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Article

Computational Biology and Machine Learning Approaches to Identify the Rubber Tree (*Hevea brasiliensis* L.) Genome Encoded Potential MicroRNAs Targeting Rubber Tree Virus 1

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Abstract: Tapping panel dryness (TPD) syndrome is a complex disease of Rubber tree (*Hevea brasiliensis* L.) which causes cessation of latex drainage upon tapping of rubber tree. Rubber tree virus (RTV1) was identified as a novel pathogen associated with rubber tree and a potential causal agent of TPD. RTV1 is a monopartite RNA virus that is linear, non-enveloped and has a single-stranded (ss) positive RNA genome of approximately 6081 nucleotides and is composed of two major open reading frames (ORFs), ORF1 (polyprotein), and ORF2 (movement protein. This study aimed to investigate the possibility of rubber genome encoded tree microRNAs (miRNAs) as novel therapeutic targets against RTV1 using *in silico* algorithms. Mature rubber tree miRNAs are retrieved from the miRBase database and are used for hybridization of RTV1 using five different five different computational algorithms including miRanda, RNA22, RNAhybrid and psRNATarget. A total of eleven common rubber tree miRNAs were identified based on consensus genomic positions. The consensus of four algorithms predicted the hybridization sites of hbr-miR396a and hbr-miR398 at common locus positions 6676, 1840 respectively. To validate the prediction, secondary structures of the consensual rubber tree miRNAs and free energy of duplex binding were calculated using the RNAfold and RNAcofold algorithms respectively. We created a plot between rubber tree miRNAs and RTV1 ORFs by using Circos algorithm. In this study, we predicted eleven consensual rubber tree miRNAs. Among these miRNAs, hbr-miR398 was identified as the most effectual miRNA that may target the ORF1 gene of the RTV1 genome. The predicted data will be important in the development of rubber trees resistant to RTV1.

Keywords: Rubber tree capillovirus 1; microRNAs; plant-virus interaction; RNAi: computational algorithms; gene silencing, minimum free energy

1. Introduction

The rubber tree (*Hevea brasiliensis* L.) is a valuable source of natural rubber (NR) which is an indispensable commercial source material for the manufacturing of the 5000 products worldwide [1]. The rubber tree genome contains a diploid set of chromosomes ($2n = 36$), and the draft genome was sequenced in 2013 [2]. The production of natural rubber is highly hampered by different pathogens that infect the rubber tree. Recently a novel capillovirus was identified first time to infect the rubber tree. The RNA genome of the novel capillovirus was isolated, sequenced and was tentatively assigned the nomenclature as rubber tree virus 1 (RTV1) in China. RTV1 has emerged as a deleterious pathogen of the genus *capillovirus* in the family *Betaflexiviridae*. The genomic organization of the RTV1 is consisted of monopartite, linear, non-enveloped, +ssRNA molecule of 6811 nucleotides [3].

Considering the RNA nature of PTV1, RNA-based molecular approaches, RNA interference (RNAi) and host-derived microRNA (miRNA) silencing are emerging as potent to cleave the viral

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mRNA in the infected host cells. RNA silencing is a conserved, sequence-specific gene silencing mechanism controlled by siRNAs [4]. It is an important line of defense against invading viruses in the host cell [5]. The Dicer and Argonaute genes are the key components of the RNAi machinery. The cleavage of the precursor dsRNA results into short 21–24 nucleotides siRNA that inhibit protein translation during infection [6]. The amiRNA-mediated RNAi has high silencing specificity and develops a single 21-nucleotide amiRNA to target corresponding sequence [7]. The plant microRNAs (miRNAs) are small endogenous, noncoding, regulatory bigwigs of 20–24 nucleotides in length which are encoded by *MIRNA* genes [8]. They can regulate complex biological processes including gene expression and regulations in plants [9]. Rubber tree plant has evolved diverse molecular mechanism and was inherited with mature miRNAs that provide immunity against biotic and abiotic stresses [10,11].

Artificial miRNA-mediated silencing technology is a novel approach to control plant viruses. The amiRNA-based construct was first time transformed in Arabidopsis to create resistance against *Tymovirus* and *Potyvirus* [7]. In the rubber tree genome, 30 mature miRNAs have been identified and characterized, and a subset of these mat-miRNAs in rubber tree should have target binding sites in the RTV1 genome. This current computational biology approach was based on a comprehensive bioinformatics analysis of RTV1 genome using rubber tree miRNAs. The current study aims to implement computational algorithms for the prediction of rubber tree genome encoded miRNAs targeting RTV1. The predicted rubber tree miRNAs can be utilized for the development amiRNA-based constructs to transform in rubber tree to control RTV1 in future.

2. Materials and Methods

2.1. Retrieval of Rubber Tree microRNAs

A total of 30 mature rubber tree (commonly called *Hevea brasiliensis*-microRNAs) (hbr-miR156-hbr-miR9387) (Accession IDs: MIMAT0025282-MIMAT0035236) **(Supplementary Table S1)** and stem-loop hairpin precursor miRNAs (hbr-MIR156-hbr-MIR9387) (Accession IDs: MI0022052-MI0028936) **(Supplementary Table S2)** were downloaded from the miRBase version 22 (http://mirbase.org/) biological database [12].

2.2. RTV1 Genome Retrieval and Annotation

The full-length genomic transcript of the RTV1 (Accession ID: MN047299) was retrieved from the NCBI biotechnological genbank database. Annotation and production of the graphical output of the RTV1 ORFs was created by the pDRAW32 DNA plasmid map analysis (AcaClone 1.1.147 software.

