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. Up till 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 fifteen randomly chosen primers and RT-PCR. Stem loop secondary structures are created using MFOLD tool. The psRNA-Target algorithm identified 7976 various protein targets of sof-miRNAs including fifty five 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: miRNA, *Saccharum officinarum*, RT-PCR amplification, Targets, Biological process.

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 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. Nearly 48860 miRNAs have been studied so far 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 between 70 and 80 percent 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), *C*ucumis *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 miRNAs.

Retrieval of candidate miRNAs

Considering the unique conserved sugarcane miRNAs via comparative homology-based search, approximately 20703 sugarcane ESTs were obtained from the EST-database (dbEST), (11 December 2019) available at 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 which 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 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 min, denaturation at 95˚C for 45 sec for 35 cycles, annealing at 60˚C for 45 sec, extension at 72˚C for 1 min and post cycling extension step at 72˚C for 5 min. 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.

Table 1. Signifies 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- AGGTGCGAGTTCCAGTAG 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- CTTGGAAGTAAAAGCCTTGC 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- TGGTTCTGGGTTTGTTTCAG R- ACAACTAAGTCTCATTCGCG	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- CTTCGATCGATCTTGCATTG 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- GATTCCTGCTCTCCGAGA	413	55.93 55.36	50.00 55.56	20 18
sof-miR9653a	DN195467	F- GATTTGCTCCCCTCCTTTC 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

Phylogenetic and conservation analyses

In this, 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). But, for conservation analysis a tool termed as 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*. The similar process was utilized for logo generation as reported by Baloch *et al*., (2018).

Targets prediction

For the prediction of 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 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,

158 6181a, 6181b, 6196a, 6196b, 6214b, 6230, 6437a, 7491, 7698, 7710, 8039, 8632, 9482, 9653a,
159 9657b and 11337 (S1 Table).

160 Furthermore, it is confirmed that these novel 50 miRNAs of sugarcane have been reported for
161 the first time and have not been mentioned earlier. Accordingly, these 50 novel miRNAs have
162 been created by the assistance of reference miRNAs of *A. lyrata* (4%), *A. thaliana* (2%), *B.*
163 *distachyon* (4%), *C. melo* (4%), *C. papaya* (2%), *G. hirsutum* (2%), *G. max* (2%), *G. raimondii*
164 (2%), *H. vulgare* (12%), *M. truncatula* (8%), *N. tabacum* (4%), *O. sativa* (22%), *P. trichocarpa*
165 (2%), *S. bicolor* (12%), *S. tuberosum* (2%), *T. aestivum* (14%) and *Z. mays* (2%).

166

167 **Sugarcane miRNAs characterization**

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

174 According to length, sugarcane pre-miRNAs range from 41 to 205 nt having an average length
175 of 88 nt. Considerably, it is found in the arrangement of pre-miRNAs lengths that 1–50 nt (6
176 out of 50), pre-miRNA and formed 12% of the overall pre-miRNA, from 51–100 nt (28 out of
177 50) 56%, 101–150 nt (14 out of 50) 28%, 151–200 nt (1 out of 50) 2% and 201–250 nt (1 out
178 of 50) 2% (Figure 2a).

179 Additionally, this work has noted that the MFE of the freshly found sugarcane pre-miRNAs
180 ranges from –74.3 to –10.1 kcal mol^{-1} having an average of –35.6 kcal mol^{-1}. In accordance
181 with class boundaries –100 to –60 kcal mol^{-1} (5) formed 10% of the overall pre-miRNA, from
182 –61 to –20 (37) 74% and from –21 to –00 kcal mol^{-1} (8) 16% of all the pre-miRNAs.

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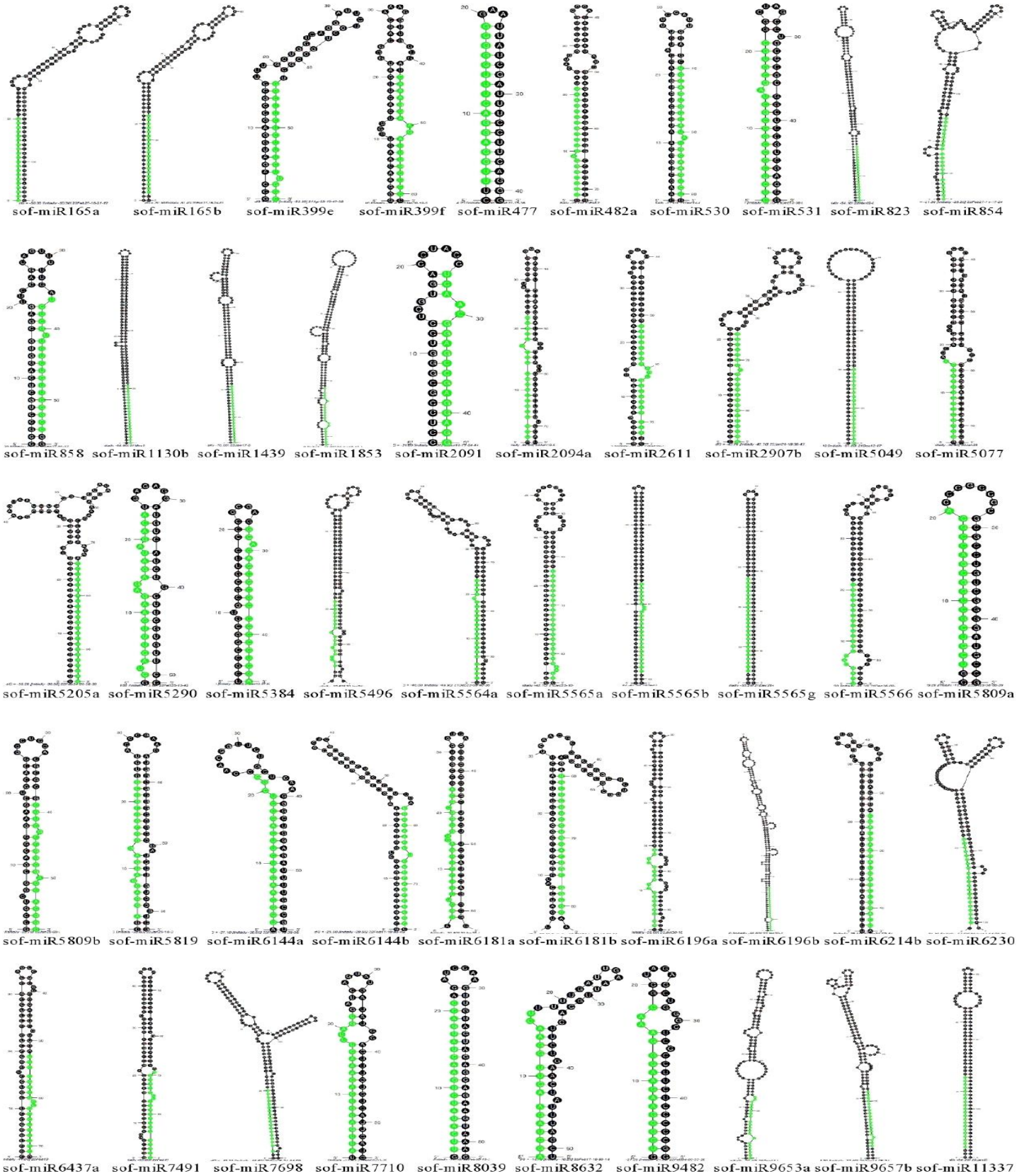

