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The In Silico Sugarcane Genome-Encoded MicroRNA and Target Network Prediction for Targeting the SCMV

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Abstract: Sugarcane mosaic virus (SCMV) is a deleterious pathogen which causes widespread Sugarcane mosaic disease (SCMD) and is classified in the genus *Potyvirus* (*Potyviridae*), disseminated by the aphid vector. RNA interference (RNAi)-mediated antiviral innate immunity is a key biological process and antiviral defence system to interfere with viral genomes for controlling plant pathogens. 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 'five algorithms' approach. The ultimate goal in this research is to mobilize the in silico endogenous predicted sof-miRNAs/ssp-miRNAs to trigger RNAi catalytic pathway experimentally and generate sugarcane cultivars for evaluating potential antiviral resistance monitoring capability and capacity for SCMV. Experimentally validated mature sugarcane (*S. officinarum*, $2n = 8X = 80$) and (*S.* spp., $2n = 100\text{-}120$) sof-miRNAs/ssp-miRNAs ($n = 28$) were acquired for alignment with the SCMV genome. Of the 28 targeting mature locus-derived sof-miRNAs/ssp-miRNAs investigated, one sugarcane miRNA homolog, sof-miR159c, was concluded to localize potential binding site at genomic nucleotide site 3847 targeting CI ORF of SCMV. In order to validate target prediction accuracy, whether the sugarcane sof-miRNA/ssp-miRNA might bind predicted SCMV mRNA target(s), we created an integrated Circos plot. Genome-wide in-silico-predicted miRNA-mediated target gene regulatory network validated interactions that warrant in vivo analysis. The current work provides valuable evidence and biological material for generating SCMV-resistant sugarcane varieties.

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

1. Introduction

Sugarcane (*Saccharum officinarum*) is a vigorous tropical and sub-tropical pertinent economically important, long-duration, biofuel cash crop, enriched with high energy roughage and also a source of agro-industrial residue [1-3]. The octaploid sugarcane (*S.officinarum*) genome ($2n = 80$; x = 10) [4,5] which is referred as "noble" cane and genome of sugarcane species and cultivars have been assembled, drafted and re-sequenced [6-11]. Sugarcane mosaic virus disease (SCMV) is highly transmissible and pathogenic potyvirus that cause sugarcane mosaic virus disease (SCMD) [12,13]. Potyviruses are observed to be spread by a common sap sucking vector—aphid species complex [14]. Innovative approaches are still needed to enhance the sugarcane productivity [15]. The genome of SCMV composed of a +ss RNA molecule 9575 nucleotides in length encoding a single large polyprotein. The genome polyprotein precursor was predicted to undergo

cleavage resulting 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), evolutionary conserved, non-coding (NC)-ss RNA molecules [20]. In higher plants, biogenesis and transcription of miRNA gene (*MIR)* is governed by RNA polymerase II which is further transcribed into single-standard polycistronic *primary* transcripts (pri-miRNAs). They govern a multitude of biological process in plants regulating gene expression, cell growth, development, differentiation and host–virus interactions [21,22]. The miRNA-mediated RNAi is a post-transcriptional gene silencing mechanism providing antimicrobial innate immunity regulating host-virus interaction for restriction of inhibition of virus infection [23].

Artificial miRNA-mediated (amiRNA) technology is an alternative, safe approach based on engineering miRNA gene to control viral infection in plants [24]. RNAi-based amiRNA construct has been deployed in research to create antiviral resistance in plants against plant viral species such as tomato [25,26], cucumber [27], rice [28] and cotton [29]. Mature miRNAs in sugarcane genome have been predicted, identified, isolated, analyzed, and validated for evaluation of host–virus interactions, gene regulation and was associated with abiotic and biotic stresses [30-40]. Recently, experimental validation of 35 conserved mature sugarcane genome-encoded, high-confidence sof-miRNAs/ssp-miRNAs and further deposition in the miRBase database were reported.