2.3. miRanda

miRanda is a seed-based standard computational scanning algorithm. It was implemented for the first time in 2003 [13]. It has been updated into a web-based tool (http://www.microrna.org/) for the prediction and analysis of miRNA [14]. It was run under well-defined standard settings. Following parameters were set for analysis: gap open penalty: (-8.00), gap extend: (-2.00), score threshold: (50.00), minimum free energy (MFE) threshold: (-20.00 Kcal/mol) and scaling parameter: (2.00).

2.4. RNA22

RNA22 is a non-seed based user-friendly, pattern-recognition algorithm that was assessed at http://cm.jefferson.edu/rna22v1.0/ [15]. It has been developed for the identification of target binding sites of miRNAs and their interrelated heteroduplexes. Characteristic parameters were set as: output format (heteroduplex's), sensitivity vs. specificity setting: (sensitivity (63%), specificity (61%)), seed region: (seed size of 7, allow maximum of I UN-paired bases in seed), minimum number of paired-up bases in heteroduplex: (12), maximum folding energy for heteroduplex: (-16.00Kcal/mol) and Maximum number of G: U wobbles allowed in seed region: (no limit).

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2.5. RNAhybrid

RNAhybrid is a seed based intermolecular hybridization algorithm which is used to predict miRNA targets in a very easy and flexible manner. It is an online available tool for the rapid prediction miRNA targets based on MFE hybridization of mRNA and miRNAs [16]. Salient parameters were set as: MFE threshold (−20 Kcal/mol), hit per target (1), no G: U in seed, helix constraint from and helix constraint to.

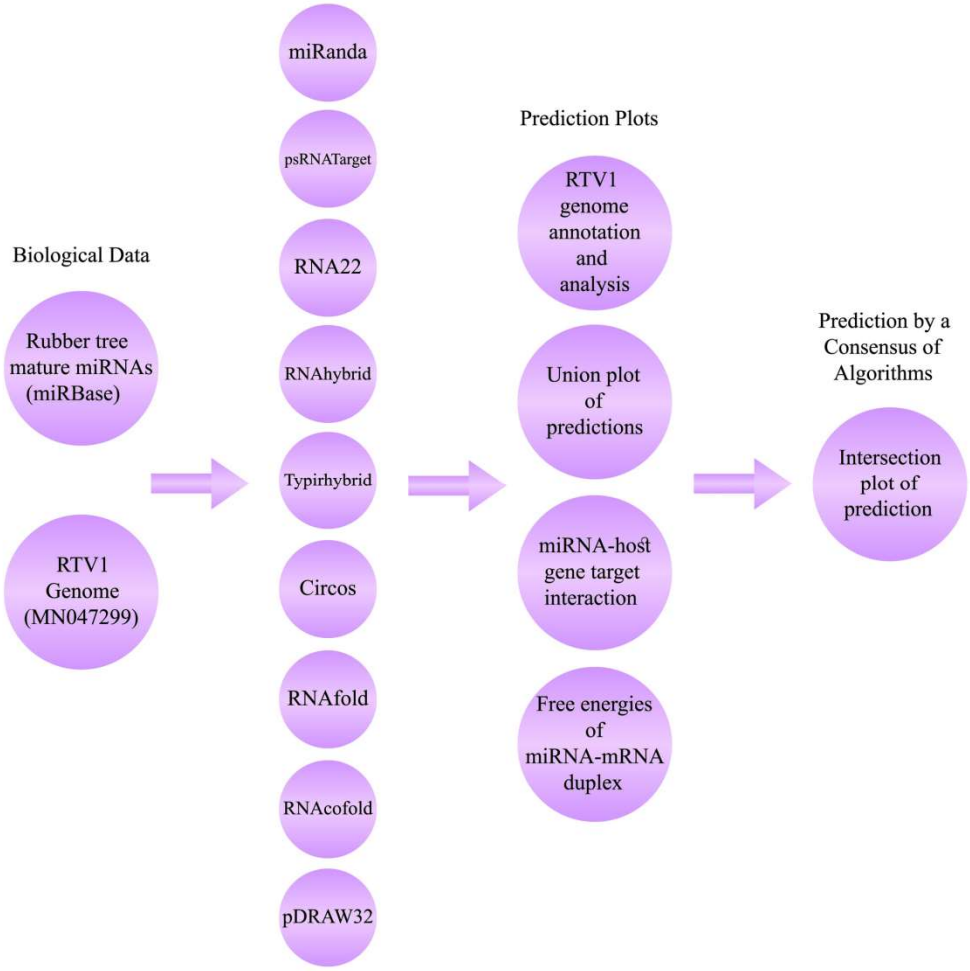

Figure1: A computational biology scheme and methodology of rubber tree miRNA prediction from the RTV1 genome.

2.6. Tapirhybrid

The Tapirhybrid is a webserver used to predict plant miRNA targets as well as target mimics using a fast and a precise algorithm [17]. Tapirhybrid was run in FASTA mode using following settings: a score cutoff score <= 9 and minimum free energy (MFE) ratio: (0.200).

2.7. psRNATarget

The psRNATarget program is newly designed a browser-based tool (http://plantgrn.noble.org/psRNATarget/) for the prediction plant miRNA targets. Complementary scoring and secondary structure prediction is the key features of psRNATarget algorithm [18]. Following standard key features were set: no. of top targets (200), expectation score (5), mode of inhibition (cleavage), penalty for G: U pair: (0.5), Penalty

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for other mismatches: (1), seed region: 2-13 (NT), no. of mismatches allowed in seed region: (2), HSP size: (19), penalty for opening gap: (2), and penalty for extending gap: (0.5)

2.8. Mapping of miRNA-Target Interaction

miRNA-target interaction was mapped using the Circos algorithm[19]. An interaction map was developed using rubber tree miRNAs and RTV1 genes.

