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IN SILICO PREDICTION OF POTENTIAL miRNAs TO TARGET ZYMV IN *CUCUMIS MELO*

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Abstract

Zucchini yellow mosaic virus (ZYMV) is the most important economically infected virus of the Cucurbitaceae family, as rigorous yield losses of up to 100% occur. Due to ZYMV the reduction occurs in the weight of fruit production and deficiency of shelf life of infected fruits. Infected plants are dwarf and have yellow mosaic-like symptoms. ZYMV is transmitted through infected seeds or by different aphid species through plant sap. For the prediction of melon-derived miRNA targets in the ZYMV genome, computational approaches were applied because miRNAs cooperate in the regulation of different cellular mechanisms. Target genes are negatively controlled by miRNA through post-transcriptionally, mRNA cleavage and translational inhibition. Different algorithms (miRanda, RNA22, psRNAtarget and tapirhybrid) were used for plant miRNA target prediction. Overall 120 mature miRNAs from mirBase were tested for ZYMV genome hybridization. Out of 120 miRNAs only 4 *Cucumis melo* miRNAs (cme-MIR396b, cme-MIR396c, cme-MIR396d (2) and cme-MIR319d) were predicted for ZYMV genome silencing. The genes of ZYMV those were targeted by these four miRNAs are PI, HC-Pro, P3, 6K1, CIP, Nia-VPg, Nia-Pro, NIB, and CP. The predicted miRNAs may be used to develop resistant ZYMV transgenic melon plants.

Key words: Chlorosis, Leaf twisting, Yield loss, GenBank, Algorithms, Zucchini yellow mosaic virus.

Introduction

Zucchini yellow mosaic virus (ZYMV) causes a severe disease in the cucurbit crops. The side effects of the infection are characteristically chlorosis, leaf twisting, disfigurement and development of projection on the upper surface of the leaf. This virus shows nearly 100% loss of the yield due to ZYMV (Malik *et al*., 2006) and is automatically transferred from infected plant to melon plant seedlings having yellow patches (Zouba *et al.*, 1997). ZYMV is also known to be transmitted by few species of aphids (Komata *et al*., 2014).

ZYMV genome is a single-stranded positive sense RNA and has 9000 nucleotides (Desbiez *et al*., 1997). It is a member of potyviridae family, causes enormous damage and decrease production of melon plant. ZYMV is thread like, 680-730 nm in length and 11-13 nm in diameter. In ZYMV genome, 12 strains of different regions with complete sequences were collected from GenBank.

MicroRNA (miRNA) is single stranded, short and 21-23 nucleotides long (ssRNA) molecules. These miRNAs are made later while proceeding of precursors miRNAs (pre-miRNAs) by enzyme (Dicer) RNase III due to presence of hair-pin loop like structure of pre-miRNAs and show negative regulation in gene expression (Brodersen *et al.,* 2008). The small RNAs (smRNAs) are non-coding, 18-30 nucleotides long and perform function in regulation (Rhoades *et al*., 2002). The smRNAs identify mRNA of virus by coordinating effectors (AGO) Argonaut protein building through base-pairing interactions with nucleic acid that generally prompts the blockage of gene expression (Lu *et al*., 2005). In host plant, RNA silencing via miRNAs used that give protection, defense and resistance to host plant from viruses (Iqbal *et al*., 2016). In genome of *Cucumis melo*, 120 mature miRNAs were obtained and the sequences of these mature miRNAs were retrieved from (http://www.mirbase.org/cgi-bin/browse.pl) mirBase and were analyzed against the genome of ZYMV by the use of four different computational tools (miRanda, psRNAtarget, RNA22 and Tapirhybrid).

The objective of present study is to apply the computational approach for the recognition of target of melon retrieved miRNAs in ZYMV genome for improving the resistance of *C. melo* to ZYMV through RNAi. The main purpose of this work was to find the *C. melo* derived miRNAs having potential attachment sites in the ZYMV genome using computational approaches of target prediction (miRanda, RNA22, psRNAtarget and tapirhybrid).

