IDENTIFICATION AND CHARACTERISATION OF VIRUS RESPONSIVE miRNAs IN BANANA *Musa* (AAB) 'NENDRAN'

by

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THESIS

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DECLARATION

I, hereby declare that this thesis entitled **"IDENTIFICATION AND CHARACTERISATION OF VIRUS RESPONSIVE miRNAs IN BANANA *Musa* (AAB) 'NENDRAN'"** is a bonafide record of research work done by me during the course of research and the thesis has not previously formed the basis for the award to me of any degree, diploma, associate ship, fellowship or other similar title, of any other University or Society.

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Athira Subramanian

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LIST OF ABBREVIATIONS

3'UTR	3'-Untranslated region
µg	Microgram
µl	Microlitre
A	Adenine
A_{260}	Absorbance at 260 nm wavelength
A_{280}	Absorbance at 260 nm wavelength
AGO	Argonaute
AFLP	Amplified fragment length polymorphism
ARF	Auxin response factor
ARG	Argonaute protein gene
BACs	Bacterial artificial chromosome
BBrMV	*Banana bract mosaic virus*
BBTV	*Banana bunchy top virus*
BLAST	Basic local alignment search tool
BSMysV	*Banana streak mysore virus*
BSV	*Banana streak virus*

bp	Base pair
bZip	Basic leucine zipper proteins
CMV	*Cucumber mosaic virus*
C	Cytosine
cDNA	Complementary DNA
cm	Centimere
Cq	Cycle quantification value
CTAB	Cetyltrimethyl ammonium bromide
dbESTs	Expressed sequence tag database
DCL 1	Dicer like enzymes
DNA	Deoxyribo nucleic acid
dNTPs	Deoxy nucleotide tri phosphates
DEPC	Diethyl pyrocarbonate
DR	Disease resistance protein
EBV	*Ebsin-barr virus*
EDTA	Ethylene diamine tetra acetic acid
ERAD	Endoplasmic reticulum associated degradation
EREBPs	Ethylene-responsive element binding protein
ERF	Ethylene-responsive transcription factor

ERQC	Endoplasmic reticulum quality control
ERT	Putative ethylene-responsive transcription factor 1 gene
ESTs	Expressed sequence tags
FAD	Flavin adenine dinucleotide
FP	Forward Primer
G	Guanine
g	Relative centrifugal force
gm	gram
GSS	Genome survey sequences
h	hour
ha	Hectare
HD-Zip	Homeo domaine-leucine zipper
HP	Hewlett-Packard
hpRNA	hairpin ribonucleic acid
ihpRNA	intron hairpin ribonucleic acid
HR	Hyper resistance
Kb	Kilo basepair
Kg	Kilogram
LEA	Late embryogenesis abundant protein

M	Molar
MFE	Minimum fold energy
Min	Minute
miRNA	micro ribonucleic acid
ml	Millilitre
mM	Millimolar
mRNA	messenger ribonucleic acid
MT	Metric tonne
MYMIV	*Mung bean yellow mosaic India virus*
MYB	Myelobast
N	Resistance gene of *Nicotiana tabacum*
NAC	No apical cotyledon
NaCl	Sodium chloride
NB-LRR	Nucleotide binding- Leucine rich repeat
NCBI	National Center for Biotechnology Information
ncRNA	Non-coding ribonucleic acid
ng	Nanogram
nm	Nanometre
nM	Nanomolar

NTC	No template control
PAL	Phenylalanine ammonium lyase
PCR	Polymerase chain reaction
PR	Pathogenesis related protein
PPV	*Plum pox virus*
PTGS	Post transcriptional gene regulation
PV	Plant viral response family protein
PVP	Polyvinyl pyrrolidone
PVX	*Potato virus X*
PVY	*Potato virus Y*
R	Resistance genes
RP	Reverse primer
RAPD	Random amplified polymorphic DNA
RFLP	Restriction fragment length polymorphism
RDR	RNAi defense response
RDV	Rice dwarf virus
RGA2	Resistant gene analogue 2
RISC	RNA-induced silencing complex
RNA	Ribonucleic acid

RNase	Ribonuclease
RNAi	RNA interference
ROS	Reactive oxygen species
rRNA	Ribosomal ribonucleic acid
RSV	*Rice streak virus*
RT	Reverse transcriptase
RT-qPCR	Reverse transcription-quantitative polymerase chain reaction
s	Second
SDS	Sodium dodecyl sulfate
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel
SER	Stress endoplasmic reticulum protein 2
siRNA	small inferring ribonucleic acid
ssRNA	single stranded ribonucleic acid
T	Thymine
TBE	Tris-borate EDTA buffer
TEV	*Tobacco etch virus*
T_m	Melting temperature
TMV	Tobacco mosaic virus
TIR	Transport inhibitor response-1 like protein

TrisHCl	Tris (hydroxy methyl) aminomethane hydrochloride
tRNA	transfer ribonucleic acid
TuMV	*Turnip mosaic virus*
TYMV	*Turnip yellow mosaic virus*
TYLCSV	*Tomato yellow leaf curl Sardinia virus*
U	unit
V	volt
viz.	namely
w/v	weight/volume
v/v	volume/volume
vi-miRNA	viral miRNAs
VSRs	viral suppressors of RNA silencing
wk	week

LIST OF SYMBOLS

%	Percent
°C	Degree Celsius

Introduction

1. INTRODUCTION

Banana is the second most important fruit crop in the world with an average production of 114.13 million tonnes. India produces around 28-31 million tonnes of banana annually and ranks first in production and productivity around the world. Kerala, Karnataka, Tamil Nadu, Maharashtra and Andhra Pradesh are the major cultivators of banana. Among these banana producing states, Tamil Nadu ranks first by 5136.2 metric tonnes (FAOSTAT, 2017).

Banana cultivation is facing serious threats in the form of biotic and abiotic stresses which affects the production and productivity. Among the biotic stresses, *Banana bunchy top virus* (BBTV), *Banana streak virus* (BSV), *Banana bract mosaic virus* (BBrMV) and *Cucumber mosaic virus* (CMV) are the most devastating viruses causing considerable yield loss in banana. Banana bract mosaic is a debilitating disease that causes about 40% yield reduction in banana plant (Cherian *et al.*, 2002). The disease is caused by *Banana bract mosaic virus*, a ssRNA virus belonging to the family Potyviridae. The virus is transmitted non-persistently by three aphids, viz., *Rhopalosiphum maidis, Aphis gossypii* and *Pentalonia nigronervosa.* In Kerala BBrMV infection was first reported in banana var. Nendran. The virus infection was limited to smaller areas however, in the present scenario almost all varieties of banana are found to be susceptible to BBrMV. So far no resistant varieties are reported against this particular disease and hence the conventional breeding programs are not able to develop any resistant varieties.

Currently the role of small RNAs in biotic and abiotic stresses in plants have become the focus of research and RNA interference (RNAi) mechanism of siRNAs and miRNA have proved to be successful in providing resistance towards biotic stresses in plants. In recent years, several studies have reported the importance of miRNAs in imparting resistance from viruses in plants. Therefore information on

miRNAs associated with virus infections and their targets may help to develop strategies to impart virus resistance in banana.

miRNAs are small regulatory, non-coding RNA of 18-22 nt in length. They play a major role in imparting post-transcriptional gene regulation in plants and animals (Bartel, 2004). The miRNAs processed via Dicer like proteins (DCL 1) binds to their complementary target mRNA. Plant miRNAs predominantly repress the target genes post transcriptionally.

Several miRNAs associated with abiotic stress conditions have been identified in banana (Wang *et al.*, 2017). But only limited studies have been conducted to identify and understand the role of miRNAs in host pathogen interactions in banana.

In a study conducted in Department of Plant Biotechnology, College of Agriculture, Vellayani during 2016-18, 52 mature miRNAs and their corresponding 124 target genes were predicted in banana genome using computational tools. From this information generated, five miRNAs and their predicted targets were selected in the present study with the objective to identify the miRNAs associated with *Banana bract mosaic virus* (BBrMV) infection in banana var. Nendran from the expression profile of selected miRNAs.

Review of Literature

2. REVIEW OF LITERATURE

2.1 BANANA: ORIGIN AND GENOME CONSTITUTION

Banana (*Musa spp.*) is an important commercial crop and is a staple source for millions of people in tropical and subtropical countries. It is the second widely consumed fruit crop next to mango in India and due to its availability, cheap cost of cultivation, taste and high nutritive value, it is well loved by people all over the world. It belongs to the order *Zingiberales* and family *Musaceae*. Today, almost all cultivated bananas come from the two triploid species *Musa acuminate* and *Musa balbisiana*, all these varieties are parthenocarpic and are propagated through suckers. The banana varieties include, diploids (AA, BB and AB), triploids (AAA, AAB and ABB) and synthetic tetraploids (AAAA, AAAB, AABB and ABBB). The origin of banana dates back to 7th century AD in the humid tropical rain forest of South East Asia. Present day, banana is cultivated throughout the humid tropical regions between 30°N and 30°S of the equator. The genome size of banana is 523 Mb with the total number of chromosomes being 11. The whole assembly comprises of 24,425 contigs and over 36,542 protein coding genes (D'Hont et al., 2012).

Average production of banana is estimated to be 114.13 million tonnes in over 120 countries and India ranks first with 30.8 million tonnes, which accounts for about 13% of the area and 33% of world's production (FAOSTAT, 2017)

2.2 VIRAL DISEASES OF BANANA

Biotic and abiotic stresses are the major threat to the production and productivity of banana all over the world. Among these biotic stresses, viral diseases are the most disastrous resulting in major reduction in the economic yield of the plant. The four infectious viruses disrupting the economic yield of banana are Banana bract mosaic virus (Banana bract mosaic), Banana bunchy top virus (Banana bunchy

top), Banana streak virus (Banana streak) and Cucumber mosaic virus (Infectious chlorosis) (Jones, 1994).

2.2.1 Virus-vector interaction

The majority of the viral diseases are transmitted by vectors. However, interaction between virus and vector varies among different viral particles and pests. According to Watson et al. (1953), there are 3 types of virus-vector relationship resulting in three modes of transmission, non-persistent, semi-persistent and persistent. In non-persistent mode of transmission the virus is only retained by the vector for a few seconds. In semi-persistent method, the virus is retained from few minutes to few hours in the vector. In persistent mode, the virus is retained for a long time and requires several hours for transmitting the disease. In b anana bract mosaic, the infection is transmitted in a non-persistent manner by Aphids, *Rhopalosipum maidis*, *Pentalonia nigronervosa*, cowpea aphid *Aphis craccivora* and *Aphis gossypii* (Magnaye and Espino, 1990; Selvarajan and Jayabaskaran, 2006).

Banana bract mosaic virus (BBrMV) infection was first recorded in the year 1988 in Phillipines (Magnaye and Espino, 1990). In India, Tamil Nadu, Kerala, Karnataka and Andhra Pradesh are the areas affected by the virus (Rodoni *et al.*, 1997; Selvarajan and Singh, 1997; Thangavelu *et al.*, 2000; Cherian *et al.*, 2002). BBrMV is a ssRNA virus belonging to the family Potyviridae. The symptoms included reddish brown spindle shaped streaks and stripes on the pseudostem and traveller's palm like leaf orientation. In severe cases, the leaves show the particular broken streaks along the primary veins, which seem to be sporadically thickened or raised. Dispersed white to yellowish streaks opposite the midrib to the edge of the leaf were also observed during severe stages. The key identifying symptoms of the disease are unusual reddish brown or necrotic streaks in the bracts of the inflorescence (Singh *et al.*, 2000). The infected plant flowers, but produce very small

bunches with curved brittle fruits. Severely affected plants may fail to flower and may die by stunted growth and necrosis of pseudostem. The male buds turn dark purple in colour and mosaic patches are also observed. There are varietal differences in the symptomatology of the disease; BBrMV is reported to cause considerable damage in Cavendish banana plantations. The disease has a great impact on the yield of the crop with maximum yield reduction in cv. Robusta (AAA) (70%), followed by cv. Nendran (AAB) (52%) (Cherian *et al.*, 2002). Besides yield reduction, the fingers become malformed and curved, which reduce the market acceptability of the fruits (Cherian *et al.*, 2002).

2.3 STRATEGIES TO COMBAT VIRAL DISEASES

Plants face a number of biotic stresses and among them viral diseases are the most devastating resulting in reduced growth and development finally affecting their yield. It is recorded that the damages caused by viruses exceeds other pathogens (Simon-Mateo and Garcia, 2011). Due to these reasons viruses are extensively studied. Presently over 6000 viruses are identified and have been studied throughout the world and among them 1300 are plant viruses (King *et al.*, 2012). Many strategies have been put forward to combat viruses, which include conventional breeding programs using natural sources of resistance and non-conventional breeding techniques like creation of transgenic plants with artificial coat protein, movement protein and *R* genes.

2.3.1 Virus resistant transgenic plants

Inducing genetic resistance in plants is one of the approaches to protect plants from virus attack. For this, virus resistance genes (*R* genes) present in the wild relatives of plants were considered. Over 200 *R* genes are reported in crops and their wild relatives which have been tagged with molecular markers including RFLP, AFLP, RAPD etc. For example, the *Bdv1* allele of wheat is linked to *R* gene *Lr34* and

Yr18 which is resistant to *Barley yellow dwarf virus* (BYDV) (Kumar *et al.*, 2014). The dominant (Hypersensitive response) alleles of *R* genes *viz.*, the *N* gene of tobacco, *Rx1* and *Rx2* of potato, $Tm2^2$ of tomato were considered to develop virus resistant varieties against *Tobacco mosaic virus, Potato virus X* and *Tomato mosaic virus* respectively.

However, the durability of this technique is entirely based on the frequency of mutations in viral genome against such resistant genes. Therefore the credibility of genetic engineering in imparting viral resistance in plants reduced over time.

2.3.2 RNA silencing mechanisms

The discovery of RNA interference (RNAi) technology or gene silencing by Andrew Fire and Craig Mello (1998) brought into light a newer concept to combat viral infection. Gene silencing/RNA interference can be explained as the regulation of the expression of certain genes at particular points in a plant's developmental phase. It occurs during the transcriptional or translational phase and it is also known as gene knockdown. It was first discovered in nematode *Caenorhabditis elegans*. The process of gene silencing is done by two small RNAs which are miRNA and siRNA. The RNAi technology in plants is called as post transcriptional gene silencing (PTGS) (Napoli *et al.*, 1990). It was first observed in petunia hybrids transgenically expressing the chalcone synthase gene.

There are three main gene silencing pathways which are miRNA mediated gene silencing, siRNA directed RNA degradation pathway and siRNA directed DNA methylation. Among these techniques miRNA mediated gene silencing assists the entry of dsRNA into the cytoplasm, which is then divided into smaller fragments with the help of Dicer like enzymes (DCL 1). These double stranded fragments can be either siRNA or miRNA.

2.4 miRNA

MicroRNAs are small noncoding regulatory RNAs which take on a fundamental role in eukaryotic post-transcriptional gene regulations by either cleavage or translational hindrance. Plant miRNAs have been known to involve in several developmental and formative processes which reach out past their capacity to respond to biotic and abiotic stress, thus indicating its involvement in plant's biotic and abiotic stresses, not just being constrained to embryogenesis, plant morphogenesis and proliferation.

Plant miRNAs are involved in modifying and restricting the expression of transcription factors and genes in plant development pathway. In addition, a number of miRNAs function as part of hormone response pathways such as the auxin mediated lateral root development pathway. They have short sequences of about 18-22nt in length, have hairpin or stemloop structure (Xia *et al.*, 2018).

In plants, MIR gene is transcribed by RNA polymerase II enzyme to form pri-miRNA. Pri-miRNA is cleaved by a member of the RNase III class enzyme, Dicer-like enzyme 1 (DCL1) to generate precursor miRNA, which contains miRNA/miRNA* sequences with a hairpin or stemloop structure (Bartel, 2004). The duplex is sliced out from nucleus to cytoplasm by HASTY, the plant orthologue of exportin V protein (Park *et al.*, 2005). In cytoplasm, pre-miRNAs are unwound into miRNAs and causes gene regulation by entering into RNA-induced silencing complex (RISC), a ribonucleoprotein complex, and this complex is assisted by Argonaut proteins. Argonaut proteins contain a guide strand RNA which leads the miRNA to bind to mRNA .One strand stands for binding and the other act as a passenger strand. The guide strand acts as gene silencing weapon once the double strand is degraded by helicase.

Biogenesis of animal miRNAs differs from plant miRNAs. In animal miRNA biogenesis, pri-miRNA is cleaved to pre-miRNA by Drosha RNase 3 endonuclease whereas in plants it is by Dicer like enzymes (DCL 1), pre-miRNA are transported

from nucleus to nucleus to cytoplasm by Exportin V and then form miRNA;miRNA duplex in animals (Hammond et al., 2000).

2.4.1 Evolution of miRNAs

miRNAs as gene regulators dates back over 400 million years ago in *Chlamydomonas reinhardtii,* which is a unicellular green algae (Zhang *et al.*, 2006), suggesting that miRNA pathway is an ancient system of gene regulation. It also indicated the occurrence of this mechanism before the evolutionary arrival of multicellurarity. This information gave concrete evidence that miRNA has a common ancestor (Zhao *et al.,* 2007).

According to a report by Floyd and Bowman (2004) two miRNAs capable of controlling the genes from HD-Zip (homeo domaine-leucine zipper) family in *Arabidopsis thaliana* are known to be conserved in all plant species. Since miRNA do not form a perfect homology with their target mRNA they were not thought to affect the stability of mRNA, but their studies have proved that they cause translational inhibition or cleavage in their target genes (Chendrimada *et al.*, 2007; Eulalio *et al.*, 2007). However, this also gave conclusive evidence as to the involvement of more than one post transcriptional gene modification method (Eulalio *et al.*, 2007; Dorner *et al.*, 2007).

Though no conservation exists between plant and animal miRNAs, connections between distant plant families exists. Several computational studies and prediction analysis have proved the evolutionary link between several major plant species because of conserved miRNA sequences. (Zhang *et al.*, 2005; Zhang *et al.*, 2006). However there exist variations in their mode of activation and function in different plant species.

According to a study conducted by Achard *et al.* (2004) in Arabidopsis, miR159 was controlled by the higher levels of gibberellins. However no such

changes were observed in miR159 of rice seedlings when treated with gibberellin (Tsuji *et al.*, 2006) which proved that miRNA of diverse species need not give the same response. There also exists specific and novel miRNAs for each plant species performing specific functions.

The miRNA gene's evolution process is similar to the evolution of protein genes, it was made possible through genome wide duplication, tandem duplication etc, followed by the diversification of species (Ma *et al.*, 2004). miRNA also performed anti-viral defense response in ancient plants and its novelty only came in picture once all necessary criteria for survival were met (Maher *et al.*, 2006).

2.4.2 Role of miRNA in biotic stress in plants

Biotic stress is a major concern due to the ever changing evolutionary advantage of biotic agents *viz.,* bacteria, fungal, viruses, insects and nematodes. In order to compensate this problem, plants have developed sRNA mediated RNAi mechanism.

During the normal functioning of a plant, the miRNA suppress the expression of stress responsive genes, however during stress, these miRNAs get downregulated. Often the pathogen miRNA interfere and suppress the action of stress responsive proteins, which may lead to susceptibility of the plant to the pathogen.