An integrative multi-network approach based on evaluation of SCMV infection, deployed to identify target-binding sites of sugarcane genome-encoded sof-miRNAs/ssp-miRNAs in the SCMV genome. Identification of several homologous amiRNAs for the creation of transgenic sugarcane cultivars—resistant to SCMV is the key objective in this study. The predicted sugarcane genome-encoded sof-miRNAs/ssp-miRNAs were further evaluated to understand complex sugarcane host plant–SCMV potyviral interactions for identification of 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 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

Predicting effective microRNA-mRNA binding sites is an initial step for understanding microRNA-regulated gene regulatory networks. The accuracy of miRNA target site prediction can be influenced by several factors, such as the specificity and sensitivity of the algorithm, the choice of reference sequence, and the length of the target sequence. To predict miRNA-mRNA target sites computationally, various in silico methods are generated for effective silencing. A computational approach refers to the use of multiple computational methods, algorithms, or tools to analyze and interpret biological data. This approach involves combining 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. Statistical Analysis

The miRNA-mRNA target prediction biological datawere further processed. Graphical representations of miRNA data was prepared using R-language [51].

3. Results

3.1. Prediction and Analyysis 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 [33,52-55]. 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 attachments sites were identified by psRNATarget. RNAhybrid predicted 28 high-probability binding sites of sugarcane miRNAs in the SCMV genomic RNA sequence (**Figure 1 and Figure 2**) (**File S1**) (**Table S2**).

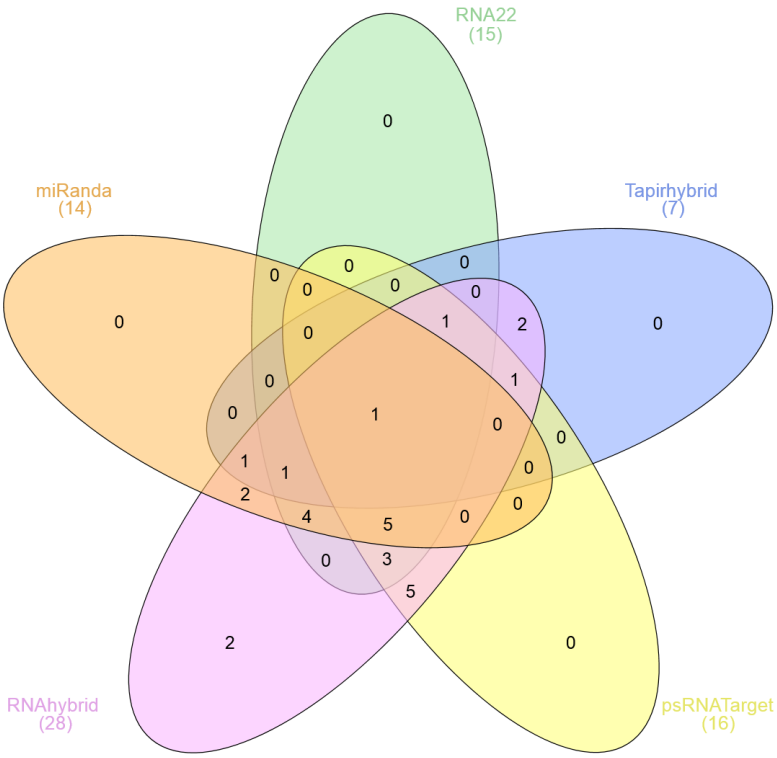

Figure 1. Five-set **v**enn diagram representing mutually common binding sites of mature sugarcane miRNAs predicted potentially targeting the SCMV genome. The in-silico prediction was established using computational tools (miRanda, RNA22, TAPIR, psRNATarget, and RNAhybrid) to identify potential targets 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 [56,57]. Host adaptation is a key process for virus genome evolution [58,59]. P1 is also related to virus-host adaptation [60]. 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**) (**Table S2-S3**).

