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In silico Analysis of miRNAs Targeting Sugarcane Mosaic Virus and Identification of Their Target Genes and Host Responses in Sugarcane (*Saccharum officinarum*)

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ABSTRACT

Sugarcane mosaic virus (SCMV) is recognized as one of the most destructive pathogens in sugarcane. In response to pathogen attacks on a host plant, the expression of miRNAs is modified, leading to changes in the expression of downstream target genes. This study aimed to bioinformatically evaluate the potential interactions between sugarcane-encoded miRNA sequences and the genome of the virus isolate, and to identify their target genes and pathways in response to SCMV. The *in-silico* analysis identified sof-miR159e as the common effective candidate, capable of targeting the HC-Pro in the SCMV genome and affecting 165 target genes in sugarcane. Singular enrichment analysis of the target genes with enrichment determined using FDR with a P-value cutoff of 0.05 revealed 25 significant Gene ontology (GO) terms in sugarcane. According to GO analysis, the pathways of biological process were related to growth and development, response to stimulus, organelle organization, reproductive-related processes, and cellular and metabolic processes. In addition, mitochondria, intracellular membrane-bounded organelles, and cytoplasmic and intracellular parts were identified as key cellular components. Furthermore, the molecular functions were primarily associated with catalytic and oxidoreductase activities. The top six enriched Kyoto encyclopedia of genes and genomes (KEGG) pathways included transcriptional regulation, plant hormone signal transduction, mitogen-activated protein kinase signaling pathway, plant-pathogen interaction, ethylene signaling, and biosynthesis of secondary metabolites, with the latter having the highest rich factor. These findings identified the key miRNA involved in the binding to the viral genome, the host target genes, and the associated pathways affected by sof-miR159e, which may provide valuable insights into the relationship between miRNAs and SCMV infection dynamics.

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Introduction

Plant viruses represent a serious challenge to global crop production and to sustainable agriculture. They significantly reduce both yield and the quality of agricultural products (Sharma, 2023). One of the important plant viruses affecting sugarcane is *Potyvirus sacchari*, the newly standardized binomial name for sugarcane mosaic virus (SCMV), as proposed by the International Committee on Taxonomy of Viruses (ICTV) in 2024. SCMV belongs to the genus *Potyvirus*, encompassing 90% of the species within the family *Potyviridae* (King *et al.*, 2011). SCMV infects several crops, including sugarcane,

maize, and sorghum, and is considered one of the most destructive pathogens causing sugarcane mosaic disease (Ricaud *et al*., 2012). SCMV leads to yield losses of over 50% reducing growth, sugar yield, juice quality, plant photosynthetic activity, sett germination, and tiller production in sugarcane (Vijai Singh *et al.*, 2003; Viswanathan and Balamuralikrishnan, 2005; Akhter *et al.*, 2018). The transmission of SCMV occurs via aphids in a non-persistent manner, characterized by flexible filamentous particles that are 700-750 nm long. These particles consist of a single-stranded RNA with positive polarity, a 5' VPg, and a polyadenylated tail at the 3' terminus (King *et al.*, 2011). The genomic RNA of SCMV is characterized by a single open reading frame (ORF) that encodes a large polyprotein. This polyprotein is autocatalytically cleaved into the following functional proteins: P1, HC-Pro, P3, P3N-PIPO, 6K1, CI, 6K2, VPg, NIa-Pro, Nlb, and CP (King *et al.*, 2011; Urcuqui-Inchima *et al.*, 2001). In Iran, SCMV has spread to most regions where sugarcane, sorghum, and maize are cultivated (Izadpanah and Kamran, 1995; Mohammadi *et al.*, 2006). The emergence of new strains, genomic variations, and recombinant isolates of SCMV has been reported across various countries (Gao *et al.*, 2011; Padhi and Ramu, 2011; Perera *et al.*, 2009; Wu *et al*., 2012), highlighting the potential threat of SCMV to the global cultivation and agricultural industry of host crops. Recently, the complete genomic sequences of the SCMV isolate, NRA, were reported from sugarcane in Iran (Moradi *et al*., 2016). On the contrary, plants have evolved sophisticated defense mechanisms to resist pathogen attacks. RNA silencing is a robust sequence-specific, RNA degradation process-based antiviral defense mechanism in plants (Lozano-Durán, 2023). It is induced by small RNAs like microRNAs (miRNAs), which play vital roles in plant post-transcriptional gene regulation (Liu *et al.*, 2017). Viral infections have the potential to activate RNA silencing mechanisms within the host plant (Huang *et al.*, 2016). Plant virus infections can induce and regulate miRNA expression, affecting defense responses (Wang *et al.*, 2016; Zhou *et al.*, 2016). The regulation of the innate immune response in plants is significantly influenced by miRNAs. They can either trigger or inhibit the transcription of particular genes that are crucial for the plant's defense against pathogens (Luo *et al.*, 2024). MiRNAs with 20 to 24 nucleotides in length, derived from imperfectly paired hairpin precursor RNAs through Dicer-like (DCL) proteins, regulate gene expression through either translational inhibition or the cleavage of complementary mRNAs (Axtell, 2013; Pérez-Quintero *et al.*, 2010). It has been reported that miRNA-mediated gene silencing plays a pivotal role in immune defense and plant resistance to viruses (Liu *et al.*, 2017; Wang and Galili, 2019). MiRNAs-mediated RNA silencing has been applied to a broad spectrum of plant species to defend against viral infections (Ali *et al.*, 2013; Petchthai *et al.*, 2018). There is growing evidence indicating that miRNAs play a role in antiviral defense mechanisms. As a result, the study of the identification of novel miRNA and miRNA targets could provide new perspectives on the sophisticated interactions between viruses and host plants. The interaction between miRNA and mRNA provides valuable insights into the molecular mechanisms of gene regulation (Qiu *et al.*, 2022), elucidating their potential regulatory and biological functions. However, there is insufficient information regarding the interaction between host plant miRNAs and the SCMV isolate NRA genome. Here, potential targets and binding sites of miRNAs were studied within the SCMV genome, which could serve as a resistance source against SCMV in sugarcane. The miRNA-based RNA silencing strategy could develop SCMV-resistant plants. Artificial miRNA-mediated (amiRNA) technology provides a powerful and innovative biotechnological approach to effectively combat viral infections in plants (Kuo and Falk, 2022). The amiRNAs expressed in transgenic plants have been effectively utilized to confer tolerance (Mitter *et al.*, 2016). Computational methods have been extensively utilized to identify plant miRNAs and their targets (Li *et al.*, 2010). Therefore, we applied computational approaches to identify novel miRNAs, predict their targets within the SCMV genome, and elucidate the responses and pathways in sugarcane. Prediction of novel miRNAs and identification of their potential targets in the SCMV genome could serve as a resistance source in sugarcane cultivars against SCMV. Therefore, this study aimed to identify sugarcane-derived miRNAs, their potential