Materials and Methods

Data source: The sequences of 120 mature miRNAs of *Cucumis melo* were retrieved from mirBase and complete sequence of ZYMV genome with accession number JN183062.1 was accessed from NCBI (http://ncbi.nlm.nih.gov).

ZYMV genome annotation and sequence analysis: The organization of the ZYMV genome, annotation and nucleotide distribution were obtained by the use of CLC genomics workbench (v.11.0) (Fig. 1). Figure 2 demonstrates a definite workflow supported to find out the miRNA regions within ZYMV genome.

Fig. 1. Figure showed genome annotation of Zucchini yellow mosaic virus. The whole genome translated into a single polyprotein. Polypeptides indicated by yellow colour.

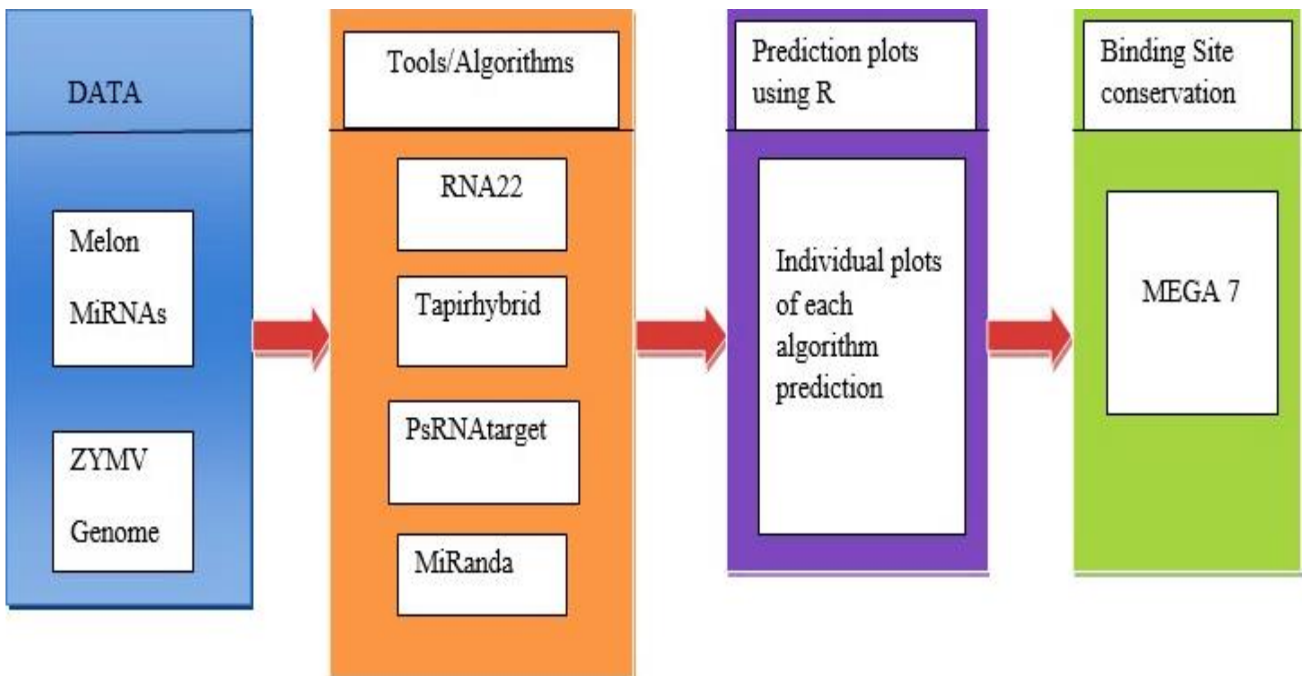

Fig. 2. Flow chart of miRNA target prediction tools in ZYMV genome pipeline.

