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Article

In Silico Identification of Sugarcane Genome-Encoded MicroRNAs Targeting Sugarcane Mosaic Virus

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Abstract: Sugarcane mosaic virus (SCMV) is a (genus, *Potyvirus*; family, *Potyviridae*) is a widespread, deleterious, and the most damaging pathogen of sugarcane ((*Saccharum officinarum* L. and *Saccharum* spp.) which causes a substantial barrier to producing high sugarcane earning. Sugarcane mosaic disease (SCMD) is caused by, single or compound infection of SCMV, disseminated by the several aphid vectors in a non-persistent manner. SCMV has flexuous filamentous particle of 700-750 nm long, which encapsidated a positive-sense, single-stranded RNA molecule of 9575 nucleotides. RNA interference (RNAi)-mediated antiviral innate immunity is an evolutionary conserved, key biological process in eukaryotes and has evolved as an antiviral defence system to interfere with viral genomes for controlling infections in plants. The current study aims to analyze sugarcane (*Saccharum officinarum* L. and *Saccharum* spp.) locus-derived microRNAs (sof-miRNAs/ssp-miRNAs) with predicted potential for targeting the SCMV +ssRNA-encoded mRNAs, using a predictive approach that involves five algorithms. The ultimate goal of this research is to mobilize the in silico- predicted endogenous sof-miRNAs/ssp-miRNAs to experimentally trigger the catalytic RNAi pathway and generate sugarcane cultivars to evaluate the potential antiviral resistance surveillance ability and capacity for SCMV. Experimentally validated mature sugarcane (*S. officinarum*, $2n = 8X = 80$) and (*S. spp.*, $2n = 100-120$) sof-miRNA/ssp-miRNA sequences ($n = 28$) were downloaded from the miRBase database and aligned with the SCMV genome (KY548506). Among the 28 targeting mature locus-derived sof-miRNAs/ssp-miRNAs evaluated, one sugarcane miRNA homolog, sof-miR159c, was identified to have a predicted miRNA binding site, at nucleotide position 3847 of the SCMV genome targeting CI ORF. To verify the accuracy of the target prediction accuracy and to determine, whether the sugarcane sof-miRNA/ssp-miRNA could bind the predicted SCMV mRNA target(s), we constructed an integrated Circos plot. A genome-wide in-silico-predicted miRNA-mediated target gene regulatory network has implicated to validate interactions necessary to warrant in vivo analysis. The current work provides valuable computational evidence for the generation of SCMV-resistant sugarcane cultivars.

Keywords: *potyvirus*; in silico tools; sugarcane mosaic virus; miRNA; RNA interference

1. Introduction

Sugarcane (*Saccharum officinarum*) is a prolific tropical and subtropical crop that is economically important, has a long life span, serves as a biofuel, is enriched with energy-rich roughage, and is also a source of agroindustrial residues [1–3]. The genome of octaploid sugarcane (*S.officinarum*) ($2n = 80$; $x = 10$) [4,5], also known as “noble” sugarcane and the genome of sugarcane species and cultivars

have been assembled, drafted and resequenced [6–11]. Sugarcane mosaic virus disease (SCMV) is a highly transmissible and pathogenic potyvirus that causes sugarcane mosaic virus disease (SCMD) [12,13]. Potyviruses are spread by a common complex of sap-sucking vectors such as aphid species [14]. Innovative approaches are still needed to increase sugarcane productivity [15]. The genome of SCMV consists of a +ss RNA molecule with a length of 9575 nucleotides encoding a single large polyprotein. The genome polyprotein precursor was predicted to be cleaved resulting in ten functional proteins: P1, HC-Pro, P3, 6 K1, CI, 6 K2, VPg, NIa, NIb and CP [16–19].

In plants, microRNAs (miRNA) are endogenously expressed small (19-25 nucleotides), evolutionarily conserved, non-coding (NC)-ss RNA molecules [20]. In higher plants, biogenesis and transcription of the miRNA gene (*MIR*) is controlled by the RNA polymerase II, which is then transcribed into single-standard polycistronic *primary* transcripts (pri-miRNAs). They control a variety of biological processes in plants by regulating gene expression, cell growth, development, differentiation and host–virus interactions [21,22]. The miRNA-mediated RNAi is a post-transcriptional gene silencing mechanism that provides antimicrobial innate immunity and regulates host-virus interactions to limit or inhibit viral infection [23].

Artificial miRNA-mediated (amiRNA) technology is an alternative, robust biotechnology based on engineering miRNA genes to control viral infections in plants [24]. RNAi-based amiRNA constructs have been used in research to induce antiviral resistance in plants against plant viruses such as tomato [25,26], cucumber [27], rice [28] and cotton [29]. Mature miRNAs in the sugarcane genome have been predicted, identified, isolated, analyzed, and validated to evaluate host–virus interactions and gene regulation, and they have been associated with abiotic and biotic stresses [30–40]. Recently, the experimental validation of 35 conserved mature locus-derived, high-confidence sof-miRNAs/ssp-miRNAs in the sugarcane genome and further deposition in the miRBase database were reported.

