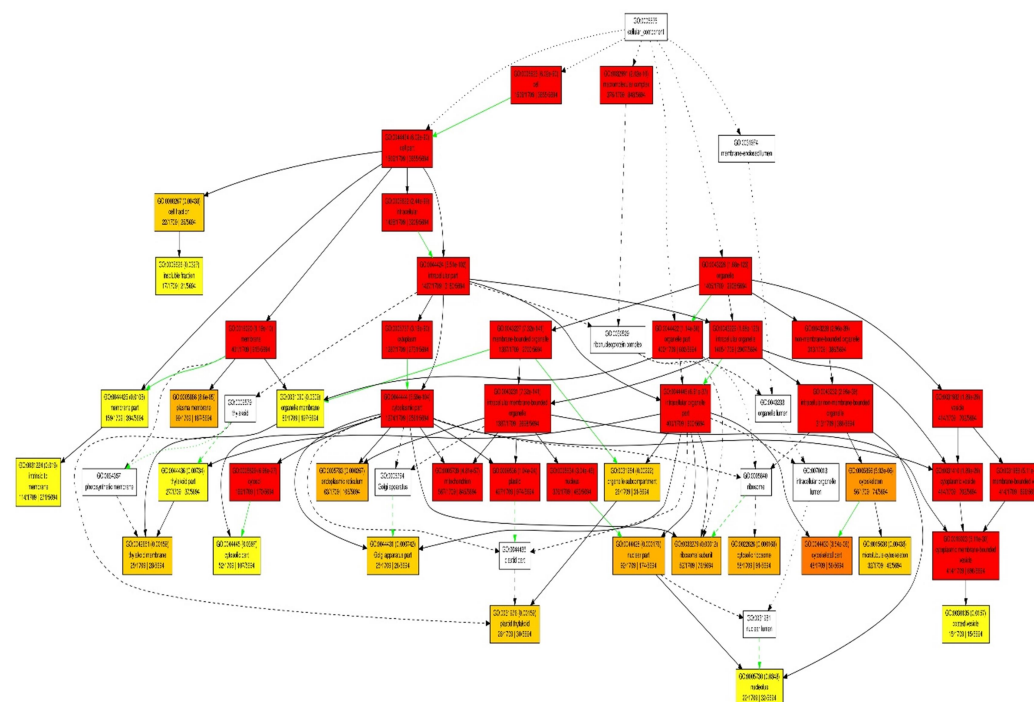


Figure 7. GO-cellular processes.

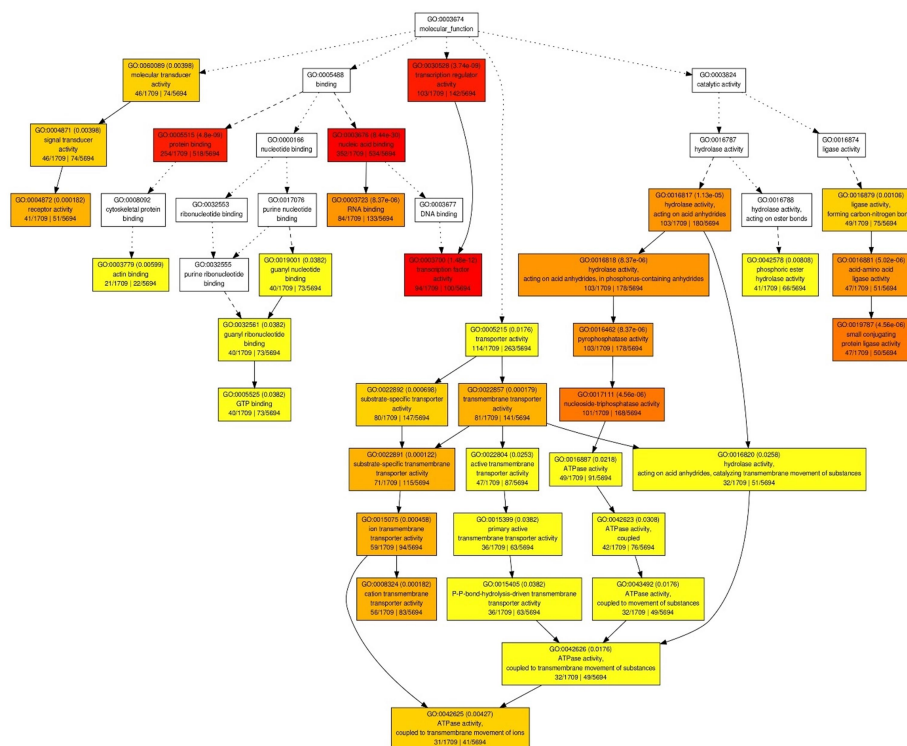


Figure 8. GO-molecular functions.



al., 2020). They are the nucleic acid binding (GO:0003676), transcription regulator activity (GO:0030528), RNA binding (GO:0003723), receptor activity (GO:0004872), ion transmembrane transporter activity (GO:0015075), signal transducer activity (GO:0004871), actin binding (GO:0003779), transporter activity (GO:0005215), ATPase activity (GO:0016887), and GTP binding (GO:0005525), which are illustrated in (S2 Table, Figure 8). Obviously, these putative related genes are targeted by sugarcane miRNAs like sof-miR6437a, sof-miR482a, sof-miR9657b, sof-miR5564a, sof-miR477, sof-miR8632, sof-miR5049, sof-miR6181a, sof-miR6181b, and sof-miR858.