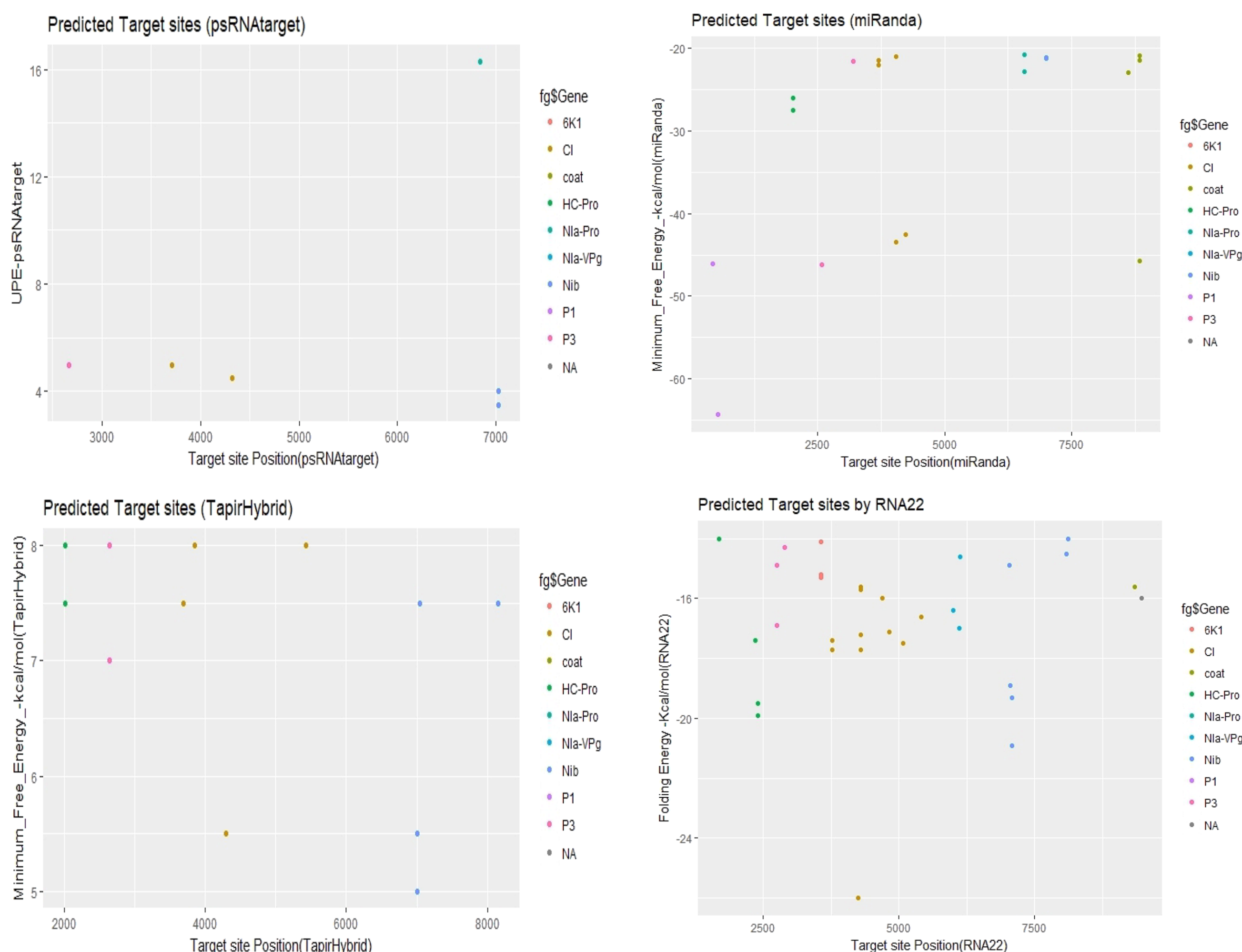

Fig. 3. miRNA target prediction result from Zucchini yellow mosaic virus; (a) indicated target prediction from miRanda (b) target prediction from psRNA target (c) target prediction from RNA22 (d) target prediction from tapirhybrid.