targets in the SCMV genome, and host target genes and pathways that might enable the development of an amiRNA antiviral strategy for generating SCMV-resistant sugarcane plants.

Materials and Methods

In silico prediction of sugarcane miRNAs-SCMV genome targets

The complete genome of SCMV isolate NRA (GenBank accession no. KT895080.1) was acquired from the NCBI GenBank database, previously isolated from infected sugarcane in Mazandaran province, Iran (Moradi *et al.*, 2016). The Iranian SCMV-NRA isolate was selected for this study based on the availability of its complete genomic sequence. Sixteen miRNA sequences of sugarcane were retrieved from miRBase (http://www.mirbase.org/). The potential miRNA sequences targeting the SCMV genome were evaluated using RNAhybrid software, version 2.2 (Rehmsmeier *et al.*, 2004). RNAhybrid functions by utilizing intermolecular hybridization to identify the effective binding sites of miRNAs in the target sequence. The parameters used in the hybridization are as follows: hits per target, 1; and the minimum free energy, -22 kcal/mol.

To further validate the predicted miRNAs targeting SCMV, the psRNATarget software (Dai *et al.*, 2011) was employed, which assesses miRNA-mRNA complementarity using a predefined scoring schema (V2, 2017 release). The analysis parameters were configured as follows: top targets: 200; expectation score: 5; mismatch penalties: 0.5 for G:U pairs, 1 for other mismatches; seed region settings: extra weight (1.5), position (2-13 NT), allowed mismatches (2); HSP size: 19; gap penalties: opening (2), extension (0.5); and translation inhibition range: 10-11 NT. Additionally, the inhibition type (cleavage or translation) mediated by sof-miR159e in viral RNA genome regulation was assessed. For miRNA target site prediction, the RNA22 web server (Miranda *et al.*, 2006; http://cm.jefferson.edu/rna22v1.0/) was used, employing a pattern-based approach combined with folding energy calculations—without cross-species conservation filtering. Key parameters included: specificity: 61%; sensitivity: 63%; seed region: 7 NT, permitting one unpaired base G:U wobbles: no restriction in seed region; and paired bases: minimum of 12, maximum folding energy of -12 kcal/mol. Finally, the Tapirhybrid web server (Bonnet *et al.*, 2010; http://bioinformatics.psb.ugent.be/webtools/tapi) was utilized for plant miRNA target prediction, with standardized parameters: score threshold: ≤ 8; MFE ratio: ≥ 0.4.