2.4.2.1 Role of miRNAs during virus infection

Plants usually function in two ways *i.e* they either create a direct attack on viral genes using endogenous miRNAs or they cleave miRNAs responsible for viral replication.

In a study conducted by Sheeba *et al.* (2013), three miRNAs (miR156, miR159, miR166) during *Banana streak mysore virus* (BSMysV) infection in banana

showed that symptom severity is correlated to the expression level of miRNAs and there was an increased expression of miR166 during the infection.

The miR168 accumulated during *Soyabean mosaic virus* strain G7 infection, got elevated and suppressed the translation of AGO1 mRNA (Pacheco *et al.*, 2012; Chen *et al.*, 2015; Varallyay *et al.*, 2017). AGO1 in *Malus hupehensis*, were also regulated by miR168 to resist the attack of *Botryosphaeria dothidea* (Yu *et al.*, 2017).

Nicotiana benthamiana when co-infected with *Potato virus X* (PVX) and *Plum pox virus* (PPV), altered the expression of miR156, miR171, miR398 and miR168 (Pacheco *et al.*, 2012).

In the susceptible varieties of *Vigna mungo,* miR156, miR159, miR160, miR166, miR398, miR1511, miR1514, miR2118 miR358 and miR169 were upregulated along with their target genes Auxin response factor (ARF), Auxin metabolic genes, Copper super oxide dismutase, NB-LRR, NAC, MYB, Zinc finger, CCAAT-box transcription factor and fructose 2–6 bisphosphate during *Mung bean yellow mosaic India virus* infection (Kundu *et al.*, 2017).

During *Mung bean yellow mosaic India virus* (MYMIV) infection in soyabean, gma-miR5787 disrupted the functioning of the viral genome and enhanced the production of AGO (Ramesh *et al.*, 2017).

The novel miRNA were accumulated along with precursors during *Rice stripe virus* (RSV), in rice, but their corresponding mRNAs were repressed (Wu *et al.*, 2015).

2.4.3 Co-evolutionary relationship of plant and viral miRNA

Plants have developed several mechanisms to combat viral diseases during the course of evolution. Along with plants, viruses have also derived a counter active measure to overcome RNA silencing. There are several host virus interactions, among them the most important are:

1) Host miRNAs targeting host genes
2) Host miRNAs targeting viral genes.
3) Viral miRNAs targeting host genes.
4) Viral miRNAs targeting viral genes to impart latency

Virus also produces non coding miRNAs called as viral miRNAs (vi-miRNA). These miRNAs responds to the host genes of plants and transforms the cell into a virus producing machinery. The first viral miRNA was reported in 2004 on *Epstein-Barr virus* (EBV) (Pfeffer *et al.*, 2004). vi-miRNA are also processed like eukaryotic miRNAs, with the help of DROSHA and DCL I. There are about 9133 vi-miRNA recorded in VIRmiRNA database. The data also includes 1908 confirmed vi-miRNA and 7283 target genes belonging to about 44 viruses (Qureshi *et al.*, 2014).

Five putative vi-miRNA were predicted from this database in *Hibiscus chlorotic ringspot virus* (HCRV). Though plants have homologous miRNA sequences among different plant species, there is no conservation of sequence information among vi-miRNA (Cullen, 2004). The vi-miRNAs are analogous to host miRNA mimicking their pathway resulting in host gene silencing (Kincaid and Sullivan, 2012).

The main purpose of vi-miRNA is to degrade mRNA/miRNA and to inhibit the formation of host miRNA so that virus can freely replicate. Throughout the evolutionary interaction between plants and viruses another important aspect developed by viruses are VSRs (viral suppressors of RNA silencing). However, it has

no sequence similarity across or within viral species. VSRs also inhibit the RNA silencing mechanism of their hosts by interacting with their siRNAs. They target conserved elements of RNA gene silencing like siRNAs or proteins like AGOs and DCL 1 (Burgyan and Havelda, 2011).

According to Azevedo (2010), P19 protein of *Tombusvirus* helps in arresting the expression of AGOs by enhancing the expression of miR168. miRNA/ siRNA mediated gene silencing technologies is in fact a co-evolution of the defense and counter defense mechanisms of plants and virus. VSRs interact with RNA silencing mechanism in many different ways and many are yet to be discovered

2.4.4 Artificial miRNAs in plants' anti-viral defense

Since plants do not have an immune system analogous to animals, it is necessary for plants to have an innate anti-viral defense system. Antisense suppression and RNAi are the main defense systems responsible for the viral defense mechanisms. siRNA/miRNA mediated gene silencing is the newly discovered process and is currently one of the most studied defense mechanism. For miRNA to function, a perfect match in the form of target mRNA is necessary which is based on the homology dependent gene silencing. This mechanism was first studied in transgenic tobacco plant containing 54kb sequence of *Tobacco mosaic virus* (TMV) containing miRNA sequence information (targets the infectious gene of the virus), no signs of infection were shown in the plant when the virus was transmitted (Carr *et al.*, 1992). Though simple in its code of conduct to withstand pathogens, it can backfire due to the high mutation frequency of viruses. However in the course of evolution siRNA mediated gene silencing emerged as a primary defense response to plants. The main advantage is the gain of immunity in some cells even when infection has spread to nearby cells. The theory was confirmed when a silenced rook stock was grafted with non silenced scions in Tobacco (Palauqui *et al.*, 1997).

In an experiment conducted by Lindbo and Dougherty (1992), transgenic tobacco plants with untranslatable sequences of coat protein gene of *Tobacco etch virus* (TEV) was created inorder to prevent the TEV replication. The plants developed immunity to the super infection of TEV and eventually became resistant to the virus. The normal plants have slower PTGS mechanisms which gives the virus sufficient time to co evolve however the transgenic plants have amplified signals which can prevent viral attack completely. Though many details regarding this technology are not yet determined, the potential possibilities of the miRNA to compete with virus is outstanding. The worth of this technology in the field of biotechnology was proved by the following experiment. A hairpin (hpRNA) construct was made from *Rice dwarf virus* (RDV). This gene construct was inserted into a vector of choice and the transgenic plants containing the vector showed high resistance to the virus (Ma *et al.*, 2004).

In a study conducted by Niu *et al.* (2007), miRNAs were modified to incorporate multiple precursors of virus inorder to compliment the target sequences of viruses *Turnip yellow mosaic virus* (TYMV) and *Turnip mosaic virus* (TuMV) in *Arabidopsis thalliana*. The genetically engineered Arabidopsis with these sequences showed explicit immunity against these viruses. Another study conducted on miR393, a host derived miRNA helped to modify the auxin signaling pathway to induce bacterial resistance (Navareo *et al.*, 2007).

The *Plum pox virus* was found to be repressed by plant derived miRNA during infection (Simon-mateo and Garcia., 2006). Arabidopsis miRNA (miR171, miR167, and miR159) target sequences incorporated into the *plum pox virus* was regulated by *Nicotiana clevelandii* and *Nicotiana benthamiana* miRNA during its infection (Simon-Mateo and Garcia., 2006).

During *Begomovirus* infection several developmental processes were upregulated by the miRNA leading to suppression of viral targets (Amin *et al.*, 2011).

The miRNA expression in rice during infection of *Rice stripe virus* (RSV) and *Rice dwarf virus* (RDV) showed that RSV virus enhanced the production of miRNA during infection (Du *et al.*, 2011)

The miRNAs, miR482 and miR2118 were found to target different *R* genes in potatoes, soyabean and *Medicago truncatula* (Zhai *et al.*,2011). In a study conducted by Li *et al.* (2012), nta-miR6019 and nta-miR6020 were found responsible for inducing disease resistance in *Nicotiana benthamiana* against TMV virus.

In a study conducted by Shekhawat *et al.* (2012), transgenic banana resistant to *Banana bunchy top virus* was created using two intron-hairpin RNA (ihpRNA) namely ihpRNA-ProRep and ihpRNA-Rep. The ihpRNA in the transformed banana plant represents the viral master replication initiation protein (Replicase gene) of BBTV.

Three miRNAs, miR169, miR156, miR2118 were found upregulated during drought stress and miR169 acted as a transcription factor for activating the dehydrin and aquaporin gene in banana cultivar, 'Saba' (Muthuswamy *et al.*, 2014). Several morphological changes were reported in transgenic banana plants over expressing MusamiRNA156 (Ghag *et al.*, 2015).

In *Pyrus pyrifolia,* a number of miRNA target genes were identified which were responsible for disease resistance and defense in the high temperature induced reduction in *Apple stem grooving virus* titers (Liu *et al.*, 2015). Studies also showed that certain miRNAs were down regulated (miR171e, miR160b and miR159f) and miR164b was upregulated in response to *Pernosclerospora cubense* infection (Weibo *et al.*, 2015).

In another experiment conducted by Wagaba *et al.* (2017) artificial miRNAs were constructed in *Nicotiana benthamiana* to combat *Ugandan cassava brown streak virus* which causes Cassava brown streak disease. When these miRNAs were expressed they inhibited the development of the virus and caused degradation.

2.5 COMPUTATIONAL PREDICTION ANALYSIS OF miRNA AND THEIR TARGETS

Complementarity between miRNA and target genes has helped the researchers to predict, validate and understand their role during plant metabolic processes. The prediction studies were focused on several algorithms which scan the genome for miRNA – mRNA complementarity. Over the years these prediction studies were refined to such a level providing more precision to the results. The miRNA has a tendency to target multiple mRNAs due to its sequence homology; plant miRNAs has a tendency to target transcription factors as they are the main elements in gene control.

Many technologies are used for the determination of miRNA among them direct cloning and deep sequencing are widely used along with homology based comparative analysis using expressed sequence tags (ESTs) and genome survey sequences (GSS). Since a lot of these miRNA sequences are conserved among different species of plants (Zhang *et al.*, 2006), the information is used for identification and prediction of these miRNAs. The miRNA predicted through ESTs can be further recovered using high throughput deep sequencing. For species whose sequences are not available, sequence databases for ESTs, GSS and BACs in public domain can be used for studying miRNA of that particular species.

Recently computational genome wide prediction of miRNAs of several plant species was conducted. Basically there are two methods to predict miRNAs, genetic approach using sequencing and computational approach where homology based

studies using ESTs and GSSs and bioinformatic prediction analysis are used (Chai *et al.*, 2014).

2.5.1 Computational method

2.5.1.1 Homology based analysis

Comparative genomic analysis helps in the prediction and identification of miRNA in non-model plants. For this ESTs and GSSs of the non model plants are utilized in homology based search against the sequenced model plants (Dehury *et al.*, 2013). The advantage is that the search algorithm uses genome matching hits between miRNA and query sequences and there are separate search for GSSs and ESTs. The BLAST analysis is compared against known unique miRNAs sequences of plants downloaded from EST database (dbEST), miRBase or GENBANK. The BLAST result with 0-3 mismatches against known miRNA is used for analysis. These sequences are again blasted against the protein database in order to pull out coding sequences. Once the results are obtained the sequences are screened to fullfill a set of criteria 1) they should be 18nt long 2) only 2-3 mismatches in sequence compared with all other known miRNA 3) in secondary structure and hairpin prediction analysis they should have 3.2 minimal folding energy (MFE) (Pani and Mahapatra, 2013). Similar works were conducted on tobacco using the known miRNA sequences of Arabidopsis, rice and poplar. Comparison was done between 349 known miRNAs against 14,20,579 GSS sequences of tobacco (NCBI) (Frazier *et al.*, 2010).

The homology based search was done for *Ecliptta prostrate,* BLAST analysis were conducted against 4802 known mature miRNAs of the plant kingdom with 84 GSS sequences of *Eclipta prostrate*. About 8 miRNAs were found out using this tool.

In an experiment conducted by Barozai *et al.* (2018), about 158 new miRNAs were found belonging to 83 families of switch grass (*Panicum virgatum* L.). The

ESTs and GSS sequence of the plant were compared against 5,744 known sequences of plant miRNAs from miRBase.

2.5.1.2 Bioinformatic prediction studies

Compared to homology based comparative analysis bioinformatic tools predict miRNAs with very high efficiency and accuracy (Dong *et al.*, 2012). Presently there are many computational tools which can identify novel miRNAs. The features used by these tools in predicting miRNAs are their length, sequence conservation or similarity among species, hairpin formation and minimal fold energy (Li *et al.,* 2010). The most important tools used to predict miRNAs are NOVOMIR, MiPred, MIR-PD and MIRFINDER.

NOVOMIR is a widely used and popularized tool that uses a set of criteria to screen miRNAs from the genome of an organism. The process is followed by a sophisticated statistical model which can predict pre-miRNAs from other miRNAs, here the genome becomes the input. The standard used here is *Arabidopsis thaliana* miRNA (positive control), tRNA, mRNA, ncRNA and DNA sequences is negative control. The sensitivity of the software is about 0.99 and specificity is 0.83. NovoMIR depends on RNA fold and RNA shapes for predicting secondary structure of RNA (Teune and Steger, 2010). They did the first prediction studies using NovoMIR in Arabidopsis genome. Over 1,477 potential miRNAs candidates were obtained.

In a study conducted in College of Agriculture, Vellayani during 2016-2018, 52 mature miRNA were predicted using NOVOMIR software and about 124 corresponding target genes were predicted using psRNATarget tool in banana genome (Mathew, 2018). These *in silico* predicted results were utilized for the present study by validating its expression through Real-Time PCR.

2.6 EXPRESSION ANALYSIS OF PREDICTED miRNAs

Identification and prediction of miRNA using computer softwares and bioinformatics tools is an indispensable approach in finding miRNA in plants and confirming these computationally predicted miRNA using wet lab procedures is essential to rule out false positives. There are two processes in which we can confirm miRNA they are: evaluation of host miRNAs target sequences and creation of amiRNA sequences (Watanabe *et al.*, 2007). The experimental validation of miRNAs includes creation of gene specific target primers and stemloop primers for miRNAs. RNA from plants are isolated and is converted to its corresponding cDNA. Real-Time PCR is carried out using cDNA samples with the gene specific primers, this process will give a complete knowledge regarding the expression profile of miRNAs. The study will be focused on the correlation between miRNA and their corresponding target genes during viral infection.

2.6.1 Expression profile analysis

The expression profiling of miRNAs helps to understand the regulatory behavior and metabolic activities of plants. By studying the genes expressed under difficult situations, their biological roles in plants during stress can be studied (Chen *et al.*, 2014). RT-qPCR is the most reliable method which can confirm and quantify the expression levels of the genes (Chen *et al.*, 2005). Though there is difficulty in extracting miRNA due to their small size and detection of the same is quite challenging, RT-qPCR devised by Chen *et al.* (2005) is highly sensitive and can provide an accurate result in quantification process.

In Real Time PCR, expression analysis is done through comparative or relative analysis between a stable gene like β-Actin (control) and gene of our choice. The results are demonstrated as fold change (increase or decrease).

In *N. benthamiana* and *A. thaliana,* the expression of miR168 and Argonaute 1 mRNA is up-regulated in response to infection by several plant viruses and was validated through the quantification analysis using RT-qPCR (Vaucheret *et al.*, 2006).

In a study conducted by Sheeba *et al.* (2013) two miRNAs, miR156 and miR159 were found to be increased in plants infected with *Banana streak mysore virus* (BSMysV). During the infection, miRNA was accumulated in abundance at and it was confirmed through Real-Time PCR.

Sly-miR6022, -miR6023/ -miR6024 and –miR6027 were found to be up-regulated during the *Tomato yellow leaf curl Sardinia virus* (TYLCSV) infection in tomato plants using RT-qPCR. These miRNAs target almost all genes belonging to R-gene families which codes for *Hcr9* resistance proteins and *Sw5* resistance protein indicating that the resistance genes in tomato during TYLSCV infection are suppressed (Miozzi *et al.,* 2014).

RT-qPCR results in cucumber showed that the expression of miR159f which targets Auxin response factor ARFI6, was found to be upregulated during *Pseudomonas syringae* infection to impart resistance (Weibo and Fangli., 2015).

In shoot apices of *Pyrillus pyrifoilus*, negative correlations were obtained for miRNAs novel345, novel482, and novel188. Target gene 2-oxoglutarate dehydrogenate mitochondrial-like protein (by novel345), disease resistance protein RGA2-like (by nove l482), and squamosa promoter-binding-like Protein13A (by novel188) were all up-regulated with relative quantitive values of 1.8, 1.42, and 2.4 in the 37 °C treatment, respectively (Liu *et al.*, 2016).

In banana var. 'Nendran' infected with *Banana bract mosaic virus* (BBrMV), three miRNAs miR-3900-5p, miR-9112 and miR-5417 were found to be upregulated and it had a positive correlation with its corresponding targets F-box/kelch-repeat

protein, Cytochrome oxidase subunit 5B protein and Glyceraldehyde-3-phosphate dehydrogenase respectively (Mathew, 2018). The following analysis was done using RT-qPCR.

2.7 SELECTION OF BIOTIC STRESS RESPONSIVE miRNAS

For the present study "Identification and characterization of virus responsive miRNAs in banana *Musa* (AAB) 'Nendran'" five miRNAs were selected from the predicted list of 52 miRNAs. The selection was based on the biotic stress responsiveness of the target genes. miR-3900-5p (targets: Plant viral response family protein gene and Putative disease resistance protein gene), miR-2172-5p (target: Putative Ethylene-responsive transcription factor 1 gene), miR-5417 (target: Stress-associated endoplasmic reticulum protein 2 gene), miR-6928-5p (target: FAD dependent oxidoreductase gene) and miR-971-5p (targets: Argonaute 1A protein gene and Transport inhibitor response 1-like protein gene) were the selected miRNAs.

2.7.1 Expression of proteins during biotic stress

Defense mechanisms in plants are confined to a form of hypersensitive response where the pathogen is localized within a set of necrotic tissues, such a mechanism acquired by the plant once encountered by a pathogen results in enhanced resistance. It also provides another layer to the pathogen and plant interaction which triggers a form of primary defense response formed only during the induction of a viral attack. The metabolic alterations formed during biotic stress are production of ethylene, activation of pathogenesis related proteins, induction of phytoalexins, hormonal regulations etc. Plants also have sophisticated system of sensory proteins which act as receptors that functions based on the presence of a hypersensitive response (HR). This response is activated in the presence of a reactive oxygen species (ROS) which in turn activates pathogenesis related proteins. These defense proteins protect the plants from several biotic and abiotic stresses (Saravanakumar and Samiyappan, 2007). Pathogenesis related proteins are defined as proteins which are

produced by plants to combat biotic stress. Other group of proteins which play a major role in anti viral RNA silencing pathways include Agronauts (AGOs) and plant Dicer proteins (DCL).

Many transcription factors are also involved in the activation of the said proteins, such as the ethylene responsive transcription factor which is produced due to the excess amounts of ethylene produced during a stress situation (Takagi and Shinshi, 1995).

