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IDENTIFICATION AND VALIDATION OF MIRNAS AND THEIR TARGETS THAT REGULATE THE RESISTANCE GENES AGAINST FUSARIUM WILT IN TOMATO

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ABSTRACT

MicroRNAs (miRNAs) are a specialized group of small RNAs (sRNAs) that regulate gene expression in plants at both transcriptional and post-transcriptional levels. Numerous families of miRNA target genes are involved in regulating plant immunity. In this study, we studied the role of miRNAs in the defensive response against a fungal pathogen, *Fusarium oxysporum* f. sp. *lycopersici*, which causes wilt disease in tomatoes. Furthermore, the expression patterns of two novel miRNAs and their targets were validated by qRT-PCR. Moreover, two new miRNAs (miR30 and miR33) were further sequenced by Applied Biosystems, using gene-specific primers. The results showed that four miRNAs, two novel (miR30 and miR33), and two known miRNAs (miR46 and miR49) and their target genes were differentially expressed during the infection with the pathogen. On the other hand, two targets (*P4*) and (*β-1,3-glucanase*) showed an inverse correlation in expression with their corresponding (miR46), and (miR33, and miR49), respectively. Our results showed that tomato cv. Pusa Early Dwarf is moderately susceptible to the fungus because its resistance is not well-expressed enough to be attributed to miRNAs. Sequences analysis showed that miR30 and miR33 are highly conserved and are found in different plant species. We predicted the secondary structures of miR30 and miR33 by minimum free energy (MFE). The total free energy of miRNA30 and miR33 was -1.2 and -0.4 kcal/mol respectively, predicted by the Vienna RNA package program V.1.7. The result of this study could improve our comprehension of the role that miRNAs play in tomato resistance to *F. oxysporum* f. sp. *lycopersici*. In addition, it will provide novel gene sources to develop resistant breeds.

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INTRODUCTION

MicroRNAs (miRNAs) are non-coding single-stranded RNA molecules with a length of 20-24 nucleotides (nt) (Llave *et al.*, 2002; Reinhart *et al.*, 2000; Park *et al.*, 2002; Pan *et al.*, 2017; Huang *et al.*, 2019). miRNAs are important in a variety of biological processes in plants, including floral morphogenesis, hormone balance, reproductive performance, and regulating growth and plant responses to abiotic (e.g., drought, salinity, and temperature) and biotic (e.g., pathogens such as fungi, bacteria, and viruses). Furthermore, in of presence of nutritional deficiency, miRNAs organize the metabolic balance of sulphur, copper, and phosphorus in the plants (Sunkar *et al.*, 2007; Padmanabhan *et al.*, 2009; Rubio-Somoza *et al.*, 2009; Chen and Cao, 2015; Xu *et al.*, 2021). During the pathogen invasion, the two-lined immune system is activated in plants to protect them from pathogens. The first line is called pathogen-associated

molecular patterns (PAMPs), which discovers conserved molecular characteristics of pathogens, to trigger immunity (pathogen-associated molecular pattern-triggered immunity [PTI]) that prevents infection of the host with pathogens (Zipfel and Felix, 2005; Boller and Felix, 2009). Successful microbes transfer effectors inside the host to inhibit PTI and establish the pathogen (Dou and Zhou, 2012). In turn, the second line of plant immunity, termed effector-triggered immunity (ETI), is initiated upon the recognition of effectors by the cognate intracellular immune receptors, such as nucleotide-binding site-leucine-rich repeat (NBS-LRR)-type proteins (Gao *et al.*, 2021). ETI is commonly associated with a hypersensitive reaction that results in programmed cell death at the infection site to prevent microbe spread (Jones and Dangl, 2006). PTI and ETI provide pre- and post-invasive resistance to the host in plant-fungus interactions, respectively. Therefore, small RNAs are needed in both PTI and ETI signaling and are represented as key fine-tuning regulators (Katiyar-Agarwal and Jin, 2010; Huang *et al.*, 2016; Zhang *et al.*, 2016).

The tomato (*Solanum lycopersicum*) belongs to the *Solanaceae* family. It is the main vegetable crop in Egypt and worldwide. However, tomatoes are greatly affected by various biotic stress factors which result in crop loss. *Fusarium oxysporum* f. sp. *lycopersici* a soil-borne plant pathogen, is an ascomycetous hemibiotrophic fungus that causes root infection by colonizing in the xylem vessels leading to wilt. Several miRNAs have been shown to respond to *F. oxysporum* f. sp. *lycopersici* infection in tomato (Jin and Wu, 2015; Srinivas *et al.*, 2019). Information about miRNA function in the plant immune system against *F. oxysporum* f. sp. *lycopersici* is limited. Therefore, this study was carried out to identify and validate *F. oxysporum* f. sp. *lycopersici*-responsive miRNAs from the roots of tomato plants.

In this study, we studied the role of miRNAs in the defensive response against a fungal pathogen, *Fusarium oxysporum* f. sp. *lycopersici*, which causes wilt disease in tomatoes. Furthermore, the expression patterns of two novel miRNAs (miR30 and miR33) and their targets were validated by qRT-PCR. Moreover, two new miRNAs were further sequenced.

MATERIALS AND METHODS

Fungal Strain, Plant Material and Culture Conditions

F. oxysporum f. sp. *lycopersici* wild-type strain 4471 and the seeds of tomato cv. Pusa Early Dwarf were obtained from Indian Agriculture Research Institute (IARI-New Delhi). *Fusarium* strain was maintained on potato dextrose agar (PDA) at 28°C in the dark (Tetorya and Rajam, 2021).