Target prediction of miRNA in ZYMV genome: For the detection of miRNA target areas against the ZYMV genome, four different algorithms (miRanda, Tapirhybrid, psRNAtarget and RNA22) were used. The arrangement of *C. melo* miRNAs and complete sequence of ZYMV genome were treated by the use of desired parameters in these four tools (Fig. 3).

miRanda: To predict the melon miRNA target, miRanda tool was used (John *et al*., 2004). Properties of miRanda are sum of match and mismatch scores for base pairs, gap penalties and free energy of RNA-RNA duplex (Iqbal *et al*., 2017). It additionally interact the miRNA target prediction regions incorporating with incomplete binding in the area of seed inside the 3´UTR of target site in this manner improving specificity (Betel *et al*., 2008; Witkos *et al*., 2011).

Tapirhybrid: Tapirhybrid tool is used for the prediction of plant miRNAs and can be downloaded from http://bioinformatics.psb.ugent.be/webtools/tapir (Bonnet *et al*., 2010). It is mostly recommended for the prediction of plant miRNA and it predicts with better specificity while distinguished to other new tools. Tapirhybrid parameters are target site accessibility, multiple target sites and seed pairing. The G:U pairs number, matches in the seed region, gaps and number of mismatches were taken to produce miRNA target scores. However this tool is used to increase the efficiency of results (Iqbal *et al*., 2017).

RNA22: RNA22 is used for determination of miRNA binding area and their comparable miRNA: mRNA structures. It may be utilized for the identification of any genome without including genome definite retraining and it has greater affectability. It is additionally unmistakable from prior revealed strategies in that it keeps the use of cross-species arrangement protection filter therefore permitting the identification of miRNA binding regions that may not be available in firmly related species (Miranda *et al*., 2006). Input parameters use in RNA22 were sensitivity 63%, specificity 61%, seed size 7,the minimum number of paired-up bases 12 and maximum folding energy -14 kcal/mol and it can be accessed from web https://cm.jefferson.edu/rna22/

psRNA target: psRNA target has two major functions i) Invert associated coordinating between miRNA and target original copy utilizing a demonstrated scoring pattern ii) target-region availability understanding by (UPE) computing unpaired energy essential to 'open' secondary arrangement in the region of miRNA target on mRNA (Dai *et al*., 2011). Software specification settings for psRNA target are the number of top targets 20,

expectation 5, penalty of G:U pairs 0.5, a penalty of other mismatches 1, extra weight in seed region 1.5, seed region 2-13nt, mismatches allowed in seed region 2, maximum UPE 25, penalty of opening gap 2, HSP size 19 and penalty of extending gap 0.5. psRNA target server can be accessed from web http://plantgrn.noble.org/psRNATarget/

Statistical analysis

By using R language through R studio, miRNA target prediction data was analyzed which obtained from four algorithms (Gandrud, 2013). For the graphical representation of data, ggplot2 and packages readxl were used.

Conservation analysis of attachment site of miRNA genome: Different types of complete genome sequence strains were accessed from NCBI in FASTA format. These strains were accessed by accession number KY225551.1, KY225549.1, KY225546.1, KX421104.1, KX499498.1, KX664482, KU528623.1 and JN183062.1 with complete genome sequence. These files were copied into notepad with a single potential miRNA and made 4 different files with four potential miRNAs separately. Sequences were examined for computationally screened miRNAs using MEGA X (Kumar *et al.*, 2016).

Phylogenetic analysis: To construct neighbor-joining tree from aligned sequences, multiple sequence alignment of all the strains of ZYMV were imported into MEGA X phylogeny.

Results and Discussion

Zucchini yellow mosaic virus (ZYMV) cause a serious outbreak of *Cucumis melo* yellow mosaic disease that result in considerable yield loss of melon (Malik *et al.*, 2006). The most important virus of family Cucurbitaceae is ZYMV and it cause major severe symptoms as well as mosaic, deformation and size reduction (kwon *et al.*, 2005). Many plant diseases occur due to plant viruses and end in yield loss to crop production. RNA silencing pathway has become a strong tool for resistant plants (Duan *et al.*, 2012). Hence the objective of this work was target the prediction of potential miRNAs in the ZYMV genome that may be targeted by particular *C. melo* miRNAs. miRNAs such as cme-MIR396b, cme-MIR396c, cme-MIR396d(2) and cme-MIR319d were found at different loci to target ZYMV. While 32 miRNAs target the CI gene pursued by VPg, NIa-Pro, CP, HC-Pro and Nib that target through 3, 5, 7, 8 and 15 miRNAs respectively.