2.7.2 Pathogenesis related proteins

The PR proteins were first identified in tobacco, where it showed hypersensitivity to *Tobacco mosaic virus* (TMV). Currently it has been detected in over 16 plant species. They consist of a set of 4 families of chitinases (*PR-3, 4, 8* and *11*), three glucanases (PR-2), one proteinase inhibitor (*PR-6*). Not every PR genes are expressed in a plant, each species have different set of genes. However collective function of these PRs is effective in delibilitating the growth and multiplication of viruses and it is also responsible for the state of induced resistance in plants (van Loon, 1997). PR proteins are not limited to infected leaves where the virus is localized, but appear in the rest of the plant, which remains without symptoms. Therefore, necrosis is not a necessity for the induction of PR, but when it comes to necrosis, PR production is increased.

A study conducted by Harish *et al.* (2009) pathogenesis related proteins and defense related enzymes were produced in abundance in BBTV infected banana var. Robusta. A high production of phenol, PAL, 1, 3-gucanase was reported when these plants were infested by viruliferous aphids. This is because some genes go through rapid activation at the time of infection while others are activated slowly. Plant viral response protein gene and Putative disease resistance protein gene belong to the family of *R* genes, which are responsible for inhibiting the growth and development of pathogens (Amaral *et al.,* 2006). Plant viral response family protein gene and

Putative disease resistance protein gene (target of miR-3900-5p) were analyzed in the present study.

2.7.3 Ethylene and GCC box of PR proteins

GCC Box is found in the promoter regions of many genes that interact with pathogens. Biosynthesis of ethylene accelerates during virus infection (Biotic stress) and activates the production of transcription factors which binds to the GCC Box of PR genes (Takagi and Shinshi, 1995; Zhou *et al.*, 1997) A number of proteins that bind to the GCC boxes were isolated and observed to be members of the EREBP (ethylene-responsive element binding protein) which is a family of DNA-binding proteins (Zhou *et al.*, 1997; Stockinger *et al.*, 1997; Lorenzo *et al.*, 2003). Thus the plant gains resistance by activating the signal pathway that runs through the EREBPs to the activation of PR genes that contain the GCC boxes. The role of Putative Ethylene-responsive transcription factor 1 gene (target of miR-2172-5p) in BBrMV infection was analyzed in the present study.

2.7.4 Role of Transport inhibitor response in stress

The gene, Transport inhibitor response 1like protein, acts as a auxin receptor that controls Aux/IAA protein's proteosomal degradation. It is involved in regulating the root length, cell elongation, lateral root formation etc. It also plays an important role in ethylene signaling in roots during a pathogen attack (Katagiri *et al.*, 2002). The role of Transport inhibitor response 1 like protein gene (target of miR-971-5p) during BBrMV infection was studied in the present research work.

2.7.5 Argonaute protein

Argonauts (AGOs) are proteins which is a part of RISCs. It acts as the molecular platform for RNA silencing machinery. These complex proteins play a vital role in plant defense response through post-transcriptional gene silencing

(PTGS) of host and pathogen genes. Antiviral defense is one of the most important aspects of AGO proteins and it is focused on RNA and certain DNA viruses.

AGO proteins has two Asp and one His residue (Asp–Asp–His) or three Asp residues (Asp–Asp–Asp) in its active catalytic sites. They bind to the miRNA of the virus thus facilitating the complementary binding of target RNA. They interact and combine, allowing the target ssRNA to be cleaved by the RNase H of AGOs. These proteins can damage viral replication and translation *via* manipulation and regulation of defense genes. AGOs can also improvise gene silencing using cleaved RNA fragments which act as substrates for RDR. The antiviral property of the AGO proteins relies on factors such as levels of expression, localization of tissue and cells, AGO cofactors, sRNA binding sites etc (Garcia-Ruiz *et al.,* 2015). The gene, Argonaute protein was considered as target for miR-971-5p in the present study, so as to understand their expression levels during BBrMV infection.

2.7.6 FAD dependent oxidoreductase family and role in oxidative stress

FAD (flavin adenine dinucleotide) is a form of redox-active flavoprotein coenzyme involved in many enzymatic reactions. They are also involved in the production of ROS species in cell. Flavins, including FAD, have a tendency to auto-oxidize oxygen molecules to superoxides during abiotic and biotic stress (Massy, 1994).

In *Mycobacterium smegmatis,* the gene MSMEG_5243 was found to be upregulated during hypoxia. MSMEG_5243 is a flavin sequestering protein (*Fsq*) which comes under the superfamily of FAD dependent oxidoreductase. It prevents the auto-oxidation of FAD molecules during stress by forming inert *Fsq*:FAD complexes (Harold *et al*., 2018).

The importance of FAD dependent oxidoreductase gene (target of miR-6928-5p) in the present study was to understand their role in inducing ROS during BBrMV infection.

2.7.7 Stress associated endoplasmic reticulum protein

The main role of endoplasmic reticulum is to secrete protein responsible for cellular functions. The proteins once secreted are passed through a series of quality checks, ER quality control (ERQC), where the protein foldings are assessed. The defective proteins are eliminated through ER associated degradation (ERAD) (Li and Liu, 2014). The stress associated protein or the bZIP factor is tethered to ER during normal functioning of the cell, but is mobilized once stress has been detected (Srivastava *et al.*, 2014). The bZIP factor activates unfolded proteins response (UPR) and the ERAD system to remove degraded and misfolded proteins. The organelle at this stage goes into overdrive to produce large quantities of heat shock proteins and LEA proteins to protect the cell from stress conditions (Tintor and Saijo, 2014).

However, several viruses have evolved sophisticated techniques to modify the UPR and ERAD systems to produce viral encoded proteins in infected cells. This up-regulates the cellular chaperon in aiding the virus protein folding process. The gene Stress associated endoplasmic reticulum protein gene (target of miR-5417) was considered in the present study to understand their expression levels during BBrMV infection.

Materials and Methods

3. MATERIALS AND METHODS

The study entitled "Identification and characterisation of virus responsive miRNAs in banana *Musa* (AAB) 'Nendran'" was conducted at the Department of Plant Biotechnology, College of Agriculture, Vellayani, Thiruvananthapuram during 2017-2019. The detailed description of the methodologies followed for this research is mentioned below:

3.1 SELECTION OF miRNAS FOR VALIDATION

In a study conducted in Department of Plant Biotechnology, 52 mature miRNAs were predicted using NOVOMIR software and over 142 targets were predicted for these miRNAs using psRNATarget in banana genome. From the computationally predicted miRNAs, five miRNAs were selected for this study, based on the role of their target gene in stress conditions (Table 1).

3.2 PRIMER DESIGNING

3.2.1 Designing of Stemloop primers for miRNAs

miRNAs are difficult to extract from the total RNA because of their small size (18-22nt). Stemloop primers are used for easy extraction of miRNA. Stable stem loop sequence of 44nt was designed by Chen *et al.* (2005) in order to lengthen the target cDNA. The miRNA specific stemloop was designed by combining stem-loop sequence (44 nt) with the reverse complement of the six 3′ nt of the mature miRNA sequence (Chen *et al.*, 2005) (5′- GTC GTA TCC AGT GCA GGG TCC GAG GTA TTC GCA CTG GAT ACG AC -3′).

3.2.2 Designing of primers for the amplification of miRNAs

miRNA specific forward primer was designed by taking the first 12 to 17 nt of the 5′ end of the mature miRNA and adding 5 to 7 additional nucleotides at 5′ nt for

adjusting the Tm to 60°C ± 1° C. The reverse primers were designed specific to the 44nt stemloop sequence proposed by Chen *et al.*, (2005). The reverse primer is universal for all miRNA (5^{I}–CCAGTGCAGGGTCCGAGGTA-3^{I}) (Kramer, 2011). The forward and reverse primer compatibility and secondary structure formation was verified with Eurofin Genomics oligo analysis tool software (Fig. 1) (https://www.eurofinsgenomics.eu/en/ecom/tools/pcr-primer-design/).

3.2.3 Primer design for target gene

Primers specific to target genes were designed using Primer3Plus software (Untergasser *et al.*, 2007) (http://www.bioinformatics.nl/primer3plus). The software comprises of an input box for sequence information and they are pasted in FASTA or EMBL format in the space provided (Fig. 2). The region required for amplification was selected using '[ ]' indicated below the dialogue box. Default parameters like length: 20-22nt, GC%: 50-60%, Melting temperature: 55- 65°C were adjusted and primers were designed (Fig. 2).

The specificity of primers was checked through NCBI Primer-BLAST software (Fig. 3). The software was specifically designed by NCBI which utilizes the BLAST program and global alignment algorithm to pick primers against the target gene sequence. It also checks the compatibility of the primers during PCR *i.e.*, to find out primer pairs causing non-specific amplifications (Ye *et al.*, 2012)

Table. 1. List of miRNAs selected for the study and their corresponding targets

Sl. No	miRNA family	miRNA Aligned Fragment	Target Aligned Fragment	Target Description	Target function
1	miR-3900-5p	CAAGGAGGU GUCACCGCC	GGCUGUGGC ACCUCUUUA	plant viral response family protein gene	Anti-viral action
		CAAGGAGGU GUCACCGCC	GUCGGUGAC AUCUCGUUG	Putative disease resistance protein gene	Anti-viral action
2	miR-6928-5p	GGGGAUUUU CAAGUACUG CA	UGCAGCAUU UGAAGAUCC UC	FAD dependent oxidoreductase gene	ROS production
3	miR-5417	AGGGGAGAA AUGGGGAUG	CGUCUCCGU CUCUCCCCU	Stress-associated endoplasmic reticulum protein 2 gene	Signals stress protein production
4		UUUGAUGAU UUGAAUUUA	CAAAUUCAA GUCAUUGAG	Transport inhibitor response 1-like protein gene	Reduces hormone availability to pathogen
	miR-971-5p	UUUGAUGAU UUGAAUUUA	UAGAAUCAA AUUAUCAAC	Protein argonaute 1A gene	RISC formation
5	miR-2172-5p	GAAAUUAUG AUUAUGCAU	AUGAUAAUC AUGAUUUU	Putative Ethylene-responsive transcription factor 1 gene	Activates pathogenesis related proteins

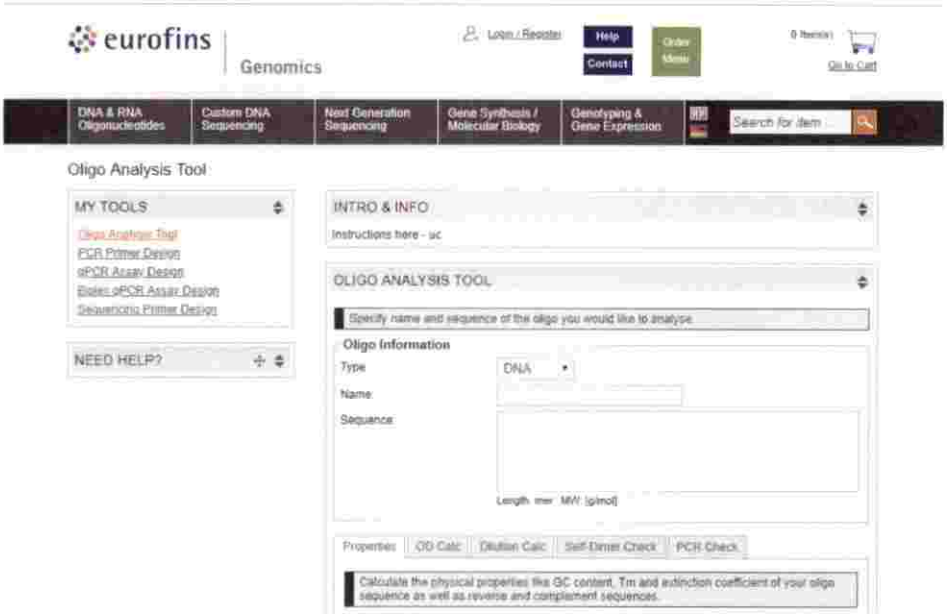


Fig. 1. Home page of Eurofins Genomics oligo analysis software.

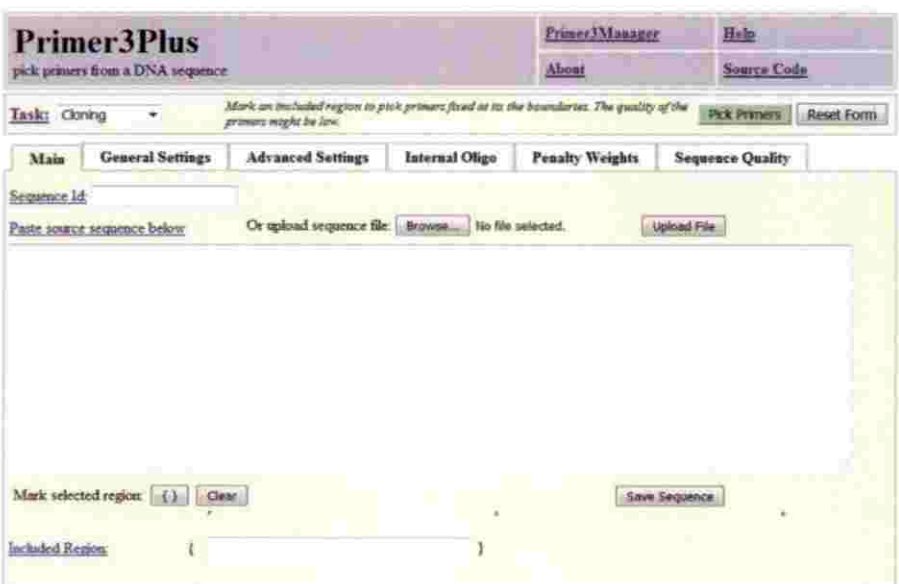


Fig. 2. Home page of Primer3Plus software

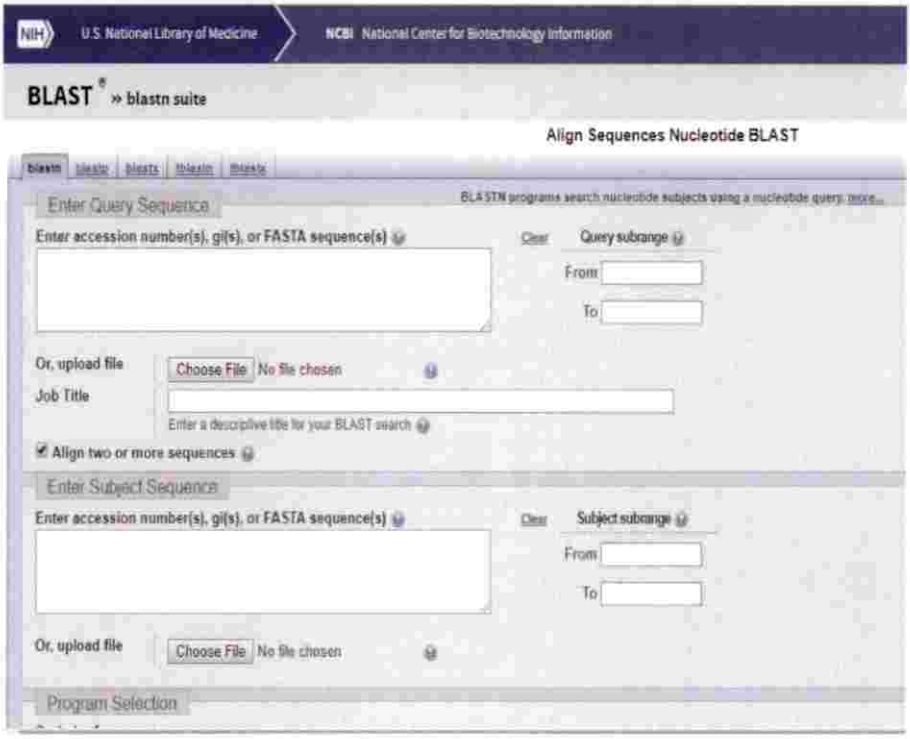


Fig. 3. Home page of NCBI Primer BLAST software

3.3 EXPERIMENTAL VALIDATION OF THE miRNAS AND THEIR TARGET GENES

3.3.1Collection and maintenance of tissue culture plants

Eight *in vitro* raised three month old banana var. Nendran plants were collected from Biotechnology Model Floriculture Center, Kazhakuttam. The plants were maintained in glasshouse and watered regularly to ensure succulence for vector transmission (Fig. 4).

3.3.2 Rearing of healthy aphids

Aphids (*Pentalonia nigronervosa*) were collected from the banana plants in Instructional farm of College of Agriculture, Vellayani, using a zero tip HP brush. The plant was slightly tapped before collection and the aphids that have stopped feeding were collected using the brush. The aphids were transferred to 5 to 6 month old healthy banana var. Nendran suckers in the glasshouse (Fig. 5). The aphid colonies were transferred to new healthy banana plants once every two months in order to maintain a thriving and vigorous aphid population.

3.3.3 Identification of infected sucker

BBrMV infected banana sucker was identified from Instructional farm of College of Agriculture, Vellayani, based on its symptoms (Fig. 6). The BBrMV infection was reconfirmed by PCR. The RNA from the BBrMV infected plant was isolated from its leaves and converted into cDNA. The BBrMV infection was detected using primers designed for its replicase gene. The sequences of replicase specific primers are given below:

Forward – 5' AGCAATGTACGCTGGGAAGA 3'
Reverse – 5' TCCGTTCCATATGCCTAAGTG 3'

PCR mix included the following components:

10X reaction buffer (1X)	2 µl
dNTP mix (100 µM each)	1 µl
Forward primer (10 µM)	1 µl
Reverse primer (10 µM)	1 µl
Taq DNA polymerase(1U/µl)	0.45 µl
cDNA (200ng/ µl)	1 µl
Nuclease free water	13.5 µl

The thermal profile used was as follows:

Step 1: 95°C for 3 mins

Step 2: 95 °C for 15s

Step 3: 55 °C for 15s

Step 4: 72 °C for 45s. The steps 2 to 4 were repeated for 30 cycles

Step 5: 72 °C for 5 mins

The integrity of the PCR products was checked using horizontal gel electrophoresis. 1gm of agarose powder was melted in 100 ml of 1X TBE buffer (Tris base 10.8 gm, Boric acid 5.5 gm, 0.5M EDTA 4 ml). 3µl ethidium bromide (EtBr) was added to the melted gel mix while lukewarm and poured into the casting tray. Comb was inserted into the gel based on the number of samples. After solidification, the comb was removed and the gel was transferred to the electrophoresis tank, which contained 1X TBE buffer. The PCR samples were loaded along with 6X gel loading dye (Bromo phenol blue). The gel was run at 75 V till the loading dye reached three-fourths of the gel. After electrophoresis was completed, the gel was documented by using Gel Doc Unit (Bio-Rad). After confirmation the infected sucker was maintained in the glasshouse for acquisition feeding.

Fig. 4. Three month old healthy tissue culture banana plants.

(a)

(b)

Fig. 5 . (a) and (b) the BBrMV infected field plant.

Fig. 6. Aphid colony in a healthy banana sucker.

3.4 EXPRESSION PROFILE OF miRNAS AND TARGET GENES DURING BBrMV INFECTION

3.4.1Vector transmission

The aphids were reared on healthy banana suckers. For vector transmission, they were collected from the suckers, starved for 30 min and transferred to BBrMV infected sucker for acquisition feeding for 2 h. After that, fifteen viruliferous aphids were released on to the leaf axils of healthy tissue culture plants with four replicates. The aphids were killed after 24 h using 0.1% quinalphos. Four uninfected plants were kept as control.

The leaves from the infected plants were collected at four time intervals *viz.*, 24 h, 48 h, 1 wk and 2 wk after infection.