Thermodynamic stability

Thermodynamic analysis of miRNA-mRNA interactions provides crucial insights into hybridization stability (Riolo *et al.*, 2020). Most miRNA target prediction methods assess these interactions by calculating the Gibbs free energy (ΔG) of the miRNA-mRNA complex. To predict the interaction ΔG between miRNA and mRNA duplexes, we used RNAcofold (Bernhart *et al.*, 2006; http://rna.tbi.univie.ac.at/cgi-bin/RNAWebSuite/RNAcofold.cgi). The tool was applied to evaluate the binding between sof-miR159e and its predicted target region in the SCMV genome, as identified by psRNATarget. The analysis was performed using default RNAcofold parameters (Mathews *et al.*, 2004) in RNAcofold 2.6.3.

Prediction of secondary structure of sof-miR159e

Secondary structure prediction of the pre-miRNA (sof-miR159e) was performed using RNAfold v2.6.3 (Lorenz *et al.*, 2011). The analysis included calculation of the thermodynamic ensemble free energy, determination of the minimum free energy (MFE) of the centroid secondary structure, estimation of the MFE structure frequency, ensemble diversity, and positional entropy. The MFE structure, thermodynamic ensemble, and centroid structure of sof-miR159e were represented. Energy parameters were adjusted for temperature (37°C) and salt concentration (1.021 M), following established RNA thermodynamics (Mathews *et al.*, 2004).

Sequence alignment of the mature miR159e in various plant species

The sequences of mature miR159e were aligned in seven plant species: *Oryza sativa*, *Malus domestica*, *Picea abies*, *Saccharum officinarum*, *Populus trichocarpa*, *Zea mays*, and *Glycine max*.

Sequence alignment was constructed using CLC Genomics Workbench 5.5.2. software.

Target gene prediction in sugarcane plants

Identification of sugarcane miRNA target genes was performed using psRNATarget, with the *S. officinarum* DFCI Gene Index (SOGI v3, 2010-04-09) serving as the reference genome. The analysis parameters were configured as follows: top targets: 200; expectation score: 5; mismatch penalties: 0.5 (G:U pairs), 1 (other mismatches); seed region settings: extra weight (1.5), position (2-13 NT), allowed mismatches (2); HSP size: 19; gap penalties: opening (2), extension (0.5); and translation inhibition range: 10-11 NT.

Gene ontology and functional analysis of the miRNA target genes

In order to determine the biological functions of miRNA-targeted genes, gene ontology (GO) enrichment analysis was conducted to identify biological processes, cellular components, and molecular functions. Singular enrichment analysis (SEA) was conducted using agriGO version 2 (https://systemsbiology.cau.edu.cn/agriGOv2/). Sugarcane locus ID (DFIC) was selected as a reference genome. The enrichment of GO terms was identified with FDR and a cut-off P-value of 0.05.

Kyoto encyclopedia of genes and genomes pathway enrichment analysis

Pathway analysis of the Kyoto encyclopedia of genes and genomes (KEGG) was used to elucidate significantly enriched pathways of the target genes. We used the clusterProfiler package in R version 4.2.2 to identify significantly enriched KEGG pathways in sugarcane. This package is widely used for functional enrichment analysis and provides robust tools for interpreting high-throughput genomic data. The results of the KEGG enrichment analysis were visualized using the ggplot2 package.

Results and Discussion

Identification of sugarcane miRNAs and their targets in the SCMV genome

The cultivation of resistant host plants has been suggested as an effective method for controlling SCMV infections (Wu *et al.*, 2012). In this regard, plant miRNAs demonstrate a superior ability to target plant viruses, highlighting their important functions in the defense strategies of plants against viral pathogens (Pérez-Quintero *et al.*, 2010). miRNAs are crucial in modulating the plant's innate immune system by either activating or inhibiting the transcription of specific genes that contribute to the plant's defense against pathogens. During pathogen invasion, miRNAs fine-tune plant immunity by dynamically adjusting hormone signaling pathways-including salicylic acid (SA), jasmonic acid (JA), and ethylene (ET)-to activate defense responses (Luo *et al.*, 2024). miRNAs can also regulate host pathways to increase or decrease the transcription of targeted genes (Bouvet *et al.*, 2021; Zhang *et al.*, 2021). Therefore, in this study, we present a bioinformatics analysis to explore the possibility of sugarcane miRNAs having a role in targeting the SCMV genome and evaluate their target genes and host responses in sugarcane. Experimental approaches for identifying miRNA-mRNA interactions can be costly and time-consuming, which emphasizes the urgent need for effective computational methods to accurately predict miRNA targets (Chipman and Pasquinelli, 2019). Bioinformatic analysis using psRNATarget and Tapirhybrid identified sof-miR159e as the only sugarcane microRNA that binds to the SCMV genome, specifically targeting the *HC-Pro* region at position 1562 (Table 1).