Inoculation of Tomato Plants

A tomato cultivar Pusa Early Dwarf (PED) was used for plant infection with the root pathogen *F. oxysporum* f. sp. *lycopersici* strain 4471. Two-month-old tomato seedlings were grown at 28±2°C with a 16/8-h photoperiod. Roots of tomato plants were removed from the soil and incubated for 30 min in a solution of *F. oxysporum* f. sp *lycopersici* conidia at a concentration of $1x10^6$/ml (Singh *et al.*, 2020). Mock-inoculated tomato plants (control tomato plants) were treated with water. Tomato plants were then replanted in soil and kept in a growth room at 25°C/24 h with constant light. The plants were then removed from the soil, and the roots were rinsed and excised with a sterile blade. The roots were frozen in liquid nitrogen and stored at -80°C.

Prediction of miRNA and Target Genes

Prediction of the miRNA target gene was conducted by following the guidelines described by Allen, (2005) (http://ted.bti.cornell.edu/cgi-bin/TFGD/sRNA/miRNA.cgi). To identify the conserved miRNAs in tomato plants, sRNA sequences obtained by deep sequencing were compared with known mature plant miRNAs in miRBase software (Griffiths-Jones *et al.*, 2007). After homology searches and further sequence analysis, a total of 103 conserved miRNAs from 24 different miRNA families were discovered, and all of which can be referred to in the important database (http://ted.bti.cornell.edu/cgi-bin/TFGD/sRNA/miRNA.cgi).

Designing oligonucleotide primers for miRNAs and target genes

The primers used in this work were designed by the OligoAnalyzer tool and are listed in Table 1.

Isolation of Total RNA

Total RNAs were isolated from uninfected control and *F. oxysporium* f. sp. *lycopersici* infected *S. lycopersicum* roots cv. Pusa Early Dwarf by TRIzol reagent according to the manufacturer's recommended protocol (Sigma, USA), followed by RNase-free DNase treatment. The quality and quantity of total RNAs were computed using a Nano-Drop ND-1000 spectrophotometer (Nano-Drop Technologies Inc., Wilmington, DE).

Stem-loop reverse transcription PCR (RT-PCR)

The total RNA (1 µg/sample) and stem-loop RT primers

were mixed in 10 µl reaction at room temperature (RT) and incubated at 65°C for 10 min, followed by a quick transfer to ice. This step was performed to relieve all secondary structures in the mixture and promote better annealing of the primers during cDNA synthesis. The DNaseI-treated total RNA was then reverse-transcribed using the RT-PCR kit (Thermo Scientific, USA). The cDNA was then used for the amplification of miRNAs using the following reaction mixture of 14.3 µl sterile dsH_2O, 2 µl 10X PCR buffer HF (Promega Corp.), 1.5 µl dNTPs (10 mM), the two primer combinations (0.5 µl each) (0.5 µM), 0.2 µl *Taq* DNA polymerase (Thermo Scientific, UK), and 1 µl of cDNA template (~400 ng) and PCR cycles of 16°C for 30 min; 60 cycles of 30°C for 30 s, 42°C for 30 s, 50°C for 15 s and 85°C for 5 min. For PCR amplification of the target genes, the cycle was 25°C/5 min; 42°C/60 min and 70°C/5 min.

Table 1. Primers used in the present study.

Primers	Single nucleotide sequence (5'-3')	Temp. melting (Tm) °C	Molecular size (bp)
miRNA 30 F	CGTCGGACCAGGCTTC		
Stem loop RT Primer 30	GTCGTATCCAGTGCAGGGTCCGAGGTATT CGCACTGGATACGAGGGAAT	63.3	55
miRNA 33 F	TCGGACCAGGCTTCA		
Stem loop RT Primer 33	GTCGTATCCAGTGCAGGGTCCGAGGTATT CGCACTGGATACGAGGGGAA	60.0	55
miRNA 46 F	GCGGAGAATCTTGATGATG		
Stem loop RT Primer 46	GTCGTATCCAGTGCAGGGTCCGAGGTATT CGCACTGGATACGAATGCAG	60.8	55
miRNA 4 9F	GGCTGGACTGAAGGGAG		
Stem loop RT Primer 49	GTCGTATCCAGTGCAGGGTCCGAGGTATT CGCACTGGATACGAAGGGAG	61.0	55
miRNA 70 F	TGTGGGTGGGGTGGA		
Stem loop RT Primer 70	GTCGTATCCAGTGCAGGGTCCGAGGTATT CGCACTGGATACGAAATCTT	63.6	55
pr1a (P4) F	GTAAGGGCGGCTCAATAAGT	62	106
pr1a (P4) R	GATTGCATGTCGTGTGATTAAGG		
*β-1,3-glucanase*mRNA F1	ATGCGCTAAGAGGATCAAACA	62	107
β-1,3-glucanase mRNA R1	GTTCTTCTGTACCCACCATCTC		
*β-1,3-glucanase*mRNAF2	CCAAAGAAACCAGGAAGGACTA	62	115
*β-1,3-glucanase*mRNA R2	GCCTCTGGTCAGGTTTAAAGA		

Quantitative RT-PCR (qRT-PCR)

MicroRNAs were detected with an all-in-one TM miRNA qRT-PCR detection kit according to the manufacturer's instructions (Thermo Scientific, USA). The qRT-PCR was carried out using a SYBR Green I Master Mix from Roche on the Light Cycler 480 II Real-Time PCR (Roche). The reaction mix was prepared in a final volume of 10 µl containing 1X Master Mix, 1 *p*mol of each of the forward and reverse primers. Specifically designed forward primers for each individual miRNA and reverse primers were used for qRT-PCR reactions (Fiedler *et al.*, 2010; Yanik *et al.*, 2013). The sequences of the primers used in the qRT-PCR are mentioned in Table 1. The qRT-PCR conditions for miRNAs were set up as follows: 50°C for 2 min, 95°C for 10 min, followed by 40 cycles at 95°C for 15 s, 60°C for 1 min, 95°C for 15 s and 60°C for 15 s. Target genes were analyzed using the following conditions, 50°C for 2 min, 95°C for 10 min; 40 cycles of 95°C for 15 s and 55°C for 1 min. The experiments were performed in four replicates and technical repeats.