3.4.2 RNA isolation

RNA was isolated from the leaf samples of both healthy and infected plants using modified CTAB method (Tsai and Harding, 2003). Four ml of the RNA extraction buffer was pre-warmed to 65° C in a 15 ml centrifuge tube. 0.5 gm of the leaf sample was ground to fine powder with liquid nitrogen in mortar and pestle and the contents were transferred to the 15 ml centrifuge tube containing the pre-warmed buffer. To the extract, 120 mg of PVP and 60 µl of mercaptoethanol were added under the chemical hood and mixed well. The tube was incubated in waterbath for 10 min at 65° C. To the lysate, 850 µl of chloroform: isoamyl alcohol (24:1) was added and centrifuged at 16000 g for 20 min at room temperature. The supernatant was transferred to a fresh tube and 850 µl of chloroform: isoamyl alcohol was added again. The mix was centrifuged at 16000 g for 20 min at 4° C. To the supernatant, 3M LiCl was added and kept overnight at -20° C. The RNA was extracted from the mix by centrifuging at 16000 g for 20 min at 4°C. The supernatant was discarded and the pellet was suspended with 100% ethanol twice at 16000 g for 10 min at 4°C. Ethanol

was removed carefully and the pellet was air dried under the LAF (laminar air flow) for 30 min. The pellet was dissolved in 30 µl DEPC water and stored in -80° C.

3.4.2.1 Qualitative Analysis of RNA

Gel electrophoresis was used to determine RNA quality. It was carried out by using horizontal electrophoresis unit. The electrophoresis was carried out in 2% agarose gel with 3 µl EtBr. The gel was run at 75 V till the gel loading dye reached three-fourth of the gel. The final results were viewed in BIO RAD's Gel Doc system.

3.4.2.2 RNA Quantification

Absorbance of RNA samples at 24 h, 48 h, 1 wk and 2 wk was taken to determine the quality and quantity of RNA. RNA quantification was done by using spectrophotometer at 260 & 280 nm. Absorbance value of 1.0 at 260 nm indicates presence of 40 µg ml^{-1} of RNA. Concentration of RNA was calculated by using the formula:

Conc. of RNA (µg ml^{-1}) = A_{260} × 40 µg ml^{-1} × dilution factor

A_{260}/A_{280} ratio indicates the quality of RNA. Ratio ranging from 1.8 – 2, indicates good quality RNA.

3.4.3 cDNA conversion

The RNA of both healthy and infected plants was converted to their complementary DNA using Verso cDNA synthesis kit. A 20 µl reaction mix was prepared using the following components:

cDNA synthesis buffer	4 µl
dNTP (500 µM)	2 µl
Oligo dT primer(10 µM)	0.5 µl
Random hexamers (400 ng/ µl)	0.5 µl
RT enhancer	1 µl
RNA (1ng/ µl)	5 µl
Reverse Transcriptase	1 µl
Water	8 µl
Total volume	20 µl

The contents were mixed well by spinning and PCR was carried out in BIO RAD thermal cycler. The thermal profile for the reverse transcriptase reaction of target gene was:

42 °C for 30 min

92 °C for 2 min.

For the reverse transcription of RNA to stemloop primers, a 10µl reaction mix was concocted using the following components:

cDNA synthesis buffer	2 µl
dNTP (500 µM)	1 µl
Stemloop primers (10 µM)	0.5 µl
RNA Sample (1ng/ µl)	2.5 µl
Reverse Transcriptase enzyme	0.5 µl
Water	3.5 µl
Total volume	20 µl

The contents were mixed well by spinning and the PCR was carried out in BIO RAD's thermal cycler. The following thermal profile was used for this reaction:

16 °C incubation: 30mins,

Step 1: 30 °C for 30s

Step 2: 42 °C for 30s

Step 3: 50 °C for 1s

Step 4: 85 °C for 5 min.

Steps 2 to 3 were repeated for 60 cycles.

The cDNA samples were maintained at -20 °C.

3.4.4 Confirmation of cDNA synthesis

cDNA of target genes were tested for quality by amplification with primers for β-Actin. The thermal profile for the following procedure was as follows.

10X reaction buffer (1X)	2 µl
dNTP mix (100 µM each)	1 µl
Forward primer (10 µM)	1 µl
Reverse primer (10 µM)	1 µl
Taq DNA polymerase (1U/ µl)	0.45 µl
cDNA (200ng/ µl)	1 µl
Nuclease free water	13.5 µl

The thermal profile used was as follows:

Step 1: 95 °C for 3 mins

Step 2: 95 °C for 15s

Step 3: 55 °C for 15s

Step 4: 72 °C for 45s. The steps 2 to 4 were repeated for 30 cycles

Step 5: 72 °C for 5 mins

After completion, the PCR products were separated on 1% agarose gel along with 50bp ladder. The electrophoresis was conducted at 75 V till the gel loading dye reached three fourth of the gel. The final results were viewed using BIO RAD's Gel Documentation system.

3.4.5 Detection of BBrMV in infected plants

Presence of BBrMV in the infected banana plants at 24 h, 48 h, 1 wk and 2 wk were confirmed by PCR using primers specific for BBrMV replicase gene. A 20 µl reaction mix was prepared using the following components:

10X reaction buffer (1X)	2 µl
dNTP mix (100 µM each)	1 µl
Forward primer (10 µM)	1 µl
Reverse primer (10 µM)	1 µl
Taq DNA polymerase (1U/ µl)	0.45 µl
cDNA (200 ng/ µl)	1 µl
Nuclease free water	13.5 µl

The thermal profile followed was:

Step 1: 95 °C for 3 mins

Step 2: 95 °C for 15s

Step 3: 55 °C for 15s

Step 4: 72 °C for 45s. The steps 2 to 4 were repeated for 30 cycles

Step 5: 72 °C for 5 mins

The PCR products were analyzed on 1% agarose gel with 100bp ladder in a gel electrophoresis unit. The gel was run at 75 V and the loading dye was allowed to

run till three-fourth of the gel. The final results were viewed using BIO RAD's Gel Documentation system.

3.5 AMPLIFICATION OF miRNAs

The stemloop of the five selected miRNAs at 24 h, 48 h, 1 wk and 2 wk were checked for amplification using stemloop sequence specific forward and reverse primers. The reaction mixture contained the following :

10X reaction buffer (1X)	2 µl
dNTP mix (100 µM each)	1 µl
Forward primer (10 µM)	1 µl
Reverse primer (10 µM)	1 µl
Taq DNA polymerase (1U/ µl)	0.45 µl
cDNA (200 ng/ µl)	1 µl
Nuclease free water	13.5 µl

The thermal profile followed was:

Step 1: 95 °C for 5 mins

Step 2: 95 °C for 5s

Step 3: 60 °C 10s

Step 4: 72 °C for 8s. The steps 2 to 4 were repeated for 30 cycles

Step 5: 72 °C for 5 mins

After completion, the PCR products were separated on 3 % agarose gel along with 50 bp ladder. The gel electrophoresis was conducted at 75 V till the gel loading dye reached three-fourth of the gel. The final results were viewed using BIO RAD's Gel Documentation system

3.6 AMPLIFICATION OF TARGET GENES

The cDNA of the infected and healthy banana plants were checked for amplification using the gene specific primers. The PCR components were added as follows:

10X reaction buffer (1X)	2 µl
dNTP mix (100 µM each)	1 µl
Forward primer (10 µM)	1 µl
Reverse primer (10 µM)	1 µl
Taq DNA polymerase (1U/ µl)	0.45 µl
cDNA (200 ng/ µl)	1 µl
Nuclease free water	13.5 µl

The thermal profile followed was:

Step 1: 95 °C for 3 mins

Step 2: 95 °C for 15s

Step 3: 55 °C for 15s

Step 4: 72 °C for 45s. The steps 2 to 4 were repeated for 30 cycles.

Step 5: 72 °C for 5 mins

After completion, the PCR products were separated on 1.5 % agarose gel along with 50bp ladder. The gel electrophoresis was conducted at 75 V till the gel loading dye reached three-fourth of the gel. The final results were viewed in BIO RAD's Gel Documentation system.

3.7 RELATIVE EXPRESSION ANALYSIS USING REAL-TIME PCR

RT-qPCR is one of the most widely used methods for gene expression analysis. This technique was used to analyse the expression levels of miRNAs and their corresponding target genes. The analysis was done in BIO RAD's CFX96 Real-time machine available at Rajiv Gandhi Centre for Biotechnology, Poojappura, Thiruvananthapuram.

For expression analysis of miRNAs and their target genes, relative expression values were analyzed using qBase plus software. Using this software, relative expression of miRNAs and target genes in healthy sample was compared to expression of miRNAs and target genes in infected sample at 24 h, 48 h, 1 wk and 2 wk. RT-qPCR reactions were carried out by using Origin's SYBR GREEN florescence dye (Chai *et al.,* 2015). Reaction mixture of 20µl was prepared using the following components:

cDNA (50 ng/ µl)	5 µl
Forward primer (10 µM)	0.6 µl
Reverse primer (10 µM)	0.6µl
SYBR Green Master mix (2X)	10 µl
Nuclease free water	3.8 µl
Total volume	20 µl

cDNA of miRNAs from both healthy and infected plants were amplified by initial denaturation for 5 min, followed by 45 cycles of denaturation at 95° C for 5 s, annealing at 60° C for 10 s and extension at 72° C for 8 s.

cDNA samples of target genes of both healthy and infected plants were amplified by initial denaturation at 95 ° C for 2 min, followed by 40 cycles of

denaturation at 95° C for 15 s, annealing at 55° C for 15 s and extension at 72° C for 30 s. Final extension was done at 72° C for 5 min. This was followed by Melt-curve analysis *i.e* fluorescence signals at 530 nm wavelength were monitored from 65° C to 95° C at the rate of 0.2° C per second to determine the product specificity.

Results were expressed as fold change (increase or decrease) in expression. The house keeping gene, β-Actin was used as reference gene for normalisation of RT-qPCR data in qBase plus software. All the reactions were conducted in two replications along with No Template Control (NTC). Threshold cycle (Cq) values were determined for all the reactions and fold changes were calculated by using ΔΔCq method (Rao *et al.*, 2013) in the software. Threshold cycle (Cq) is the cycle at which the fluorescence value of a sample reaches an arbitrary threshold florescence. The difference between Cq values of control or infected sample and reference gene was considered as ΔCq value.

$$\Delta Cq = Cq \text{ (Reference gene)} - Cq \text{ (Control/Infected)}$$

Results

4. RESULTS

The study entitled "Identification and characterisation of virus responsive miRNAs in banana *Musa* (AAB) 'Nendran'" was conducted at the Department of Plant Biotechnology, College of Agriculture, Vellayani, Thiruvananthapuram during 2017-2019. The results of the study are presented in this chapter.

4.1 VECTOR TRANSMISSION AND DETECTION OF BBrMV IN HEALTHY AND INFECTED TISSUE CULTURE PLANTS

4.1.1 Confirmation of BBrMV in the infected banana sucker

The banana sucker collected from Instructional Farm showed the symptoms of BBrMV. PCR analysis with replicase specific primers yielded amplicons of size 347 bp which confirmed the presence of BBrMV in the selected plant (Plate 1).

4.1.2 Vector transmission of virus using *Pentalonia nigronervosa*

Fifteen viruliferous aphids, after 2 h of acquisition feeding were collected and allowed to feed on the leaf axils of healthy tissue culture banana var. Nendran plants and samples were collected for further analysis.

4.1.3 RNA isolation

RNA was isolated from the leaf samples of healthy and infected banana tissue culture plants at 24 h, 48 h, 1 wk and 2 wk after infection using modified CTAB method (Tsai and Harding, 2003). RNA profile on gel electrophoresis at 1.5% agarose gel (Plate 2) showed the presence of 28 rRNA and 16 rRNA bands indicating good quality. Spectrophotometric analysis showing A_{260}/A_{280} ratios ranging from 1.85 to 2.00 also indicated good quality RNA (Table 2).

4.1.4 cDNA synthesis

cDNA synthesis for both healthy and infected plants at 24 h, 48 h, 1 wk and 2 wk was done using Thermo Fisher Scientific's Verso cDNA synthesis kit. The quality of cDNA was determined by doing PCR with β-Actin specific primers. The presence of amplicons of Actin gene (125 bp) indicated good quality cDNA (Plate 3).

4.1.5 Detection of BBrMV in infected samples

BBrMV infection was detected using virus specific replicase primer in infected plants at 24 h (Plate 4), 48 h (Plate 5), 1 wk (Plate 6) and 2 wk (Plate 7). The presence of amplicons of replicase gene of size 347bp at these four time intervals indicated the presence of BBrMV infection. The presence of the virus was detectable from 24 h onwards.

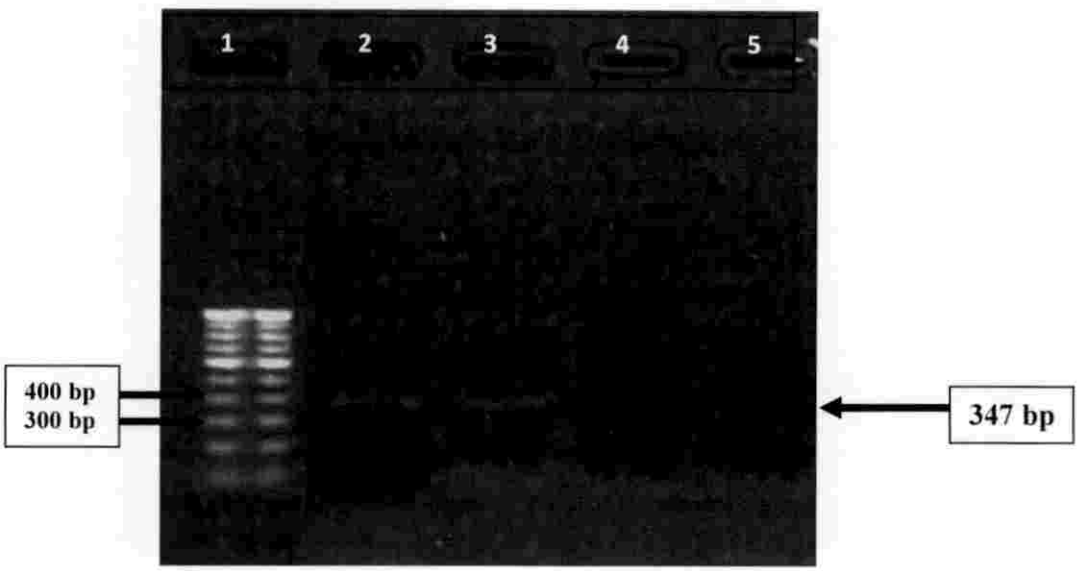


Plate 1. PCR based detection of BBrMV using replicase gene specific primers in infected sucker. Lane 1: 100 bp ladder, Lane 2,3: Infected plants, Lane 4, 5: Healthy control

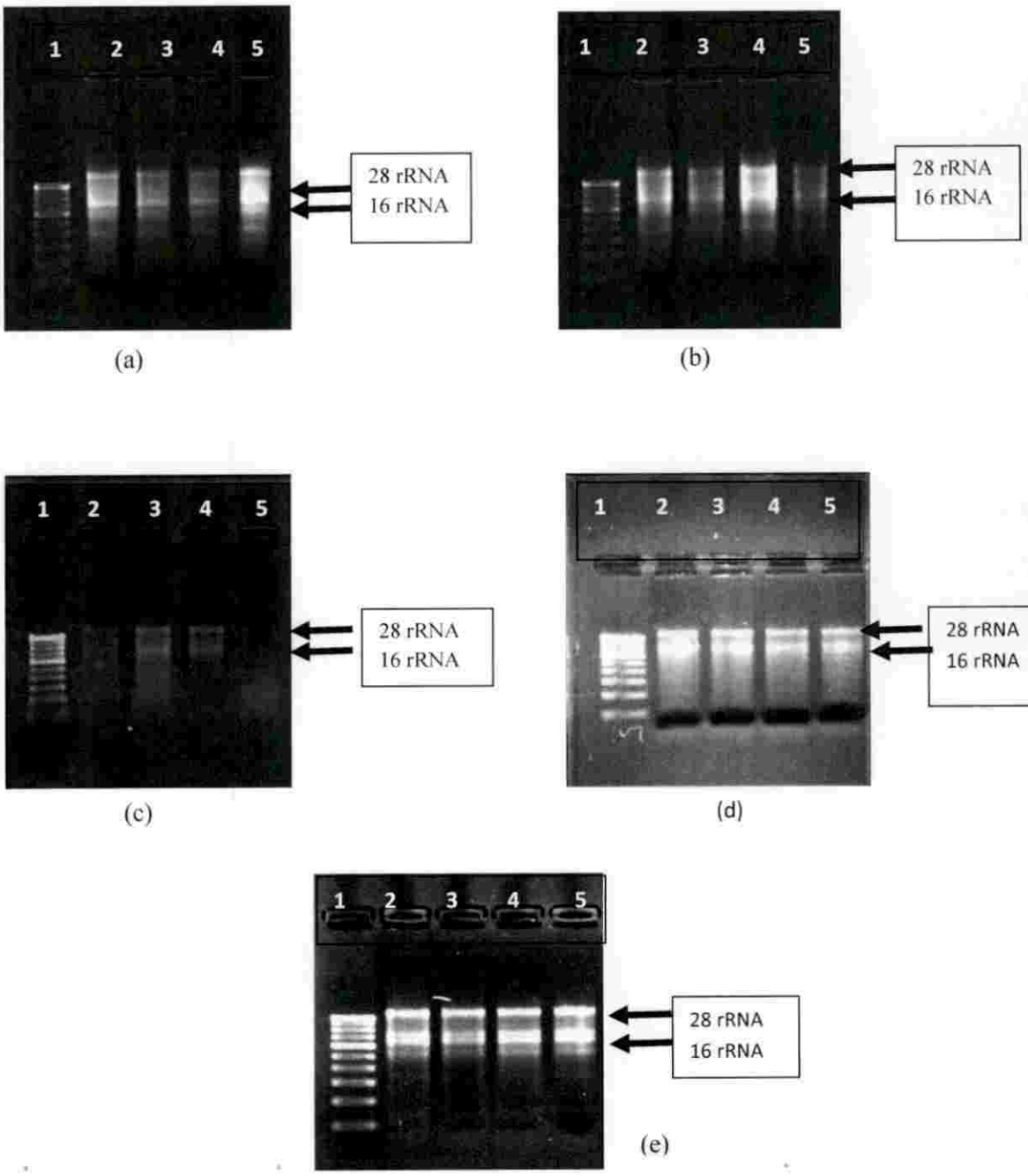


Plate 2. Gel profile of RNA isolated from (a) Uninfected plants, (b) infected plants at 24 hour after infection, (c) infected plants at 48 hour after infection, (d)) infected plants at 1 week after infection and (e) infected plants at 2 week after infection.

Lane 1: 100bp ladder, Lane 2: R1, Lane 3: R2, Lane 4: R3, Lane 5: R4

Table 2. Quantity of RNA measured through spectrophotometer.

