EXPRESSION PROFILING OF microRNAs ASSOCIATED WITH VIRUS INFECTION IN BANANA CULTIVAR RED BANANA (*Musa* AAA)

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EXPRESSION PROFILING OF microRNAs ASSOCIATED WITH VIRUS INFECTION IN BANANA CULTIVAR RED BANANA (*Musa* AAA)

by

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(2018-11-171)

THESIS

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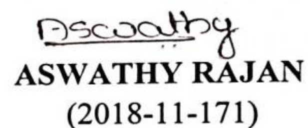
COLLEGE OF AGRICULTURE
VELLAYANI, THIRUVANANTHAPURAM-695 522
KERALA, INDIA
2020

DECLARATION

I, hereby declare that this thesis entitled **"EXPRESSION PROFILING OF microRNAs ASSOCIATED WITH VIRUS INFECTION IN BANANA CULTIVAR RED BANANA (*Musa* AAA)"** 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, associateship, fellowship or other similar title, of any other University or Society.



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Certified that this thesis entitled **"EXPRESSION PROFILING OF microRNAs ASSOCIATED WITH VIRUS INFECTION IN BANANA CULTIVAR RED BANANA (*Musa* AAA)"** is a record of bonafide research work done independently by Ms. Aswathy Rajan under my guidance and supervision and that it has not previously formed the basis for the award of any degree, diploma, fellowship or associateship to her.

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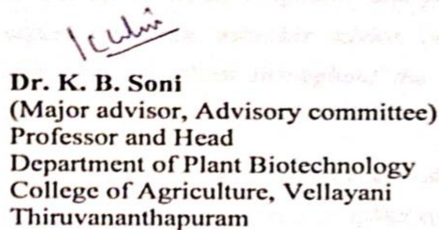

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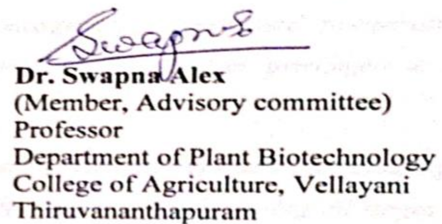

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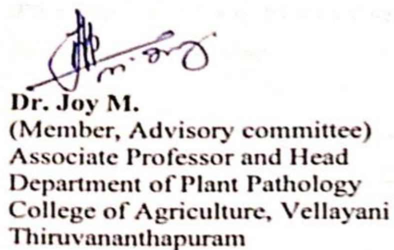

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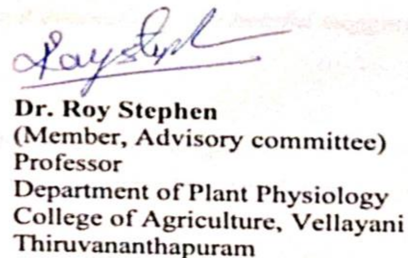

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LIST OF ABBREVIATIONS

%	Percentage
A genome	*Musa acuminata* genome constituent
A_{260}	Absorbance at 260 nm wave length
A_{280}	Absorbance at 280 nm wave length
AGO	ARGONAUTE
B genome	*Musa balbisiana* genome constituent
Bp	Base pairs
BSV	Banana streak virus
CTAB	Cetyl trimethylammonium bromide
cv.	Cultivar
DCL 1	Dicer like enzymes
dsDNA	Double stranded circular DNA
EST	Expressed sequence tag
FAD	Flavin adenine dinucleotide
FAOSTAT	Food and Agriculture Organization Corporate Statistical Database
HEN1	HUA enhancer 1
Min	Minutes
miRNA	microRNA
miRNA*	microRNA star strand
Ml	Millilitre
mM	Millimolar
mRNA	Messenger RNA
NCBI	National Center for Biotechnology Information
ng	Nanogram

nM	Nanomolar
nt	Nucleotides
PCR	Polymerase chain reaction
Pre-miRNA	Precursor microRNA
Pri-miRNA	Primary microRNA
PTGS	Post transcriptional gene silencing
RISC	RNA-induced silencing complex
RNA	Ribonucleic acid
RNAi	RNA interference
RNase	Ribonuclease
RNA-Seq	RNA sequencing
ROS	Reactive oxygen species
rRNA	Ribosomal RNA
RT	Reverse transcriptase
siRNA	Small interfering RNA
Tm	Melting temperature
tRNA	Transfer RNA
U	Unit
V	Volt
w/v	Weight per volume
µl	Microliter

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Introduction

1. INTRODUCTION

Banana is the second most important commercial fruit crop in the tropical and subtropical regions of the world with an average production of 115.73 million tonnes. India is the largest producer of banana with around 30 million tonnes of production (FAOSTAT, 2018).

Biotic and abiotic stresses are the major constraints in banana cultivation. Among them, viruses are the most damaging which cause serious loss to yield and quality of banana. *Banana bract mosaic virus* (BBrMV), *Banana bunchy top virus* (BBTV), *Banana streak virus* (BSV) and *Cucumber mosaic virus* (CMV) are among the most destructive viruses causing huge economic losses in banana all over the country. Since most of the popular edible bananas are triploids and natural resistance source is not available, traditional breeding for virus resistance is difficult. Genetic engineering techniques based on coat protein mediated resistance have been tried in a few crops.

RNA mediated resistance is comparatively a new approach for virus resistance by regulating the expression of a gene at a particular point in the plant's developmental phase. It was first observed in the transparent soil nematode *Caenorhabditis elegans* in which gene silencing was done by two small RNAs such as miRNA and siRNA. Gene silencing technology in plants is also known as post-transcriptional gene silencing (PTGS) (Napoli *et al*., 1990).

microRNAs (miRNAs) are an abundant class of endogenous non-protein-coding small RNAs with 20-24 nucleotide length (Bartel, 2004). Due to their role in gene regulation, miRNAs have been shown to take part in a wide variety of plant metabolic and biological processes (Palatnik *et al*., 2003). Studies have shown the role of miRNAs in plant-pathogen interactions. But only limited studies have been conducted in banana to understand the role of miRNAs in virus infection. Expression of the miRNAs and their target genes during the infection process will help to understand the functions of these genes in stress responses.