Agarose Gel Electrophoresis

The amplified PCR products for miRNAs and target genes were electrophoresed on 5% and 2% agarose respectively, containing ethidium bromide (0.5 µg/ml) in 1X TBE buffer at 75 volts and visualized using a UV transilluminator. The size of each amplicon was determined with reference to a *GeneRuler Ultra Low Range DNA Ladder* (Thermo Scientific, USA).

Validation of miRNAs

Four miRNAs (miR30, miR33, miR46, and miR49) were validated by miRBase v. 20 (http://www.mirbase.org) to provide integrated interfaces to comprehensive microRNA sequence data, annotation, and predicted gene targets.

Cloning of the miRNA30 and miRNA33 Genes

Two fragments of the expected size, 55 bp for two new miRNAs (miR30 and miR33) were cut from the agarose gels and further purified using a Gene Jet Gel DNA purification kit (Thermo Scientific, USA). The concentrations of the purified products were measured by a NanoDrop ND-1000 spectrophotometer. The purified DNA fragments were ligated into pGEM®-T Easy vector (Promega, Mannheim, Germany). Ligated plasmids were transformed into *E. coli* XL-1 Blue competent cells. Isolation of plasmid DNA from the putative colonies obtained was done by the alkaline lysis method according to Sambrook *et al.* (1989). Plasmid DNA was digested by *Eco*RI restriction enzyme at 37ºC overnight to confirm the presence of positive intact clones.

Sequencing

The positive clones of two new miRNAs (miR30 and miR33) were further sequenced by Applied Biosystems (Inst model/Name 3100/3130XL-1468-009, India) using gene-specific primers. The sequence was aligned with corresponding sequences from the database using miRBase software.

The Secondary Structure of miRNAs

The secondary structures of miR30 and miR33 sequences obtained after cloning were predicted by Vienna RNA package program V.1.7. Institute for Theoretical Chemistry, University of Vienna (http://rna.tbi.univie.ac.at//cgi-bin/RNA), which uses a free energy minimization algorithm (Mathews and Turner, 2006).

Statistical Analysis

The qRT-PCR results are expressed as a standard deviation. Statistical analysis was carried out to determine *P* values by one-tailed paired Student's t-test analysis using Microsoft *Excel* 2019. *P* values < 0.05 were considered to be statistically significant.

RESULTS

Expression Profiling of *F. oxysporum* f. sp. *lycopersici*-Responsive miRNAs and Target Genes in Tomato

We have investigated the expression of miRNAs in tomato roots (cv. PED -moderately susceptible) during infection with the wilt fungus *F. oxysporum* f. sp. *lycopersici*, compared with the control (treated with water) (Figure 1). Our goal was to identify differentially expressed miRNAs in tomato upon infection with the pathogen.

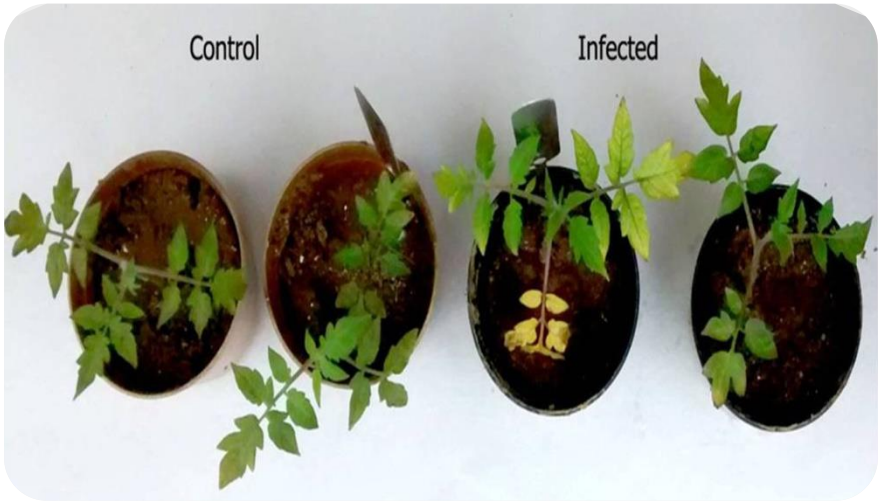

Figure 1. Symptoms of *F. oxysporium* on tomato cultivar PED compared with the control.

The literature survey and *in silico* analysis provided with four miRNAs in tomato, two novel (miR30 and miR33) and two known (miR46 and miR49) and their target genes. Therefore, the expression pattern of four miRNAs (miR30, miR33, miR46, and miR49) and two target genes (*p4* and *β-1,3-glucanase*) was analyzed in tomato plants infected with *F. oxysporum* f. sp. *lycopersici* and control by qRT-PCR 30 dpi (days post-inoculation). The expression

levels were dramatically changed during the infection (Figure 2). The transcript level of miR30 was not significantly altered in tomato plants infected with the pathogen (fold= 3.61), compared with the control. However, miR46 was down-regulated with a fold decrease of 33.33, upon fungal infection, compared with the control. On the contrary, miR33 and miR49 were up-regulated, with a fold increase in the expression of 0.4 and 0.15, respectively. The target gene's relative expression compared with control displayed down-regulation with a fold decrease of 2.43 and 9.09 for *p4* and *β-1,3 glucanase*, respectively. The target genes showed an inverse correlation in expression with miR30 and miR46 (Figure 2). This expression pattern suggests that the target mRNAs are susceptible factors during plant defense in tomato (cv. PED) upon infection (Figure 2).