	SAMPLE	CONCENTRATION (µg/ml)	A_{260}/A_{280}
UNINFECTED PLANT/ CONTROL	R1	1792.8	1.85
	R2	2570.2	1.80
	R3	1120.0	1.90
	R4	2147.8	1.90
24 HOURS	R1	1720.0	1.90
	R2	2156.7	1.90
	R3	1475.3	1.90
	R4	1674	2.0
48 HOURS	R1	1860.7	1.80
	R2	2560.5	1.88
	R3	1678.8	1.90
	R4	2164.8	1.9
1 WEEK	R1	1475.3	1.8
	R2	1720.0	1.9
	R3	1674.4	2.0
	R4	2570.8	1.85
2 WEEK	R1	1175.3	1.80
	R2	1674.0	2.00
	R3	2458.2	1.85
	R4	1292.8	1.80

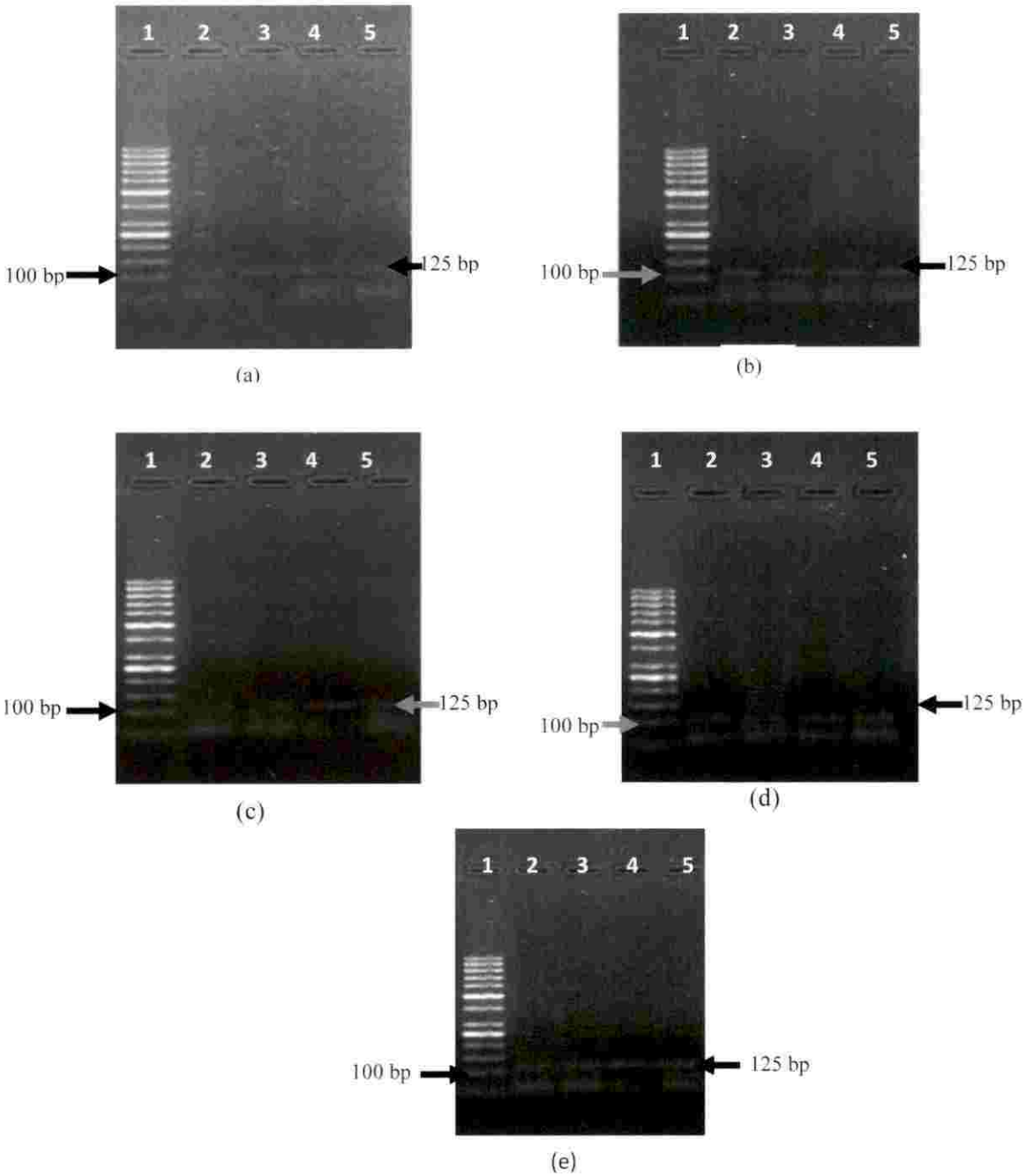


Plate 3. β-Actin gene specific fragments amplified from cDNA. (a) Uninfected plants, (b) infected plants at 24 hour after infection, (c) infected plants at 48 hour after infection gene (d) infected plants at 1 week after infection and (e) infected plants at 2 week after infection

Lane 1: 100bp ladder, Lane 2: R1, Lane 3: R2, Lane 4: R3, Lane 5: R4

Fig. 7. Plants before vector transmission treatment plants R1, R2, R3 and R4.

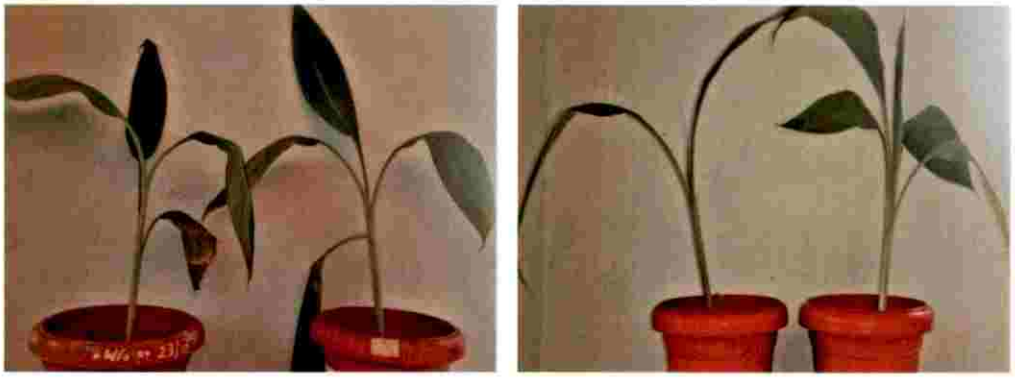


Fig. 8. Infected plants after 2 weeks: R1, R4, R2 and R3.

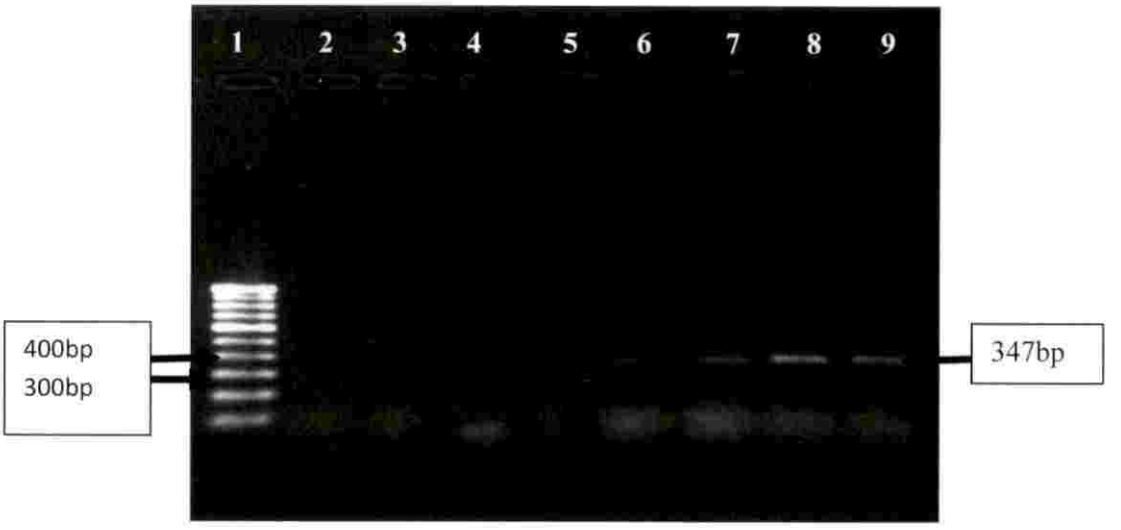


Plate 4. Detection of BBrMV using replicase gene specific primers 24 h after infection. Lane 1: 100 bp ladder, Lane 2 to 5: Uninfected plants, Lane 6 to 9: Infected plants

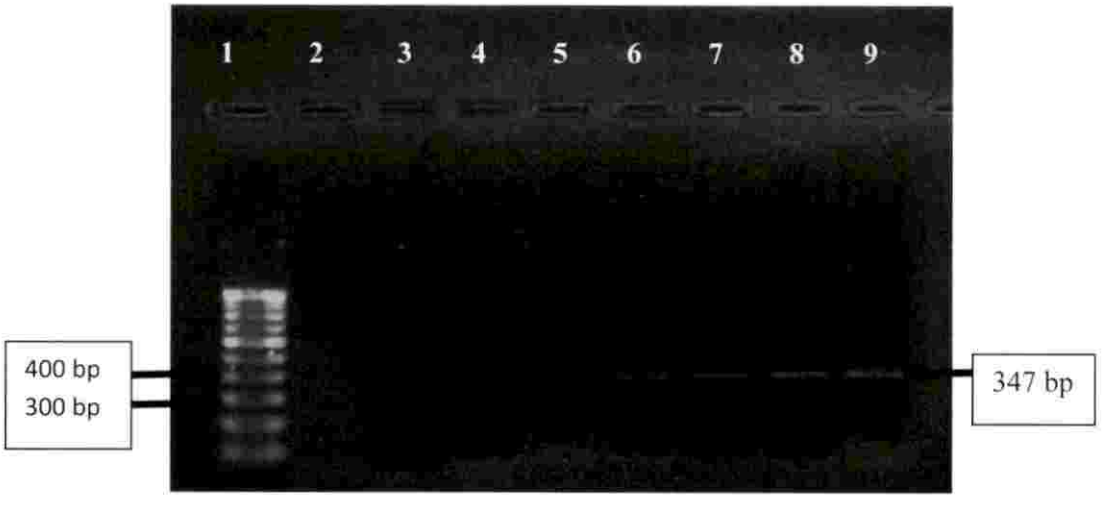


Plate 5. Detection of BBrMV using replicase gene specific primers 48 h after infection. Lane 1: 100 bp ladder, lane 2, 3, 4, 5: Uninfected plant, lane 6, 7, 8, 9: Infected plant

Plate 6. Detection of BBrMV using replicase gene specific primers 1 wk after infection. Lane 1: 100 bp ladder, lane 2, 3, 4, 5: Uninfected plant, lane 6, 7, 8 , 9: Infected plant.

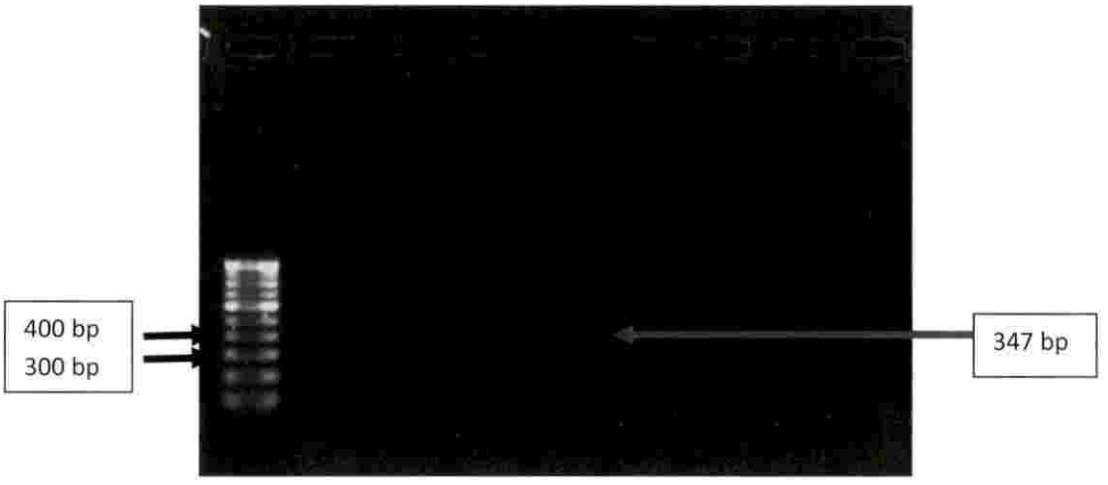


Plate 7. Detection of BBrMV using replicase gene specific primers 2 wk after infection. Lane 1: 100 bp ladder, lane 2, 3, 4, 5: Infected plant, Lane 6, 7, 8 , 9: Uninfected plant.

4.2 AMPLIFICATION EFFICIENCY OF PRIMERS DESINGED for miRNAs AND TARGET GENES

Primers were designed for the five selected according to the reports of Chen *et al.* (2005). The details of primers are shown in Table 3 and 4. Primer specificity check was done using PCR. The PCR products were run on 3% agarose gels and the results were visualized under BIO RAD's Gel Documentation system.The miRNA, miR-2172-5p did not show any expression in the uninfected control, the amplicon size of the PCR products were 61 bp (Plate 8). miR-3900-5p, miR-5417, miR-6928-5p and miR-971-5p showed expression in control as well as in infected samples at 24 h, 48 h, 1 wk and 2 wk, their amplicon sizes were 58 bp (Plate 9), 62 bp (Plate 10), 65 bp (Plate 11) and 62 bp (Plate 12) respectively.

Target gene specific primers were designed using Primer3Plus software. The primers showing the best complimentarity to banana genome were synthesized and used for validation. The list of target gene specific primers is shown in Table. 5. Amplification efficiency of these primers were checked by conducting PCR. The PCR products were run on 1.5% agarose gels and the results were visualized under BIO RAD's Gel Documentation system. The target genes Plant viral response protein gene (target of miR-3900-5p), Putative disease resistance protein gene (target of miR-3900-5p), Putative Ethylene-responsive transcription factor 1 gene (target of miR-2172-5p), FAD dependent oxidoreductase gene (target of miR-6928-5p), Argonaute protein gene (target of miR-971-5p) and Transport inhibitor response 1 like protein gene (target of miR-971-5p) showed expression in all samples and their amplicon size were 165 bp (Plate 13), 233 bp (Plate 14), 206 bp (Plate 15), 274 bp (Plate 16), 300 bp (Plate 17) and 177 bp (Plate 18) respectively. No expression was observed for the gene Stress-associated endoplasmic reticulum protein 2 gene (target of miR-5417) (Plate 19).

Table 3. The sequence of stemloop primers

Sl. No	miRNA name	miRNA sequence (5'..3')	miRNA specific stem loop sequence (5'..3')
1	miR-3900-5p	CAAGGAAGGTG TCACCGCC	GTCGTATCCAGTGCAGGGTCCGAGGTATTC GCACTGGATACGACGGCGGT
2	miR-6928	GGGGATTTTCA AGTACTGCA	GTCGTATCCAGTGCAGGGTCCGAGGTATTC GCACTGGATACGACTGCAGT
3	miR-5417-5p	AGGGGAGAAAT GGGGATG	GTCGTATCCAGTGCAGGGTCCGAGGTATTC GCACTGGATACGACCATCCC
4	miR- 971-5p	TTTGATGATTTG AATTTA	GTCGTATCCAGTGCAGGGTCCGAGGTATTC GCACTGGATACGACTAAATT
5	miR-2172-5p	GAAATTATGATT ATGCAT	GTCGTATCCAGTGCAGGGTCCGAGGTATTC GCACTGGATACGACATGCAT

Table 4. Sequence of forward and reverse primers designed for miRNA

Sl. No	miRNA name	miRNA sequence(5'..3')	Forward primer(5'..3')	universal reverse primer(5'..3')
1	miR-3900-5p	CAAGGAAGGTG TCACCGCC	CGTCGCCAAG GAAGGTGTC	CCAGTGCAGGGTCCGAGG TA
2	miR-6928	GGGGATTTTCA AGTACTGCA	GAGGGCCGGG GATTTTCAAGT	CCAGTGCAGGGTCCGAGG TA
3	miR-5417-5p	AGGGGAGAAAU GGGGAUG	GCTGGCGAGG GGAGAAATG	CCAGTGCAGGGTCCGAGG TA
4	miR- 971-5p	TTTGATGATTTG AATTTA	GGGGGGCGTT TGATGATTTG	CCAGTGCAGGGTCCGAGG TA
5	miR-2172-5p	GAAATTATGATT ATGCAT	GGCGGGGCGG AAATTATGATT	CCAGTGCAGGGTCCGAGG TA

Table 5. Forward and reverse primers designed for target genes.

Sl. No	Target name	Forward primer(5'...3')	Reverse primer(5'...3')	Expected amplicon size (bp)
1	Plant viral response family protein gene (*PV*)	TCATGTAGCACC AGGCTTTG	GCCAGGCTGCC TAGTATGAT	165
2	Putative disease resistance protein gene (*DR*)	ACAATTGCCCT GTGCTCATT	GCACAGAGTTT GGAGGAAGG	233
3	FAD dependent oxidoreductase gene(*FAD*)	TGTGCAAATCG TTTGCTTTC	GTATTGTTGCC CATCCATGA	274
4	Stress-associated endoplasmic reticulum protein 2 gene (*SER*)	CCAAATATTGAT CCGAAGACG	AAAGACGAAG AACCCAAGCA	230
5	Transport inhibitor response 1-like protein gene (*TIF*)	CCACCGAACCG ATGTTTATC	AGGTCATCACC CTGAATTGC	177
6	Argonaute protein gene (*ARG*)	TACTGGACAAA AGCCCCAAC	GACGTTTTTGG ACAACCACA	300
7	Putative Ethylene-responsive transcription factor 1 gene (*ERT*)	AAATCAACTGC ATGGGAAGC	CTTGTCCTGGG TTCCTCAGA	206

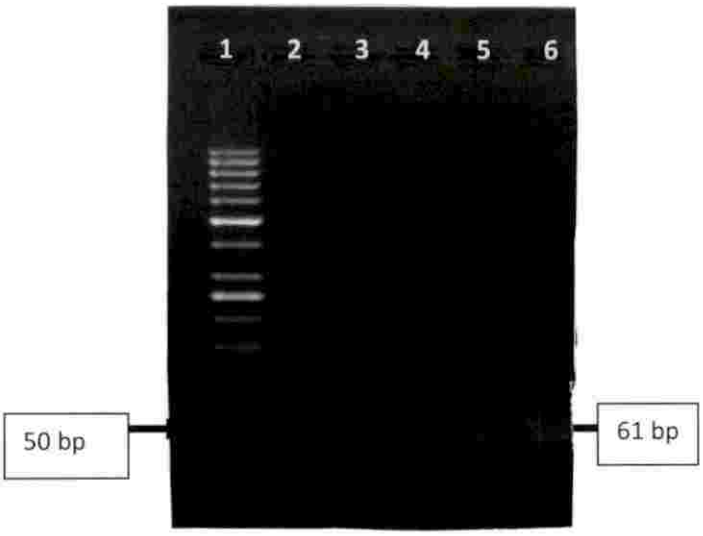


Plate 8. Amplification of miR-2172-5p in BBrMV infected plants. Lane 1: 50 bp ladder, Lane 2: Uninfected control, Lane 3to 6: Infected (24 h, 48 h, 1 wk and 2 wk after infection).