In a study conducted in the Department of Plant Biotechnology, 52 mature miRNAs were predicted in the banana genome using NovoMIR software and over 142 targets were identified using psRNATarget. During the validation of computationally predicted miRNAs, a few miRNAs and their targets have been found to be differentially expressed in tissue culture raised plants infected with BBrMV(Subramanian, 2019). But the expression of these miRNAs in naturally infected field grown plants was not studied.

In this context,two miRNAs and their predicted targets that showed differential expression in virus infected plants were selected with an objective to study the expression of selected miRNAs in BBrMV and BBTV infection in banana cultivar red banana (*Musa* AAA). The results of the study will provide information on the regulatory genes involved in the host defense against viruses in banana. The information will help to develop RNA mediated strategies for virus resistance.

Review of Literature

2. REVIEW OF LITERATURE

2.1 BANANA: ORIGIN AND GENOME CONSTITUTION

Banana (*Musa spp*.) is the most important fruit crop and staple fruit for millions of people in tropical and subtropical countries. It is the second extensively consumed fruit crop next to mango in India and due to its availability, low- cost of cultivation, taste and high nutritive value, and the fruit is favored by people all over the world. It belongs to the order *Zingiberales* and family *Musaceae*. Almost all edible seedless, parthenocarpic triploids bananas come from two diploid seedy wild species *Musa acuminata* and *Musa balbisiana* and all these varieties are propagated using suckers (Ortiz, 2013). The banana varieties include diploid cultivars like AA and AB, triploids with the genome constitution as AAA, AAB and ABB, synthetic tetraploids like 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. The genome size of banana is 600 Mb with the total number of chromosomes being 11. The whole assembly comprises 24,425 contigs and it has over 36,542 protein coding genes (D'Hont *et al*., 2012).

Average production of banana is estimated to be 115.73 million tonnes in over 120 countries in which India rank first with 30.8 million tonnes, which accounts for 33% of the world's production (FAOSTAT, 2018).

2.2 VIRAL DISEASES OF BANANA

Biotic and abiotic stresses are the significant limitations in banana cultivation. One of these, the viral diseases is regarded as a significant concern for banana production due to their impacts on quality and yield, in addition to constraints to the global germplasm exchange of banana. BBrMV, BBTV, BSV and CMV are one of the most damaging viruses causing enormous financial losses in banana all around the nation. One of those, BBTV and BSV are significant threats for banana production. Of both, BSV exists as episomal and endogenous types and more broadly spread globally than BBTV,

though the latter is up to now the most economically harmful virus resulting in a yield decrease of around 100 per cent.

In general, most of the viral diseases are transmitted by vectors. However, the interaction between virus and vector varies among different viral particles and pests. There are three types of virus-vector interaction 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 however, requires few hours for acquisition feeding (Watson *et al*., 1953).

2.2.1 Banana bract mosaic virus disease

BBrMV is an ssRNA virus belongs to potyviridae family and the disease was first noted in the year 1988 in Philippines (Magnaye and Espino, 1990). In India, the virus mostly affected the areas of Tamil Nadu, Kerala, Karnataka and Andhra Pradesh (Thomas *et al*., 1997; Selvarajan *et al*., 1997; Rodoni *et al*., 1999; Thangavelu *et al*., 2000; Singh, 2002; Kiranmani *et al*., 2005). The symptoms include mosaic patterns on the bract of the inflorescence, spindle shaped reddish brown streaks, stripes on the pseudostem and traveler's palm like leaf orientation. In severe cases, the leaves show particular broken streaks along the primary veins and also dispersed white to yellowish streaks opposite the midrib to the edge of the leaf. This viral disease is transmitted in a non-persistent manner by different species of Aphids, *Rhopalosipum maidis*, *Pentalonia nigronervosa*, cowpea aphid *Aphis craccivora* and *Aphis gossypii* (Magnaye and Espino, 1990; Selvarajan *et al.,* 2006). Banana bract mosaic disease incidence in cv. Nendran in Kerala ranges from 5 to 36 per cent and the infected plants flower, but produce very small bunches with curved brittle fruits. Very severely affected plants may fail to flower and may die by stunted growth and necrosis of pseudostem. The male buds are dark purple in colour with mosaic patches. There are varietal differences in the symptomatology of the disease. BBrMV has been 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). The accessions with ABB genome have a maximum incidence of the disease. Besides yield reduction, the fingers become malformed and curved, which reduces the market acceptability of fruits (Cherian *et al.,* 2002). The economic loss is however found to be 100% due to the presence of shriveled and malformed fingers (Selvaraj and Jeyabhaskaran, 2006).

2.2.2 Banana bunchy top virus disease

Banana bunchy top disease (BBTD) is the devastating and important most disease of banana (Dale, 1987). BBTD is caused by *Banana bunchy top virus* (BBTV) which is a multicomponent, circular, single stranded DNA virus. BBTV is also the type member of the genus Babuvirus in the family Nanoviridae (King *et al*., 2012). The isometric virion measures 18-20 nm in diameter. BBTV genome is made up of minimum of six circular single stranded (ss) DNA molecules and each component encoding for a protein from its single open reading frame (ORF) in the virion sense. These are designated as DNA-R (rolling circle replication initiation protein), DNA-S (coat protein peptide of 19.6 kDa), DNA-M (movement protein), DNA-C (cell cycle link protein), DNA-N (nuclear shuttle protein), and DNA-U3 (protein of unknown function) (Beetham *et al.,* 1999; Burns *et al*., 1995; Vetten *et al*., 2012). Each DNA component has two common regions: one common stem-loop region of 69 nucleotides with 62 per cent conserved between viral components and 2 major common regions of 92 nucleotides with 76 per cent conserved between viral components (Burns *et al.,* 1995). The disease was first reported in Fiji in 1889 (Magee, 1953). And these days it is common in most banana growing areas in the world. Banana bunchy top was most likely brought to India from Sri Lanka during 1940 (Varghese, 1945; Elangovan *et al*., 1990).