ZYMV genome target prediction of miRNA: Four different algorithms were used for miRNA target prediction in ZYMV genome. miRanda, psRNA target, Tapirhybrid and RNA22 were used in combination to filter false positive results and optimize miRNA target prediction accuracy (Iqbal *et al.*, 2017; Jabbar *et al.*, 2019).

***Cucumis melo* miRNAs for silencing ZYMV genome:** Four miRNAs (cme-MIR396b, cme-MIR396d (2), cme-MIR396c and cme-MIR319d) were predicted for silencing of ZYMV genome by at least three algorithms. Such miRNAs were classified as the *C. melo* miRNAs best matched against the genome of ZYMV. In case of *Oryza sativa*, seven miRNAs were predicted and their conservation of target site was evaluated (Jabbar *et al.*, 2019), 10 miRNAs for silencing of MCMV genome in maize (Iqbal *et al.*, 2017) while 2 miRNAs for silencing plum pox virus in peach plant (Shahid *et al.,* 2021).

Proteins encoded by ZYMV genome

P1: The first potyviral protein that translated and least studied was P1 protein. After HC-Pro and Nia, P1 protein was the last identified peptidase (Verchot *et al.*, 1991). In sub-genomic RNA sequence, the P1 protein is attached at the 5′ end. It was targeted by two different miRNAs were cme-MIR858 (1) at locus 432 and cme-MIR7130 (1) at locus 530.

HC-Pro (helper component proteinase) gene: HC-Pro has been shown to cause symptoms of plant developmental defects and is a suppressor of the silencing of genes. Proteinase is the output of HC-Pro (Carrington *et al.*, 1998) and it is targeted by 8 miRNAs at different loci such as cme-MIR156a, cme-MIR156c, cme-MIR156d and cme-MIR156i targeted at locus 2353. Similarly miRNAs cme-MIR159a (2) and cme-MIR159b (2) were targeted at locus 2013 and other than these two miRNAs, cme-MIR167f and cme-MIR168 targeted hc-pro gene at locus 2395.

P3 protein: P3 protein is targeted by 11 miRNAs at different gene loci. Three miRNAs cme-MIR164a (1), cme-MIR164c (2) and cme-MIR164d(2) targeted P3 gene at locus 3208. Similarly two miRNAs cme-MIR167b and cme-MIR167c targeted at locus 2894. Other than these two miRNAs cme-MIR828 (1) and cme-MIR858 (2) targeted at locus 2753. While cme-MIR171b (1), cme-MIR171d (1) and cme-MIR171i targeted at locus 2649 and single miRNA cme-MIR828 (2) targeted P3 gene at locus 2580.

6K1: 6k1 was targeted by 4 miRNAs (miRNAs cme-MIR172a, cme-MIR172b, cme-MIR172c and cme-MIR172d) at same gene locus. These miRNAs targeted 6k1 gene at locus 3560.

miRNAs targeting CIP (Cylindrical Inclusion Protein) gene: Among all of these, 32 miRNAs targeted the CI gene. The gene facilitates cell to cell movement and has helicase activity (Carrington *et al.*, 1998). These 32 miRNAs are (cme-MIR156b, cme-MIR159b, cme-MIR 169b, cme-MIR319a, cme-MIR319b, cme-MIR319c, cme-MIR319d, cme-MIR159a(1), cme-