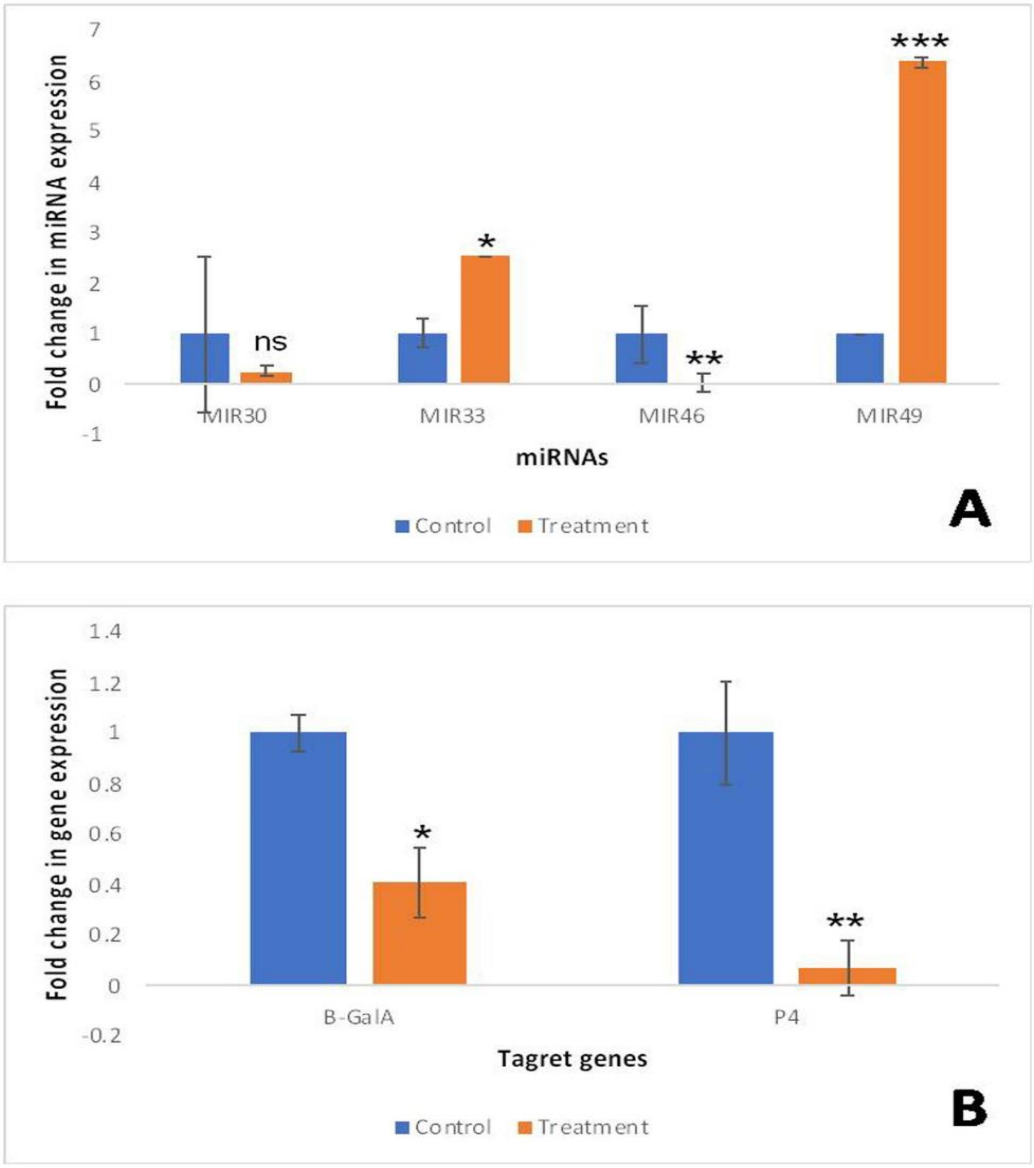

Figure 2. Relative expression of four miRNAs (a) and two target genes (b) in tomato cultivar PED using qRT-PCR, compared with the control. Expression was normalized to that of Actin. All values represent as standard deviation of results obtained with four replicates in each group (n=4).*P value < 0.05, **P value < 0.01, ***P value < 0.001, ns= not significant.

Amplification of Target Genes (*p4* and *β-1,3-glucanase*) and miRNA

The amplicon size of target gene *p4* was 106 bp (Figure 3A), while the fragment sizes of target gene *β-*

1,3-glucanase were 107 and 115 bp, were obtained by using two different gene-specific primers (Figures 3B and 3C). The four miRNAs (miR30, miR33, miR46, and miR49) were amplified with primers designed to anneal to undergo the stem-loop RT-PCR. Four miRNAs were amplified with the expected product size ~55 bp with no additional non-specific amplicons (Figure 4).