An integrative multi-network approach based on SCMV infection assessment used to identify target binding sites of sugarcane genome-encoded sof-miRNAs/ssp-miRNAs in the SCMV genome. The identification of multiple host-derived miRNA binding sites in SCMV genome for the creation of transgenic sugarcane varieties resistant to SCMV is the main objective of this study. In this study, several miRNA prediction tools were evaluated and used to identify microRNA–mRNA binding sites in the SCMV genome for use in developing transgenic or non-transgenic modified sugarcane plants with resistance to SCMV and, potentially, closely related potyviruses. Potential targets of the most promising sugarcane miRNAs for breeding were also of interest to better understand potyvirus–sugarcane plant interaction during infection. Until now, there have been no reports of the use of an amiRNA-based strategy to develop SCMV tolerance in sugarcane plants, which is based on the prediction of homologous amiRNAs for silencing SCMV. The predicted locus derived sof-miRNAs/ssp-miRNAs in the sugarcane genome were further evaluated to understand the complex interactions between sugarcane host plant and SCMV Potyviruses and to identify novel antiviral targets.

2. Materials and Methods

2.1. Sugarcane MicroRNAs and SCMV Genome Data Retrieval and Processing

Experimentally validated high-confidence mature sugarcane microRNAs (sof-miRNA156-sof-miR11892/ssp-miR156-ssp-1432) (Accession ID: MIMAT0001656-MIMAT0001671/ MIMAT0020291-MIMAT0020290) and (*Saccharum* sp.-microRNAs) (ssp-miR166-ssp-miR1432) (Accession ID: MIMAT0030451- MIMAT0020290) (**Table S1**) were retrieved from the miRNA registry (miRBase, version 22) [41]. The full-length SCMV +ssRNA genome sequence (9575 bases) (Accession number KY548506) was acquired from the NCBI GenBank database [42].

2.2. Potential Targets of Sugarcane MicroRNAs in SCMV Genome

Prediction of effective microRNA–mRNA binding sites is a first step toward understanding microRNA-regulated gene regulatory networks. The accuracy of miRNA target site prediction can be

affected by several factors, such as the specificity and sensitivity of the algorithm, the choice of reference sequence, and the length of the target sequence. Various in silico methods for effective silencing are developed for computational prediction of miRNA-mRNA target sites.

. A computational approach refers to the use of multiple computational methods, algorithms, or tools to analyze and interpret biological data. This approach combines different types of publicly available in silico algorithms, miRanda[43,44], RNA22[45,46], TAPIR[47], psRNATarget[48,49] and RNAhybrid [50] (**Table 1**).

Table 1. Different features and parameters of algorithms applied for miRNA target predictions.

Algorithms	Features	Organism	Parameters	Source
miRanda	Seed-based interaction, multiple target sites, free energy of miRNA-mRNA duplex, conservation	Human, rat, fly, worms	Score threshold= 140, Free energy=-20 Kcal/mol, Gap open penalty=-9.00, Gap extend penalty=-4.00	http://www.microrna.org/ (retrieved 14 August 2019)
RNA22	Pattern recognition, folding energy, heteroduplex,	Human, mouse, fly and worms	Number of paired-up bases= 12, Sensitivity (63%), Specificity (61%), Folding energy=-15 Kcal/mol	https://cm.jefferson.edu/rna22/Interactive/ (retrieved on 22 June 2019)
TAPIR	Sees pairing, target site accessibility, multiple sites	Plants	Free energy ratio=0.2 Score= 9	http://bioinformatics.psb.ugent.be/webtools/tapir (retrieved on 25 June 2021)
psRNATarget	Complementarity scoring, multiple target sites, translation inhibition	Plants	Expectation Score= 6.5, Penalty for G:U pair= 0.5 HSP size= 19 Penalty for opening gap= 2	https://www.zhaolab.org/psRNATarget/analysis?function=2 (accessed on 26 May 2022)
RNAhybrid	Seed pairing and free energy	Any	Free energy=-20 Kcal/mol, Hit per target= 1	http://bibiserv.techfak.uni-bielefeld.de/rnahybrid (accessed on 26 May 2022)

2.3. miRanda

The miRanda is one of the first miRNA target predictors, a highly versatile algorithm based on seed-based interaction of miRNA target duplexes [43]. It was implemented as a standard tool to detect potential miRNA binding sites. RNA-RNA duplex dimerization and sequence complementarity are features considered by the miRanda algorithm. It considers cross-species conservation of target site that distinguishes it from other algorithms[44]. The miRanda algorithm has been implemented in C and first version was published in 2003. The default parameters were selected for nalaysis (**Table 1**).

2.4. RNA22

The RNA22 algorithm has a diverse, web-based application implemented interactive exploration. It uses a pattern-recognition based approach to serve as miRNA target discovery tool. It predicts statically significant target patterns using maximum folding energy (MFE)[45,46]. Site complementarity and non-seed-based interaction are important features. Prediction is also based on highly sensitive and significant target patterns. The default parameters were chosen (**Table 1**).

2.5. TAPIR

The TAPIR algorithm is used to assess seed-based interaction of plant miRNAs in the target sequence. It is highly precise plant miRNA target prediction algorithm to detect target binding sites in the target sequence. It is used to deliver precise miRNA target predictions, including target mimics, with FASTA and RNAhybrid search options[47]. The default parameters were chosen (**Table 1**).

2.6. psRNATarget

The psRNATarget algorithm is a highly sensitive, newly designed web-based tool developed for plant miRNA prediction. Target binding sites of plant miRNAs were predicted based on

complementary scoring schema. The algorithm predicts the inhibition pattern of cleavage [48,49]. The default parameters were chosen (Table 1).