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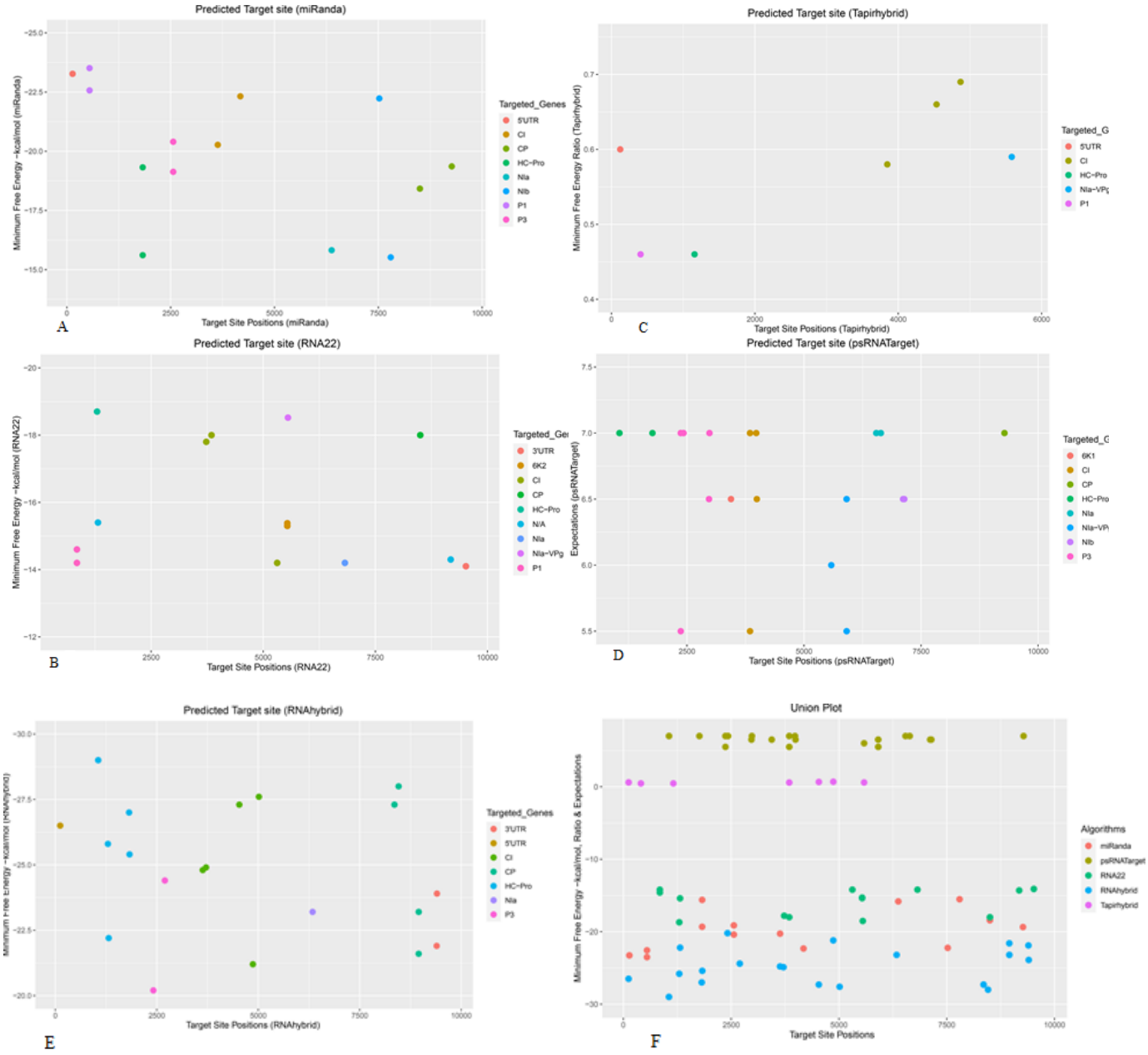

Figure 2. Individual sugarcane sof-miRNA/ssp-miRNA and their predicted high-confidence binding sites in the SCMV genome were predicted based on 'five algorithms' approach. (**A**) miRNA-sites were detected by miRanda. (**B**) Several miRNA-target sites were detected by RNA22. (**C**) TAPIR identified 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 nt) 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 [61-66]. 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 nt position 1296 also (**Figure 2A-2B**).