The initial symptom bearing leaf develops deep green streaks of variable length in the leaf veins, petioles and midribs. This dotting and streaking are typically called a "morse code" pattern (Magee, 1940). Hook like extension of the leaf lamina with deep

green color will be able to be found in the mid rib and leaf lamina. BBTV in the phloem tissue of banana causes cytological changes, as a result, the successive leaves become progressively short and develop marginal chlorosis or yellowing and further become upright, crowded and bunched at the apex of the plant giving a "rosette" or "bunchy top" appearance (Iskra-Caruana, 1990). Probably the most diagnostic symptoms are actually quite short green dots and dashes along the minor leaf veins, which form hooks as they get into the edge of the midrib. This particular symptom is actually best seen as soon as the leaf is looked at from the underside, towards the light. Symptoms appear exclusively on leaves formed after infection as well as in the basal part of the lamina or even on the petiole. Plants infected at an early stage of development rarely produce a bunch; with later infections a distorted bunch might be formed. When several abnormal leaves emerge, extreme congestion is actually noticeable at the crown, rather than the normal symmetrical disposition of leaves, rosette condition develops (Singh, 2002).

Banana aphid (*Pentalonia nigronervosa*) is recognized to be the main vector of BBTV (Magee, 1927). Banana aphid has a worldwide distribution and transmits the virus in a circulative and persistent manner (Anhalt and Almeida, 2008; Selvarajan *et al.,* 2006). The main spread of BBTV was reported to be through conventional planting materials including suckers and corms. All the infected suckers from infected stool eventually become infected. Cent per cent spread of BBTV was recorded in the case of vegetative propagules such as the suckers and corms (Magee, 1927). One more species of banana aphid, *P. caladii* has been found to transmit BBTV at a lower efficiency than *P. nigronervosa* (Watanabe *et al*., 2013).

2.2.3 Banana Streak Disease

Banana streak disease is recognized to be the most commonly distributed in banana plantations all over the world. The disease was first located in the Nieky Valley on the Ivory Coast in 1958 (Lockhart and Jones, 2000). Banana streak virus (BSV) a part of the genus Badnavirus of family Caulimoviridae stands out as the causal virus of the disease. The virions of BSV are bacilliform shaped, double stranded circular DNA

(dsDNA) genome roughly 7.2-7.8 kb longer that makes use of a virus encoded reverse transcriptase (RT) to replicate (Lockhart and Olszewski, 1993).

Symptoms of BSV are actually discontinuous usually chlorotic yellowish dots or maybe streaks that turn necrotic which run out of the midrib to the leaf margin. In some cases, spindle or eye shaped pattern or maybe blotches are actually noticed. At times the leaf lamina might be distorted. In the later stages, the streaks darken to orange and in most cases become black or brown. Necrosis has been observed to take place on the leaf midrib as well as petiole particularly below the temperature that is low as well as short day conditions. Signs may be concentrated or sparse in distribution. In general, symptoms are erratically distributed on the plant and not shown on all leaves. Some isolates are really severe that they produce stunting with serious necrotic streaks, leading to the death of the plant (Thangavelu *et al*., 2000). Stunting, decreased bunch sizes in addition to distortion of fingers have been reported (Gauhl and Pasberg_Gauhl, 1994). The often wide yellow line of leaf lamina parallel to the midrib, purple margin on leaf lamina, leaf twisting, and also an abnormal arrangement of the leaves comparable to traveler's palm were also noticed in plants infected with BSV. Symptomless and symptomatic stages alternate in infected plants that are infected, but virus can easily be recognized at all stages (Harper *et al*., 2002).

2.2.4 Banana Mosaic or Infectious Chlorosis Disease

Banana mosaic or infectious chlorosis disease initially discussed in 1930 from Australia (Magee, 1930) is actually among the typical viral diseases affecting plantain and then banana anywhere. Banana mosaic is likewise referred to as cucumber mosaic, sheath rot, heart rot, and infectious chlorosis. Typical strains of the virus have not been reported to result in serious pandemic or maybe loss to banana plantation. Nevertheless, serious strain (heart rot) of the virus is actually recognized to cause significant economic harm (Niblett *et al*., 1994). The condition is realized in the majority of the banana growing areas of the world (Jones and Lockhart, 2000). Banana mosaic illness is brought on by the *Cucumber mosaic virus* (CMV) that is a part of Cucumovirus team (OCEAH,

1995). Spherical virus particles of 28-30 nm of size have single stranded positive sense RNA genome. The majority of the CMV genome consists of 3 genomic and one subgenomic RNA species (Fauquet *et al*., 2005).

The virus causes adjustable indicators from gentle chlorosis to acute chlorotic streaks on leaf lamina based on the pathogen strain as well as the weather conditions. Symptoms are recognized to fluctuate throughout the growing season based on the temperature as well as rainfall. Leaf deformation and curling are occasionally found in the infected plants. This particular virus brings about noticeable signs sporadically in the area, and vast majority of leaves did not show any symptom. Typically mosaic indications have been noticed on fruits of infected vegetation. In general, the symptoms are definitely more severe in wintertime when temperatures fall under 24 °C in the tropics as well as subtropics. Symptoms tend to be more pronounced which include necrosis of emerging inner cells and leaves of pseudostem when banana vegetation is actually infected with serious strains of the virus. Fruits might show mosaic symptoms, as well as bunches, might bear no fruit or maybe malformed fruit. Plant death might happen in severe cases particularly when plants get infected with serious strain right after growing.