2.7. RNAhybrid

The RNAhybrid is a seed-based scanning algorithm based on intermolecular hybridization to predict effective binding sites of miRNAs in the target sequence. It predicts target binding sites in a very easy and flexible manner[50]. It is an online available tool. It is used for rapid prediction of miRNA targets based on MFE hybridization of mRNA and miRNAs. The default parameters were chosen (Table 1).

2.8. RNAfold

The RNAfold algorithm is available on a web server implemented in the ViennaRNA package[51].

2.9. Statistical Analysis

The miRNA-mRNA target prediction biological data were further processed. Graphical representations of miRNA data was prepared using R-language [52].

3. Results

3.1. Prediction and Analysis of Sugarcane MicroRNAs Targeting SCMV Genome

An integrative computational approach to identify possible interactions of high- confidence target sites of sugarcane mature miRNAs located in the SCMV positive-sense single-stranded (+ssRNA) genome from among the 28 sugarcane miRNAs (sof-miRNAs/ssp-miRNAs) revealed sof-miRNAs/ssp-miRNAs—derived MIR genes at high proportion of sugarcane miRNA gene loci [33,53–56]. The predicted SCMV +ssRNA encoded mRNA sequences were localized hypothetically best sof-miRNAs/ssp-miRNAs—annealing sites predicted by miRanda algorithm (19 miRNA-mRNA target pairs) and RNA22 (15 sugarcane sof-miRNAs/ssp-miRNAs and 20 loci). The TAPIR identified 7 binding sites of sugarcane mature sof-miRNA-target/ssp-miRNA-target pairs. Twenty nine sugarcane miRNAs targeting thirty three cleavable attachments sites were identified by the psRNATarget algorithm. RNAhybrid predicted 28 high-probability binding sites of sugarcane miRNAs in the SCMV genomic RNA sequence (Figures 1 and 2) (File S1) (Table S2).

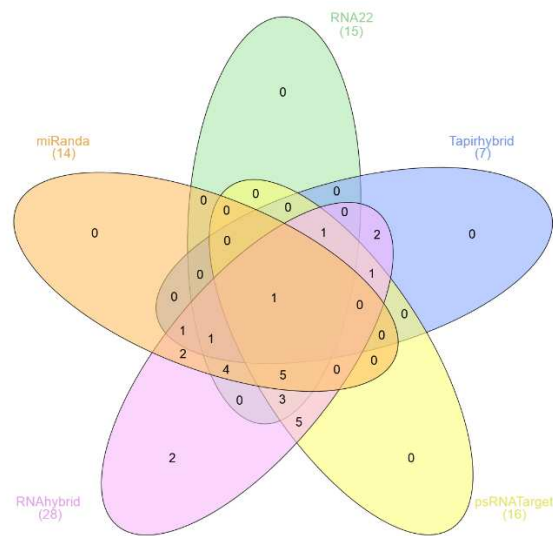


Figure 1. Five-set venn diagram representing mutually common binding sites of mature sugarcane miRNAs predicted potentially targeting the SCMV genome. The in-silico prediction was established

using computational tools (miRanda, RNA22, TAPIR, psRNATarget, and RNAhybrid) to identify potential targets of sugarcane-encoded miRNAs. The area of overlap among computational tools showed miRNA-binding sites. The high-order intersection of five algorithms revealed the most potent sugarcane mature miRNA—ssp-miR1444c-3p.

3.2. Sugarcane miRNAs Targeting P1

The potyviral first protease protein P1 encoded by P1 ORF (149-847) (698 bases), the least conserved, hypervariable, modulates host responses and essential for replication of viral +ssRNA genome [57,58]. Host adaptation is a key process for virus genome evolution [59,60]. P1 is also related to virus-host adaptation [61]. The miRanda and RNA22 algorithms predicted bindings of two sof-miRNAs: sof-miR168 (a, b) at nucleotide positions 547 and 846, respectively as shown in (Figure 2A-2B). The sof-miRNA168a was targeted at nucleotide position 406, by the TAPIR algorithm (Figure 2C). No sof-miRNA/ssp-miRNA was predicted targeting the P1 region, by the psRNATarget and RNAhybrid algorithms (Figure 2D-2E) (File S1) (Tables S2 and S3).