2.9. RNAfold

RNAfold is one of the latest web-based algorithms used for the prediction secondary structures from the precursor sequences using MFE as standard [20].

2.10 RNAcofold

RNAcofold is a web-based newly developed algorithm used to estimate the free energy (ΔG) of duplex binding [21]. It is used to evaluate the mRNA and miRNA duplex interaction.

2.10.1 Graphical Representation

R (version 3.1.1) studio was used to process all the biological data into graphical representations [22].

3. Results

3.1. Rubber Tree miRNA's Loci on RTV1 Genome

A biological computational framework was designd using the plant miRNA prediction algorithms and host-derived miRNAs from the miRBAse **(Figure 1).** We investigated the possibility of the rubber tree (host) miRNAs with a potential to target RTV1 genome. We generated the RTV1 genome from the NCBI database and computational annotation of different ORFS were performed **(Figure 2).** We employed five different miRNA prediction algorithms (miRanda, RNA22, RNAhybrid, Tapirhybrid and psRNATarget) to predict the rubber tree miRNA binding strenght in the genome of the RTV1 ssRNA molecule. The miRanda algorith has predicted 12 rubber miRNAs targeting 12 loci. RNA22: 6 rubber tree miRNAs: 8 loci. RNAhybrid: 29 rubber tree miRNAs: 29 loci. Tapirhybrid has predicted that 5 rubber tree miRNAs targeted 5 loci. psRNATarget: 14 rubber tree miRNAs: 23 loci **(Figure 3) (Supplementary Table 3)**.

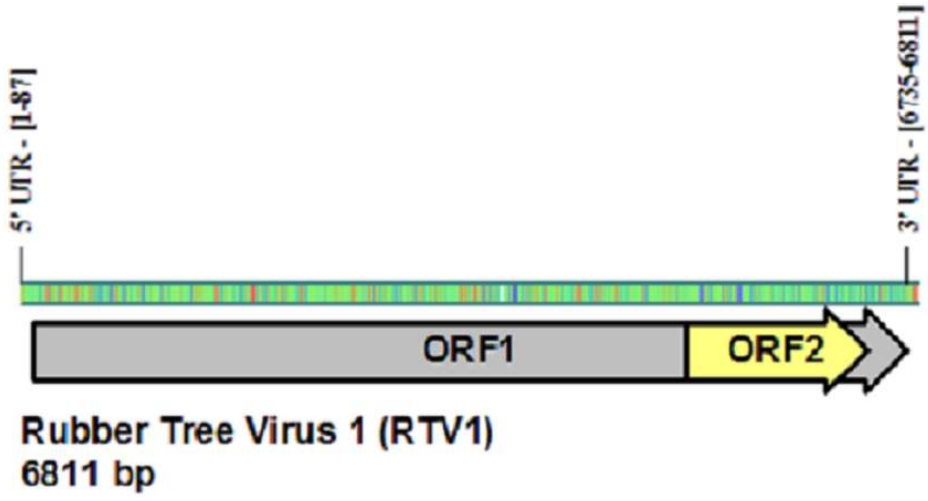

Figure 2: Schematic representation of the RTV1 genome. Coordinates are designed based on Accession number of the RTV1 genome.

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3.2 ORF1 encoding Polyprotein

ORF1 encodes a polyprotein (2215 amino acids (AA) of the RTV1 genome. ORF1 was targeted by seven rubber tree miRNAs: hbr-miR159a (locus 2178), hbr-miR319 (locus 2992), hbr-miR396 (a, b) (locus 6678), hbr-miR398 (locus 1839), hbr-miR408b (locus 6498), and hbr-miR6171 (locus 1633), as indicated by miRanda algorithm. Potential hybridization sites were identified for ORF1of the RTV1 genome by the RNA22 algorithm. These include: hbr-miR319 (loci 569, 3204), hbr-miR396a locus (6675), hbr-miR398 (locus 1838), hbr-miR482a (locus 3951), hbr-miR6167 (locus 1058), and hbr-miR6168 (loci 145, 647). Tapirhybrid has predicted target binding of only four rubber tree miRNAs: hbr-miR396a (locus 6674), hbr-miR6167 (locus 2716), hbr-miR6171 (locus 1633), and hbr-miR6483 at (locus 106).