The spread of the disease occur in nature via vegetative planting material and also by more than sixty distinct species of aphid vector such as *A. gossypii, R. maidis*, *A. craccivora, R. prunifolium,* and *Myzus persicae* (Rao, 1980). CMV features a broad host range, infecting more than 900 species in nearly every region of the globe. Aphids generally develop the virus from other crops and diseased weed dispersed to banana plantation as a result of the migration of viruliferous vectors from diseased places. Nevertheless, the majority of the aphid species don't colonize on banana, though they could be in a position to transmit the CMV with fairly a lesser amount of performance throughout their exploratory trip to banana. A higher incidence of CMV seen in a freshly planted field could be due to the lack of alternate hosts for viruliferous aphid vectors to the field.

2.3 STRATEGIES TO COMBAT VIRAL DISEASES

The major constraints include various abiotic and biotic stresses. Among them viral diseases cause severe problems resulting in reduced growth and development finally leading to lesser production. It is recorded that the damage caused by viruses is more compared to other pathogens (Simon-Mateo and Garcia, 2011). Currently, over 6000 viruses are identified and have been studied throughout the world and among them, 1300 are plant viruses (King *et al*., 2012). Different strategies have been used to combat viruses, which include conventional breeding programs using natural sources of resistance and non conventional breeding technique like development of transgenic plants with coat protein, movement protein and *R* genes.

2.3.1 Virus resistant transgenic plants

Genetic resistance induction in plants is one of the techniques which were used to protect plants from virus attack. And it was done by considering the virus resistance (*R* genes) present in the wild relatives. For example, *RSV* and *Rsc* genes of soybean were linked to *R* genes which was resistant to soybean mosaic virus (SMV) (Widyasari *et al.*, 2020). The dominant allele of *R* genes *viz*. the *N* genes of tobacco. *Rx1* and *Rx2* of potato were considered to develop virus resistant against *Tobacco mosaic virus* and *Potato virus X.*

The durability of this technique was 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

RNA interference technology or gene silencing brought new light to combat viral infection (Fire *et al*., 1998). Gene silencing or RNA interference is known as the regulation of gene expression at a particular point in a plant's developmental phase. And when it occurs during the transcriptional and translational phase it is said as gene knockdown. It was first observed in the transparent soil nematode *Caenorhabditis*

elegans where gene silencing was done by two small RNAs such as miRNA and siRNA. Gene silencing technology in plants is known as post transcriptional gene silencing (PTGS) (Napoli *et al*., 1990). There are different gene silencing pathways which include miRNA mediated gene silencing, siRNA directed RNA degradation pathway and siRNA directed DNA methylation. Among these pathways miRNA mediated gene silencing helps in the invasion of dsRNA into cytoplasm and then it is converted to small fragments using Dicer like enzymes (DCL 1). The fragments formed can be either miRNA or siRNA.

2.4 microRNAs

microRNAs (miRNAs) are an extensive class of endogenous, non-coding small regulatory RNAs that bind complementary to the mRNA molecules and negatively regulate gene expression at the post-transcription levels and they have a length of 20-24 nucleotides length in plants (Bartel, 2004; Zhang *et al*., 2006). Each miRNA is encoded by miRNA genes that are located mainly in intergenic regions. The first miRNA was discovered in the transparent soil nematode *C. elegans* in 1993 (Lee *et al*., 1993; Wightman *et al*., 1993). The discovery of extensive miRNAs in animals prompted scientists to search for miRNAs in plants. In 2002, several research groups independently identified more than 100 miRNAs in *Arabidopsis* by using direct cloning technology (Reinhart *et al*., 2002).

In plants, biogenesis of miRNAs starts in the nucleus. miRNA genes are transcribed by RNA polymerase II into long primary transcripts (pri-miRNAs) followed by cleavage to precursor mRNA (pre-miRNA) by the nuclear RNase III-like enzyme, called DICER-LIKE (DCL1). The imperfect stem-loop secondary structures of the pre-miRNA hairpins are cut by DCL1 enzymes to generate the miRNA: miRNA* duplexes. The duplexes are methylated by S-adenosyl-L-methionine dependent RNA methyl transferase, HUA enhancer 1 (HEN1); and exported to the cytoplasm by HASTY where they undergo RNA-induced silencing complex (RISC) loading. The miR* is released and the mature miRNA loads onto ARGONAUTE (AGO) ribonucleases in the RISC

complex. Extensive base-pairing with mRNA targets is required for plant miRNA function to regulate gene expression. The miRNAs guide AGO proteins to their specific targets through sequence complementarity which then leads to silencing by degrading the target mRNA transcript or by repressing its translation. On binding to a target mRNA with perfect complementarity, the Ago-miRNA complex induces its cleavage and degradation. An Ago-miRNA complex binding imperfectly to the 3′ UTR of the target mRNA induces translational inhibition, or deadenylation and subsequent decapping (DCP1, 2) and degradation of the target mRNA (Park *et al*., 2002; Djami *et al*., 2017).

The biogenesis of animal miRNAs varies from plant miRNAs. In animals, pri-miRNA is cleaved to pre-miRNA using DroshaRNase 3 endonuclease whereas in plants it is done using Dicer like enzymes (DCL 1). After cleavage, pre-miRNA is transported to cytoplasm from nucleus by Exportin-5 and form miRNA: miRNA duplex in animals (Hammond *et al*., 2000).

2.4.1 microRNA Identification

Experimental and bioinformatics strategies have been widely employed to identify miRNAs in both plant and animal kingdoms. miRNAs have been validated using different experimental approaches, including deep sequencing, direct cloning approach, which is widely used to discover animal miRNAs, and is also used to discover miRNAs in different plant species. Bioinformatics approaches, including Computational and Homologue-based analysis (Zhang and Wang, 2015).