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**) (**Table S2-S3**).

3.4. Sugarcane miRNAs Targeting P3

The P3 ORF (2228-3268 nt) encodes a P3 membrane-associated protein participating directly in the genomic RNA replication mechanism of SCMV. It is also involved in potential cell-to-cell spread (movement and transport) and is responsible to determine host-range and symtoms [67-69]. The miRanda algorithm predicted binding of sof-miRNAs: sof-miR168 (a, b) at nucleotide position 2562 (**Figure 2A**). No sof-miRNA/ssp-miRNA was predicted targeting the P3 by the RNA22 and TAPIR algorithms (**Figure 2B-2C**). 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**). Further, RNAhybrid identified sof-miR167(a, b), ssp-miR437b—target sites at nt positions 2699 and 2416, respectively (**Figure 2E**) (**File S1**) (**Table S2-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 [70,71]. The 6K1 had the least number of predicted sugarcane sof-miRNAs ssp-miR444c-3p at nulceotide position 3441, by the psRNATarget algorithm (**Figure 2D**) (**File S1**) (**Table S2-S3**).

3.6. Sugarcane miRNAs Targeting CI

The CI ORF (3470-5383 nt) encodes a multifunctional cylindrical inclusion (CI) protein that is essential for ATP-binding and RNA helicase activity [72-74]. CI was targted by two miRNAs: sof-miR396, ssp-miR166 at nt positions 3634, 4178 respectively, as indicated by miRanda (**Figure 2A**). RNA22 predicted two miRNAs: sof-miR159c, ssp-miR444b at nt positions 3730, 5311 respectively(**Figure 2B**). Further, TAPIR predicted three sugarcane miRNAs: sof-miR159c, ssp-miR437a and ssp-miR1128 at nucleotide positions 3847, 4869 and 4534, respectively (**Figure 2C**). The psRNATarget 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**) (**Table S2-S3**).

3.7. Sugarcane miRNAs Targeting 6K2

The potyviral 6K2 (5384-5542 nt) encoded 6K2 multifunctional protein, induces formation of RE-derived complexes and develop resistance against drought [75,76]. The RNA22 identified five sugarcane sof-miRNAs: sof-miR408 (a, b, c, d, e) on locus position 5538 (**Figure 2B**) (**File S1**) (**Table S2-S3**).

3.8. Sugarcane miRNAs Targeting NIa-VPg

The potyviruses *NIa-VPg* ORF (5543-6109 nt) encodes viral genome-linked protein (VPg) which functions as virulence determinant and genome translation [77-81]. It also invovlved in replication, translation and movement [82-84]. The RNA22 and TAPIR predictd binding of ssp-miR444c-3p on locus position 5552 (**Figure 2B-2C**). The psRNATarget 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 (**Figure 2E**) (**File S1**) (**Table S2-S3**).

3.9. Sugarcane miRNAs Targeting NIa

The potyviruses *NIa* ORF (6110-6835 nt) encodes nuclear inclusion a (*NIa*) protein that is involved in RNA-binding and also interacts with NIb [85,86]. miRanda, RNA22 and RNAhybrid predicted binding of only sugarcane miRNA: ssp-miR528, sof-miR396 and ssp-miR827 at nucleotide positions 6376, 6821 and 6338, respectively (**Figure 2A-2B, 2E**). The psRNATarget idendified 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**) (**Table S2-S3**).

3.10. Sugarcane miRNAs Targeting NIb

The potyviruses *NIb* ORF (6836-8398) encodes nuclear inclusion b (*NIb*) protein that is involved in translocation activity and also interacts with *NIa* [87]. It contains nuclear signals and also called as RdRp [88]. The miRanda algorithm detected 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 by the RNA22, TAPIR and RNAhybrid algorithms, (**Figure 2B-2C-2E**) (**File S1**) (**Table S2-S3**).