## CONCLUSIONS

In short, this study is the first to disclose the existence of 50 novel potential sugarcane miRNAs that are members of 44 different miRNA families. To predict and analyze these miRNAs, new and sophisticated bioinformatics techniques have been employed. Additionally, 15 miRNAs were chosen at random to serve as primer templates, and RT-PCR was used to validate the primers. Taking into account the key targets, the newly found sugarcane miRNAs revealed 7,976 different protein targets using the psRNA Target method. This resulted in the achievement of 55 GO terms that are further integrated into the key targets like localization, response to salt stress, response to radiation, immune response, regulation of nitrogen compound metabolic process, response to biotic stimulus, substrate-specific transporter activity, ligase activity, forming carbon-nitrogen bonds, intracellular, cytoplasmic vesicle, cytoplasmic vesicle, thylakoid membrane, vesicle, and organelle membrane having specific GO terminology as (GO:0051179), (GO:0009651), (GO:0009314), (GO:0006955), (GO:0051171), (GO:0009607), (GO:0022892), (GO:0016879), (GO:0005622),

(GO:0031410), (GO:0031410), (GO:0042651), (GO:0031982), and (GO:0031090), respectively. Hence, these results demonstrated that sugarcane miRNAs target a variety of related genes and have the capacity to affect the environment and system in order to improve the productivity of the sugarcane plant.

## ACKNOWLEDGEMENTS

The work for this research was carried out at the Center for Applied Molecular Biology, University of Punjab, Lahore, Pakistan. The authors are thankful to the faculty members of CAMB for encouragement and providing the necessary facilities for conducting experiments. The authors also acknowledge the Department of Botany, University of Balochistan, for providing the necessary resources.

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### اعتبار سنجی تجربی و خصوصیات MicroRNA های کدگذاری شده با ژنوم نیشکر و

#### اهداف آنها با استفاده از روش بیانی مبتنی بر PCR

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#### چکیده

MicroRNA ها (miRNA ها)، معمولاً مولکول های RNA های کوچک، درون ژا و غیر کدکننده هستند

که بیان ژن را در سطح پس از رونویسی با تخریب mRNA یا سرکوب ترجمه تنظیم می کنند. آنها از ۱۸ تا ۲۶

نوکلئوتید تشکیل شده اند و در طول تکامل برای توسعه miRNA های جدید در گیاهان مختلف حفظ می شوند. نیشکر (*Saccharum officinarum*) به طور کلی یک محصول با ارزش غذایی و علوفه ای است که در سراسر جهان رشد می کند. تاکنون، miRNA های مختلف نیشکر برای رشد گیاه و پاسخ به استرس مشخص شده اند. در این تحقیق، ۵۰ miRNA منحصر به فرد نیشکر حفظ شده از ۴۴ خانواده miRNA مختلف با استفاده از انواع ابزارهای مبتنی بر ژنومیک پیش‌بینی شده است. miRNA های پیش‌بینی شده نیشکر با استفاده از مجموعه ای از پانزده آغازگر به طور تصادفی انتخاب شده و RT-PCR اعتبارسنجی شدند. ساختارهای ثانویه حلقه ساقه با استفاده از ابزار MFOLD ایجاد می شوند. الگوریتم psRNA-Target 7976 هدف پروتئینی مختلف از SOF-miRNA ها از جمله پنجاه و پنج عبارت GO خاص را شناسایی کرد. آنها اهداف قابل توجهی در عملکردهای بیولوژیکی، سلولی و مولکولی دارند. علاوه بر این، SOF-miR5205a فرآیند بیوسنتزی ترکیب گوگرد را تنظیم می کند و a۹۶۵۳ فرآیند کاتابولیک پروتئین وابسته به یوبیکوئیتین را هدایت می کند. در نتیجه، اتصال RNA و غشای تیلاکوئید به ترتیب توسط SOF-miR9657b و ۲۰۹۱ کنترل می شوند. در نتیجه، نتایج miRNA های جدید نیشکر، انواع مختلفی از ژن های قابل توجهی را هدف قرار می دهد که به کنترل محیط نیشکر برای تولید محصول با کیفیت بالاتر کمک می کند.