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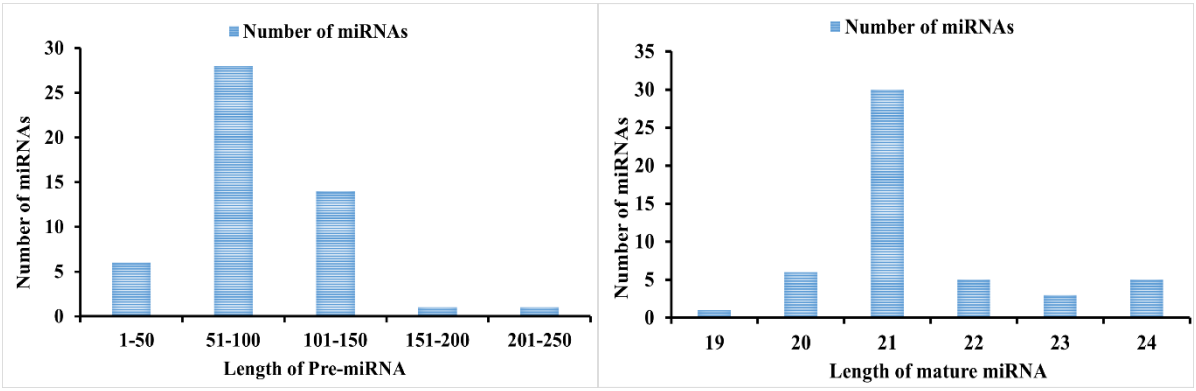
185 **Figure 1.** The newly identified sugarcane miRNAs secondary structures (mature in green).

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 having an average of 2 mismatches. Henceforth, with 3 mismatches (13 miRNAs out of 50) are sought 26% of whole miRNAs, 2 mismatches (9 miRNAs out of 50) with 18%, 4 mismatches (24 miRNAs out of 50) with 48% and 1 mismatch were 8% (4 miRNAs out of 50).

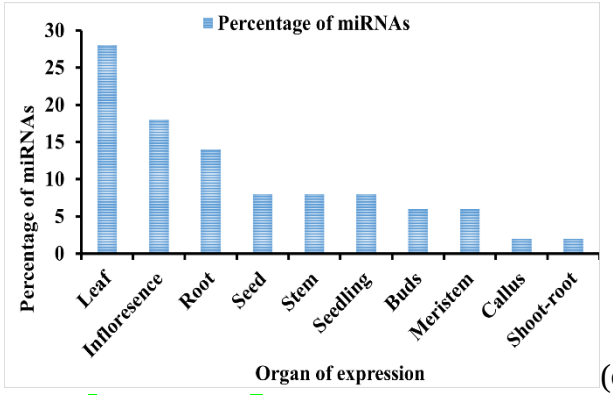
Accordingly, the mature lengths of sugarcane miRNAs, which have a minimum and maximum of 19 and 24 nt, respectively, with an average of 21 nt, were found. Now assuming the class boundaries, the lengths of mature sequences now range from shortest to longest are; 19 nt have (1 out of 50) formed 2% of total, 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 2b). 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 are observed to have been created in an anti-sense strand orientation that produced 38% of the whole 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 was found to range 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 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 has also been calculated for their ESTs. The majority of miRNAs are found in the leaf (14 out of 50), which accounts for 28% of the total and followed by inflorescence 18%, root 14%, seed 8%, stem 8%, seedling 8%, buds 6%, meristem 6%, callus 2% and shoot-root 2% (Figure 2c). 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).



(a) (b)



(c)

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

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 fifteen 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-miR9653a) and 15 (sof-miR9657b). Among the fifteen sugarcane miRNAs, fourteen miRNAs have been validated through RT-PCR in an appropriate way and results are shown (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 fifteen products. Such outcomes were used by numerous researchers studying various plant types (Din *et al*., 2016; Zhang *et al*., 2008).