3.10.1. Sugarcane miRNAs Targeting CP

The potyviruses *CP* ORF (8399-9337) encodes multistaking protein, coat (CP) that is involved in the development of virion assembly. The CP is involved in all steps of potyviral life cycle [89-91]. The miRanda predicted binding of three sugarcane ssp-miRNAs (ssp-miR444 (a, b, c-3p) (start site 8501). ssp-miR444c-3p also targetd CP region at nt position 9268(**Figure 2A)**. RNA22 predicted binding of ssp-miRNA444 family at nt positions 8502 and 9181(**Figure 2B)**. The psRNATarget predicted bind of ssp-miR444c-3p at nt position 9282. Potential binding sites of sugarcane miRNAs: sof-miR159 (a, b, d, e), sof-miR408 (a, b, c, d), and ssp-miR169 were detected by RNAhybrid at nt 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 [92,93]. 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 identifed 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 preicted 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**) (**Table S2-S3**).

3.5. Identification of Consensual Sugarcane MicroRNAs

The current study was concluded on the basis of consensus genomic target binding sites of sugarcane miRNAs detected 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) that were detected on the basis of 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) respectively (**Table 2 and Table 3**). Of nine consensus sugarcane-encoded locus-derived sof-miRNAs/ ssp-miRNAs investigated in this study, only one sof-miRNA (sof-miR159c at nt position 3847 targeting CI) was detected by union of consensus genomic positions by at least three

algorithms (RNA22, TAPIR and psRNATarget) (**Figure 3, Table 2 and Table 3**) (**File S1**) (**Table S2-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

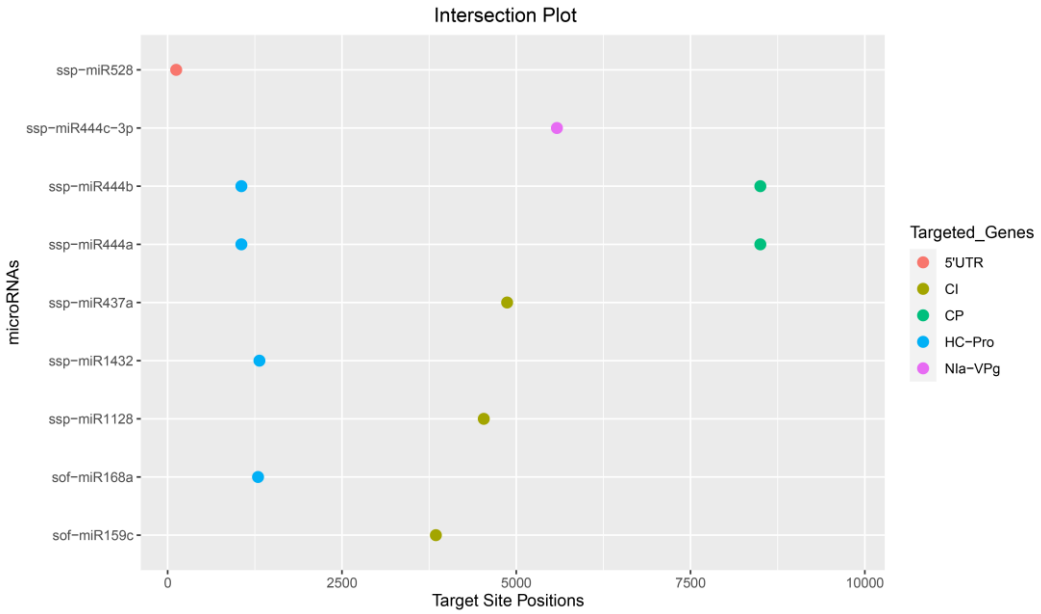

Figure 3. Intersection plot show consensus high-confidence binding sites of sugarcane mature miRNAs predicted by at least two computational tools. The colored dots represent sugarcane miRNA-binding sites targeting different genes of SCMV.