MIR160a(2), cme-MIR160c, cme-MIR160d, cme-MIR164c(1), cme-MIR164d(1), cme-MIR 164d(3), cme-MIR169k, cme-MIR169p(2), cme-MIR169q(2), cme-MIR169s(2), cme-MIR171a, cme-MIR171e, cme-MIR171g, cme-MIR171h, cme-MIR394b, cme-MIR396d(1), cme-MIR396e(1), cme-MIR398a, cme-MIR398b, cme-MIR399g, cme-MIR477b(3), cme-MIR 408, cme-MIR 477b(2) and cme-MIR 477b(1). Seven miRNAs (cme-MIR156b, cme-MIR159b, cme-MIR 169b, cme-MIR319a, cme-MIR319b, cme-MIR319c and cme-MIR319d) targeted CIP at locus 4301 and cme-MIR160a (2), cme-MIR160c, cme-MIR160d targeted at locus 3779 and other miRNAs targeted at different loci.

NIa-VPg: Three miRNAs (cme-MIR164b, cme-MIR530a and cme-MIR854) were found having the potential to disrupt this gene's regular functioning. These miRNAs targeted at different loci. cme-MIR164b targeted at locus 6119, cme-MIR530a targeted at locus 6135 and cme-MIR854 targeted at locus 6004.

NIa-Pro (C-Terminal Proteinase Domain of NIa) gene targeting miRNAs: The 5 miRNAs (cme-MIR169n, cme-MIR169h, cme-MIR169f, cme-MIR828 (3) and cme-MIR169e) were potentially targeted at NIa-pro gene. Four miRNAs (cme-MIR169n, cme-MIR169h, cme-MIR169f and cme-MIR169e) targeted NIa-Pro gene at locus 6579 and cme-MIR828 (3) targeted at locus 6820.

Nib (Nuclear Inclusion Protein b) gene targeting miRNAs: Fifteen separate miRNAs (cme-MIR164c(3), cme-MIR164a(2), cme-MIR169p(1), cme-MIR169q(1), cme-MIR169s(1), cme MIR171c, cmeMIR171f, cme-MIR396a, cme-MIR396b, cme-MIR396c, cme-MIR396d(2), cme-MIR396e(2), cme-MIR845 and cme-MIR7130(2) and cme-MIR162) targeted the NIb gene. In these five miRNAs (cme-MIR396b, cme-MIR396c, cme-MIR396d (2), cme-MIR396e (2), cme-MIR396a) targeted Nib gene at locus 7007. Two miRNAs (cme-MIR164c (3) and cme-MIR162) targeted at locus 7087. Three miRNAs (cme-MIR169p (1), cme-MIR169q (1) and cme-MIR169s (1) targeted at locus 8150 and other miRNAs targeted NIb gene at different loci. It is involved in viral replication and it encodes the RNA-dependent RNA polymerase (Iqbal *et al*., 2016).

Coat protein: Seven miRNAs (cme-MIR160a (1), cme-MIR169g, cme-MIR169r, cme-MIR169t, cme-MIR171b (2), cme-MIR171d (2) and cme-MIR160b) targeted the CP gene. miRNAs (cme-MIR160a (1) and cme-MIR160b) targeted at locus 9340. Other miRNAs (cme-MIR169g, cme-MIR169r and cme-MIR169t) targeted at locus 8835. cme-MIR171b (2) and cme-MIR171d(2) targeted at locus 8617.

Data analysis: Attachment of genomic sites to the respective screened miRNAs showed the information about conservation level of binding sites in different strains of ZYMV. Intersection plot of *C. melo* miRNA target site in ZYMV genome predicted from miRNA target predicted algorithms with the inclusion of Tapirhybrid (Fig. 4). Sequences where alignment occurs between potential miRNA and complete genome sequence were shown in figure 5. The phylogenetic analysis of these sequences represented in the neighbor-joining tree showed the phylogeny of all strains of ZYMV (Fig. 6). The secondary structure of predicted miRNAs precursors has been shown in figure 7.