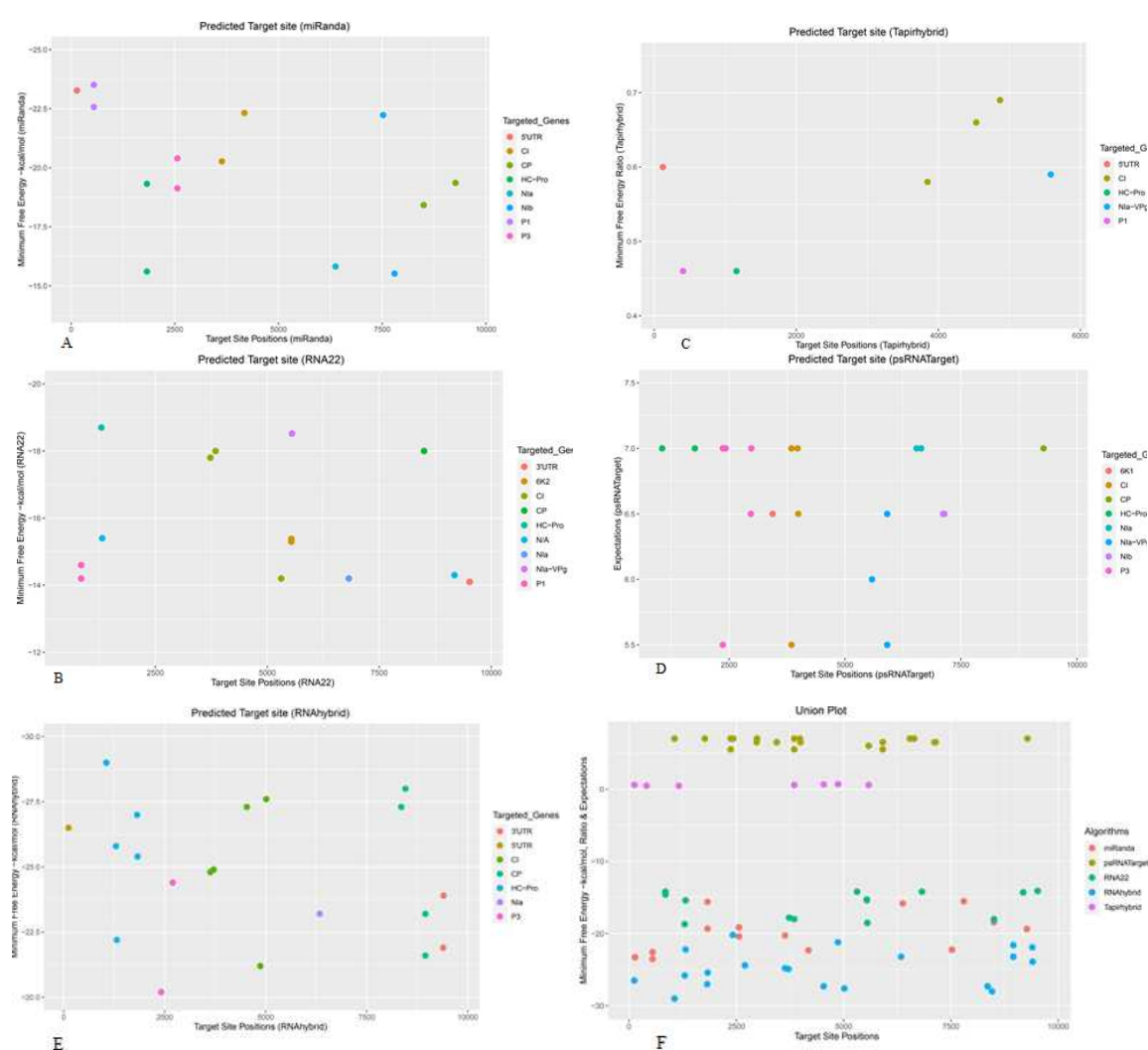


Figure 2. Individual sugarcane sof-miRNA/ssp-miRNA and their predicted high-confidence binding sites in the SCMV genome were predicted based on ‘five algorithms’ approach. (A) miRNA-sites were detected by miRanda. (B) Several miRNA-target sites were detected by RNA22. (C) TAPIR identified sugarcane miRNA-binding sites. (D) psRNATarget predicted several binding sites of sugarcane miRNAs. (E) Prediction of miRNA-sites by RNAhybrid. (F) Union plot representing all predicted binding sites detected by all the algorithms used. Multiple copies of miRNA target binding sites were represented by colored dots. Targeted genes of SCMV were indicated by different colors.

3.3. Sugarcane miRNAs Targeting HC-Pro

The HC-Pro ORF (848-2227 nucleotides) encodes a multifunctional, non-structural dimeric—helper component—proteinase. It has been reported as a viral suppressor. Enhanced expression by fusion of P1, symptoms development and viral replication are the key functions [62–67]. The miRanda and RNA22 algorithms predicted target site of sof-miR168 (a, b) at nucleotide position 1827. Both the algorithms binding of sof-miR168a at nucleotide position 1296 also (**Figure 2A,B**).

TAPIR predicted attachment site of sof-miRNA159e at locus 1159 (**Figure 2C**). The psRNATarget algorithm detected binding of ssp-miR444 (a, b, c-3p) at nt positions (1058 and 1763) (**Figure 2D**). The RNAhybrid algorithm predicted—sof-miR159c, sof-miR168 (a, b), ssp-miR444 (a, b, c-3p) and ssp-miR1432 at nucleotide positions 1830, 1296, 1818, 1057 and 1316, respectively (**Figure 2E**) (**File S1**) (**Tables S2 and S3**).

3.4. Sugarcane miRNAs Targeting P3

The P3 ORF (2228-3268 nt) encodes a membrane-associated P3 protein that is directly involved in the mechanism of SCMV genomic RNA replication. It is also involved in potential cell-to-cell spread (movement and transport) and is responsible for determining host-range and symptoms [68–70]. The miRanda algorithm predicted the binding of sof-miRNAs: sof-miR168 (a, b) at nucleotide position 2562 (**Figure 2A**). The RNA22 and TAPIR algorithms did not predict sof-miRNA/ssp-miRNA targeting to P3 (**Figure 2B,C**). Potential target sites of sof-miR167 (a, b), sof-miR168a, ssp-miR437c, ssp-miR444 (a,b, c-3p) at nucleotide positions 2427, 2971, 2981 and 2367, respectively, were detected by psRNATarget (**Figure 2D**). In addition, RNAhybrid identified sof-miR167(a, b), ssp-miR437b target sites at nucleotide positions 2699 and 2416, respectively (**Figure 2E**) (**File S1**) (**Tables S2 and S3**).