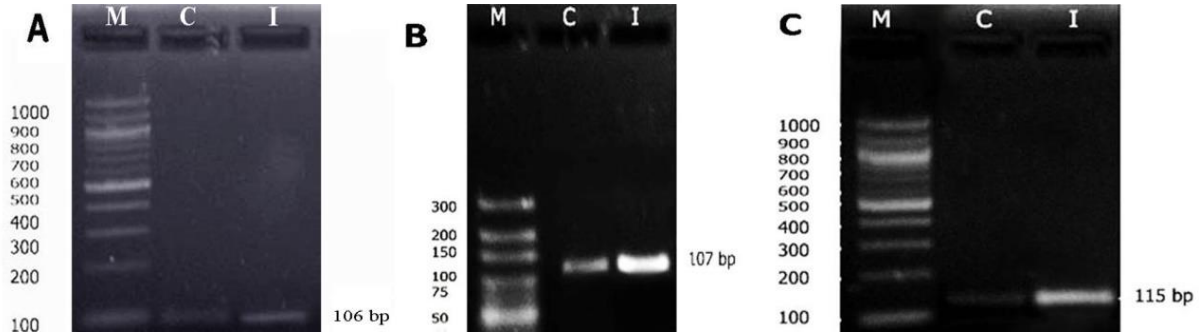

Figure 3**A**. PCR product of the *pr1a* (*P4*) gene from tomato roots cv PED using specific primer pr1a (P4). Lane M: DNA ladder100 bp, lane C: The control (106 bp) and lane I: PCR amplicon of *P4* gene (106 bp) from tomato plants infected with *F. oxysporium*. **B**. PCR amplification of *β-1,3-glucanase* gene from tomato roots cv PED using primers *β-1,3-glucanase* mRNA F1 and *β-1,3-glucanase* mRNA R1. Lane M: DNA size marker 100 bp. Lane C: The control (107 bp) and lane I: PCR product of *β-1,3-glucanase* (107 bp) from tomato plants infected with *F. oxysporium*. **C**. PCR amplification of *β-1,3-glucanase* gene from tomato roots cv PED using primers *β-1,3-glucanase* mRNA F2 and *β-1,3-glucanase* mRNA R2. Lane M: DNA size marker 100 bp. Lane C: The control (115 bp) and lane I: PCR product of *β-1,3-glucanase* (115 bp) from tomato plants infected with *F. oxysporium*.

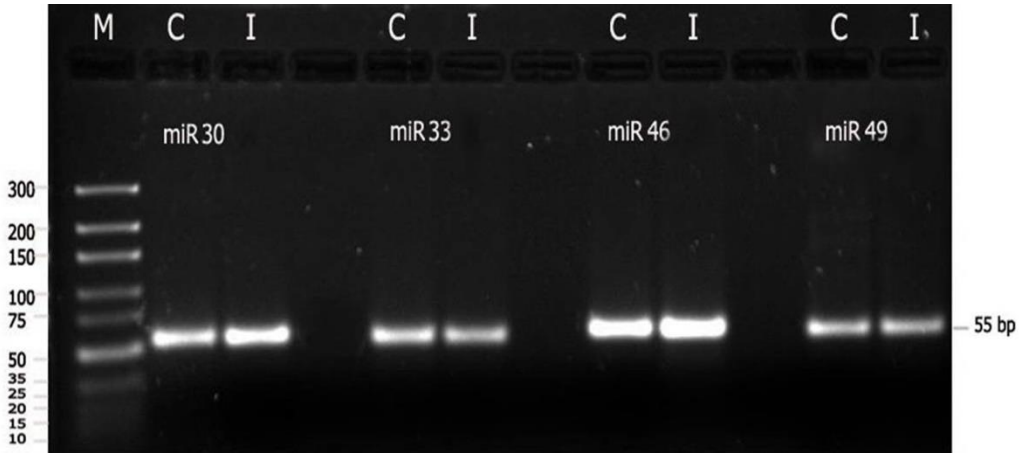

Figure 4. PCR amplification of miRNA30, miRNA33, miRNA46, and miRNA49 from tomato roots cv PED by stem loop RT design primers. Lane M: A GeneRuler Ultra Low Range DNA Ladder. Lane C: The control (expect size: 55 bp) and lane I: PCR product of four miRNAs (55 bp) from tomato plants infected with *F. oxysporium*.

Cloning of miR30 and miR33

Since miR30 and miR33 were novel miRNAs, the sequence validation was carried out by cloning the miRNAs. Five colonies were screened for the desired insert. All colonies having miR30 and miR33 gave one specific band with a molecular size of ~55 bp (Figures S1 and S2). A positive colony was then utilized for restriction digestion using *Eco*RI, which showed the expected size of 55 bp release of miR30 and miR33 (Figures S3 and S4).

Multiple Sequence Alignments

The sequence analysis using BLAST showed % similarity with the sequence reported in the database. The nucleotide sequences of miR30 and miR33 were 20 and 21 nt, respectively (Figure S5). Unknown readings were

analyzed with miRBase (Release 21.0) to find miRNA homologs. Homology analysis showed that miR30 and miR33 had 100% homology to mature conserved miRNA166 found in various plant species such as *Arabidopsis lyrata*, *Hordeum vulgare*, *Citrus sinensis*, *Phaseolus vulgaris*, *Glycine max*, *Nicotiana tabacum*, *Helianthus paradoxus*, *Cucumis melo*, *Linumusit atissimum, Solanum tuberosum*, and *Prunus persica*.

The Primary Sequence and Secondary Structure of miRNAs

The primary structures of two novel miRNAs (miR30 and miR33) were determined using the sequences (Figure S5), while the secondary structures were predicted by the Vienna RNA package program V.1.7 (Figure 5). Two miRNAs formed an intra-molecular base pairing among their bases, which included interior loops, hairpin loops, and external loops (Figure 5 and Table 2). The bracket notation for miR30 and miR33 secondary structures was represented in the space-efficient bracket notation (Figure S5). The symbols «(and)» correspond to the 5′ and 3′ bases in the base-pair, respectively. However, «.» represents an unpaired base. On the other hand, it was observed that miR30 (20 bp) has a mutation in the stem-loop region. This mutation was found between G and U, while miR33 (21 bp) has not displayed any mutations (Figure 5).