PsRNATarget analysis revealed that 14 out of 21 nucleotides of sof-miR159e (66.67% complementary) could bind to the viral genome (Table 1). It has been shown that the psRNATarget software offers enhanced accuracy in binding predictions (Amirnia *et al.*, 2016). RNAhybrid analysis showed that all 16 miRNAs could bind to the SCMV genome (Supplement 1 and 2). The RNA22 algorithm identified 14 miRNAs capable of binding 1-3 target regions (33 binding sites) within the viral genome (Supplement 1 and 3). Sof-miR159e was the common miRNA predicted by the multiple algorithms (Tapirhybrid, RNA22, RNAhybrid, and psRNATarget) used in this study. Based on these findings, we selected sof-miR159e for further analysis of target genes and pathways in sugarcane. In another study led by Wenzhi *et al.* (2024), only one sof-miRNA (sof-miR159c) was identified as the most effective candidate for targeting the nucleotide position 3847 of the

cylindrical inclusion (*CI*) ORF in the SCMV genome.

Table 1. Sof-miR159e target (SCMV isolate NRA, GenBank accession no. KT895080.1) prediction using tapirhybrid and psRNATarget algorithms

Tapirhybrid		**PsRNATarget**	
Score	6.5	Expect	5.0
mfe_ratio	0.41	UPE	N/A
Start	1562	Inhibition	Cleavage
Seed_gu	1	Multiplicity	1
Mismatch	5	miRNA 21-1	UUCUCGAGGAAAGUUAGGUUU
gu	1	Target 1562-1582	CGUAACACGUUUUAAUCCAAA
miRNA_3'	UUCUCGAGGAAAGUUAGGUUU	-	-
Target_5'	CGUAACACGUUUUAAUCCAAA	-	-

The score denotes the global score value. The parameters mfe_ratio, start, seed_gu, mismatch, and gu represent the free energy ratio of the miRNA:mRNA duplex, the starting position of the alignment on the mRNA sequence, the count of G:U pairs in the seed region, the number of mismatches outside the seed region, and the count of G:U pairs outside the seed region, respectively. The expectation value (expect) serves as the penalty for mismatches between the mature small RNA and the target sequence. A higher value signifies a lower degree of similarity (and likelihood) between the small RNA and the target candidate. UPE indicates the maximum energy required to unpair the target site, reflecting the target accessibility. The accessibility of the mRNA target site to small RNA is regarded as a crucial factor in target recognition. Inhibition refers to the silencing mechanism of the mRNA targets of miRNA, which occurs through either the cleavage of mRNA or translational repression. Multiplicity indicates the number of target sites associated with each small RNA/target pair.

They also identified the target binding site for sof-miR159e located at nucleotide position 5535 within the sugarcane bacilliform virus (SCBV) genome (Wenzhi *et al.*, 2024). The study led by Ashraf *et al.* (2022) highlighted the sof-miR159e as the top effective candidate against SCBV. The computational analysis revealed that miR159e could target the ORF3 region of the SCBV genome that encodes the largest polyprotein (Ashraf *et al.*, 2022). In our study, sof-miR159e could target the coding regions (nucleotide positions 1562-1582; helper component proteinase region; HC-Pro) in the SCMV genome (Supplement 4). The function of potyviral HC-Pro in suppressing post-transcriptional gene silencing and interfering with miRNA functions has been documented (Ivanov *et al.*, 2016; Kasschau *et al.*, 2003). Additionally, it has been reported that HC-Pro encoded by SCMV could suppress the RNA silencing and regulate the accumulation of different siRNAs (Zhang *et al.*, 2008). It appears that sof-miR159e could interfere with SCMV HC-Pro function, which requires further investigation. It has been revealed that the accumulation of miR159 family was negatively regulated by SCMV infection in maize (Xia *et al.*, 2018). However, rice black streaked dwarf virus infection up-regulated miR159 in rice leaves (Sun *et al.*, 2015). Additionally, the accumulation of phased siRNAs derived from miR159 precursors in rice was enhanced by rice stripe virus infection (Du *et al.*, 2011). These findings suggest that miR159 targets a broad spectrum of viral genomes, implying potential coevolution between plants and viruses, as well as a certain level of conservation across various viral strains (Hajieghrari *et al.*, 2019). Considering the essential role of HC-Pro in inhibiting gene silencing, its potential regulation by miRNAs may strengthen the miRNA-mediated defense mechanisms in host plants. The analysis of potential binding of miR159e sequences from six plant species to the SCMV genome showed that none of the miR159e sequences could bind to the SCMV genome. Nucleotide sequence alignments showed that the sof-miR159e sequences targeting SCMV are not conserved (Supplement 5). Analysis of the selected miRNA sequences from non-host plants for binding to the SCMV genome showed that sugarcane has evolved miRNA-based defense mechanisms against the virus. This study demonstrates that the binding sites of sof-miR159e in the SCMV genome are specific to either the miRNA type (plant species-dependent) or the particular virus isolate, highlighting the sophisticated interaction between host miRNAs and viral genomes.