3.5. Sugarcane miRNAs Targeting 6K1

The 6K1 ORF (3269-3469 nucleotides) encoding a 6K1 protein which functions viral genome replication. It mediates cell-to-cell movement, controlling defense mechanism and gene regulation. It is a key component of 6K2-induced viral replication complex (VRC), and regulation [71,72]. The 6K1 had the least number of predicted sugarcane sof-miRNAs ssp-miR444c-3p at nucleotide position 3441, by the psRNATarget algorithm (**Figure 2D**) (**File S1**) (**Tables S2 and S3**).

3.6. Sugarcane miRNAs Targeting CI

The CI ORF (3470-5383 nt) encodes a multifunctional cylindrical inclusion protein (CI) essential for ATP-binding and RNA helicase activity [73–75]. CI was targeted by two miRNAs: sof-miR396, ssp-miR166 at nt positions 3634 and 4178 respectively as indicated by the miRanda algorithm (**Figure 2A**). The RNA22 algorithm predicted two miRNAs: sof-miR159c, ssp-miR444b at nt positions 3730 and 5311 respectively (**Figure 2B**). In addition, TAPIR predicted three sugarcane miRNAs: sof-miR159c, ssp-miR437a and ssp-miR1128 at nucleotide positions 3847, 4869, and 4534, respectively (**Figure 2C**). The psRNATarget algorithm identified seven miRNAs: sof-miR159 (a, b, c, d, e), ssp-miR444b, ssp-miR1432 at nt positions 3847, 3992 and 3980, respectively (**Figure 2D**). Five miRNA-binding sites were detected by RNAhybrid: sof-miR396 (start site 5016), sof-miR408e (3633), ssp-miR166 (3714), ssp-miR437a (4868) and ssp-miR1128 (4533) (**Figure 2E**) (**File S1**) (**Tables S2 and S3**).

3.7. Sugarcane miRNAs Targeting 6K2

Potyvirus 6K2 (5384-5542 nt) encodes the multifunctional protein 6K2, induces the formation of RE-derived complexes, and develops resistance to drought [76,77]. The RNA22 algorithm identified five sugarcane sof-miRNAs: sof-miR408 (a, b, c, d, e) at locus position 5538 (**Figure 2B**) (**File S1**) (**Tables S2 and S3**).

3.8. Sugarcane miRNAs Targeting NIa-VPg

Potyvirus NIa-VPg ORF (5543-6109 nt) encodes a viral genome-linked protein (VPg) that functions as a virulence determinant and genome translator [78–82]. It is also involved in replication, translation and movement [83–85]. The RNA22 and TAPIR algorithms predicted the binding of ssp-miR444c-3p at locus position 5552 (**Figure 2B,C**). The psRNATarget algorithm predicted six miRNAs: sof-miR156, sof-miR159 (a, b, c, d), ssp-miR444c-3p (**Figure 2D**). No sof-miRNA/ssp-miRNA was predicted targeting the NIa-VPg region by The RNAhybrid algorithm did not predict sof-miRNA/ssp-miRNA targeting the NIa-VPg region (**Figure 2E**) (**File S1**) (**Tables S2 and S3**).

3.9. Sugarcane miRNAs Targeting NIa

Potyvirus NIa ORF (6110-6835 nt) encodes nuclear inclusion a protein (NIa) which is involved in RNA-binding and also interacts with NIb [86,87]. miRanda, RNA22 and RNAhybrid predicted the binding of only sugarcane miRNA: ssp-miR528, sof-miR396 and ssp-miR827 at nucleotide positions 6376, 6821 and 6338, respectively (**Figure 2A,B,E**). The psRNATarget identified three sugarcane miRNAs: sof-miR408e, ssp-miR444 (a, b) at nucleotide positions 6544 and 6641, respectively (**Figure 2D**). No miRNA-target pair was identified by TAPIR (**Figure 2C**) (**File S1**) (**Tables S2 and S3**).

3.10. Sugarcane miRNAs Targeting NIb

Potyvirus NIb ORF (6836-8398) encodes the nuclear inclusion b (NIb) protein, which is involved in translocation activity and also interacts with NIa [88]. It contains nuclear signals and is also referred as RdRp [89]. The miRanda algorithm detected the binding of two sugarcane ssp-miRNAs: ssp-miR169 and ssp-miR1432 at nucleotide positions 7798 and 7523 respectively (**Figure 2A**). The psRNATarget algorithm predicted binding of two sugarcane ssp-miRNAs: sof-miR396 and ssp-miR444b at nucleotide positions 7798 and 7523, respectively (**Figure 2D**). No miRNA-target pairs were identified using the RNA22, TAPIR, and RNAhybrid algorithms (**Figure 2B-2C-2E**) (**File S1**) (**Tables S2 and S3**).

3.10.1. Sugarcane miRNAs Targeting CP

Potyvirus CP ORF (8399-9337) encodes a multistaking protein, coat (CP) which is involved in the development of virion assembly. The CP is involved in all steps of the potyviral life cycle [90–92]. The miRanda algorithm predicted the binding of three sugarcane ssp-miRNAs (ssp-miR444 (a, b, c-3p) (start site 8501). ssp-miR444c-3p also targeted the CP region at nucleotide position 9268(**Figure 2A**). The RNA22 algorithm predicted the binding of ssp-miRNA444 family at nt positions 8502 and 9181(**Figure 2B**). The psRNATarget algorithm predicted the binding of ssp-miR444c-3p at nt position 9282 (**Figure 2D**). Potential binding sites of sugarcane miRNAs: sof-miR159 (a, b, d, e), sof-miR408 (a, b, c, d), and ssp-miR169 were detected by the RNAhybrid algorithm at nucleotide positions 8953, 8355, and 8458 respectively (**Figure 2E**) (**File S1**) (**Table S2-S3**).