2.4.2 Role of microRNA in plants

miRNAs play critical roles in almost all biological and metabolic processes that lead to plant growth and development. They regulate plant development, plant phase transition and plant response to abiotic and biotic stresses. microRNAs control plant development and morphology through controlling plant cell differentiation and proliferation. It includes fruit and seed development, leaf development and leaf morphology, shoot and root development, apical dominance and plant biomass (Pantaleo

et al., 2010; Zhang *et al*., 2011). For example, overexpressed miR165, miR166 acting on HD-ZIPIII transcription factor improved leaf and vascular development in maize and *Arabidopsis* (Williams *et al*., 2005).miR 160 is one of the foremost vital quality controllers for root development and gravitropism. Overexpression of miR160 caused root tip deformity and further caused abnormality in *Arabidopsis* root development and growth (Wang *et al*., 2005).

2.4.2.1 Role of microRNA in response to stresses

Abiotic and biotic environmental stresses are the major factors limiting plant growth, yield and sustainable agriculture worldwide. The common abiotic stresses include drought, high salinity, high and low temperature, nutrient deficiency, hypoxia, and pollutants. These limiting factors, particularly for drought and high salinity, are exacerbated by global climate change, which causes marginal lands to become more susceptible to arid and brackish conditions. Thus, breeding new plant cultivars with improving tolerance to environmental stresses is necessary for maximizing plant biomass and crop yield. miRNAs have been identified to be promising targets for increasing plant's tolerance to a diversity of abiotic and biotic stresses (Zhang *et al*., 2005).

2.4.2.2 Roles of miRNAs in abiotic stress in plants

Stress-induced miRNAs lead to down-regulation of negative regulators of stress tolerance and stress-inhibited miRNAs allow the accumulation and function of positive regulators. For example, overexpression of miR169a exhibited more sensitivity to drought stress in transgenic arabidopsis plants than its wild-type plants due to enhanced leaf water loss (Li *et al*., 2008). In creeping bentgrass overexpression of miR 319 led to enhanced drought and salt tolerance by increased leaf wax content and water retention (Zhou *et al*., 2013). Overexpression of miR394 improved tolerance to drought in *Arabidopsis* majorly due to lowered leaf water loss (Ni *et al.,* 2012) in an ABA dependent way (Song *et al*., 2013). Nevertheless, exactly the same transgenic plant line with overexpression of miR 394 showed delicate to salinity (Song *et al.,* 2013).

Overexpression of osamiR 396c decreased salt as well as alkali anxiety tolerance in both Rice and *Arabidopsis* (Gao *et al*., 2010).

2.4.2.3 Roles of miRNAs in biotic stress in plants

Insects and diseases are two major biotic stresses limiting plant growth and development and further plant biomass and yield. Several miRNAs are aberrantly expressed during insect, bacterial, and virus infection (Barah *et al*., 2013).

During the normal functioning of a plant, the miRNA suppresses the expression of stress responsive genes. However, during stress conditions, these miRNAs get downregulated. Often the pathogen miRNA interference suppresses the action of stress responsive proteins, which may lead to the susceptibility of the plant to the pathogen (Hewezi *et al*., 2012).

2.4.2.3.1 Roles of miRNAs during virus infection

Involvement of miRNAs in biotic stresses has been reported in many crops. Plants function either by creating a direct attack on the viral genes using endogenous miRNAs or by cleaving the miRNA responsible for the replication of virus. The miRNA 398 levels were found down regulated in *Arabidopsis* during biotic stress (Jagadeeshwaran *et al*., 2009). During *Begomoviruses* infection, several developmental processes were up regulated by the miRNA and it caused the suppression of viral targets (Amin *et al*., 2011).

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

In a study conducted by Sheeba *et al*. (2013), eighteen banana miRNAs have been predicted from ESTs and BAC sequences. Expression profiling of three miRNAs (miR156, miR159, and miR166) after the infection of *Banana streak mysore virus* (BSMYV) in banana showed an increased expression of miR166. Ghag *et al*. (2015)

observed several morphological changes in transgenic banana plants overexpressing *Musa* miRNA156.

In *Pyrus pyrifolia,* a number of miRNA target genes were identified which are 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 up regulated in response to *Pernosclerospora cubense* infection (Jin *et al*., 2015).

In rice, miR444, a monocot-specific miRNA, has been reported to regulate resistance against *Rice stripe virus* (RSV) infection by up regulation of the *OsRDR1* expression (Wang *et al*., 2016). It has been reported that the induction of miR319 by *Rice ragged stunt virus* (RRSV) infection suppressed jasmonic acid (JA)-mediated defense to facilitate virus infection (Zhang *et al*., 2016). Based on the sRNA transcriptome data and qRT-PCR verification, Shun *et al*. (2016) observed that some miRNAs were differentially expressed between *Fusarium* wilt-resistant and susceptible banana varieties. A total of 293 and 31 target genes were predicted based on the draft maps of banana genome and *Fusarium oxysporum* genomes, respectively. They reported that two important pathogenic genes in *F. oxysporum* genomes, namely, feruloyl esterase gene and proline imino peptidase gene, were targeted by banana miRNAs.

During *Sugarcane mosaic virus* (SCMV) infection in maize plant, up regulation of miR168 and miR528, and down regulation of miR159 have been observed (Xia *et al.,* 2018).

2.5 COMPUTATIONAL PREDICTION OF miRNA AND THEIR TARGETS

Experimental and bioinformatics strategies have been widely employed to identify miRNAs in both plant and animal kingdoms. miRNAs have been validated using different experimental approaches, including deep sequencing, direct cloning approach, which is widely used to discover animal miRNAs, and is also used to discover miRNAs in different plant species. Basically, there are two approaches to predict miRNAs, the

genetic approach using sequencing and the computational approach using homology based analysis using ESTs and GSSs (Chai *et al*., 2015).