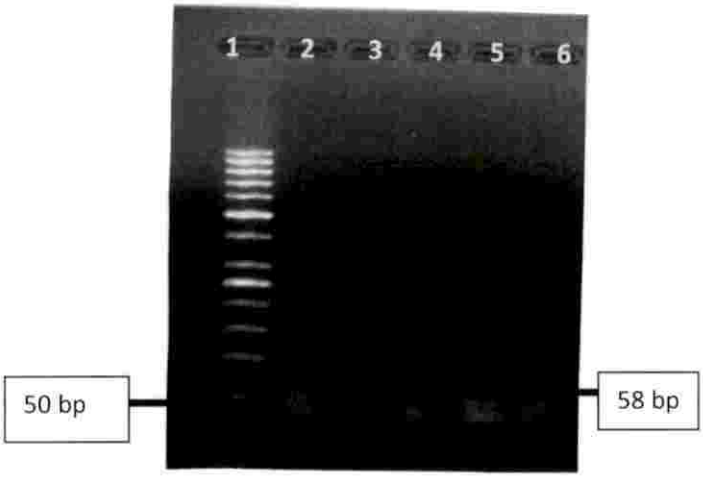


Plate 9. Amplicons of miR-3900-5p in BBrMV infected plants. Lane 1: 50 bp ladd·, lane 2: Uninfected control, lane 3 to 6: infected (24 h, 48 h, 1 wk and 2 wk after infection).

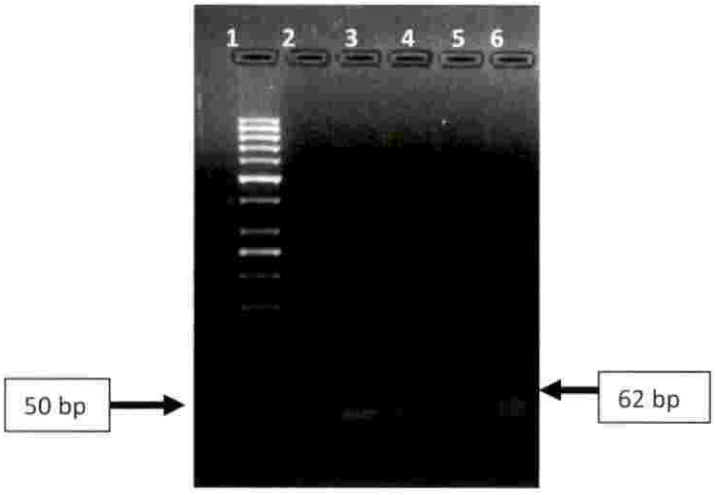


Plate 10. Amplicons of miR-5417 in BBrMV infected plants. Lane 1: 50 bp ladder, lane 2: Uninfected control, lane 3 to 6: infected (24 h, 48 h, 1 wk and 2 wk after infection).

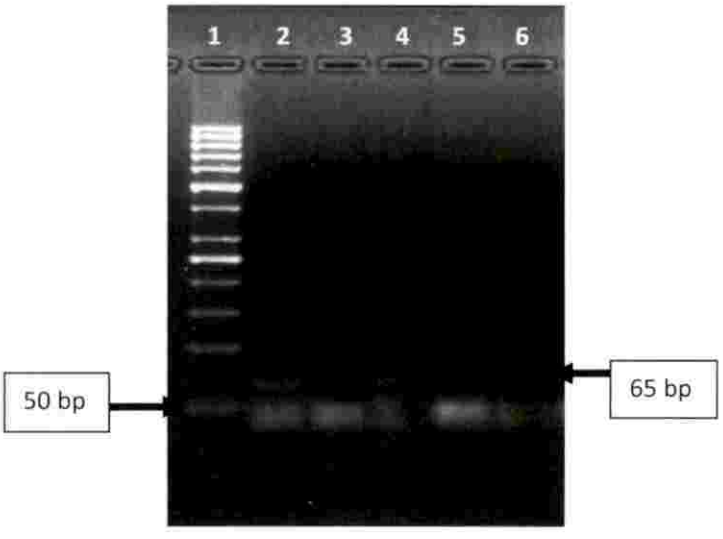


Plate 11. Amplicons of miR-6928-5p in BBrMV infected plants. Lane 1: 50 bp ladder, lane 2: Uninfected control, lane 3 to 6: infected (24 h, 48 h, 1 wk and 2 wk after infection).

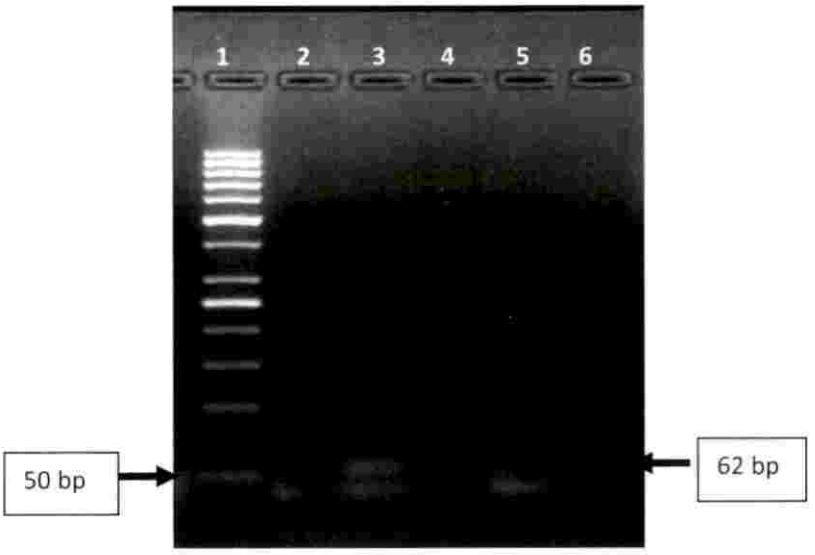


Plate 12. Amplicons of miR-971-5p in BBrMV infected plants. Lane 1: 50 bp ladder, lane 2: Uninfected control, lane 3 to 6: infected (24 h, 48 h, 1 wk and 2 wk after infection).

Plate 13. Amplicons of Plant viral response protein gene (target of miR-3900-5p) .Lane 1: 50 bp ladder, lane 2: Uninfected control, lane 3 to 6: infected (24 h, 48 h, 1 wk and 2 wk after infection).

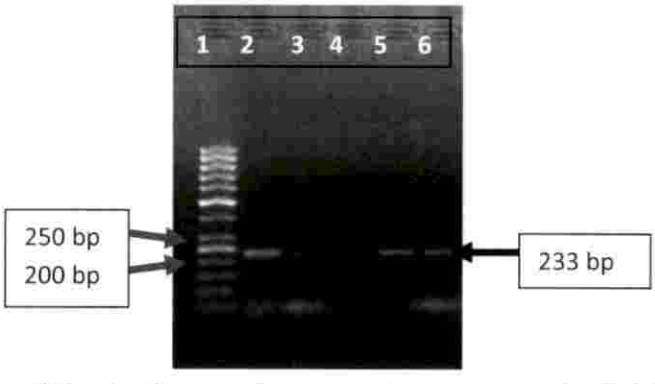


Plate 14. Amplicons of Putative disease resistance protein gene (target of miR-3900-5p) . Lane 1: 50 bp ladder, lane 2: Uninfected control, lane 3 to 6: infected (24 h, 48 h, 1 wk and 2 wk after infection).

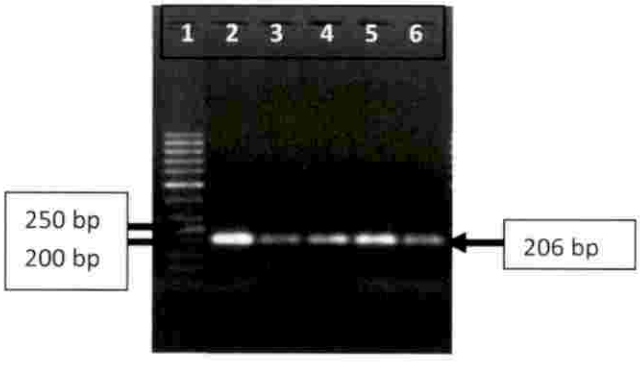


Plate 15. Amplicons of Putative Ethylene-responsive transcription factor 1 gene(Target of miR-2172-5p). Lane 1: 50 bp ladder, lane 2: Uninfected control, lane 3 to 6: infected (24 h, 48 h, 1 wk and 2 wk after infection).

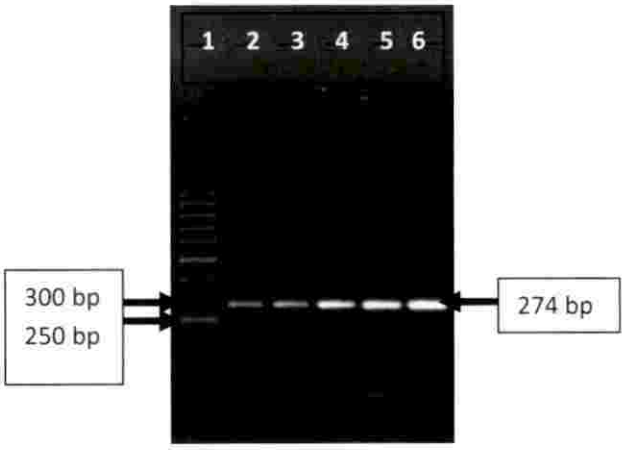


Plate 16. Amplicons of FAD dependent oxidoreductase gene (target of miR-6928-5p). Lane 1: 50 bp ladder, lane 2: Uninfected control, lane 3 to 6: infected (24 h, 48 h, 1 wk and 2 wk after infection).

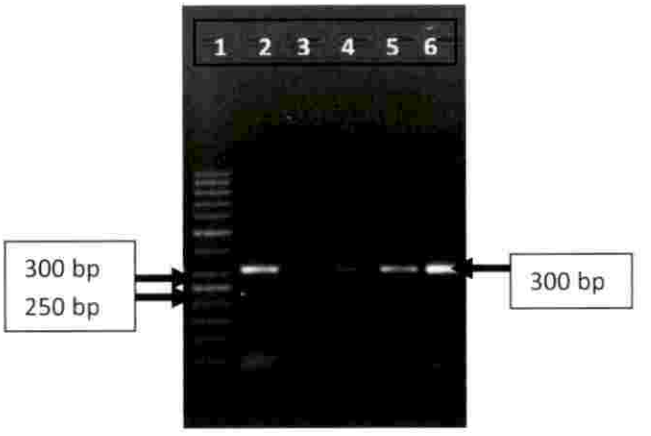


Plate 17. Amplicons of Argonaute protein gene (target of miR-971-5p). Lane 1: 50 bp ladder, lane 2: Uninfected control, lane 3 to 6: infected (24 h, 48 h, 1 wk and 2 wk after infection).

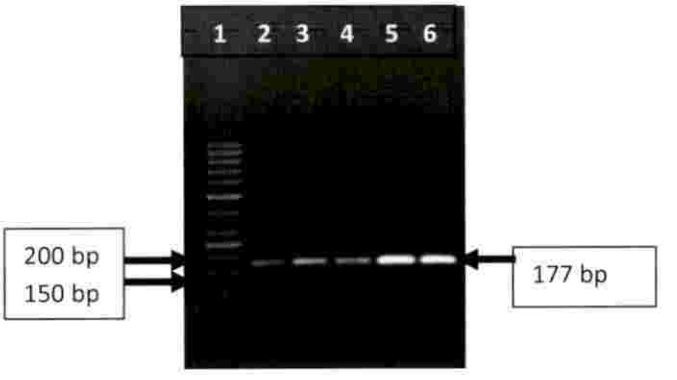
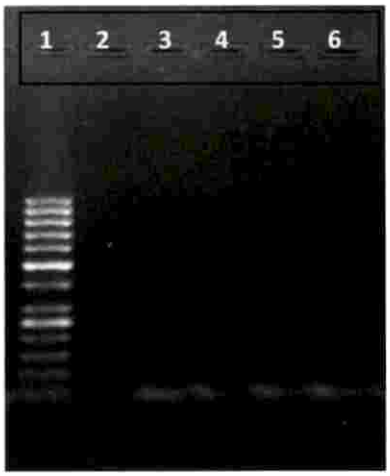


Plate 18. Amplicons of Transport inhibitor response 1 like protein gene (target of miR-971-5p). Lane 1: 50 bp ladder, lane 2: Uninfected control, lane 3 to 6: infected (24 h, 48 h, 1 wk and 2 wk after infection).



No bands

Plate 19. Amplicons of Stress-associated endoplasmic reticulum protein 2 gene (target of miR-5417). Lane 1: 50 bp ladder, lane 2: Uninfected control, lane 3 to 6: infected (24 h, 48 h, 1 wk and 2 wk after infection).

4.3 REAL-TIME PCR

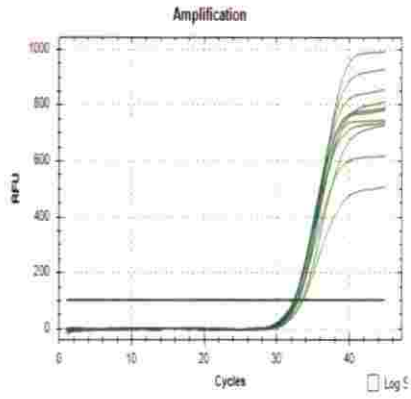
Expression of miRNAs and their corresponding target genes in the BBrMV infected plants was analysed 24 h, 48 h, 1 wk and 2 wk after infection using RT-qPCR and compared with that of uninfected control plants.

4.3.1 Raw expression data and Cq values of miRNAs and target gene

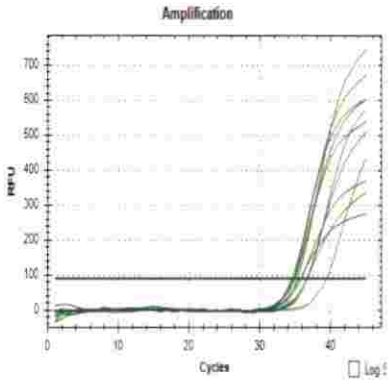
The RT-qPCR for each gene was conducted with 2 technical replicates at each time interval. Amplification plot for each miRNAs and their target genes were developed. Showed the raw expression data of the particular miRNAs and target gene. The Cq values represented the amplification point and it varied for each gene. For miRNAs the values ranged from 32 to 38 (Fig. 9.) and target genes it ranged from 26 to 40 (Fig. 11). Lower the amplification higher the Cq value vice versa. The Cq values of miRNAs and target gene are represented in Table 6 and 7.

4.3.2 Melt curve analysis

The specificity of the RT-qCR was determined through the Melt-curve analysis. The change in fluorescence to change in temperature ($\Delta F/\Delta T$) was measured against temperature (T) and the prominent peak in the graph represents the efficiency of the primers during the amplification of the gene. The peak below the threshold line represented poor amplification and the ones shifting to the left represented primer dimer formation. All miRNAs showed prominent peaks indicating good amplification (Fig. 10). All target genes except Stress-associated endoplasmic reticulum protein 2 gene (target of miR-5417) showed prominent peaks (Fig. 12).



(a) miR-3900-5p



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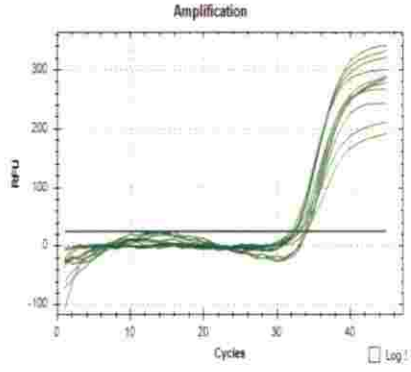
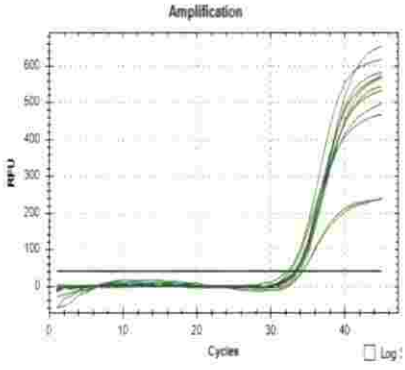
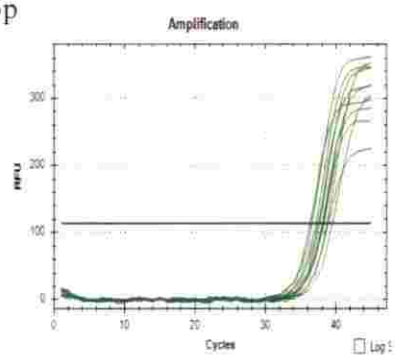


(c) miR-971-5p

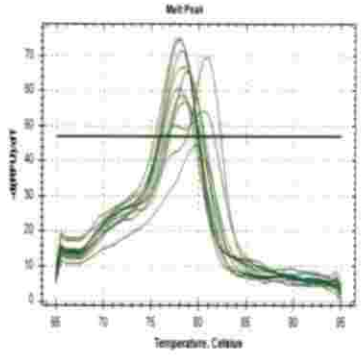


(d) miR-6298-5p

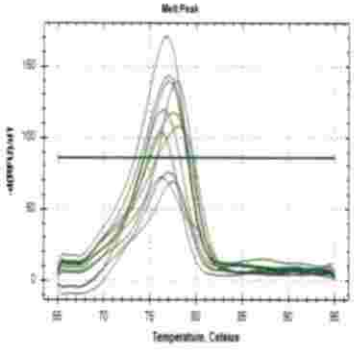


(e) miR-5417

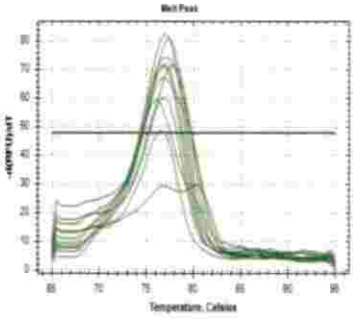
Fig. 9. Raw expression data of miRNAs



(a) miR-3900-5p



(b) miR-2172-5p



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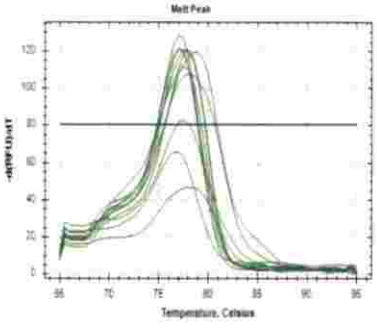

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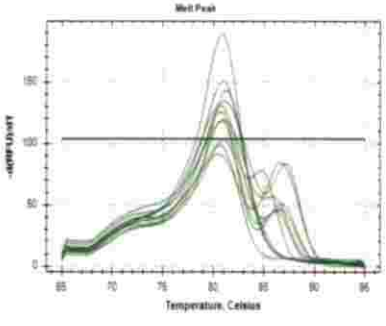
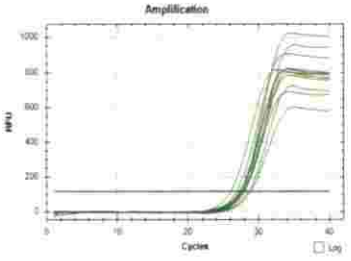
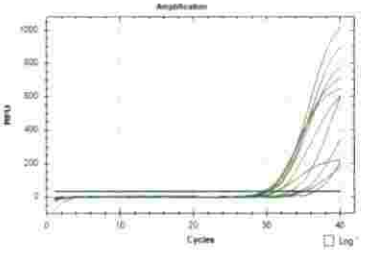