3.10.2. Sugarcane miRNAs Targeting UTR

The potyviruses 5' untranslated region (5' UTR) (1-148 nt) and 3' UTR (9341-9575 nt) are involved in replication and translocational activities of the ORFs [93,94]. The sof-miR408 (a, b, c, d) was predicted target the 5' UTR at nt positions 139 by miRanda (**Figure 2A**). Similarly, ssp-miR528 was identified to target the 5' UTR at nt position 122 by TAPIR and RNAhybrid (**Figure 2C-2E**). RNA22 predicted binding of sof-miR168 (a, b) at nt position 9520 in the 3' UTR (**Figure 2B**). RNAhybrid predicted binding of two sugarcane miRNAs in the 3'UTR: sof-miR156 and ssp-miR437c at nt positions 9402 and 9395 respectively (**Figure 2E**) (**File S1**) (**Tables S2 and S3**).

3.5. Identification of Consensual Sugarcane MicroRNAs

The present study was concluded based on consensus genomic target binding sites of sugarcane miRNAs determined by different algorithms. Among them, we selected 9 sugarcane miRNAs (sof-

miR159c, sof-miR168a, ssp-miR437a, ssp-miR528, ssp-miR444 (a, b), ssp-miR444c-3p), ssp-miR1128, and ssp-miR1432), which were based on consensus genomic positions 3847 (target gene CI), 1296 (HC-Pro), 4869 (CI), 122 (5' UTR), 8502/1058 (CP/HC-Pro), 5583 (NIa-VPg), 4534 (CI) and 1316 (HC-Pro) were detected (Tables 2 and 3). Of the nine consensus locus-derived sof-miRNAs/ ssp-miRNAs in the sugarcane genome investigated in this study, only one sof-miRNA (sof-miR159c at nt position 3847 targeting CI) was identified by the union of genomic consensus positions by at least three algorithms (RNA22, TAPIR, and psRNATarget) (Figure 3, Tables 2 and 3) (File S1) (Tables S2 and S3).

Table 2. Predicted high-confidence binding sites of consensus sugarcane miRNAs targeting SCMV genome were detected by different computational algorithms.

Sugarcane miRNA	Position miRanda	Position RNA22	Position TAPIR	Position psRNATarget	Position RNAhybrid	MFE * miRanda	MFE ** RNA22	MFE Ratio TAPIR	Expectation psRNATarget	MFE* RNAhybrid
sof-miR159c		3847	3847	3847			-18.00	0.58	5.50	
sof -miR168a		1296			1296		-18.70			-25.80
ssp -miR437a			4869		4868			0.69		-21.20
ssp-miR528			122		121			0.60		-26.50
ssp-miR444a	8501	8502		1058	1057	-18.42	-18.00		7.00	-29.00
ssp-miR444b	8501	8502		1058	1057	-18.42	-18.00		7.00	
ssp-miR444c-3p			5583	5583				0.59	6.00	
ssp-miR1128			4534		4533			0.66		-27.30
ssp-miR1432		1315			1316		-15.40			-22.20