Prediction of miRNA Secondary Structure using Minimal Free Energy (MFE)

The secondary structures of miR30 and miR33 were predicted from their primary sequences by summing the energy contribution of all base pairs, hairpin loops, external loops, and interior loops at 37°C. Therefore, the MFE algorithm, which estimates experimental thermodynamic values was used to calculate the miRNA secondary structures. Table 2 illustrates ΔG°_{37} (Gibbs free energy) of external loops, interior loops, and hairpins depending on the predicted free-energy values (kcal/mol at 37°C). The total free energy of miR30 and miR33 was found to be -1.2 and -0.4 kcal/mol respectively, using Vienna RNA package V.1.7. (Table 2).

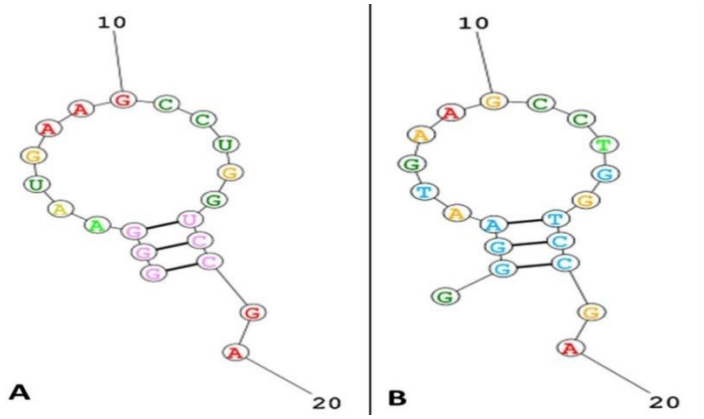

Figure 5. Prediction of the secondary structure of miR30 and miR33 using Vienna RNA package program V.1.7. (**A**). Hairpin structure of miR30. (**B**). Hairpin structure of miR33.

Table 2. Illustrated thermodynamic of a secondary structure for miRNA30 and miRNA33.

miRNA	Secondary structure	Nucleotides number	ΔG°_{37} (kcal/mol)
miRNA30	External loop		-0.120
	Interior loop	(1,12) GC; (2,11) GC	-0.330
	Hairpin loop	(2,11) GC	+0.330
*MFE			-1.2
miRNA33	External loop		-0.110
	Interior loop	(3,19) GC; (4,18) GC	-0.330
	Interior loop	(4,18) GC; (5,17) AU	-0.240
	Hairpin loop	(5,17) AU	0.640
MFE			-0.4

*MFE=minimum free energy. ΔG°_{37} = (Gibbs free energy**)**.

DISCUSSION

Both plants and animals have miRNAs, which are a class of small RNAs. The miRNAs are single-stranded RNA molecules, non-coding, and composed of ~20-24 nucleotides in length. miRNAs are generated from stem-loop structures harbored in primary miRNA transcripts (Bartel, 2004). miRNA links to its mRNA target primarily through a sequence complementary to the 3′-untranslated region (3′UTR), triggering the case of either translational suppression or degradation of the mRNA transcript.

In this study, the role of miRNAs in the defense response of tomato plants against fungus *F. oxysporum* f. sp. *lycopersici* was studied. The expression of four miRNAs, two unknown (miR30 and miR33) and two known miRNAs (miR46 and miR49) related to disease development and the two target genes *p4* and *β-1,3-glucanse* in tomato were quantified by qRT-PCR. Furthermore, qRT-PCR results showed variations in their expression, wherein miR46 was down-regulated and miR33 and miR49 were up-regulated compared with the control. However, miR30 was not significantly changed in tomato plants infected with *F. oxysporum* f. sp *lycopersici*, compared with the control. Moreover, the expression of two target mRNAs (*p4*) and (*β-1,3-glucanase*) displayed a negative relationship in the expression with their corresponding miRNAs (miR46) (miR33 and miR49), respectively. On the contrary, there were no significant differences in the level of expression among the other genes and mock. Therefore, miR33 and miR49 may have influenced the expression of their target genes *β-1,3-glucanase* in the presence of fungal infection, the latter belongs to pathogenesis-related proteins (PR), which causes inhibition of root pathogens. Furthermore, *β-1,3-glucanase* is an important plant defense enzyme involved in *F. oxysporum* f. sp. *lycopersici* fungus cell wall destruction (Kavroulakis *et al.*, 2005). Also, our results showed that tomato variety PE is moderately susceptible to Fusarium wilt because its resistance is not well-expressed enough to be attributed to miRNAs. These results were in agreement with Chopada Chopada *et al.* (2014) who mentioned that tomato variety PED is moderately susceptible to Fusarium wilt infection with a percent wilt incidence of 66.67%. Ouyang *et al.* (2014); Ji *et al.* (2021) investigated the production of miRNAs after infection with the wilt fungus *F. oxysporum* f. sp *lycopersici* in two tomato cultivars, Motelle (resistant) and Moneymaker (susceptible). The results of the study showed that target mRNA was up-regulated in a resistant cultivar Motelle as a defense response against fungus, but not Moneymarker after infection. In addition, the miRNAs were down-regulated in the resistant cultivar Motelle after infection, but the study did not identify any miRNAs that were decreased or increased after infection in the susceptible cultivar Moneymarker. Our findings showed that plant miRNAs are important in the defense response against fungal infections and provide a platform for differentially expressed miRNAs in tomato after infection with fungus.