Thermodynamic characteristics of miRNA-mRNA complexes

The miRNA-mRNA complex exhibits considerable thermodynamic stability. A lower

(more negative) free energy (ΔG) enhances the regulatory effect of miRNA on the target mRNA (Bernhart *et al.,* 2006). The free energy of the thermodynamic ensemble was -4.44 kcal/mol, with an MFE structure frequency of 18.45% and a heterodimer binding ΔG of -3.61 kcal/mol (Table 2). miRNA-mRNA binding is typically considered stable when ΔG is more negative in the range of -15 to -30 kcal/mol for strong interactions (reflecting high-affinity binding) (Bergman *et al.,* 2020). However, even weaker interactions can still be biologically relevant, particularly in miRNA target recognition, where partial complementarity often plays a role. However, experimental validation remains essential to confirm the functional results of such interactions in viral repression.

Table 2. The thermodynamic characteristics of the miRNA-mRNA complex and the secondary structure of pre-miRNA (sof-miR159e) from the precursors of the mature miRNA

Minimum free energy prediction	miRNA-mRNA complex	Secondary structure
The free energy of the thermodynamic ensemble	-4.44 kcal/mol	-112.00 kcal/mol
The frequency of the MFE structure in the ensemble	18.45 %	0.07 %
Delta G for heterodimer binding	-3.61 kcal/mol	
The ensemble diversity	-	28.51
The minimum free energy of the centroid secondary structure	-	102.80 kcal/mol

We evaluated the stability of miRNA-mRNA duplexes through free energy calculations, a critical determinant of binding site accessibility and secondary structure formation (Peterson *et al.,* 2014). Our validation approach incorporated minimum free energy (MFE) analysis as a primary metric for assessing interaction viability (Pinzón *et al.,* 2017), with RNAhybrid analysis employing a stringent MFE threshold of -20 kcal/mol. MFE analysis confirmed structural stability (Supplement 6; Lorenz *et al.,* 2011). Thermodynamic ensemble free energy measured -112.00 kcal/mol (Table 2), indicating exceptional duplex stability. Key parameters calculated for sof-miR159e precursor: MFE structure frequency: 0.07% in ensemble, ensemble diversity: 28.51, centroid structure MFE: 102.80 kcal/mol. ΔG serves as an indicator of a biological system's stability. When the binding between a miRNA and a potential target mRNA is predicted to be stable, this suggests a higher likelihood of it being a genuine miRNA target. Since directly measuring free energy is challenging, the change in ΔG during a reaction is typically analyzed. Reactions with a negative ΔG release energy, leading to greater system stability because less energy remains available for further reactions. By modeling the hybridization of a miRNA and its candidate target, regions with varying free energy levels can be identified, and the overall ΔG provides insight into the binding strength between them (Yue *et al.,* 2009). The binding pattern and the MFE serve as effective indicators of miRNA-target interaction with its target gene, as well as the stability of the hybrids (Dandare *et al.,* 2021). More negative MFE values correlated with enhanced binding stability in human miRNA interactions with *Flavivirus* 3′ UTRs (Avila-Bonilla and Salas-Benito, 2024), highlighting the stability of the miRNA-mRNA duplex, with lower values showing a more stable and thus stronger interaction.

Identification of target genes and pathways in sugarcane plants

The potential binding of sugarcane sof-miR159e with sugarcane genes and determination of host pathways were evaluated. Sof-miR159e affected 165 sugarcane target genes and regulated multiple genes and cell phenotypes. The mechanism of repression (RNA degradation or translation repression) associated with the miRNA demonstrated that gene expression in sugarcane is primarily (87%) regulated by the degradation of the viral genome (Supplement 7). The SEA identified 25 significant GO terms for sof-miR159e targets. Enriched biological processes included growth and development, response to stimulus, organelle organization, reproductive-related processes, and cellular and metabolic processes (Fig. 1).

The suppression of miR159 in *Arabidopsis*, tobacco, and rice leads to a range of pleiotropic abnormalities, notably including reduced growth (Zheng *et al.,* 2020). In addition, mitochondria, intracellular membrane-bounded organelles, and

cytoplasmic and intracellular parts were identified as key cellular components (Fig. 2).