(e) miR-5417-5p

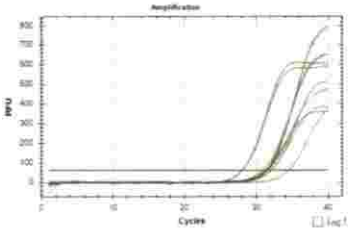
Fig, 10. Melt curve of miRNAs



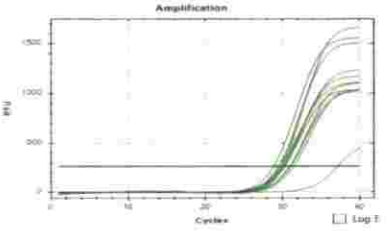
(a) β-Actin



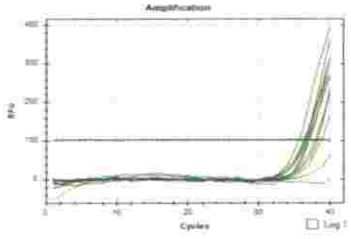
(b) Plant viral response family like protein



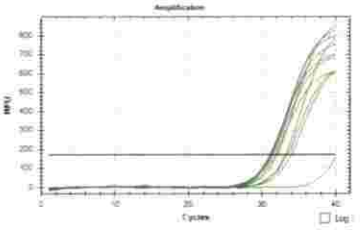
(c) Putative disease resistance protein gene



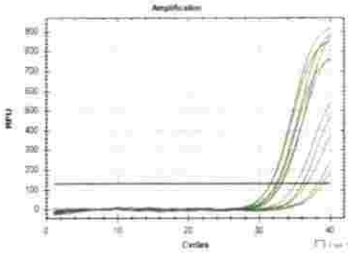
(d) Putative Ethylene-responsive transcription factor 1 gene



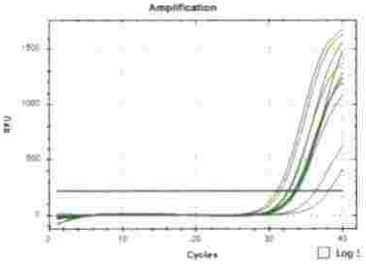
(e) Stress-associated endoplasmic reticulum protein 2 gene



(f) Transport inhibitor response 1-like protein gene

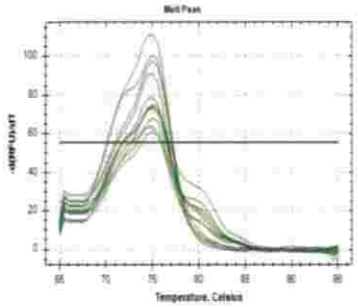


(g) Argonaute protein gene

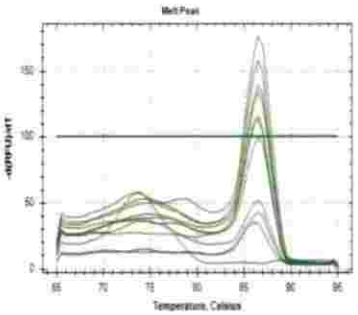


(h) FAD dependent oxidoreductase gene

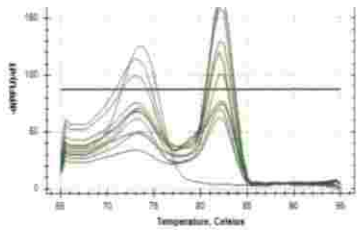
Fig. 11. Raw expression data of target genes



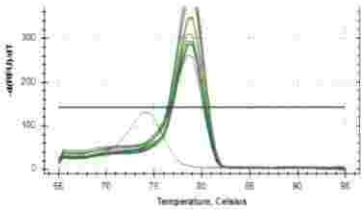
(a) β-Actin



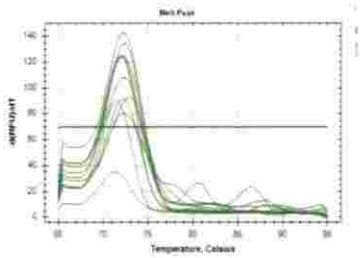
(b) Plant viral response family like protein gene



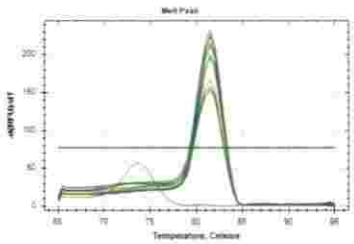
(c) Putative disease resistance protein gene



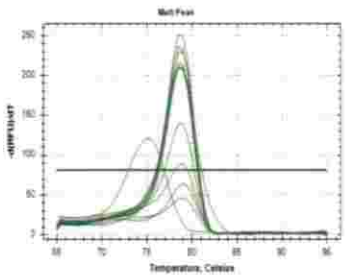
(d) Putative Ethylene-responsive transcription factor 1 gene



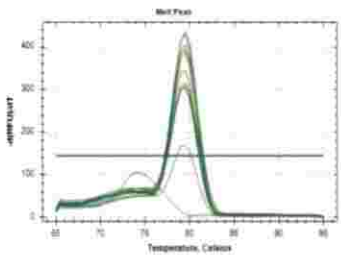
(e) Stress-associated endoplasmic reticulum protein 2 gene



(f) Transport inhibitor response 1-like protein gene



(g) Argonaute protein gene



(h) FAD dependent oxidoreductase gene

Fig. 12. Melt curve analysis of target genes.

Table 6. The Cq values of miRNAs.

	miR-2172-5p	miR-3900-5p	miR-5417	miR-6928-5p	miR-971-5p
C	38.05	33.31	37.51	33.56	33.06
24 h	34.75	32.38	38.66	33.22	32.01
48 h	36.03	32.62	36.84	32.78	32.58
1 wk	34.83	32.54	37.27	33.28	32.58
2 wk	35.8	33.09	38.68	33.37	33.09

Table 7. The Cq values of target genes.

	β-*Actin*	*PV*	*DR*	*ERT*	*SER*	*ARG*	*TIF*	*FAD*
C	27.94	30.32	31.16	31.19	40.00	33.83	32.41	36.3
24 h	28.65	31.57	28.08	30.14	40.00	35.71	30.5	33.51
48 h	27.75	32.15	31.91	29.60	40.00	37.76	30.89	33.21
1 wk	26.4	29.73	31.45	29.50	40.00	31.92	30.27	31.29
2 wk	27.46	33.05	31.68	30.35	40.00	32.29	30.04	33.08

4.4 RELATIVE EXPRESSION VALUES OF miRNAs AND TARGET GENE

The Cq values of uninfected sample and infected sample at 24 h, 48 h, 1 wk and 2 wk were analyzed by qBase plus software. The software generates a relative expression value of miRNAs and target genes with respect to the uninfected sample. For target gene, the Cq values were normalized with the Cq values of β-Actin (housekeeping gene). The relative expression values are shown in Table 8 and 9. The values were then represented in graphs and their comparative analysis is depicted below

Table 8. NRQ values of miRNAs.

	miR-971-5p	**miR-2172-5p**	**miR-3900-5p**	**miR-5417**	**miR-6928-5p**
C	1.00	1.00	1.00	1.00	1.00
24 h	2.07	1.98	1.21	0.78	1.27
48 h	1.39	1.53	1.14	0.87	1.72
1 wk	1.39	1.95	1.17	1.04	1.21
2 wk	0.98	1.60	1.04	0.78	1.14

Table 9. NRQ values of target genes

	β-Actin	***ARG***	***DR***	***ERT***	***FAD***	***PV***	***SER***	***TIF***
C	1.00	1.00	1.00	1.00	1.00	1.00	0.00	1.00
24h	1.00	0.44	13.83	3.39	13.13	0.69	0.00	6.15
48h	1.00	0.06	0.52	2.64	7.46	0.25	0.00	2.51
1wk	1.00	1.29	0.28	1.11	11.08	0.52	0.00	1.52
2wk	1.00	2.08	0.50	1.28	6.08	0.11	0.00	3.71

Out of the five miRNAs selected in this study, four miRNAs, miR-3900-5p, miR-2172-5p, miR-6928-5p and miR-971-5p showed differential expression in the BBrMV infected plants. After 24h of infection, the expression of miR-3900-5p was increased by 1.27 folds. But after that it showed a decreasing trend (1.14, 1.17 and 1.04 fold at 48 h, 1 wk and 2 wk respectively), but still showing higher expression compared to control. mRNA transcript encoding putative disease resistance protein (DR), one of its predicted target, showed a drastic increase in expression by 13 folds after 24 h of BBrMV infection and then gradually reduced. On the other hand, the second target i.e., mRNA transcript encoding plant viral response family protein (PV) showed an inverse correlation with the miR-3900-5p in infected plants, with 0.69 to 0.11 fold reduction after 24 h to 2 wk (Fig. 13). This indicated a miRNA-target interaction responsive to BBrMV interaction.

miR-2172-5p and its target did not show any inverse correlation. Both miRNA and its target remained upregulated in the infected samples throughout the period of observation, not showing the predicted interaction between them. miRNA miR-2172-5p showed induction of 1.98 and 1.6 fold after 24 h and 2 wk respectively. mRNA transcripts of putative ethylene-responsive transcription factor 1 (ERT) showed 3.39 fold induction at 48 h and then reduced to 1.28 fold after 1 wk (Fig. 14).

There was a 2 fold induction of miR-971-5p at 24 h of infection, which was reduced gradually. Among the two targets, mRNA transcripts encoding Protein argonaute 1A gene (ARG) showed an inverse correlation with miR-971-5p. There was a reduction in the ARG transcripts upto 48 h, but as the infection progressed it was increased upto 2.08 fold by 2 wk (Fig. 15).There was a drastic induction of mRNA coding for transport inhibitor response-1 like protein (TIR1) in the infected plants (6.15 fold) at 24 h. Even though it was reduced to 2.5 fold at 48 h, it remained elevated in the infected plants compared to control.

miR-6928-5p showed an increased expression in the infected plants to a maximum of 1.72 folds at 48 h, then slowly decreased. On the other hand, its target mRNA transcript encoding FAD dependent oxidoreductase showed 13 fold increase at 24 h which was maximum and it remained at a higher level compared to control in the succeeding time intervals also (Fig. 16). Here an inverse correlation in the expression could not be found between miR-6928 and its target.

miR5417 did not show any response to BBrMV infection. Its mRNA target encoding stress-associated endoplasmic reticulum protein 2 showed no expression in Real-Time PCR (Fig. 17).

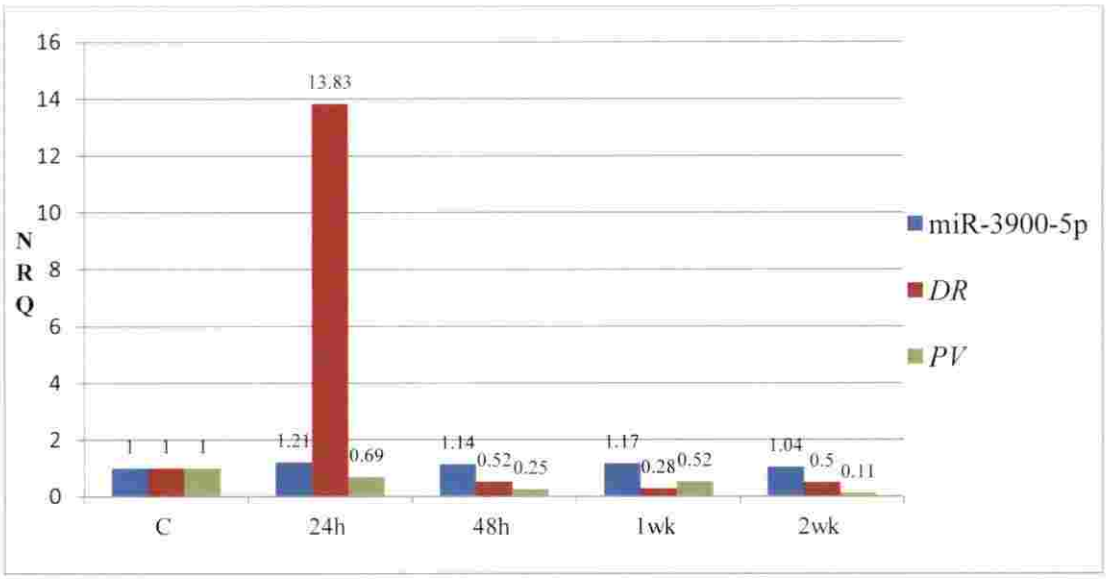


Fig. 13. Relative expression of miR-3900-5p and its targets Plant viral response family protein (PV) and Putative disease resistance protein (DR) genes

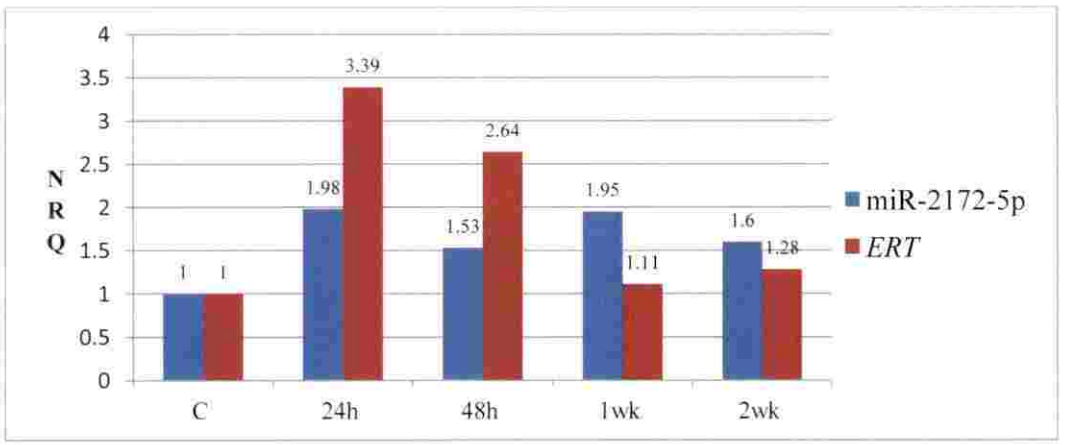


Fig. 14. Relative expression of miR-2172-5p and its target Putative ethylene responsive transcription factor gene

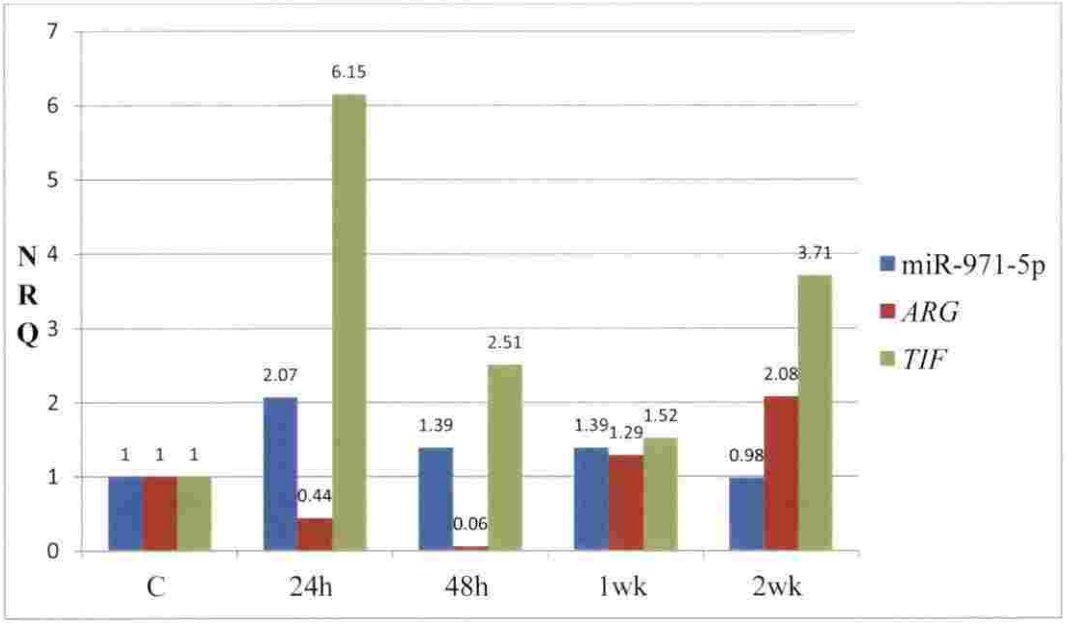


Fig. 15. Relative expression of miR-971-5p and its target Argaunte protein and Transport inhibitor response 1-like protein genes.

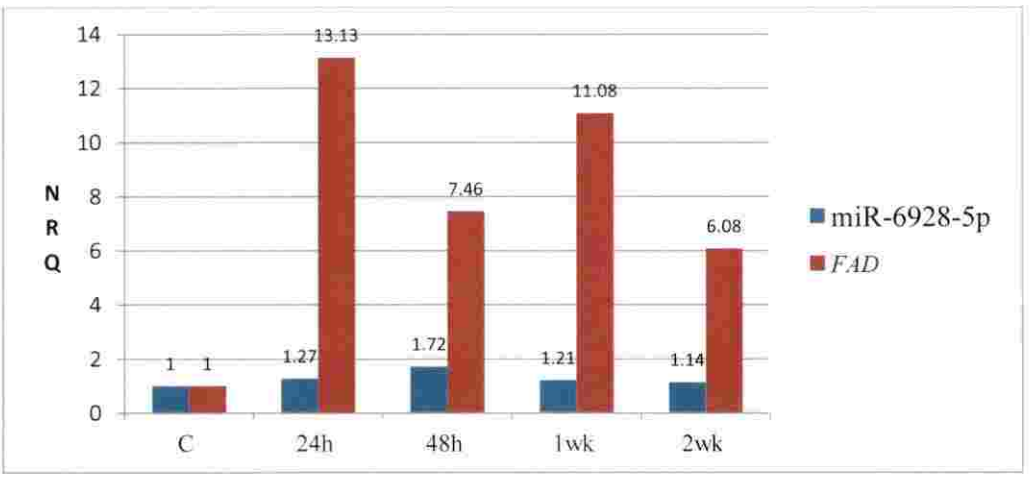


Fig. 16. Relative expression of miR-6928-5p and its target FAD dependent oxidoreductase gene.