2.5.1 Computational approach for miRNA prediction

2.5.1.1 Homology based analysis

Comparative genomic studies help in identifying and characterizing miRNAs in non-model plants. Homology based analysis is based on the search against the sequenced model plant ESTs and GSSs of non model plants (Dehury *et al*., 2013). The advantage of the study is that the search algorithm uses genome matching hits to check between miRNA and query sequences and a separate search for GSSs and ESTs is done. The BLAST analysis is compared against known specific miRNAs sequences of plants downloaded from EST database (dbEST), miRBase or GENBANK. The BLAST result with 0-3 mismatches used for analysis against known miRNA and sequences are blasted against the protein database in order to pull out coding sequences. Similar works were conducted in tobacco using the known miRNA sequences of *Arabidopsis*, rice and poplar. A comparison was done between 349 known miRNAs against 14, 20,579 GSS sequences of tobacco (NCBI) (Frazier *et al.,* 2010).

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

There are several computational tools that are used to identify novel miRNAs. They predict the miRNAs based on their length, sequence similarity among the species, hairpin formation and minimal fold energy (Li *et al*., 2010). NOVOMIR, MiPred, MIR-PD and MIRFINDER are the most commonly used tools to predict miRNAs. Among these NOVOMIR is popularly used because it has a set of criteria to screen miRNAs from the genome of an organism. Then it is followed by a sophisticated statistical model

that predicts pre-miRNAs from the other miRNAs, using genome as the input. The sensitivity and specificity of the software are about 0.99 and 0.83. And it depends on the RNA fold and shapes for predicting secondary structure of RNA (Teune and Steger, 2010). The first prediction using NOVOMIR was done in Arabidopsis genome and obtained 1477 miRNAs.

In a study conducted in the Department of Plant Biotechnology, 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 A 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

miRNAs are predicted and identified using computational and bioinformatic tools in plants and they are validated in wet lab experiments. miRNAs can be confirmed using two processes such as the evaluation of host miRNAs target sequence and artificial miRNA (amiRNA) sequence creation (Watanabe *et al*., 2007). The experimental validation includes the creation of gene specific target primers and stemloop primers for miRNAs. Isolated plant RNA is converted to the corresponding cDNA. Then Real-Time PCR is done using cDNA samples with gene specific primers, to get complete information regarding the expression profile of miRNAs. The present study was focused on the expression profiling of the selected miRNAs and their corresponding target genes during viral infection.

2.6.1 Expression profile analysis

Expression profiling of miRNAs is done to understand the regulatory role and metabolic functions of plants. By studying the expression of gene under stress conditions, their biological role in plants can be understood (Chen *et al*., 2014)**.** RT-qPCR is the most accurate method that is used for confirmation and quantification of the gene expression level (Chen *et al*., 2005).

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

In the case of rice, overexpressed OsmiR397 acting on OsLAC target gene caused increased grain size and panicle branching (Zhang *et al*., 2013). In *Arabidopsis* down regulation of miR396 caused nematode resistance (Hewezi *et al*., 2012).

Sheeba *et al*. (2013) predicted eighteen banana miRNAs from ESTs and BAC sequences. Expression profiling of three miRNAs (miR156, miR159, and miR166) after the infection of *Banana streak mysore virus* (BSMYV) in banana showed an increased expression of miR166.

In banana var. Nendran infected with *Banana bract mosaic virus* (BBrMV), three miRNAs such as 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 glyceraldehydes-3-phosphate dehydrogenase respectively (Mathew, 2018).

2.7 IDENTIFICATION OF BIOTIC STRESS RESPONSIVE miRNAs

For the present study "Expression profiling of microRNAs associated with virus infection in banana cultivar Red Banana (*Musa* AAA)" two miRNAs were selected from the previous work. The selection was based on the relative expression of the selected miRNAs which include miR-6928-5p (target: FAD dependent oxidoreductase gene) and miR-971-5p (target: Argonaute 1A protein gene).

2.7.1 FAD dependent oxidoreductase family and role in oxidative stress

FAD (flavin adenine dinucleotide) is a kind of redox-active flavoprotein coenzyme involved in a lot of enzymatic reactions. It also functions in the generation of ROS species in cells. Flavins, including FAD, have a tendency to auto oxidise oxygen molecules to superoxide during biotic and abiotic stress (Massy, 1994). The importance of FAD dependent oxidoreductase gene (target of miR-6928-5p) in the current study was to understand its role in inducing ROS during BBrMV and BBTV infection in field-grown plants.

2.7.2 Argonaute protein

Argonauts (AGOs) are proteins that are a part of RISC. It acts as the molecular platform for RNA silencing machinery. These complex proteins play an important role in plant's defense response through post-transcriptional gene silencing (PTGS) of host and pathogen genes. Antiviral defense is among the most significant features of AGO proteins and it is centered 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 siRNA of the virus therefore 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 might also damage viral replication and translation by manipulation and regulation of defense genes. AGOs can also improvise gene silencing using cleaved RNA fragments which serve as substrates for RDR. The antiviral property of the AGO proteins depends on factors including levels of expression, localization of tissue and cells, AGO cofactors, sRNA binding sites etc. (Garcia-Ruiz *et al*., 2015). The Argonaute protein gene was considered as target for miR-971-5p in the present study, in order to understand its expression levels during BBrMV and BBTV infection in field grown plants.

Materials and Methods

3. MATERIALS AND METHODS

The study entitled "Expression profiling of microRNAs associated with virus infection in banana cultivar Red Banana (*Musa* AAA)" was conducted at the Department of Plant Biotechnology, College of Agriculture, Vellayani, Thiruvananthapuram during 2018-2020. The detailed description of the methodologies followed for this research is mentioned below:

3.1 COLLECTION OF SAMPLES

Banana plants of cultivar red banana infected with BBrMV (Plate 1) and BBTV (Plate 2) were identified from the Instructional Farm, College of Agriculture, Vellayani, based on the visual symptoms. Leaf samples were collected from healthy uninfected and infected plants. The BBrMV and BBTV infection were reconfirmed by RT-PCR and normal PCR respectively using the primers specific to coat protein genes of the viruses.