Mitochondria activate immune responses through the oxidative burst of reactive oxygen and nitrogen species, triggering programmed cell death. Mitochondria also play a role in SA-mediated resistance and contribute to phytohormone interplay (Wang *et al.*, 2022). The role of mitochondria-related genes in plant defense has been revealed against viruses (Shahriari *et al.*, 2025). Furthermore, the molecular function was primarily associated with catalytic and oxidoreductase activities (Fig. 3).

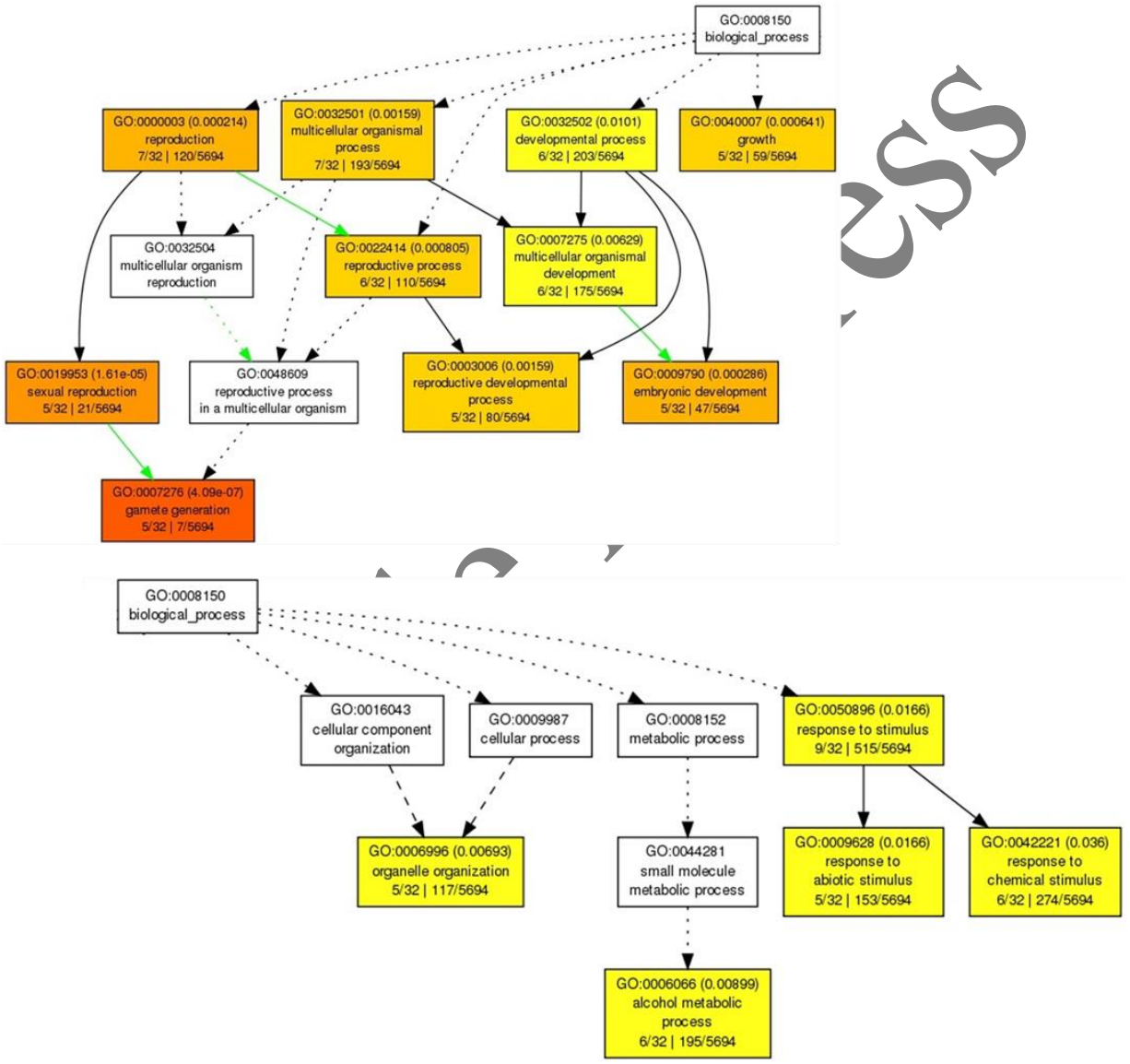

Fig. 1. GO biological process enrichment analysis of the target genes was performed using agriGO. Each box represents a GO term, displaying its identifier, p-value (in parentheses), and the term description. Two sets of numerical values accompany each term: the first indicates the number of genes associated with the term in the dataset versus the total genes in the dataset, while the second shows the database-wide count of genes linked to the term versus all GO-annotated sugarcane genes in the reference database. Statistical significance is color-coded: yellow = 0.05, orange = 1e-05, and red = 1e-09, with white boxes indicating non-significant results. The intensity of the color reflects the strength of term enrichment. Connecting lines represent enrichment relationships, where solid lines link two enriched terms, dashed lines link one, and dotted lines indicate no enriched terms.