In the present work, the full length of two novel miRNAs (miR30 and miR33), from tomato infected with *F. oxysporum* f. sp. *lycopersici* was 20 and 21 nt, respectively. These findings were in agreement with those obtained by Chen *et al.* (2009) who stated that small RNA 21 and 24 nt classes were dominant in the plants. Our results demonstrated that miR30 and miR33 sequences had 100% identity to mature conserved miR166 represented in different plant species, including *Arabidopsis lyrata*, *Hordeum vulgare*, *Citrus sinensis* and *Phaseolus vulgaris*. These results are similar to those reported by other authors Jones-Rhoades (2012); Sun (2012) and Cardoso *et al.* (2018) who reported that miRNAs in plant species are conserved as well as non-conserved (unique) and belong to miRNA families. For example, miR156 is recognized as a conserved class that is known in several plant species. Zhang *et al.* (2008) identified miRNAs from many plant species against tomato nucleotide sequences and predicted 13 miRNA candidates grouped into nine miRNA families (miR159, miR157, miR167, miR162, miR172, miR395, miR171, miR399, and miR319) from over 57,8000 tomato sequences. Furthermore, mature miRNAs as well as miR171, miR162, and miR319 precursors have been cloned (Itaya *et al.*, 2008). Yin *et al.* (2008) identified 21 conserved miRNAs from 14 miRNA classes (miR159, miR160, miR156/157, miR162, miR168, miR169, miR399, miR172, miR167, miR403, miR869.1, miR1030, miR437, and miR830), seven of which were known in the Expressed Sequence Tag (EST) database and 14 in the Genome Survey Sequences (GSS) database, while some of them were not found in the *S. lycopersicum* (Zuo et al., 2011). The entire class of miRNAs has been identified in cultivated tomato through whole-genome sequencing. However, a few numbers of miRNAs have been found to be involved in tomato-specific processes,

like fruit ripening (Sato *et al.*, 2012; Wang *et al.*, 2011).

In the current study, the total free energy, or Gibbs free energy (Δ*G*) of miR30 and miR33 was -1.2 and -0.4 kcal/mol respectively, predicated by the Vienna RNA package program V.1.7. These results are in accordance with Trotta (2014) who mentioned that minimum free energy (MFE) has been applied for different aims. For instance, normalization was applied to progress the secondary structure prediction by removing fragments with normalized equilibrium free energies less than a threshold value. Normalized MFE was also applied to compare evolutionary relationships between miRNA genes and their functions (Zhu *et al.*, 2012), and its advantage in identifying new non-coding RNAs was compared with other criteria (Eva and Vincent, 2005). Free energy can be applied as a measure of biological system stability. If the binding of a miRNA: target mRNA interaction is predicated to be stable, it is considered more probable to be a true target of the miRNA (Yue *et al.*, 2009). Normalized MFE assisted in indicating thermodynamic variables between nuclear-encoded miRNAs located in the cytosol and mitochondria (Bandiera *et al.*, 2011). The MFE was used in the search to predict actual miRNAs precursors (Loong and Mishra, 2007), to improve RNA folding prediction algorithms, and for comparing the thermodynamic stability (Ni *et al.*, 2010).

CONCLUSIONS

In summary, a total of four different miRNAs: two novel miRNAs (miR30 and miR33) and two known ones (miR46 and miR49) were identified from tomato cv. PED infected with *F. oxysporum* f. sp. *lycopersici*. After infection with the pathogen, qRT-PCR analysis revealed that the expression of four miRNAs and two target genes (*p4* and *1,3-glucanase*) were differentially expressed. We also observed that (*p4*) and (*β-1,3-glucanase*) displayed an inverse relationship in expression with their corresponding miRNAs (miR46) (miR33 and miR49) respectively. As a result, tomato cultivar PED appeared to be moderately susceptible to fungus because its resistance was not expressed sufficiently to be attributed to miRNAs. The result of this work could improve our understanding of the role of miRNAs in resistance to fusarium wilt disease in tomato.

COMPETING INTERESTS

The authors declare that they have no competing interests.

FUNDING INFORMATION

Not applicable

AUTHORS CONTRIBUTIONS

MVR conceived and designed the work. SAM and HAM wrote the manuscript and MVR and SY corrected and edited the manuscript. All the authors read and approved the manuscript.

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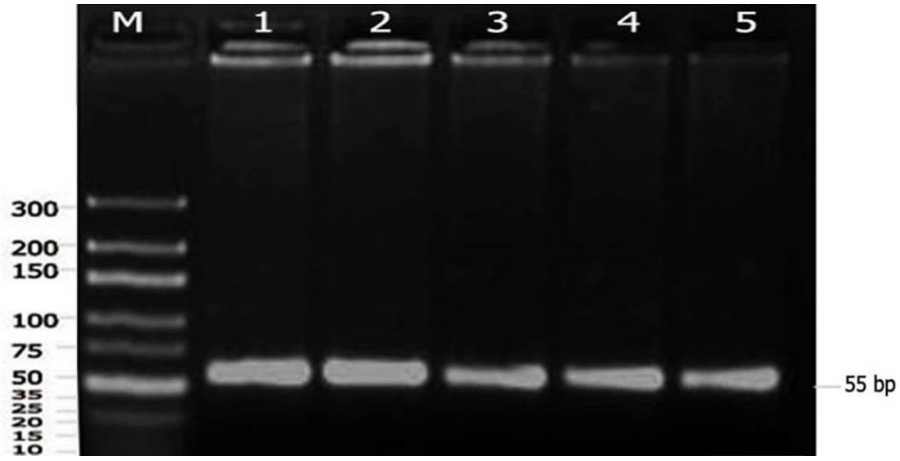

Figure S1. Screen for the positive clones containing miR30 fragment by colony PCR. Lanes 1-5 five colonies gave positive band with the expected size (55 bp). Lane M: GeneRuler Ultra Low Range DNA Ladder.