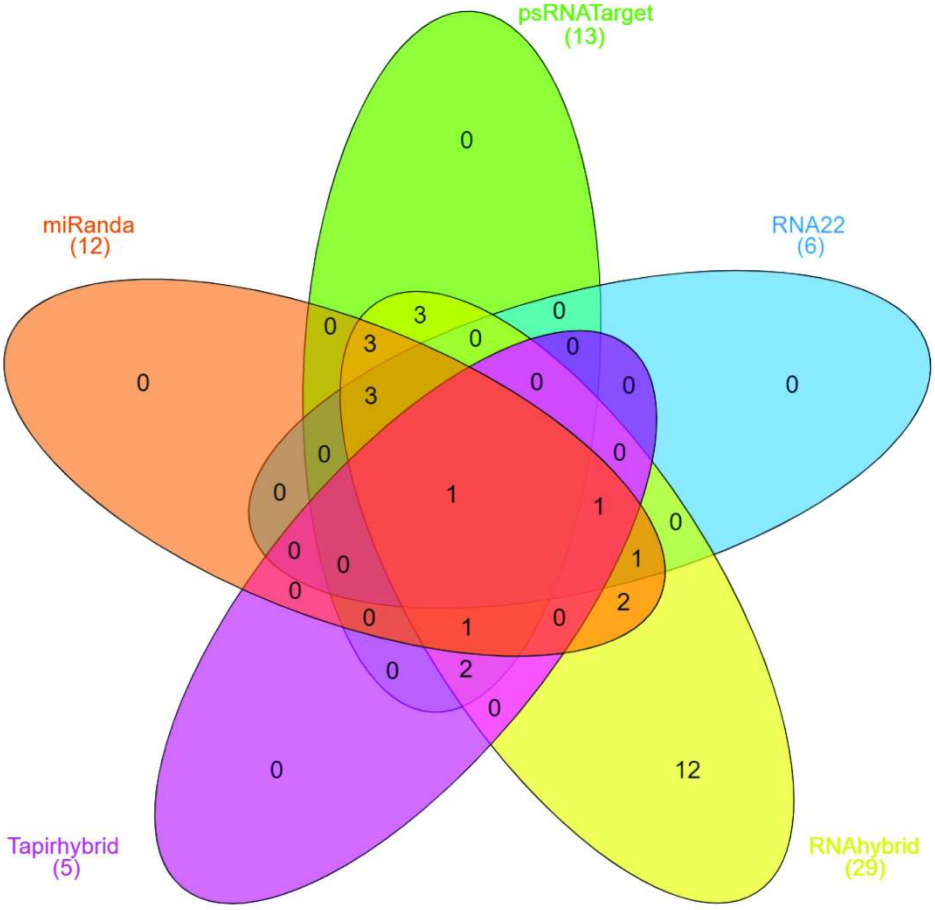

Figure3: Venn diagram plot of RTV1 representing common rubber tree genome-encoded miRNAs calculated by all the algorithms used in this study. Furthermore, single rubber tree miRNA (hbr-miR6167) is predcited by all the algorithms used in this study.

RNAhybrid predicted twenty two rubber tree miRNAs: hbr-miR156 (locus 6492), hbr-miR159a (locus 2177), hbr-miR166a (locus 1060), hbr-miR396a (locus 6675), hbr-miR396b (locus 823), hbr-miR398 (locus 1840), hbr-miR408a (locus 573), hbr-miR408b (locus 6497), hbr-miR482b (locus 6500), hbr-miR2118 (locus 6592), hbr-miR6166 (locus 1515), hbr-miR6168 (locus 1641), hbr-miR6169 (locus 3882), hbr-miR6170 at (locus 6660), hbr-miR6171 (locus 2043), hbr-miR6172 (locus 4500), hbr-miR6173 (locus 3749), hbr-miR6174 (locus 4303), hbr-miR6482 (locus 2163), hbr-miR6483 (locus 1267), hbr-miR6485 (locus 3042), and hbr-miR9386 (locus 370).

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Twelve rubber tree miRNAs were preidcted for the silencing of the RTV1 genome by psRNATarget algorithm: hbr-miR159a (loci 2177, 3505, 2549, 4786), hbr-miR319 (locus 2548), hbr-miR396b (locus 6677), hbr-miR398 (locus 1838), hbr-miR482a (locus 627), hbr-miR6167 (loci 1459, 980), hbr-miR6169 (locus 1693), hbr-miR6171 (locus 1633, 4540), hbr-miR6482 (locus 399), hbr-miR6483 (locus 106, 3183), hbr-miR6484 (locus 4295), and hbr-miR9386 locus (4422) (Table 1) (Supplementary Table S3 and S4).

3.3. ORF2 Encoding Movement Protein

ORF2 (5055-6437 bp) encoded a movement protein (MP) of 460 AA size in the RTV1 genome. miRanda predicted five rubber tree miRNAs: hbr-miR482a (locus 5332), hbr-miR2118 (locus 5480), hbr-miR6167 (locus 5985), hbr-miR6168 (locus 5056), and hbr-miR6484 (locus 5994). No rubber tree miRNAs were identified to target the ORF2 gene with the RNA22 algorithm. Potential rubber tree miRNAs targeting ORF2 gene were predcited: hbr-miR166b (locus 6125), hbr-miR319 (locus 5323), hbr-miR476 (locus 6176), hbr-miR482a (locus 5334), hbr-miR6167 (locus 5984), hbr-miR6175 (locus 5118), and hbr-miR6484 (locus 5993). Only one rubber tree miNRA was predicted by the Tapirhybrid algorithm: hbr-miR6169 (locus 5291). psRNATarget has predicted six rubber tree miRNAs: hbr-miR396b (locus 5836), hbr-miR482a (locus 5331), hbr-miR6169 (locus 5291), hbr-miR6171 (locus 6066), hbr-miR6174 (locus 5542) and hbr-miR6483 (locus 5666) (Table 1) (Supplementary Table S3 and S4).

3.4. Visualization of miRNA-target Interaction Network

Circos ploting is widely used to understand host-virus interaction using the biological data in a precise manner. The mapped consenual rubber tree miRNAs are depicted in the RTV1 genomic ORFs **(Figure 4)**.