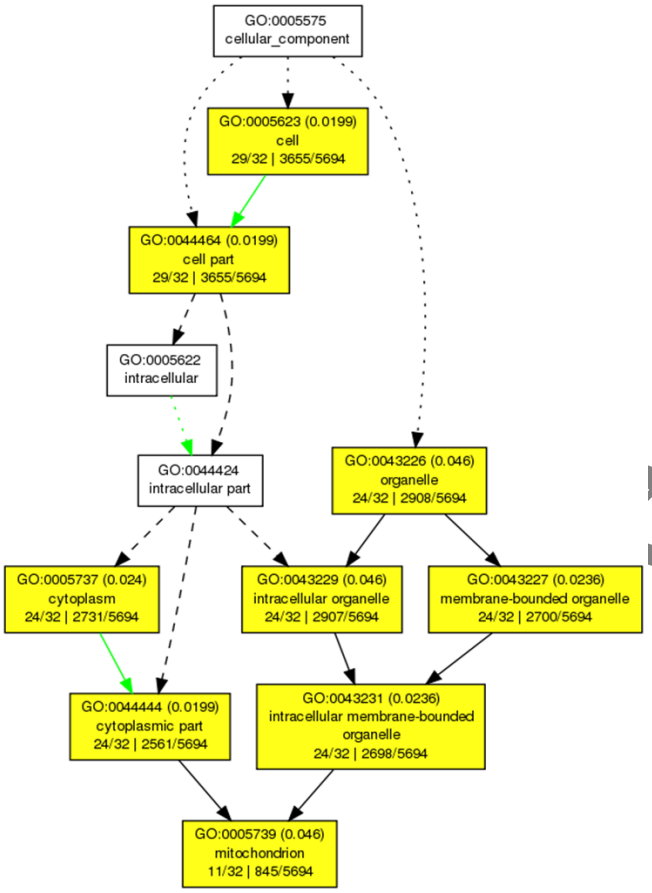

Fig 2. GO cellular component enrichment analysis of the target genes was conducted using agriGO. Each box includes the GO term identifier, its p-value (in parentheses), and the term description. Two gene count ratios are provided for each term: the first represents the number of genes associated with the term in the dataset versus the total dataset genes, while the second indicates the database-wide number of genes linked to the term versus all GO-annotated sugarcane genes in the reference database. Statistical significance is color-coded as follows: yellow ($p \leq 0.05$) with white boxes showing non-significant results. Connecting lines illustrate enrichment relationships, where solid lines indicate two enriched terms, dashed lines indicate one enriched term, and dotted lines indicate no enriched terms.

Findings showed that Toll/interleukin-1 receptor-catalyzed 2'-(5''-phosphoribosyl)-5'-adenosine monophosphate and diphosphate act as a missing link in Toll/interleukin-1 receptor signaling. These molecules likely act as second messengers via Enhanced Disease Susceptibility 1-Phytoalexin Deficient 4 in plant immunity (Huang *et al.*, 2022). It has been shown that oxidoreductase enzymes are involved in plant defense mechanisms through ROS accumulation (Das and Sen, 2024). KEGG pathway enrichment analyses were conducted on the target genes with p-values lower than 0.05. Analysis of target genes ($p< 0.05$) revealed 12 enriched metabolic pathways, including the top six KEGG pathways: transcriptional regulation, plant hormone signal transduction, mitogen-activated protein kinase (MAPK) signaling pathway, plant-pathogen interaction, ET signaling, and biosynthesis of secondary metabolites, with the highest rich factor (Fig. 4).

In this study, it appears that sof-miR159e modulates transcriptional regulation, plant hormone signal transduction, MAPK signaling pathway, plant-pathogen interaction, ET signaling, and biosynthesis of secondary metabolites that could contribute to defense responses against SCMV. The function of plant hormones, including JA, SA, and ET, has been extensively documented in plant defense against viral infections (Zhao *et al.*, 2021). ET accumulation is linked to defense responses in

infected plants, and exogenous application of ET compounds improved plant resistance to viruses (Alazem *et al.*, 2015; Zhao *et al.*, 2021).