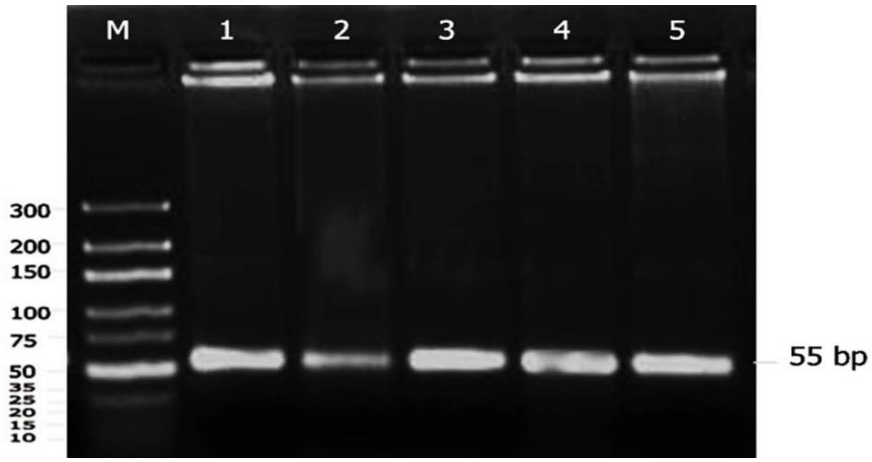

Figure S2. Verification of the positive clone containing miR33 fragment by colony PCR.
Lanes 1-5 five colonies picked for screening, every colony showed positive band and the size of the all the bands matched up to the expected size (55 bp). Lane M: GeneRuler Ultra Low Range DNA Ladder.

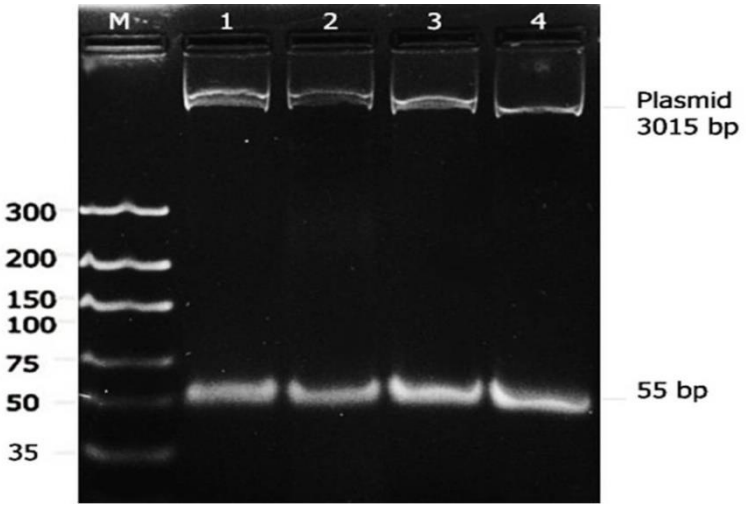

Figure S3. A gel showing digests of the pGEMT plasmid by restriction enzyme *EcoR1* (3015 bp) contained miR30 insert (55 bp). Lanes 1-4 featured the correct insert orientation digested clones.

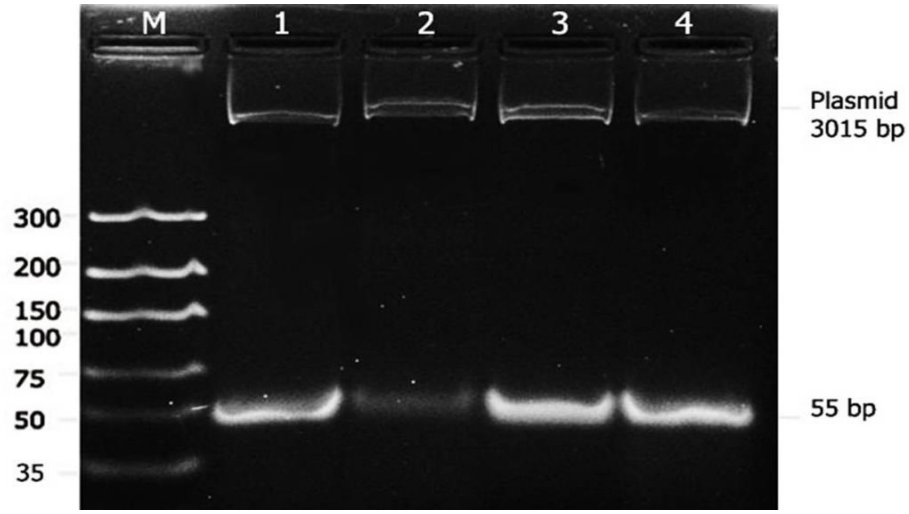

Figure S4. A gel showing digests of the pGEMT plasmid using restriction enzyme *EcoR1* (3015 bp) contained miR33 insert (55 bp). Lanes 1-4 digested clones featured the correct insert orientation digested clones.

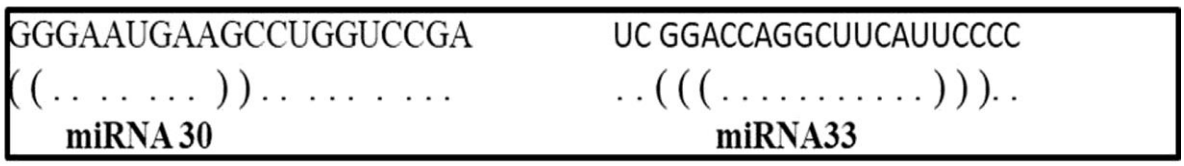

Figure S5. Nucleotide sequencings of primarily and secondary structure (by dot brackt notation) of miRNA30 (20 nt) and miRNA33 (21 nt). « (and) » corresponding to the ˈ5 and 3ˈ bases in the base-pair, respectively, «.» represents an unpaired base.