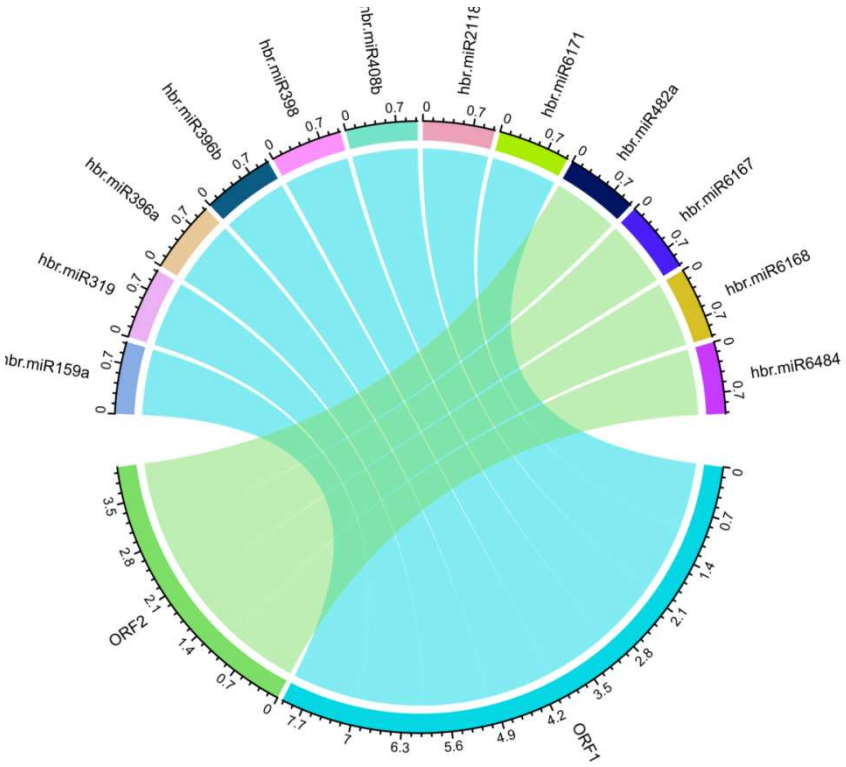

Figure 4: A schematic interaction circos map representing the rubber tree miRNAs and RTV1 ORFs. The colored lines represent the RTV1 genomic components (ORF1-2).

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3.5. Predicting Common Rubber Tree miRNAs

On the basis of predicted rubber tree miRNAs that have the potential to silence the RTV1 genome, hbr-miR6167 was predicted as a common miRNA by all five algorithms (miRanda, psRNATarget, RNA22, RNAhybrid, and Tapirhybrid) **(Figure 3).** Furthermore, three rubber tree miRNAs (hbr-miR482a, hbr-miR398, and hbr-miR319) were predicted by union of consensus between the multiple algorithms (miRanda, RNA22, RNAhybrid and psRNATArget). Three rubber tree miRNAs such as hbr-miR396b, hbr-miR159a, and hbr-miR6484 were identified by three algorithms used in this study (Figure 3) (Table 1).

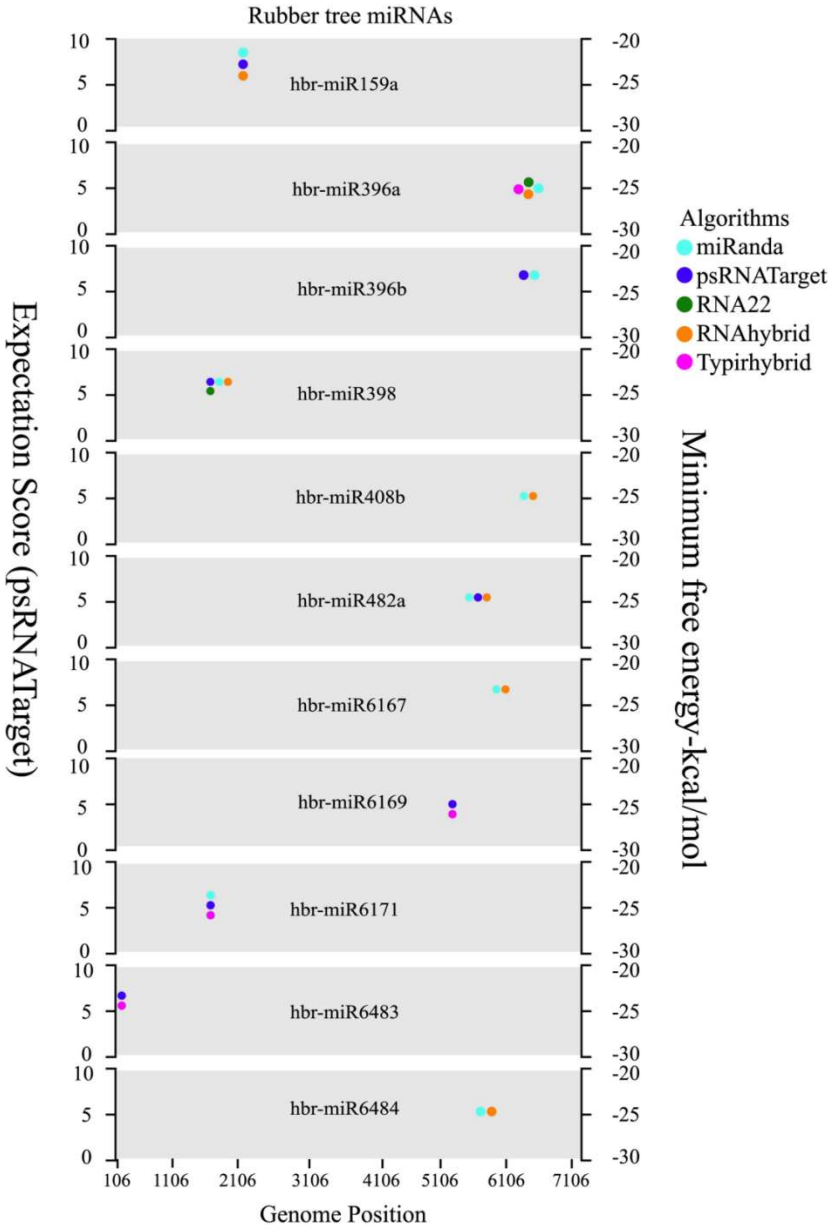

Figure 5: Intersection plot representing consensual rubber tree miRNAs predicted by different algorithms at common locus positions.