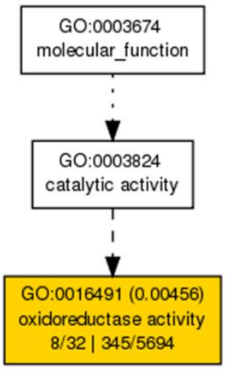

Fig 3. GO molecular function enrichment analysis of the target genes was performed using agriGO. Each box displays the GO term identifier and the corresponding functional term. Two gene ratios are provided for each term: dataset-specific ratio: number of genes associated with the term versus total genes in the dataset. database-wide ratio: number of genes linked to the term versus all GO-annotated sugarcane genes in the reference database. Statistical significance is indicated by color, with yellow ($p \leq 0.05$) marking significant terms and white boxes denoting non-significant results. Dashed lines connect single enriched terms, while dotted lines indicate no enriched terms.

The ET pathway is also involved in MYB4L-mediated resistance against tobacco mosaic virus in *Nicotiana benthamiana* (Zhu *et al.*, 2022). Another enriched KEGG pathway was the biosynthesis of secondary metabolites. Secondary metabolites are directly involved in pathogen defense. Plant secondary metabolites induce systemic acquired resistance. As an example, reticine A induced a hypersensitive reaction and elevated the accumulation of H_2O_2, SA, and PR proteins in tobacco against tobacco mosaic virus (Kumar *et al.*, 2023). It has also been revealed that most secondary metabolites, including alkaloids, flavonoids, and phenolics, demonstrate antiviral activities (Reichling, 2010). In our study, the MAPK signaling pathway was another KEGG pathway.

MAPK pathways play a key role in antiviral defense. For instance, they contribute to resistance against potato virus Y. In this case, MAPK-mediated immunity is activated via *Nicotiana benthamiana* phospholipase Dα1 and its derived phosphatidic acid that bind to WIPK/SIPK/NTF4 and induce phosphorylation of WRKY8, resulting in the activation of defense-related genes (Lin *et al.*, 2024).

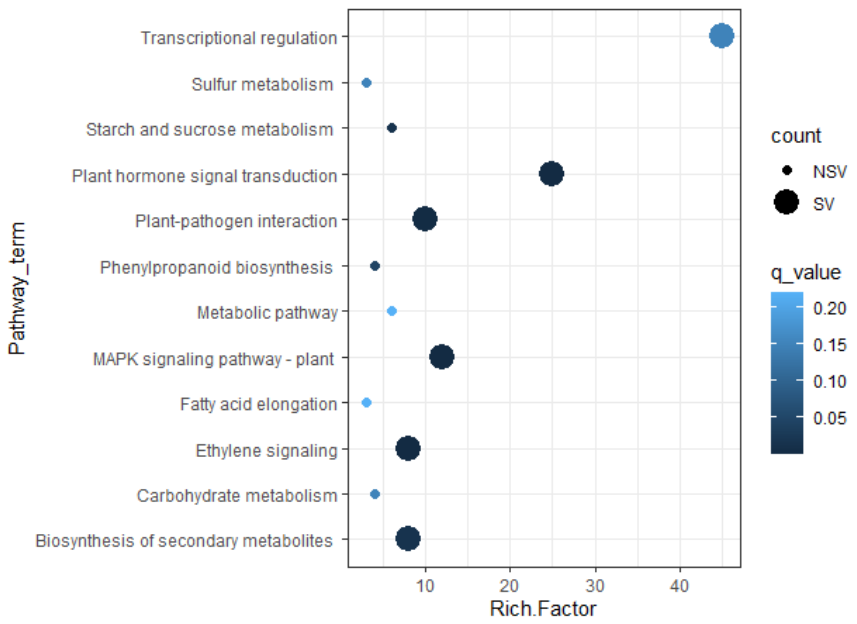

Fig 4. KEGG pathway enrichment analysis of sugarcane target genes regulated by sof-miR159e. The rich factor indicates the ratio of target genes to all genes within a given pathway. Color gradients correspond to varying P-value significance levels, as shown in the legend.

Conclusions

These findings identified the key miRNA that binds to the viral genome, regulates host target genes, and alters associated pathways-all of which may significantly influence the plant's response to SCMV. Identifying additional miRNA-target modules will elevate miRNAs to prominence in future resistance breeding strategies. These findings demonstrate how miRNAs regulate SCMV infection and provide a molecular basis for understanding host defense responses. The amiRNA approach enhances current molecular techniques by providing target-specific control of SCMV disease. Further studies should validate *in silico* predictions experimentally, elucidate miRNA regulatory networks, quantify miRNA accumulation, analyze transient miRNA expression, and optimize sugarcane transformation. Genome editing advancements enable the genetic engineering of miRNAs for pathogen-resistant crops. These miRNAs may enable the development of amiRNA constructs that enhance sugarcane's SCMV resistance.

Conflict of interest

The authors have no conflicts of interest to declare.

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