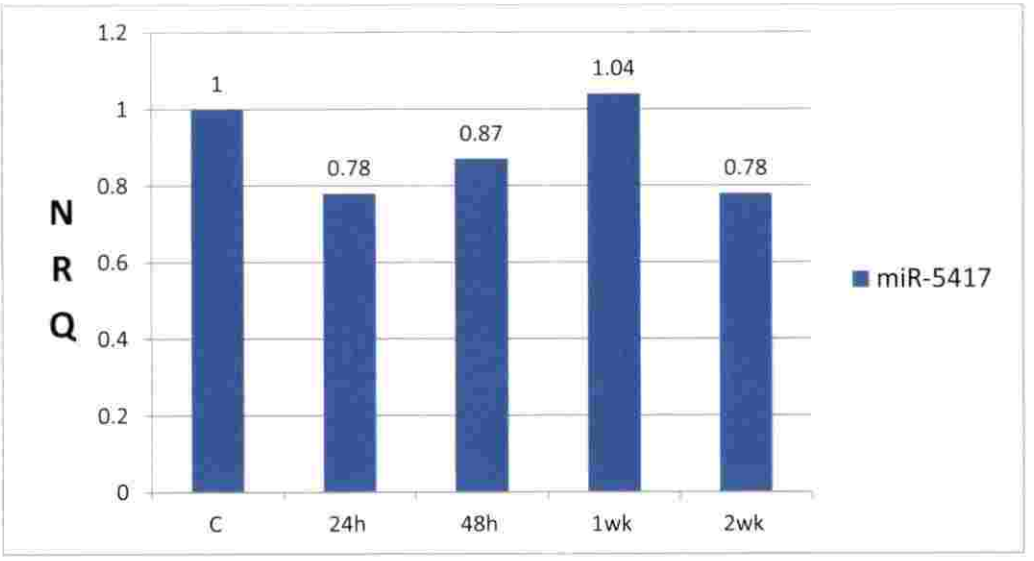


Fig. 17. Relative expression of miR-5417.

Discussion

5. DISCUSSION

Banana is the second most important fruit crop in the world having a gross production of 114.13 million metric tonnes. It is grown in both tropical and subtropical regions of the world and is cultivated throughout the year. Due to its cheap cost of cultivation and high nutritive value it is the most preferred crop for cultivation by farmers. Abiotic and biotic stresses are the major constraints affecting the production and productivity of banana. Among the biotic stresses, viruses pose a major threat to banana cultivation, resulting in serious economic loses. *Banana bract mosaic virus* (BBrMV) is one of the destructive viruses affecting banana, which causes upto 40% yield loss. In Kerala, almost all varieties of banana are susceptible to BBrMV infection. Since no wild virus resistant relatives of banana are available, conventional breeding techniques are not suitable for developing resistant varieties.

Plants have evolved several defense responses to resist the invasion of pathogenic microorganisms. Recent studies have indicated changes in the expression of endogenous miRNAs in biotic stress conditions.

Nicotiana benthamiana when co-infected with *Potato virus X* (PVX) and *Plum pox virus* (PPV), altered the expression of miR156, miR171, miR398 and miR168 (Pacheco *et al.*, 2012). *Banana streak mysore virus* (BSMysV) infection upregulated the expression of miR166, miR156 and miR159 in banana during the course of infection (Sheeba *et al.*, 2013). The levels of miR168 elevated during *Soyabean mosaic virus* strain G7 infection, leading to suppression of translation of AGO1 mRNA (Pacheco *et al.*, 2012; Chen *et al.*, 2015; Varallyay *et al.*, 2017). AGO1 in *Malus hupehensis*, was also regulated by miR168 to resist the attack of *Botryosphaeria dothidea* (Yu *et al.*, 2017). *Mung bean yellow mosaic India virus* (MYMIV) infection in soyabean was disrupted by gma-miR5787, which enhanced the production of AGO (Ramesh *et al.*, 2017).

As plants do not have an immune system to combat virus, miRNA mediated gene regulation is a new strategy to control plant viruses. miRNAs are non-coding endogenous single stranded RNA molecules of 18-22 nt length and they negatively regulate gene expression via mRNA cleavage or translational inhibition of their targets, thus playing an important role in adaptation to stresses. In banana very less number of miRNAs has been identified and studies on the role of miRNAs in biotic stress are also very less. Identification of differentially expressed miRNAs during viral infection can help development of a more targeted genetic transformation or gene editing approach for imparting virus resistance in banana.

In a previous study, 52 mature miRNAs and their target genes were predicted using computational approach from the banana genome using the NOVOMIR and psRNATarget software (Mathew, 2018). This study was an attempt to validate these miRNAs by wet lab experiments for identifying the miRNAs that can be targeted for development of virus resistance. Hence, among the 52 mature miRNAs predicted *in silico,* five were selected for *in vivo* validation based on their predicted relation of target genes to stress adaptation. The selected miRNAs for the study were, miR-3900-5p (targets: Plant viral response family protein gene and Putative disease resistance protein gene), miR-2172-5p (target: Putative ethylene responsive transcription factor1gene), miR-6928-5p (target: FAD dependent oxidoreductase gene), miR-5417 (target: Stress endoplasmic reticulum protein 2 gene) and miR-971-5p (targets: Argonaute protein gene and Transport inhibitor response-1 like protein gene).

The experiment was carried out in three month old *in vitro* raised banana var. Nendran plants to study their response towards BBrMV infection. The plants were infected with BBrMV through 15 viruliferous aphids (*Pentalonia nigronervosa*). They were released on to the healthy tissue culture plants after 2 h of acquisition feeding from the infected sucker. In a study conducted by Dhanya (2004), a pre-acquisition fasting of 1 h was proposed along with 30 min acquisition feeding for transferring BBrMV in healthy banana plants. In the present study, 30 min starvation

period followed by 2 h acquisition feeding was found sufficient for transmitting the virus. The aphids were retained in the infected plants for 24 h and were killed using 0.1 per cent quinalphos. The leaf samples were collected from the infected plants at 24 h, 48 h, 1 wk and 2 wk of infection. PCR analysis with BBrMV replicase gene specific primers confirmed the presence of virus in the infected plants by yielding the amplicons of expected size. The presence of virus was detectable from 24h onwards.

Expression analysis of miRNAs and their target genes was done by RT-qPCR. Due to the short length and absence of common sequence features like polyA, the quantification of mature miRNA expression is difficult. Chen *et al.* (2005) have designed stem-loop primers for the detection of miRNAs. Stem-loop qRT-PCR has proven a reliable technique for the detection of mature miRNAs.RNA isolated from the leaves of infected and healthy plants at 24 h, 48 h, 1 wk and 2 wk was found to be of good quality. cDNA was synthesized using oligo-dT primers for target genes and stem loop primers for miRNAs. The results of RT-qPCR were normalised using the house keeping gene β-Actin with reference to the uninfected control in qBase plus software. The results showed upregulation of miR-3900-5p, miR-2172-5p, miR-6928-5p and miR-971-5p (1.2, 2.0, 1.27, 2.0 fold respectively) at 24 h after infection.

In this study, miR-3900-5p showed an increased expression in the BBrMV infected plants throughout the period of observation. Out of the two predicted targets, mRNA transcript encoding plant viral response family protein (PV) showed an inverse correlation with the miR-3900-5p in infected plants. Its expression was reduced by 0.44 folds at 24 h when compared to the uninfected sample and remained down regulated throughout the period of observation. Though the mRNA transcripts encoding putative disease resistance protein (DR) showed a drastic increase in expression by 13 folds at 24 h after BBrMV infection, in the subsequent stages its expression was reduced, showing an inverse correlation with the miRNA. PV and DR genes belong to the family of pathogenesis related protein genes, which trigger the production of avirulence proteins that may arrest the growth and development of

viruses (Amaral *et al.,* 2006). Both the target genes were suppressed during the course of infection, which may be an indication of susceptibility of the var. Nendran to BBrMV.

miR-2172- 5p and its target Putative ethylene-responsive transcription factor 1 gene (ERT) were found upregulated in BBrMV infected plants by 1.98 and 3.39 folds respectively at 24 hours. Both remained upregulated throughout the period of observation. ERT functions as a transcription activator and is responsible for the activation of PR protein genes (Zhou *et al.*, 1997; Stockinger *et al.*, 1997; Lorenzo *et al.,* 2003). As mentioned earlier, 24 h after infection there was a drastic increase in the expression of mRNA transcripts encoding DR. When the infection was manifested, ethylene produced in the host might have led to the activation of ERF 1, subsequently activating the DR leading to 13 fold increase in its expression at 24 h.

Among the two predicted targets of miR-971-5p, only mRNA transcripts coding for Protein argonaute 1A showed an inverse correlation with the miRNA. It remained downregulated upto 48 h and showed induction upto 2 fold as the infection progressed. The Argonaute protein is involved in the RISC formation (Garcia-Ruiz *et al.*, 2015) which is essential for the synthesis of miRNAs. But only from the pattern of expression of this gene obtained in this study, it is not able to establish its correlation with virus infection. The other target gene which codes for transport inhibitor response-1like protein (TIR) showed a 6.15 fold expression at 24 h in infected plants. Transport inhibitor response-1like protein regulates the level of auxin during pathogen attack and mediate ethylene signaling in roots of plants (Katagiri *et al.*, 2002).Involvement of auxin has been shown in disease susceptibility to viral pathogens (Padmanabhan *et al.*, 2006; Robert-Seilaniantz *et al.*, 2007).

The target mRNA transcripts of miR-6928-5p showed upregulation in the infected plants, with a maximum of 13.13 fold at 24 h. It remained elevated compared to control throughout the period of observation. The expression of miR-6928-5p

increased at 48 h by 1.72 folds; however its levels reduced in the consecutive stages. But an inverse correlation could not be found between miR-6928-5p and its target. FAD dependent oxidoreductase tends to auto oxidise oxygen to superoxides (ROS) during stress (Massy, 1994). The target gene's expression remained high throughout the different time intervals as compared to the uninfected sample, indicating that the FAD molecules during the course of infection are getting auto oxidised to ROS molecules (Harold *et al.*, 2018) and in order to compensate the loss, newer FAD molecules need to be synthesized by the plants. This may be the reason for the abundance of FAD transcripts in the infected plants.

The study could demonstrate the biological validation of five computationally predicted miRNAs in banana. Out of them miR-3900-5p, miR-2172-5p, miR-6928-5p and miR-971-5p showed differential expression in BBrMV infected plants. During virus infection, miR3900-5p and its target encoding plant viral response family protein and miR971-5p and its target encoding protein Argonaut 1 showed an inverse expression pattern, indicating the regulation of the targets by these miRNAs.

The differentially expressed miRNAs need to be further analyzed at different time intervals to understand their role during BBrMV infection. Degradome analysis may be helpful to identify candidate targets of these miRNAs which can aid in the development of a miRNA targeted approach for BBrMV resistance in banana.

Summary

6. SUMMARY

The study entitled "Identification and characterisation of virus responsive miRNAs in banana *Musa* (AAB) 'Nendran'" was conducted at the Department of Plant Biotechnology, College of Agriculture, Vellayani, Thiruvananthapuram during 2017-2019. The objective of the study was to identify the miRNAs associated with *Banana bract mosaic virus* (BBrMV) infection in banana var. Nendran from the expression profile of selected miRNAs.

Banana is the second most widely consumed fruit crop next to mango in India. The area under production for banana is about 8.58 lakh ha and the annual production is 30.8 MT. Among the biotic stresses rendering banana's production and productivity, plant viruses cause the most serious damage all over the world. Among them, Banana bract mosaic virus causes about 40% yield loss in Nendran banana. Due to the absence of a innate viral defense mechanism in banana plant almost all banana varieties are susceptible to the disease. Since conventional breeding techniques failed to produce a virus resistant variety, transgenics were developed to combat virus infection (Sagi et al., 1995). Due to continuous mutation in viral genome, transgenic banana plants with modified viral coat protein and replicase gene gained less importance throughout the years. RNA interference technology (RNAi) discovered in the past decade, is now considered to be the most important technique in developing a virus resistant banana plant. The mechanism is based on small RNA (siRNA and miRNA) mediated post transcriptional gene silencing, that can impart resistance towards stress in plants. The present study was focused on identifying miRNAs responsive to BBrMV infection in banana through RT-qPCR, so as to understand their role during biotic stress. The salient findings of the study are summarized below:

Five miRNA were selected from computationally predicted miRNAs based on their target's role in biotic stress. They were miR-3900-5p (targets: Plant viral response family protein gene and Putative disease resistance protein gene), miR-2172-5p (target: Putative Ethylene-responsive transcription factor 1gene), miR-5417 (target: Stress-associated endoplasmic reticulum protein 2 gene), miR-6928-5p (target: FAD dependent oxidoreductase gene) and miR-971-5p (targets: Argonaute 1A protein gene and Transport inhibitor response 1-like protein gene).

For studying the expression of these miRNAs, leaf samples were collected from the infected plants 24 h, 48 h, 1 wk and 2 wk after infection. RNA was isolated using CTAB method, reverse transcribed to cDNA and PCR was done. PCR analysis confirmed the presence of BBrMV from 24 h onwards.

Gene expression analysis was conducted using "qBase plus" software. The miRNAs and their target genes were taken with reference to the uninfected control plant. Raw Cq values were converted to relative expression values by qBase plus software.

Expression analysis with RT-qPCR confirmed the presence of all five miRNAs in the banana plant. Among them, miR-3900-5p, miR-2172-5p and miR-971-5p maintained higher expression upto one week compared to uninfected control. miR-6928-5p showed an increase in expression at 48 h. The expression of miR-5417 was down regulated by BBrMV infection at 24 h of infection.

Plant viral response family protein gene (target of miR-3900-5p) and Putative disease resistance protein gene (target of miR-3900-5p) showed differential expression during BBrMV infection. Putative disease resistance protein gene showed a peak in expression at 24 h and then a drastic reduction in expression during the course of infection. Plant viral response family protein gene showed a continuous decrease in expression throughout the period of observation, indicating that both genes were suppressed by the miRNA during BBrMV infection.

Ethylene responsive transcription factor gene (miR-2172-5p) was upregulated at 24 h, however at 1 wk and 2 wk, the gene expression was reduced drastically, indicating the negative regulation of miR-2172-5p in Ethylene responsive transcription factor gene expression.

FAD dependent oxidoreductase gene (target of miR-6928-5p) showed maximum expression at 24 h after infection and maintained a higher level upto 1wk. Argonaute protein gene (target of miR-971-5p) showed a lower expression upto 48 h and a drastic increase in expression as the infection progressed, suggesting the possible role of miR-971-5p in regulating the gene during stress. The gene coding for Transport Inhibitor response-1like protein gene (target of miR-971-5p) was peaked at 24 h and decreased in 48 h and 1 wk respectively, indicating negative regulation by miR-971-5p.

The study suggested that miR3900-5p, miR2172-5p, miR6928-5p and miR971-5p were associated with BBrMV infection in banana var. Nendran. Their targets *viz*., Putative disease resistance protein gene, Putative ethylene responsive transcription factor 1 gene, FAD dependent oxidoreductase gene and Transport inhibitor response-1 like protein gene showed significant increase immediately after the infection. The targets, Plant viral response family protein gene and Argonaute protein gene were suppressed during the course of BBrMV infection, indicating the possible role of miR-3900-5p and miR-971-5p in regulating the infection process.

For the future studies, the expression of miRNAs responding to BBrMV infection may be further studied in detail, by including more time intervals. This may help in understanding the role of miRNAs during BBrMV infection.

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7. REFERENCE

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Abstract

IDENTIFICATION AND CHARACTERISATION OF VIRUS RESPONSIVE miRNAs IN BANANA *Musa* (AAB) 'NENDRAN'

by

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Abstract of the thesis

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ABSTRACT

The study entitled "Identification and characterization of virus responsive miRNAs in banana *Musa* (AAB) 'Nendran'" was carried out during 2017-2019, in the Department of Plant Biotechnology, College of Agriculture, Vellayani. The objective of the study was to identify the miRNAs associated with *Banana Bract Mosaic Virus* (BBrMV) infection in banana var. Nendran from the expression profile of selected miRNAs.

Five miRNAs were selected from the 52 computationally predicted miRNAs in a previous study conducted in the Department of Plant Biotechnology. Selection was based on the function of their target genes i.e. their role in biotic stress conditions. The miRNAs selected were miR-3900-5p (targets: Plant viral response family protein and Putative disease resistance protein genes), miR-2172-5p (target: Putative ethylene responsive transcription factor1gene), miR-6928-5p (target: Flavin adenine dinucleotide dependent oxidoreductase gene), miR-5417 (target: Stress endoplasmic reticulum protein 2 gene) and miR-971-5p (targets: Argonaute protein and Transport inhibitor response-1 like protein genes).

For studying the expression of miRNAs, three month old *in vitro* raised banana var. Nendran plants were infected with BBrMV through viruliferous aphids (*Pentalonia nigronervosa)* by acquisition feeding method. Fifteen to twenty aphids reared in healthy banana suckers were transferred to the BBrMV infected sucker for 2 hour acquisition feeding, after starving for 30 min. These aphids were released on to the leaf axils of the tissue culture plants for a period of 24 h. After this time period the aphids were killed using an insecticide. Infection was confirmed by PCR using replicase specific primers and the results showed the presence of BBrMV specific amplicons from 24h onwards in all the samples.

For studying the expression of these miRNAs, leaf samples were collected from the infected plants 24 h, 48 h, 1 wk and 2 wk after infection. RNA was isolated using CTAB method, reverse transcribed to cDNA and PCR was done. PCR analysis confirmed the presence of all the five computationally predicted miRNAs in banana. The expression profiles of the miRNAs and their target genes were studied by RT-qPCR. The results showed upregulation of miR-3900-5p, miR-2172-5p, miR-6928-5p and miR-971-5p (1.2, 2.0, 1.27, 2.0 fold respectively) 24h after BBrMV infection. Among them miR-3900-5p, miR-2172-5p and miR-971-5p maintained higher expression upto one week compared to uninfected control. miR-6928-5p showed a 1.72 fold increase in expression 48 h after infection. Expression of miR-5417 was down regulated by BBrMV infection at 24 h of infection.

The two targets of miR-3900-5p (Plant viral response family protein and Putative disease resistance protein genes) showed contrasting trends in their expression after BBrMV infection. While Putative disease resistance protein showed a drastic increase (13 fold) 24 h after infection, Plant viral response family protein expression showed a continuous reduction throughout the period of observation. miR-2172- 5p and its target Putative Ethylene-responsive transcription factor 1gene (3.39 fold) were found upregulated during the infection. While miR-6928-5p showed maximum expression at 48 h, its target FAD (Flavin adenine dinucleotide) dependent oxidoreductase gene showed maximum expression at 24 h after infection and maintained a higher level upto 48 h. Among the two targets of miR-971-5p, Transport inhibitor response-1like protein gene showed a quick response to virus infection with a 6.15 fold expression at 24 h, while Argonaute protein gene showed a lower expression upto 48h and a drastic increase upto 2 fold as the infection progressed.

The study suggested that miR3900-5p, miR2172-5p, miR6928-5p and miR971-5p were associated with BBrMV infection in banana var. Nendran. Their targets *viz.*, putative disease resistance protein gene, putative ethylene responsive transcription factor 1gene, FAD dependent oxidoreductase gene and transport

inhibitor response-1 like protein gene showed significant increase immediately after the infection. Suppression of the targets *viz*., Plant viral response family protein and Argonaute protein genes during BBrMV infection suggested the possible role of miR-3900-5p and miR-971-5p in regulating the infection process.

Appendices

APPENDIX I

CTAB Extraction Buffer

CTAB	2.5% (w/v)
Tris-HCl	100 mM
EDTA	25 mM
Nacl	1.5 M
β-mercaptoethanol	0.2% (v/v)
PVP	4% (w/v)