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	UCGCUUGGUGCAGAUCGGGAC	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
ssp-miR444a (1)	MIMAT0020284	UGCAGUUGUUGCCUCAAGCUU	HC-Pro	1058–1078
ssp-miR444b	MIMAT0020285	UGCAGUUGUUGCCUCAGGCUU	CP	8501–8521
ssp-miR444b (1)	MIMAT0020285	UGCAGUUGUUGCCUCAGGCUU	HC-Pro	1058–1079
ssp-miR444c-3p	MIMAT0020286	UGCAGUUGUUGUCUCAAGCUU	NIa-VPg	5583–5604
ssp-miR1128	MIMAT0020289	UACUACUCCCUCCGUCCCAAA	CI	4534–4555
ssp-miR1432	MIMAT0020290	CUCAGGAAAGAUGACACCGAC	HC-Pro	1315–1336

3.7. Identification of miRNA-mRNA Regulatory Network

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 [94]

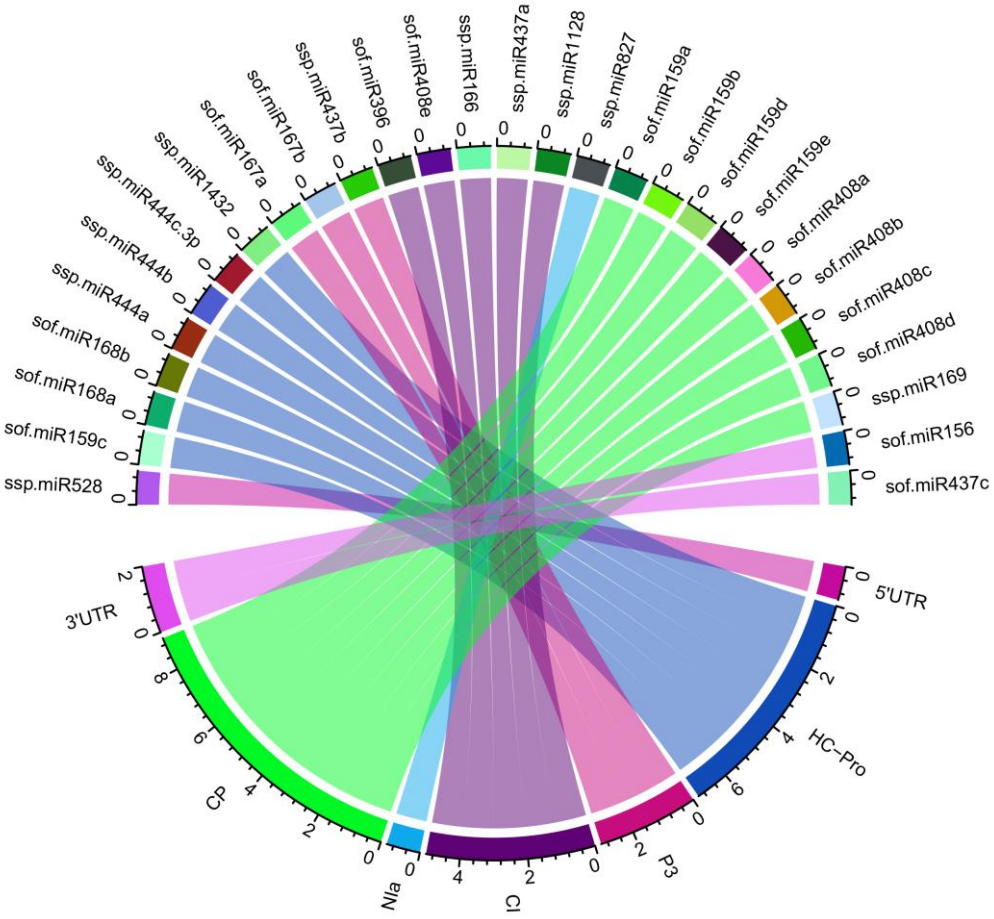

Figure 4. Integrated Circos plot demonstrate multiple targets of sugarcane-encoded miRNAs. The colored connection lines are targeted genes (ORFs) in SCMV genome. Construction, exploration, target predictions and interactions between the sugarcane miRNAs and SCMV genes are mapped.