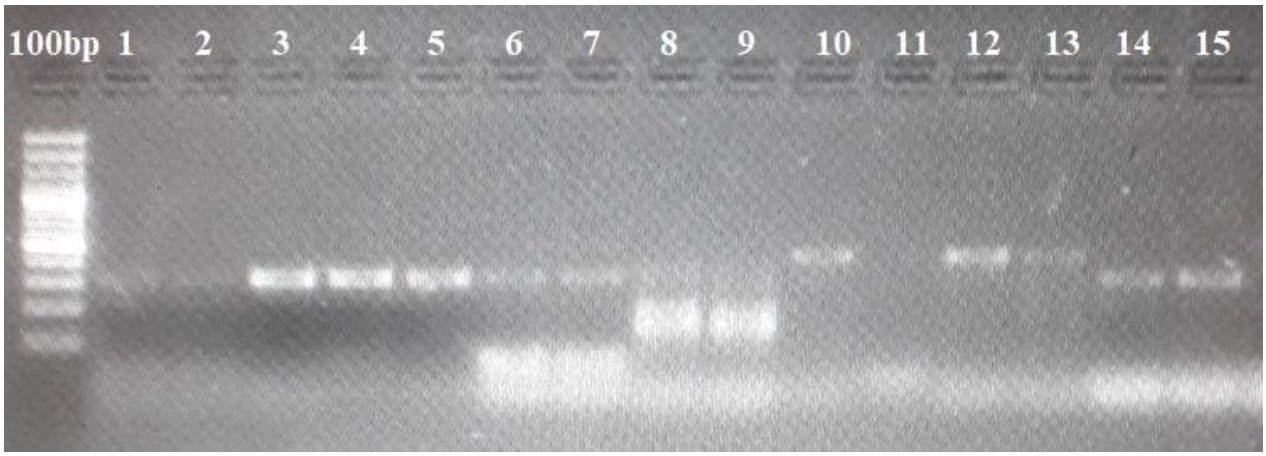


Figure 3. Sugarcane miRNAs RT-PCR expression validation.

Phylogenetic and conservation studies of sugarcane miRNAs

The phylogenetic tree and conservation studies for sugarcane miRNAs are generated and displayed (Figures 4 and 5). Sugarcane and a grass specie termed as 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*.

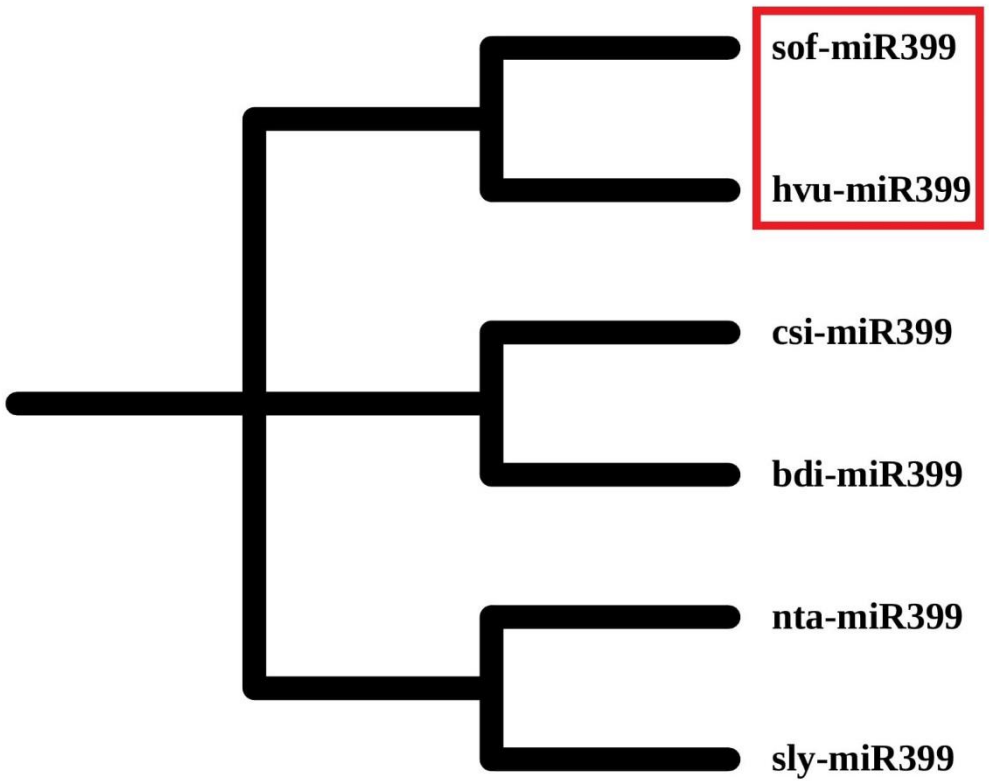


Figure 4. Sugarcane miRNA and their phylogenetic analysis.

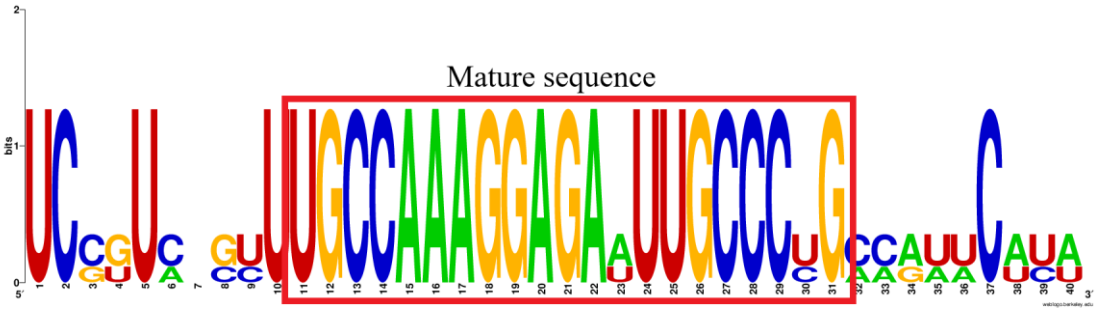


Figure 5. Conservation analysis of the miRNA in sugarcane. Mature miRNA sequences and their conserved nature are shown in the red boxed area that has been highlighted.