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3.6. Prediction of consensual rubber tree miRNAs

Out of 30 rubber tree miRNAs, only two rubber tree miRNA: hbr-miR396a at common locus position (6674) and hbr-miR398 at common locus position (1838) were predicted by the four algorithms used in this study. Furthermore, three consensual rubber tree miRNAs were predicted by al least three of the algorithms used to have potential unique target binding sites at common locus: hbr-miR159a (locus 2177), hbr-miR482a (locus 5331) and hbr-miR6171 (locus 1633). Interesitngly, seven miRNAs were predicted to have unique hybridization binding sites at common locus position: hbr-miR396b (locus 6677), hbr-miR408b (locus 6497), hbr-miR6167 (locus 5984), hbr-miR6169 (locus 5291), hbr-miR6483 (locus 106) and hbr-miR6484 (locus 5993) (Figure 5).

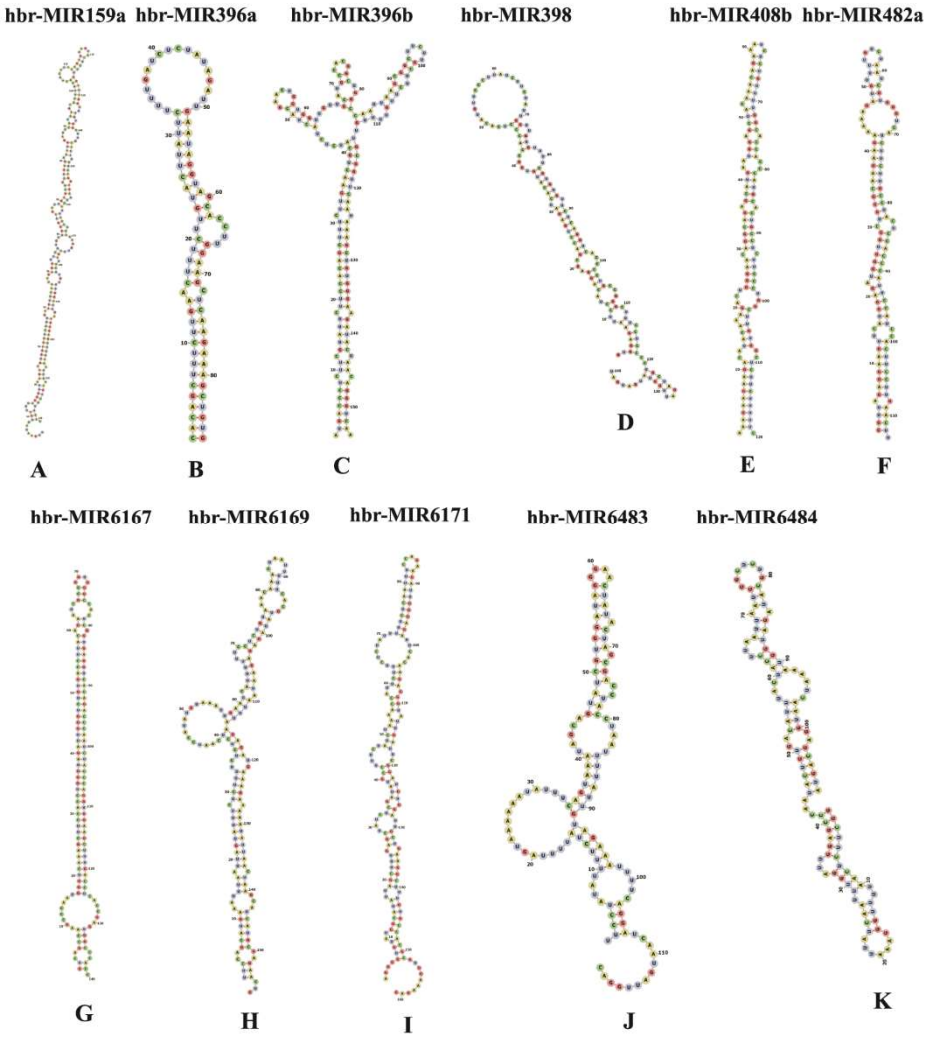

Figure 6: *Seven pre-miRNA* secondary structures were predicted in this study.

3.7. Prediction of Consensual Secondary Structures

The validtaion of the predicted consensual rubber tree miRNAs was monitored by the production their seondary structure from the precursor squences. The precursor of mature rubber tree miRNAs are mnaually curated. The MFE is the key factor to determine the secondary structures. In this study, the significant parameters of eleven consensual secondary structures of precursor miRNAs were also identified such as length miRNA, length precursor, MFE, GC content, AMFE, and MFEI. In our studies, the

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length precursor ranges from 86–221 nucleotides, along with MFE (-16.50 to -101.30 kcal/mol), GC content of 27-57%, AMFE of -13.86 to -46.50, and MFEI ranges from -0.49 to -1.26 (Figure 6).

Table 2: Rubber tree miRNAs and their target binding sites in the RTV1 genome predicted by different algorithms in this study.