3.8. RNA Secondary Structures

The Computationally predicted consensual sugarcane mature miRNAs were analyzed by generating their secondary structures using original precursor sequences. The pre-miRNA hairpin sequences were used to perform manual curation. Salient parameters of predicted stable secondary structures were evaluated (**Table 6**). Stable secondary structures of potential consensual sugarcane precursors sequences were predicted by RNAfold algorithm [95].

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, suggested as etiological agent that spread to Pakistan and China due to highly transmissible pathogen, becoming an increasing potential, long lasting, threat to sugarcane and maize production over the past two decades [13,17,96]. In our previous studies, we have investigated experimentally validated sugarcane genome-encoded mature microRNAs that were predicted to target SCBGAV, SCYLV and SCBV based on in silico criteria [37-39]. Several studies have identified complex host-virus interactions and have investigated host-plant miRNAs targeting plant viruses using an in silico approach [97-103]. The miRNAs have evolved as novel endogenous targets for multiple layers of miRNA-gene level regulation [52,104,105]. In order to abate host plant-virus infection, several studies have indicated that efficacy of amiRNA-based RNA interference resulting specific gene silencing in transgenic crops [27,28,106-108]. In this computational research work, mature sugarcane sof-miRNAs were aligned with the target, SCMV genomic sequence 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.

Based on our reported finding, the SCMV genome (HC-Pro, CI, NIa-VPg and CP) is vulnerable to be targeted nine consensus sugarcane miRNAs. We revealed nine miRNAs could theoretically derive from sugarcane genome (**Table 3** and **Figure 4**). In silico tools—RNA22, TAPIR and psRNATarget—screened a consensus genomic high-confidence binding of base-pairing complementarity sof-miR159c at nucleotide position 3847, (**Figure 1 and Table 2**). While all five algorithms identified ssp-miR444c-3p as the unique sugarcane miRNA (**Figure 1 and Table 2**). We identified the maximum folding energy of consensus functional miRNA-mRNA target pair which is −18.00 Kcal/mol using RNA22. RNA22 is a highly sensitive algorithm uses a pattern-based approach to screen target sites of miRNAs. While, we estimated expectation score 5.50 of consensual target pair by psRNATarget (**Table 2**). The [109]RNA22 and psRNATarget algorithms predict target sites using a non-seed-based approach. These results provide support for predicted consensus miRNA-mRNA duplex to represent a 'true target'. Our findings demonstrated that sugarcane - miRNAs probably have putative role in host-virus pathogenesis interaction. Our results highlight the interaction of SCMV ss-RNA on the sugarcane miRNA: target interaction network.

The potyviral cylindrical inclusion helicase (CI) is required to initiate viral replication mechanism. It also controls cell-to-cell movement and plant-host protein-virus interaction [72,73]. Computational prediction and analysis implicated the sugarcane consensus sof-miR159c high-confidence target site potentially targeting the CI ORF. The conserved precursor MIR159 is reported to govern plant growth, and fertility [110]. The consensus sof-miR159e (Accession ID: MIMAT0001661), that have predicted effective target binding site at nucleotide position 5535 in SCBV genome, was identified as top effective miRNA by miRanda, RNA22 and RNAhybrid algorithms.

While miRNA-mRNA target pair interactions between sugarcane genome-encoded ghr-miRNAs and SCMV have been determined, development of amiRNA-based construct and further transformation in sugarcane to control SCMV is yet to fully understand. We reported first time a comprehensive analysis of SCMD-associated *Potyvirus* which is an initial step to construct miRNA-based anti-viral therapy. The amiRNA construct is based on highly specificity of nucleotide base-pairing to control detrimental off-target effects. The small size of amiRNA is a unique feature to develop a single gene expression vector to control multiple Potyviruses in transgenic sugarcane. 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: www.mdpi.com/xxx/s1, 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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