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 have been 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 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 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 have been 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 by a number of studies (Din *et al*., 2016; Baloch *et al*., 2015; Bibi *et al*., 2017). The MFE of the freshly noted sugarcane miRNAs have range from –74.3 to –10.1 kcal mol^{-1} having an average of –35.6 kcal mol^{-1}. Several researchers earlier 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 having an average of 2 mismatches. So, 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 having 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 better crop management by controlling the environment for sugarcane.

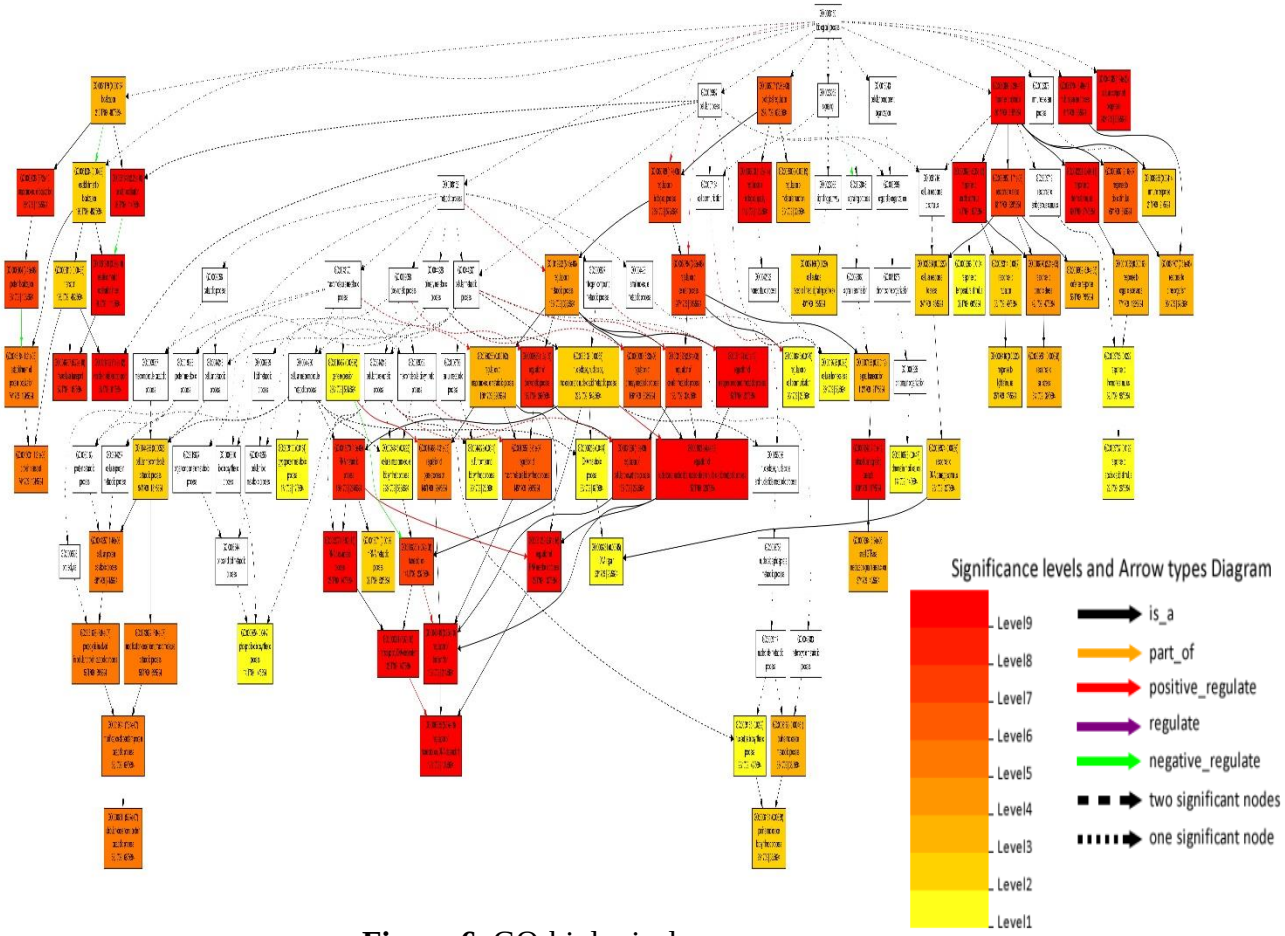


Figure 6. GO-biological processes.

In 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.