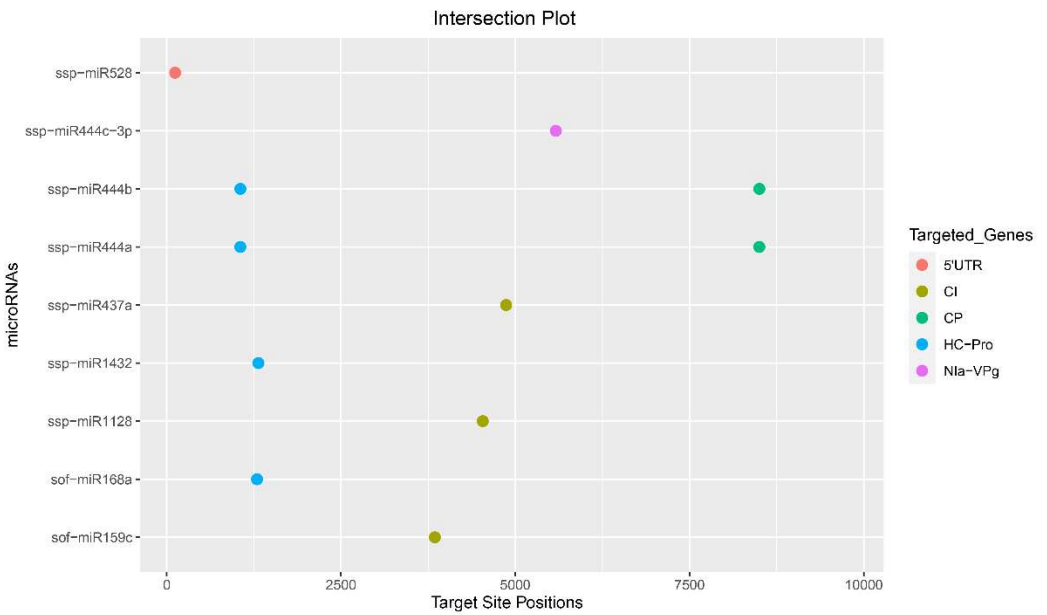
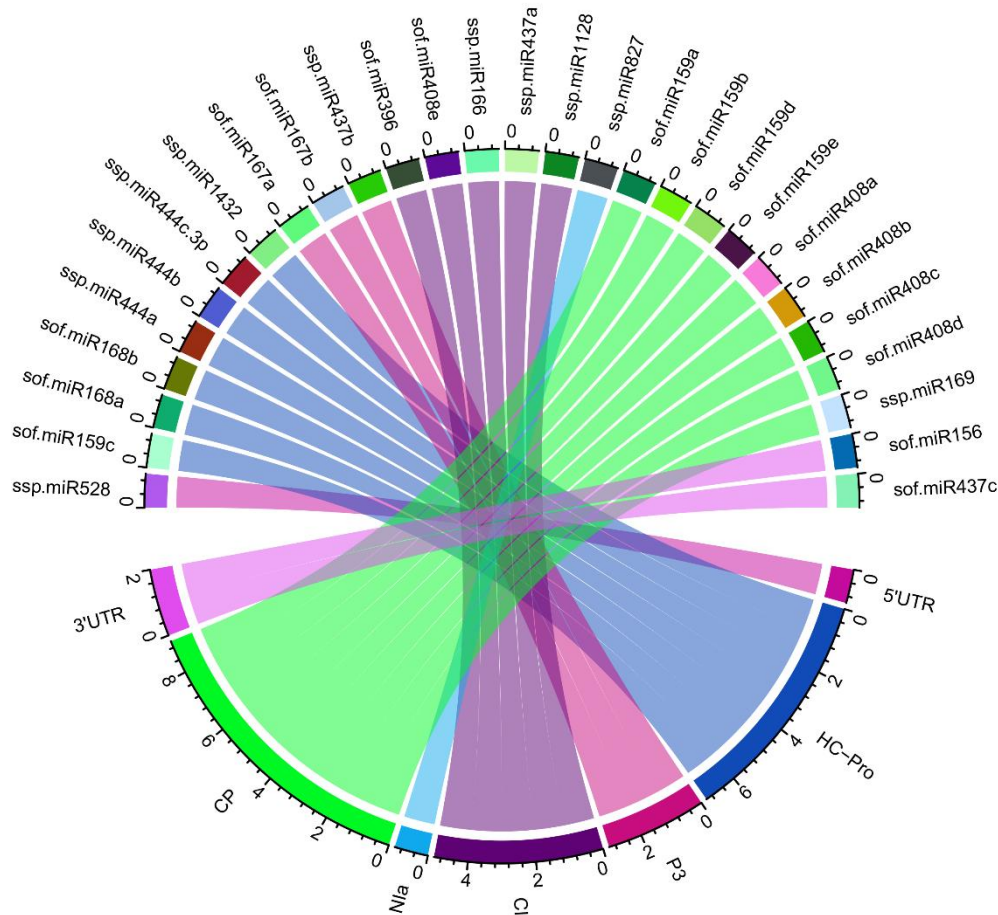


Figure 3. Intersection plot show consensus high-confidence binding sites of sugarcane mature miRNAs predicted by at least two computational tools. The colored dots represent sugarcane miRNA-binding sites targeting different genes of SCMV.

Table 3. Predicted consensus sugarcane-encoded miRNA-target sites localized in different target genes of SCMV-SO.

miRNA ID	Accession ID	Mature Sequence (5'–3')	Target Genes ORF(s)	Target Binding Locus Position
sof-miR159c	MIMAT0001662	CUUGGAUUGAAGGGAGCUCCU	CI	3847–3868
sof-miR168a	MIMAT0001665	UCGCUUGGUGCAGAUCCGGAC	HC-Pro	1296–1317
ssp-miR437a	MIMAT0020280	AAAGUUAGAGAAGUUUGACUU	CI	4869–4890
ssp-miR528	MIMAT0020288	UGGAAGGGGCAUGCAGAGGAG	5'UTR	122–143
ssp-miR444a	MIMAT0020284	UGCAGUUGUUGCCUCAAGCUU	CP	8501–8521

Circos plot represents predicted host–virus interactions of sugarcane miRNAs and SCMV target genes. The Circos plot was generated to visualize comprehensive master miRNA regulatory network with novel antiviral targets (**Figure 4**). Generation of miRNA-mRNA Regulatory Network was conducted using ‘Circos’ software [95].



3.8. RNA Secondary Structures

The computationally predicted locus-derived mature miRNAs in the sugarcane genome were analyzed by generating their secondary structures using the original precursor sequences. The pre-miRNA hairpin sequences were used for manual curation. The main parameters of the predicted stable secondary structures were evaluated (**Table 4**). The stable secondary structures of the potential consensus sugarcane precursor sequences were predicted by the RNAfold algorithm [51].