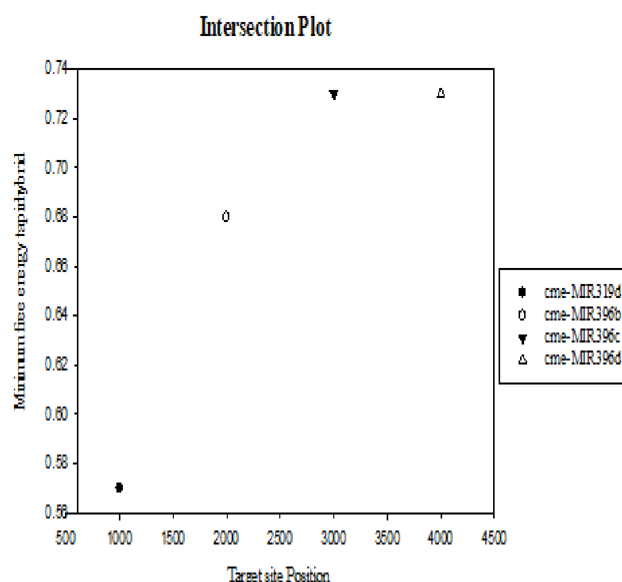

Fig. 4. Intersection plot of *Cucumis melo* miRNA target site in ZYMV genome predicted from miRNA target predicted algorithms with the inclusion of tapirhybrid.

Conclusion

This study used an *In silico* approach to detect host-derived miRNAs, with the goal of silencing the virus genome that affected the host plant through RNA interference. The current study used bioinformatics tools (miRanda, RNA22, psRNAtarget and tapirhybrid) to identify the miRNAs of *Cucumis melo*, which targeted ZYMV. The putative miRNAs of *C. melo* identified as the short-listed miRNAs that may be used for the production of ZYMV resistant melon plants. This study provides a better way to computationally analyze the best-candidate miRNAs against viruses, prior to cloning. A narrow-range of match-mismatch in miRNA-mRNA attachment has been shown in this study.

Conflict of interest statement: The authors state that the research was steered in the absence of any commercial or financial relationships that could be taken as a potential conflict of interest.

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Fig. 5. Multiple sequence alignment of ZYMV strains genome showed the conservation of attachment site of the respective miRNAs (sites are numbered according to the accession number JN183062).

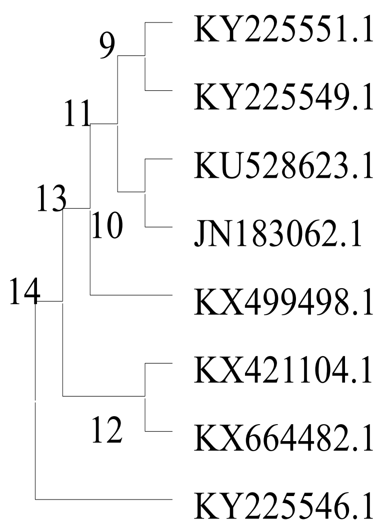

Fig. 6. Phylogenetic analysis represented in neighbor joining tree form showed the phylogeny of all strains of ZYMV.

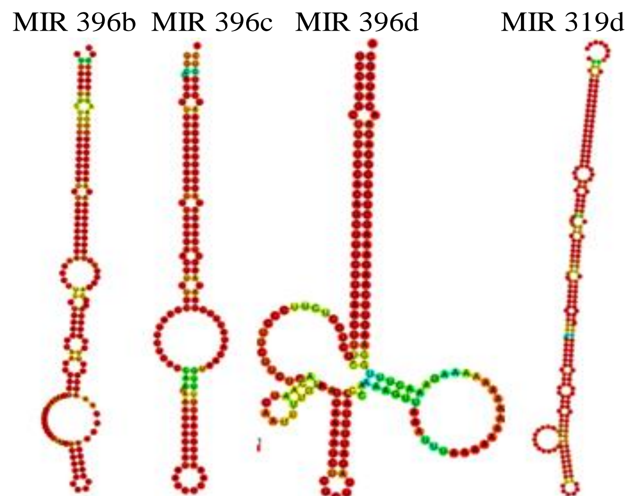

Fig. 7. Secondary pre-miRNAs structures, precursors of the mature miRNAs (MIR396b, MIR396c, MIR396d and MIR319d) found in the analysis as the miRNAs found by an algorithm consensus.

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