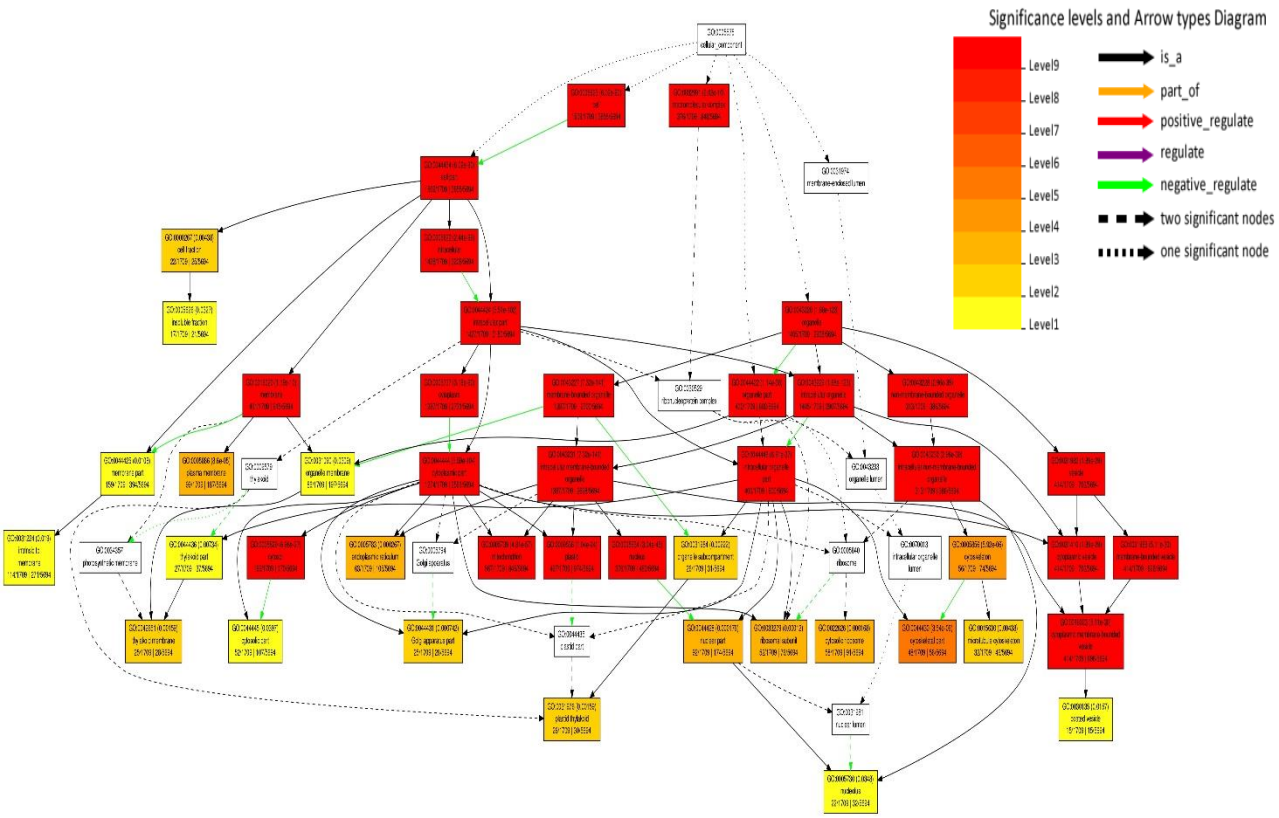


Figure 7. GO-cellular processes.

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 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 (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.

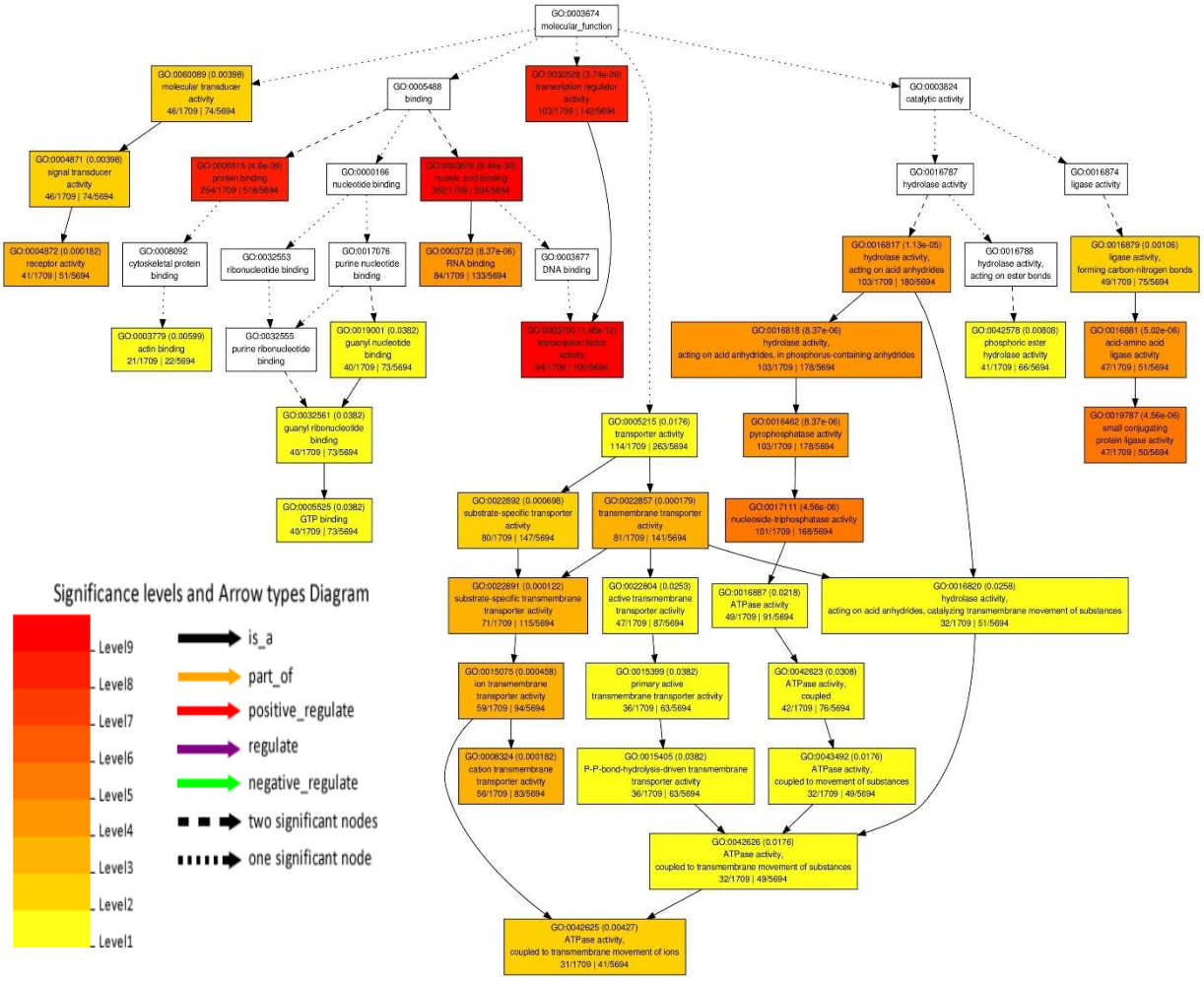


Figure 8. GO-molecular functions.