Rubber tree miRNAs	Locus miRanda	Locus RNA22	Locus RNAhybrid	Locus TAPIR	Position psRNAT-arget	MFE* miRanda	MFE** RNA22	MFE* RNAhybrid	MFE-ratio TAPIR	Expectation psRNATarget
hbr-miR156			6492					-20.6		
hbr-miR159a	2178		2177		2177	-20.2	-15.2	-24.8		6.5
hbr-miR159a(1)					3505					6
hbr-miR159a(2)					2549					6.5
hbr-miR159a(3)					4786					7
hbr-miR166a			1060					-25		
hbr-miR166b			6125					-28.5		
hbr-miR319	2992	569	5323		2548	-20.2	-18.9	-29.8		7
hbr-miR319(1)		3204					-16.7			
hbr-miR396a	6676	6675	6675	6674		-24.7	-21.3	-27.2	0.66	
hbr-miR396b	6678		823		6677	-20.2		-25.1		7
hbr-miR396b(1)					5836					7
hbr-miR398	1839	1838	1840		1838	-21.3	-18.1	-25.1		7
hbr-miR408a			573				-14.2	-25.8		
hbr-miR408b	6498		6497			-23.2	-14.9	-25.3		
hbr-miR476			6176					-21.3		
hbr-miR482a	5332	3951	5334		5331	-26.1	-17.1	-29.3		5.5
hbr-miR482a(1)					627					6.5
hbr-miR482b			6500					-24.3		
hbr-miR2118	5480		6592			-20		-27.8		
hbr-miR6166			1515					-21.7		
hbr-miR6167	5985	1058	5984	2716	1459	-23	-16.9	-26.9	0.5	6.5
hbr-miR6167(1)					980					7
hbr-miR6168	5056	145	1641			-21.1	-19.8	-28.6		
hbr-miR6168(1)		647					-18.1			
hbr-miR6169			3882	5291	5291		-14.5	-23.3	0.55	5
hbr-miR6169(1)					1693					5
hbr-miR6170			6660					-20.2		
hbr-miR6171	1633		2043	1633	1633	-20.4	-15.8	-25.8	0.64	5.5
hbr-miR6171(1)					6066		-15.8			5.5
hbr-miR6171(2)					4540		-15.8			7
hbr-miR6172			4500				-15.3	-22.3		
hbr-miR6173			3749					-24.3		
hbr-miR6174			4303		5542			-24		7

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hbr-miR6175			5118				-14.9	-25.5		
hbr-miR6482			2163		399			-24.9		7
hbr-miR6483			1267	106	106			-20.1	0.41	6
hbr-miR6483(1)					5666					7
hbr-miR6484	5994		5993		4295	-20.7		-24.7		6
hbr-miR6485			3042					-22.4		
hbr-miR9386			370		4422			-24		6.5
hbr-miR9387										

3.8. Evaluation of free energy (ΔG) of mRNA-miRNA interaction

The predicted consensual rubber tree miRNAs were evaluated by caculating the free energies of miRNA-target duplexes binding. The free energies (ΔG) of eleven consensual rubber tree miRNAs were predicted: hbr-miR159a (ΔG: -18.90 kcal/mol), hbr-miR396a (ΔG: -22.50 kcal/mol), hbr-miR396b (ΔG: -19.40 kcal/mol), hbr-miR398 (ΔG: -18.80 kcal/mol), hbr-miR408b (ΔG: -19.9.50 kcal/mol), hbr-miR482a (ΔG: -24.20 kcal/mol), hbr-miR6167 (ΔG: -21.10 kcal/mol), hbr-miR6169 (ΔG: -16.70 kcal/mol), hbr-miR6171 (ΔG: -19.20 kcal/mol), hbr-miR6483 (ΔG: -11.80 kcal/mol), hbr-miR6484 (ΔG: -17.50 kcal/mol) by the RNAcofold algorithm.

4. Discussion

We examined the efficiency of different computational algorithmic tools used here to evaluate the prediction of rubber tree miRNA target sites for the screening of false positive findings. In the RTV1 viral genome, the prediction of computational algorithms provides quick ways to predict potential host-derived miRNA target sites. At the individual, intersection, and union levels, we developed a way of analyzing rubber tree miRNA prediction findings. Using several prediction algorithms, default algorithm characteristic parameter were efficient for filtering out false-positive target binding sites of the miRNAs. Default algorithms indicate optimum identification for a miRNA to its appropriate viral genome target site. The miRanda algorithm was used to test a variety of factors, ranging from target site preservation to miRNA target prediction across the entire genome. Conservation level is the key feature of the miRanda algorithm [23]. While, the psRNATarget algorithm was utilized to predict miRNA target binding sites using unique plant-based features[18]. RNA22 algorithm was distinct from other algorithms due to its unique feature which is pattern based recognition of miRNA-mRNA interactions[15]. RNAhybrid was implemented for the calculation of minimum free energy (MFE) of rubber tree miRNAs[16]. Tapirhybrid algorithm was used for rubber tree miRNAs analysis [17].