Table 4. Features of predicted precursors of sugarcane were determined.

miRNA ID	Accession ID	MFE */Kcal/mol	AMFE **	MFEI ***	(G+C)%
sof-MIR159c	MI0001760	-110.60	-46.47	-0.87	53.36
sof-MIR168a	MI0001763	-66.20	-63.65	-0.83	75.96
ssp-MIR437a	MI0001763	-57.10	-32.62	-1.29	25.14
ssp-MIR528	MI0001763	-48.50	-52.71	-0.86	60.84
ssp-MIR444a	MI0001763	-57.70	-54.94	-1.28	42.86
ssp-MIR444b	MI0001763	-63.70	-60.09	-1.38	43.39
ssp-MIR444c	MI0001763	-61.80	-57.22	-1.31	43.52
ssp-MIR1128	MI0001763	-101.70	-36.98	-1.18	31.27
ssp-MIR1432	MI0001763	-57.10	-64.88	-1.14	56.82

4. Discussion

The SCMV is a monopartite potyvirus suspected as an etiological agent that has spread to Pakistan and China due to its high transmissibility and has become an increasing potential long - lasting threat to sugarcane and maize production in the last two decades [13,17,96]. In our previous studies, we have investigated experimentally validated mature locus-derived microRNAs in the sugarcane genome which were predicted to be targets of SCBGAV, SCYLV and SCBV based on in silico criteria [37–39]. Several studies have identified complex host-virus interactions and have investigated miRNAs targeting plant viruses using an in silico approach [97–103]. The miRNAs have emerged as novel endogenous targets for multiple levels of miRNA gene level regulation [53,104,105]. Several studies have shown that the efficacy of amiRNA-based RNA interference leads to resulting specific gene silencing in transgenic crops to reduce host plant virus infection [27,28,106–108]. In this computational research , mature sugarcane sof-miRNAs/ssp-miRNAs were aligned with the genomic sequence of the SCMV target to identify miRNA-mRNA binding sites hypothesized to understand complex host-virus specific interactions with the P1, HC-Pro, P3, 6K1, CI, 6K2, NIa-VPg, NIa-Pro, NIb, CP of SCMV. Until now, the potential for exploiting the regulation of sugarcane genome-encoded miRNA to abate infection by SCMV has not been investigated as strategy for developing tolerant or resistant sugarcane cultivars. The results of this study provide the first computationally-based evaluation of mature locus-derived miRNAs in the sugarcane plant genome to enable prediction of effective miRNA-binding sites and provide new tools for better understanding the molecular and omic interactions between sugarcane plant host cells and SCMV-encoded mRNAs/protein.

Based on our findings, the SCMV genome (HC-Pro, CI, NIa-VPg and CP) is susceptible to nine consensus sugarcane miRNAs. We found that nine miRNAs could theoretically originate from the sugarcane genome (Table 3 and Figure 4). In silico tools—RNA22, TAPIR, and psRNATarget identified a genomic consensus base pair complementarity sof-miR159c at nucleotide position 3847 (Figure 1 and Table 2). All five algorithms identified ssp-miR444c-3p as the only sugarcane miRNA (Figure 1 and Table 2). We identified the maximum folding energy of the consensus functional miRNA-mRNA target pair, which is -18.00 Kcal/mol, using RNA22. RNA22 is a highly sensitive algorithm that uses a pattern-based approach to target miRNAs. Using the psRNATarget algorithm, we estimated expectation score 5.50 for a consensus target pair (Table 2)[109]. The RNA22 and psRNATarget algorithms predicted target sites using a non-seed-based approach. These results suggest that the predicted consensus miRNA-mRNA duplex represent a ‘true target’. Our results indicate that sugarcane-miRNAs likely play a role in the interaction between host and virus. Our results highlight the interaction of SCMV ss-RNA with on the sugarcane miRNA target interaction network.

Potyvirus cylindrical inclusion helicase (CI) is required for the initiation of the viral replication mechanism, cell-to-cell movement and plant-host protein-virus interaction [73,74,110]. Computational prediction and analysis revealed that the sugarcane consensus sof-miR159c is high-confidence target site potentially targeting the CI ORF. The conserved precursor MIR159 is considered to be controlled by plant growth, and fertility [111]. The consensus sof-miR159e (Accession ID: MIMAT0001661), predicted to have an effective target binding site at nucleotide

position 5535 in the SCBV genome, was identified as the most effective miRNA by the miRanda, RNA22 and RNAhybrid algorithms.

While miRNA-mRNA target pair interactions between locus-derived miRNAs in the sugarcane genome and SCMV have been determined, the development of amiRNA-based constructs and further transformation in sugarcane to control SCMV is not fully understood. We have performed a comprehensive analysis of SCMD-associated *Potyvirus* for the first time which is a first step toward the development of miRNA-based antiviral therapy. The amiRNA construct relies on high-level specificity of nucleotide base pairing to control deleterious off-target effects. The small size of amiRNA is a unique feature for the development of a single gene expression vector to control multiple potyviruses in transgenic sugarcane. This approach offers specificity and sensitivity and complements existing molecular approaches for analyzing targets for SCMV disease abatement. Results indicate that the use of in silico tools provides better results than a single algorithm when developing amiRNA-based mdm-miRNA therapeutics to target SCMV and other plant viruses as well. The in silico analysis has been designed for experimental validation to show whether these predicted miRNAs could make the plants resistant to SCMV. Future work is focused on transiently expressing these miRNAs or injecting RNA hairpins in *N. benthamiana* to show its efficacy against SCMV.

Supplementary Materials: The following supporting information can be downloaded at the website of this paper posted on Preprints.org, Table S1: Sugarcane mature microRNA sequences used for prediction binding sites in the SCMV genome Table S2: Identification of high-confidence binding sites of sugarcane miRNAs in the SCMV; Table S3: Gene wise prediction; File S1: Prediction results by computational tools.

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