Conclusions

In a nutshell, 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, fifteen 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 7976 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.

Acknowledgement

The work for this research has been carried out at the Center for Applied Molecular Biology, University of Punjab, Lahore and the authors are thankful to the faculty members of CAMB for encouragement and providing 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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474 **S1 Table:** Explanation of freshly profiled sugar cane preserved miRNAs**.** The sugar cane projected miRNAs are categorized in respect of reference
475 miRNAs, precursor miRNA length (PL), minimum free energy (MFE), mature sequences (MS), number of mismatches (denoted in bold and red)
476 NM, mature sequence length (ML), source EST (SE), mature sequence arm (MSA), GC percentage (GC%), strand orientation (SO) and organ of
477 expression (OE).

***Saccharum officinarum* miRNAs**	**Source miRNAs**	**PL**	**MFE**	**MS**	**NM**	**ML**	**SE**	**MSA**	**GC%**	**SO**	**OE**
sof-miR165a	aly-miR165a	109	-50.30	GGAAUGUUGUCUGG**U**UC**A**AG	2	20	CN607727	5′	45	+	Leaf
sof-miR165b	aly-miR165a	115	-51.90	UCGGACCAGGCUUCAU**U**CCC	1	20	CN607727	3′	60	+	Leaf
sof-miR399e	zma-miR399e	60	-22.20	**G**GGCUUCUCUUUCUUGGC**AGG**	4	21	CF570161	3′	57	+	Stem
sof-miR399f	hvu-miR399	61	-14.40	**UGC**CAAAGGA**A**GAUUUGCCCC	4	21	CA272977	3′	52	+	Root
sof-miR477	cpa-miR477	41	-14.20	**A**UUGGAGGACUUUGGGGG**AGC**	4	21	CA299640	5′	57	+	Inflorescence
sof-miR482a	gma-miR482b	72	-25.70	**UA**UGGGGGGAUUGGG**UG**GGAAU	4	22	CA264644	5′	55	+	Buds
sof-miR530	tae-miR530	61	-21.40	UGCAGUGGCAUAUGCAA**G**UCU	1	21	CA257041	3′	48	+	Leaf
sof-miR531	tae-miR531	50	-30.70	C**U**CUCGCCGG**C**GC**U**AGCGUGC	3	21	CA082279	5′	76	+	Meristem
sof-miR823	ath-miR823	149	-54.60	**C**GGGUGGUGAUC**G**U**C**UAAG**U**U	4	21	CA103350	3′	52	-	Seedlings
sof-miR854	cme-miR854	126	-21.39	GAUGAGG**UAG**AUAGUGAGGAG	3	21	CA285817	3′	48	+	Seeds
sof-miR858	cme-miR858	56	-17.30	UCUCGUUGUCUGUUCG**G**CC**UU**	3	21	CA225244	3′	52	-	Leaf
sof-miR1130b	tae-miR1130b	139	-60.90	UCUUAUA**G**U**U**UGG**A**A**U**GGAGG	4	21	CF574551	3′	38	-	Stem
sof-miR1439	osa-miR1439	149	-70.50	**A**UUUGGAACGGAG**G**GAGUAUU	2	21	CA198902	3′	43	-	Leaf
sof-miR1853	osa-miR1853	163	-41.50	**U**AAUU**U**G**C**GAUGUUCGGUUGCU	3	22	CA191199	3′	41	-	Root
sof-miR2091	osa-miR2091	43	-21.00	UCAAC**G**GAGCCGAGGAGG**AGG**	4	21	CA268697	3′	67	+	Root
sof-miR2094a	osa-miR2094	73	-31.00	UGGCUGCU**G**GG**U**U**G**CU**U**GGUG	4	21	DN195720	5′	62	+	Inflorescence
sof-miR2611	mtr-miR2611	74	-20.90	UAUU**C**GUCAGUGUUUGAU**C**AA	2	21	CA089603	3′	33	-	Meristem
sof-miR2907b	osa-miR2907a	86	-42.70	GGCAG**G**C**CG**GCGAGGGCCUCGG	3	22	CA104808	3′	86	-	Seedlings
sof-miR5049	tae-miR5049	109	-52.00	AAU**U**UGGA**A**CGGAGGGAGUA**C**	3	21	CN608955	3′	48	-	Leaf
sof-miR5077	osa-miR5077	73	-39.50	**GUUC**GCGUCGGGUUCACCA	4	19	CA109931	5′	63	+	Seedlings
sof-miR5205a	mtr-miR5205a	96	-35.28	C**C**UA**U**AAUUUGGGACGGA**A**GGAG	3	23	CA214238	3′	48	-	Inflorescence
sof-miR5290	mtr-miR5290	51	-13.60	GUA**G**UUUG**A**AGAGAGAUAGACA**UA**	4	24	CA114045	5′	33	-	Buds
sof-miR5384	tae-miR5384	46	-37.70	G**GA**GAGCGCGCCGCCGUCGA**G**	3	21	CA266415	3′	81	+	Buds
sof-miR5496	osa-miR5496	108	-32.60	**CC**AGCCGGUGGCAUA**C**U**G**CUC	4	21	CA254292	5′	67	-	Leaf