The current study was designed on the basis of three algorithmic approaches to evaluate the prediction results. These are individual level, union level and intersection level of prediction. Union approach depends upon the combination of one or more prediction tools to predict the false positive results. This approach increases the sensitivity level of prediction as compare to the specificity of the results. Whereas, the intersection approach is entirely rely on the combination of algorithmic tools and enhances the specificity of the prediction results [24]. We have observed the best prediction results using both computational approaches **(Figure 3 and Figure 5)**. These miRNAs are selected and predicted as the most potent rubber tree miRNAs against the RTV1 genome. These predicted consensual miRNAs were concluded after setting the standard setting parameters of the algorithms: MFE, seed pairing, target site accessibility, minimum folding energy and pattern recognition. So integrating all major aspects of miRNA target prediction was considered during prediction. Therefore, they are considered the most effective selections

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for the silencing of the RTV1 genome. Rubber tree miRNA target binding sites in the RTV1 genome and miRNA-mRNA duplex was consensually identified by all five algorithms. In this study, RTV1 genome (ORF1 and ORF2) is vulnerable to targeting by eleven consensus rubber tree miRNAs (Figure 5). Out of eleven predicted consensual rubber tree miRNAs, hbr-miR398 was identified to target ORF1 by four computational algorithms such as miRanda, psRNATarget, RNA22, and RNAhybrid (Figure 5 and Table 1). We predicted the evaluation of the free energy of mRNA-miRNA duplex using RNAcofold. The free energy of miRNA-mRNA duplex binding is highly stable in this study. In the previous studies on host-virus interaction, in silico algorithms were used to study the potato virus Y (PVY) [24], and maize chlorotic mottle virus (MCMV)[25]. We have designed the similar studies against sugarcane viruses using computational algorithms to understand the host-virus interaction as well finding the best target in our previous research [26-28].

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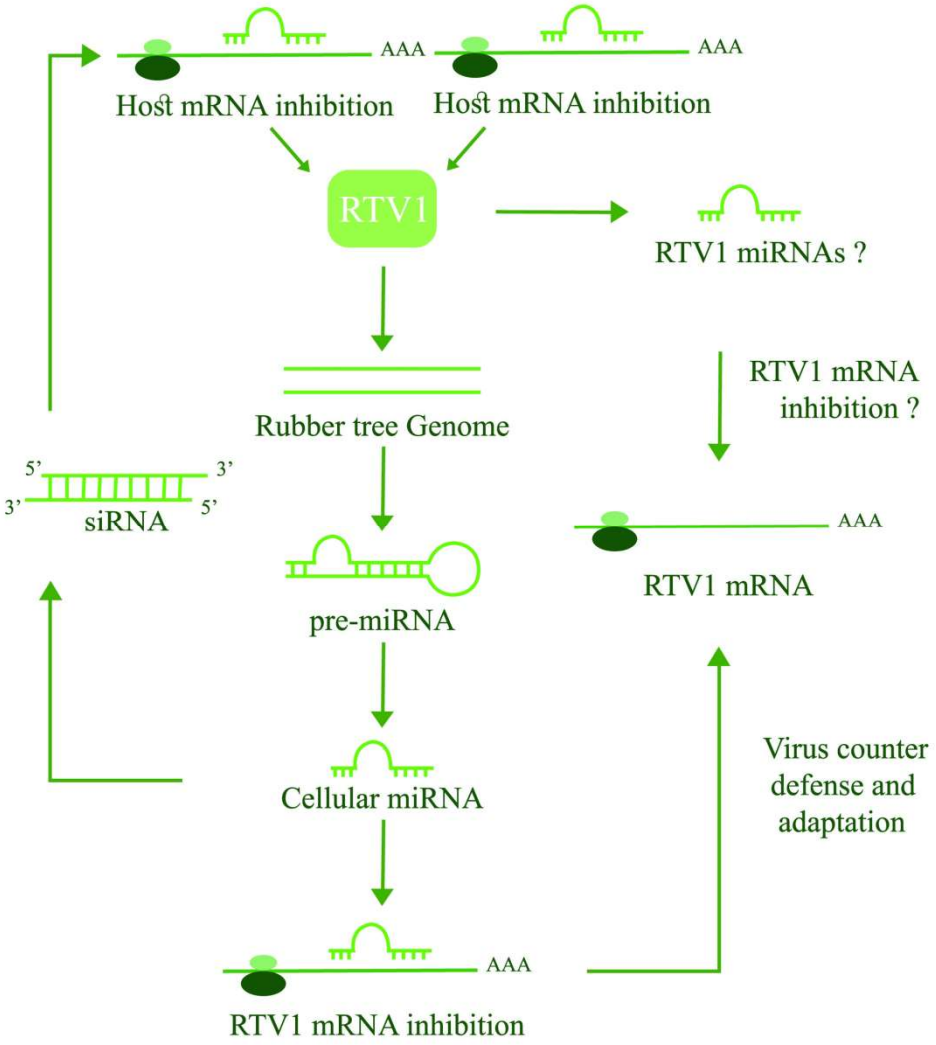

Figure 7: Schematic RNAi based mechanistic model design for miRNA-mediated gene silencing representing host-virus interaction.

We predicted that hbr-miR398 would be an excellent choice for targeting the RTV1 genome in this work. To establish RTV1 replication experimentally, it is critical to evaluate the function of predicted consensual rubber tree miRNAs for the detection of *Capillovirus* replication. Rubber tree-derived miRNAs were shown to suppress RTV1 mRNA and rubber tree genes against RTV1 in this schematic model (**Error! Reference source not**

found.7). Our computational study on RTV1 genome silencing might pave the way for a novel approach to antiviral effects of host-derived miRNAs against RTV1.

5. Conclusions and Recommendations

Since the application of RNAi based artificial miRNA constructs, many researchers have investigated the silencing of gene using host-encoded miRNAs against plant viruses. In this study, hbr-miR396a and hbr-miR398 were predicted as the most potent rubber tree miRNAs to target the RTV1 genome. Pathological studies are required to validate the rubber tree miRNA-RTV1 genomic interaction. The designed amiRNA constructs are highly suitable for the development of RTV1-resisitant transgenic rubber tree plants after rubber tree transformation in the future.

Supplementary Materials: The following supplementary data is provided: Table S1, Table S2, Table S3, Table S4 and File S1.

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