3.2 DNA ISOLATION FROM BBTV INFECTED SAMPLES

DNA was isolated from the leaf samples of both control and infected plants using Doyle and Doyle (1990) protocol. Samples (200 mg) were taken in a pre-chilled mortar and ground to a fine powder using liquid nitrogen. The powder was transferred to a centrifuge tube and 2 ml of extraction buffer (Appendix I) was added to make a homogenate. To this 1 µl (10 mg/ml) RNase was added and incubated in a water bath at 65 ºC for 30 min. After cooling the mixture, an equal volume of chloroform-isoamyl alcohol (24:1) was added and mixed well by inversion. The mixture was centrifuged at 10,000 rpm, for 5 minutes at 4 ºC. To the supernatant 400 µl isopropyl alcohol/ ethanol was added and kept overnight at -20 ºC. The mixture was centrifuged at 10000 rpm, 4 ºC, for 5 minutes and the pellet recovered was washed twice with 70 percent ethanol and dissolved in 50 µl sterile DNase free water or TE buffer.

3.2.1 Agarose gel electrophoresis

The quality of DNA was analysed on 0.8 per cent agarose gel in a horizontal gel electrophoresis unit. Agarose was weighed out and melted in 1X TBE (Tris base 10.8 g, Boric acid 5.5 g, 0.5 M EDTA 4 ml). Ethidium bromide 0.5 $\mu g ml^{-1}$ was added to the agarose after cooling it to about 50 ºC. The mixture was then poured into a casting tray fitted with the appropriate comb. After solidification, the comb was removed and the gel was placed in an electrophoresis tank containing 1X TBE buffer filled to around 1 mm above the gel. DNA samples (5 µl) were mixed with gel loading dye (1 µl) 6X Bromophenol blue (0.25%) and Glycerol (30%) and loaded into the wells. Electrophoresis was carried out at 75 V until the loading dye reached the three-fourth length of the gel. The gel was documented using the Gel Doc^{TM}XR+ documentation system (BIO-RAD).

3.2.2 Quantification of DNA

Quantification of DNA was carried using NanoDrop spectrophotometer. The optical density of the samples was recorded at both 260 and 280 nm. The absorbance value of 1.0 at 260 nm indicates the presence of 50 $\mu g\ ml^{-1}$ of DNA. The concentration of DNA was calculated by using the formulae:

Conc. of DNA ($\mu g\ ml^{-1}$) = A_{260} x 50 $\mu g\ ml^{-1}$ x dilution factor

A_{260}/A_{280} ratio indicates the quality of DNA. Ratio ranging from 1.8-2 indicates good quality DNA.

3.3 RNA ISOLATION FROM BBrMV AND BBTV INFECTED SAMPLES

RNA was isolated from the leaf samples of BBrMV and BBTV control and infected plants using the method of Rodrigues-Garcia modified by Ekatpure *et al.* (2019). In this modified protocol incubation time for the lysis was increased from 10 min to 30 min at 65 ºC. RNA was precipitated with 3 M LiCl incubating overnight at 4 ºC instead of -20 ºC.

Samples (0.5 gm) were taken in a pre-chilled mortar and ground to a fine powder using liquid nitrogen. To this 3 ml of extraction buffer (Appendix II) was added to make a homogenate and incubated in a water bath at 65 ºC for 30 min. The homogenate (approx. 750 µl) was transferred to a sterile 2 ml microfuge tube using the cut tip to avoid the RNA damage and was precipitated using 5 mM potassium acetate (66 µl) and absolute ethanol (150 µl). The tube was vortexed briefly for 1 min. An equal volume of chloroform-isoamyl alcohol (49:1) was added, vortexed briefly, and centrifuged at 16000 g for 20 min at room temperature. The supernatant was collected in a fresh tube and repeated this process to eliminate any cell debris that was suspended. The supernatant was collected in a fresh tube and 850 µl of fresh phenol chloroform-isoamyl alcohol (25:24:1) was added. It was then vortexed for 10 sec and centrifuged at 16000 g for 15 min at room temperature. The supernatant was collected in a fresh sterile tube and 850 µl of chloroform-isoamyl alcohol (49:1) was added and the tube was briefly vortexed for 10 sec and centrifuged at 16000 g for 15 min at 4 ºC. To the supernatant, 3 M LiCl was added and kept overnight at 4 ºC. The content was then spun at 16000 g for 20 min at 4 ºC. The supernatant was discarded and the pellet recovered was washed twice with 70 percent ethanol. Pellet was air-dried at room temperature under the Laminar Air Flow (LAF) and dissolved in 30 µl DEPC treated sterile distilled water. RNA was quantified in a UV-light spectrophotometer and stored at -80 ºC until use.

3.3.1 Agarose gel electrophoresis

The quality of RNA was analysed on 2 per cent agarose gel in a horizontal gel electrophoresis unit. Agarose was weighed out and melted in 1X TBE (Tris base 10.8 g, Boric acid 5.5 g, 0.5 M EDTA 4 ml). Ethidium bromide 0.5 $\mu g ml^{-1}$ was added to the agarose after cooling it to about 50 ºC. The mixture was then poured into a casting tray fitted with the appropriate comb. After solidification, the comb was removed and the gel was placed in an electrophoresis tank containing 1X TBE buffer filled to around 1 mm above the gel. RNA samples (5 µl) were mixed with (1 µl) 6X gel loading dye (Bromophenol blue 0.25% and Glycerol 30%) and loaded into the wells. Electrophoresis

was carried out at 75 V until the loading dye reached the three-fourth length of the gel. The gel was documented using the Gel Doc^{TM} XR+ documentation system (BIO-RAD).