sof-miR5564a	sbi-miR5564a	96	-46.00	UGGGGAAGCAAUUCGUCGAACG	1	22	CA222602	5′	55	+	Inflorescence
sof-miR5565a	sbi-miR5565a	87	-42.70	UACACACGUGGAUUGAUGUGAAUC	4	24	CA238969	3′	42	+	Inflorescence
sof-miR5565b	sbi-miR5565a	101	-43.20	AAUACAUGUGGAUUGAGGCGAAUC	1	24	CO373749	3′	42	-	Root
sof-miR5565g	sbi-miR5565g	98	-55.20	UUCAUCUCAAUCCACAUGUGUUGA	4	24	CA296316	5′	38	+	Seeds
sof-miR5566	sbi-miR5566	85	-50.20	UCAGCACCACCUCCCUAUUGU	2	21	CA222783	5′	52	+	Leaf
sof-miR5809a	osa-miR5809	47	-19.20	UCGUCGCCGGCGACCGCAGC	3	20	CA258383	5′	80	+	Root
sof-miR5809b	osa-miR5809	57	-25.70	UCGUCGCCGGCGACCACGGC	2	20	CA153085	3′	80	-	Shoot-root
sof-miR5819	osa-miR5819	63	-35.60	AGGACGAGGAGGGCGGCGGCG	3	21	DN194911	5′	81	+	Leaf
sof-miR6144a	nta-miR6144	58	-27.10	UGGCAACUUCUUCAGCCUCGG	4	21	CA297687	5′	57	+	Seeds
sof-miR6144b	nta-miR6144	78	-29.50	UGGCAACUUCUUCAGCCUCGG	4	21	CA297687	3′	57	+	Seeds
sof-miR6181a	hvu-miR6181	62	-23.80	UGCUCUUCCUGGACUGCGGCGCA	4	23	CA235019	5′	65	+	Leaf
sof-miR6181b	hvu-miR6181	83	-33.60	UGCUCUUCCUGGACUGCGGCGCC	2	23	CA259150	3′	70	+	Root
sof-miR6196a	hvu-miR6196	95	-28.60	AGGACGAGGAGGUGGAAGAGG	2	21	CA212234	5′	62	+	Seedlings
sof-miR6196b	hvu-miR6196	205	-66.60	AGGACGAGGAAAUGGAGAGGA	4	21	DV733872	3′	52	+	Stem
sof-miR6214b	hvu-miR6214	74	-38.60	CGACGACGACGAGCCCGACG	2	20	CA131343	3′	75	+	Root
sof-miR6230	sbi-miR6230	121	-51.39	UUCUAGGUCACUAAACUUGUU	3	21	CA074068	5′	46	-	Meristem
sof-miR6437a	ptc-miR6437a	72	-44.00	UUACGGACGGCGGCUCC-GGCG	4	21	CA298737	3′	76	+	Inflorescence
sof-miR7491	ghr-miR7491	89	-35.00	UGGGAUCUUCGAGAGG--UGGGCC	4	22	CA242522	3′	64	+	Inflorescence
sof-miR7698	mtr-miR7698	135	-43.30	UUUUCAUCAAAGUUUUCUGG-	3	20	CA094693	5′	30	-	Callus
sof-miR7710	bdi-miR7710	55	-14.10	AUUGAUGUCACAAACUUUAGUAGC	4	24	CA254800	5′	33	+	Inflorescence
sof-miR8039	stu-miR8039	52	-10.10	UUUCAUAUCUGAACUAUGACC	3	21	CA221928	5′	33	-	Inflorescence
sof-miR8632	gra-miR8632	51	-10.10	AUGAGCUAGAAGUUGGAACUC	4	21	CN612112	5′	43	+	Stem
sof-miR9482	bdi-miR9482	47	-21.20	CCUUUGGGGAAGAAGGGAAAC	4	21	DN192807	5′	52	-	Leaf
sof-miR9653a	tae-miR9653a	134	-27.10	UUUGAGACUUUGUCCAAGGCC	2	21	DN195467	3′	48	-	Leaf
sof-miR9657b	tae-miR9657b	139	-74.30	CCUGCUUCCUCGUCGAGCGGU	4	21	CA201285	3′	67	+	Leaf
sof-miR11337	osa-miR11337	114	-60.70	UGCGAGACGAAUCUAUUAAGC	4	21	CA255688	5′	43	-	Inflorescence

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481 **S2 Table:** Evaluation of possible sugar cane target enrichment in GO terms. A biological process is denoted by BP, a molecular function by MF,
482 a cellular component by CC and false discovery rates by FDR.

miRNA	Target ACC.	GO term	Ontology	Description	Gene No.	p-value	FDR
sof-miR531	CA118749	GO:0044085	BP	Cellular component biogenesis	243	8.80E-27	1.80E-23
sof-miR9482	TC122025	GO:0050896	BP	Response to stimulus	301	2.20E-21	2.30E-18
sof-miR7491	CA123127	GO:0032774	BP	RNA biosynthetic process	125	2.30E-17	8.00E-15
sof-miR6214b	TC115674	GO:0065008	BP	Regulation of biological quality	113	4.30E-17	1.30E-14
sof-miR11337	TC121611	GO:0051171	BP	Regulation of nitrogen compound metabolic process	152	2.20E-14	5.10E-12
sof-miR8039	CA254555	GO:0051704	BP	Multi-organism process	81	2.20E-13	3.50E-11
sof-miR7698	TC130026	GO:0009628	BP	Response to abiotic stimulus	114	3.60E-13	5.30E-11
sof-miR5566	CA140247	GO:0009889	BP	Regulation of biosynthetic process	158	1.70E-11	1.60E-09
sof-miR399e	CA105920	GO:0016070	BP	RNA metabolic process	156	1.60E-11	1.60E-09
sof-miR5809a	TC138721	GO:0050789	BP	Regulation of biological process	269	1.90E-10	1.70E-08
sof-miR165a	CA117384	GO:0006950	BP	Response to stress	181	2.00E-10	1.70E-08
sof-miR2091	CA099275	GO:0065007	BP	Biological regulation	287	1.10E-09	7.90E-08
sof-miR5496	CA288945	GO:0009607	BP	Response to biotic stimulus	66	3.20E-09	2.20E-07
sof-miR9653a	CA200668	GO:0006511	BP	Ubiquitin-dependent protein catabolic process	58	9.40E-09	6.00E-07
sof-miR165b	CA150682	GO:0015031	BP	Protein transport	74	2.20E-08	1.20E-06
sof-miR6196b	TC143487	GO:0006952	BP	Defense response	56	1.20E-06	5.90E-05
sof-miR399f	CA118211	GO:0051179	BP	Localization	210	3.90E-06	0.00017
sof-miR854	CA103622	GO:0009651	BP	Response to salt stress	34	8.00E-06	0.00033
sof-miR5384	TC148451	GO:0009314	BP	Response to radiation	35	0.00011	0.0037
sof-miR8632	TC131567	GO:0006955	BP	Immune response	21	0.00012	0.0041
sof-miR5205a	TC144558	GO:0044272	BP	Sulfur compound biosynthetic process	20	0.0012	0.034

sof-miR6437a	TC120339	GO:0003676	MF	Nucleic acid binding	352	1.20E-32	8.40E-30
sof-miR5290	CA124167	GO:0003700	MF	Transcription factor activity	94	4.20E-15	1.50E-12
sof-miR482a	TC112952	GO:0030528	MF	Transcription regulator activity	103	1.60E-11	3.70E-09
sof-miR5819	TC145783	GO:0005515	MF	Protein binding	254	2.70E-11	4.80E-09
sof-miR9657b	TC119382	GO:0003723	MF	RNA binding	84	1.20E-07	8.40E-06
sof-miR5564a	TC113250	GO:0004872	MF	Receptor activity	41	3.90E-06	0.00018
sof-miR477	TC138466	GO:0015075	MF	Ion transmembrane transporter activity	59	1.00E-05	0.00046
sof-miR6196a	CA151361	GO:0022892	MF	Substrate-specific transporter activity	80	1.70E-05	0.0007
sof-miR823	TC142460	GO:0016879	MF	Ligase activity, forming carbon-nitrogen bonds	49	2.70E-05	0.0011
sof-miR5809b	TC122900	GO:0060089	MF	Molecular transducer activity	46	0.00011	0.004
sof-miR8632	TC147589	GO:0004871	MF	Signal transducer activity	46	0.00011	0.004
sof-miR5049	TC142251	GO:0003779	MF	Actin binding	21	0.00019	0.006
sof-miR6181a	TC151233	GO:0005215	MF	Transporter activity	114	0.00061	0.018
sof-miR6181b	TC112784	GO:0016887	MF	ATPase activity	49	0.00084	0.022
sof-miR858	TC144289	GO:0005525	MF	GTP binding	40	0.0019	0.038
sof-miR5077	TC133977	GO:0043227	CC	Membrane-bounded organelle	1387	2.70E-143	7.30E-141
sof-miR2094a	CA161804	GO:0043229	CC	Intracellular organelle	1405	9.20E-126	1.70E-123
sof-miR2611	TC133178	GO:0043226	CC	Organelle	1405	1.20E-125	1.70E-123
sof-miR1439	TC131194	GO:0044444	CC	Cytoplasmic part	1274	5.10E-106	5.60E-104
sof-miR1130b	CA129594	GO:0044424	CC	Intracellular part	1427	6.00E-104	5.50E-102
sof-miR5565b	CA238969	GO:0005623	CC	Cell	1509	1.10E-91	6.00E-90
sof-miR2907b	CA164291	GO:0005739	CC	Mitochondrion	567	9.60E-59	4.80E-57
sof-miR11337	TC119617	GO:0005634	CC	Nucleus	376	6.60E-47	3.00E-45
sof-miR530	TC135096	GO:0005829	CC	Cytosol	169	2.70E-28	7.00E-27
sof-miR6230	TC153936	GO:0009536	CC	Plastid	497	4.20E-26	1.00E-24
sof-miR5565a	TC150092	GO:0016020	CC	Membrane	401	5.00E-12	1.20E-10
sof-miR1853	CA166708	GO:0005622	CC	Intracellular	1428	3.10E-101	2.40E-99

sof-miR6144b	CA097986	GO:0031410	CC	Cytoplasmic vesicle	414	6.90E-31	1.90E-29
sof-miR6144a	CA097986	GO:0031410	CC	Cytoplasmic vesicle	414	6.90E-31	1.90E-29
sof-miR2091	CA183755	GO:0042651	CC	Thylakoid membrane	25	9.70E-05	0.0016
sof-miR5565g	TC119068	GO:0031982	CC	Vesicle	414	6.90E-31	1.90E-29
sof-miR6196b	TC145867	GO:0031090	CC	Organelle membrane	86	0.0024	0.031
sof-miR530	CA222771	GO:0005626	CC	Insoluble fraction	17	0.0026	0.033
sof-miR5077	CA069236	GO:0005730	CC	Nucleolus	22	0.0028	0.035

اعتبار سنجی تجربی و خصوصیات MicroRNA های کدگذاری شده با ژنوم نیشکر و اهداف آنها با استفاده از روش بیانی مبتنی برPCR

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MicroRNA ها (miRNA ها)، معمولاً مولکول های RNA های کوچک، درون زا و غیر کدکننده هستند که بیان ژن را در سطح پس از رونویسی با تخریب mRNA یا سرکوب ترجمه تنظیم می کنند. آنها از 18 تا 26 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، 50 miRNA منحصر به فرد نیشکر حفظ شده از 44 خانواده miRNA مختلف با استفاده از انواع ابزارهای مبتنی بر ژنومیک پیش‌بینی شده است. miRNA های پیش بینی 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-miRNAها 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 a9653 فرآیند کاتابولیک پروتئین وابسته به یوبیکوئیتین را هدایت می کند. در نتیجه، اتصال RNA و غشای تیلاکوئید به ترتیب توسط SOf-miR9657b و 2091 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.