3.3.2 Quantification of RNA

The quantification of RNA was carried using a NanoDrop spectrophotometer. The optical density of the samples was recorded at both 260 and 280 nm. The absorbance value of 1.0 at 260 nm indicates the presence of 40 µg ml^{-1} of RNA. The concentration of RNA was calculated by using the formulae:

$$\text{Conc. of RNA } (\mu g\ ml^{-1}) = A_{260} \times 40\ \mu g\ ml^{-1} \times \text{dilution factor}$$

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

3.3.3 cDNA synthesis and its confirmation

The RNA isolated from healthy control and those of BBrMV and BBTV infected samples were converted to their complementary DNA using Verso cDNA synthesis kit (Thermo Scientific) according to the manufacturer's protocol. The kit contained reverse transcriptase enzyme and oligodT primers and an RNase inhibitor which prevents the degradation of isolated RNA. It also contains an RT enhancer for the removal of DNA contamination. A 20 µl reaction mix was prepared using the following components.

Components	**Volume (µl)**
cDNA synthesis buffer (5X)	2 µl
dNTP (500 µM)	1 µl
RNA primer (2 µM)	0.5 µl
RT enhancer	0.5 µl
RNA (1 µg/µl)	3 µl
Reverse transcriptase (1U/µl)	0.5 µl
Water	2.5 µl
Total volume	**10 µl**

The contents were mixed well after giving a flash spin; PCR was carried out in a thermal cycler (BIO-RAD). The thermal profile for the reverse transcription reaction of the target gene was:

42 ºC for 30 min

95 ºC for 2 min

The cDNA prepared was checked for genomic DNA contamination as well as successful conversion to cDNA by RT-qPCR with β-actin gene primers. The primers were designed spanning an intron. Genomic DNA contamination will produce a ~700 bp larger amplicon whereas cDNA produces ~100 bp amplicon. A standard PCR mix was prepared for 20 µl total volume containing:

Components	**Volume (µl)**
SYBR Green Master Mix (2X)	10 µl
Forward primer (10 µM)	0.6 µl
Reverse primer (10 µM)	0.6 µl
cDNA (500 ng/ µl)	2 µl
Nuclease free water	6.8 µl
Total volume	**20 µl**

The thermal profile used was as follows

Step 1: 95 ºC for 3 min

Step 2: 95 ºC for 15 sec

Step 3: 55 ºC for 15 sec

Step 4: 60 ºC for 45 sec. Steps 2 to 4 were repeated for 30 cycles

Step 5: 60 ºC for 5 min

After completion, the PCR products were separated on 1.5 per cent agarose gel in a horizontal gel electrophoresis unit along with a 100 bp ladder. The electrophoresis was conducted at 75 V till the gel loading dye reached three-fourth of the gel and the final results were viewed using Gel Doc^{TM}XR+ documentation system (BIO-RAD).

3.4 CONFIRMATION OF BBTV INFECTION IN SAMPLES USING COAT PROTEIN GENE-SPECIFIC PRIMER

Presence of BBTV in the infected banana plants were confirmed by PCR using coat protein gene-specific primer. The following primer was used (Ponsubrya, 2014).

BBTV coat protein forward primer – ATGGCTAGGTATCCGAAGAAATCC
BBTV coat protein reverse primer- ACTCCAGAACTACAATAGAATGCC

The reaction mixture contained the following

Components	**Volume (µl)**
10X reaction buffer (1X)	2.5 µl
dNTP mix (100 µM each)	2.0 µl
Forward primer (10 µM)	0.5 µl
Reverse primer (10 µM)	0.5 µl
Taq DNA polymerase(1U/µl)	0.5 µl
DNA (10 ng/µl)	5.0 µl
Nuclease free water	14.0 µl
Total volume	25.0 µl

The PCR was carried out by denaturing the template at 95 ºC for 3 min followed by 30 cycles of denaturation at 92 ºC for 30 sec, annealing at 60 ºC for 30 sec, and extension at 72 ºC for 45 sec. A final extension was given at 72 ºC for 5 min.

The integrity of the PCR products was checked by on 1.4 per cent agarose gel 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^{TM}XR+ documentation system (BIO-RAD).

3.5 CONFIRMATION OF BBrMV INFECTION IN SAMPLES USING COAT PROTEIN GENE-SPECIFIC PRIMER

The infection was confirmed by doing RT-PCR using the primer specific to coat protein gene of BBrMV (Jadhav, 2019). The cDNA synthesized as mentioned in section 3.3.3 was used as the template. The primer used for amplification is shown below:

BBrMV coat protein forward primer - ATGTCAGCTCCATCTTCATC
BBrMV coat protein reverse primer – TATCACGCTTCACATCTTCA

The components of the reaction mixture are given in the table shown below

Components	**Volume (µl)**
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)	0.5 µl
cDNA (200 ng/µl)	1 µl
Nuclease free water	13.5 µl
Total volume	20.0 µl

The thermal profile used was as follows:

Step 1: 95 ºC for 3 min

Step 2: 95 ºC for 15 sec

Step 3: 55 ºC for 15 sec

Step 4: 72 ºC for 45 sec. Steps 2 to 4 were repeated for 30 cycles

Step 5: 72 ºC for 5 min

The integrity of the PCR products was checked on 1 per cent agarose gel 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™ XR+ documentation system (BIO-RAD).

3.6 ISOLATION AND AMPLIFICATION OF miRNAs

Two miRNAs and their targets which were found differentially expressed during BBrMV infection in the previous study were selected. The details of miRNA and target shown in table 1.

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-6928-5p	GGGGAUUUUC AAGUACUGCA	UGCAGCAUUU GAAGAUCCUC	FAD-dependent oxidoreductase	Protection from ROS
2	miR-971-5p	UUUGAUGAUU UGAAUUUA	UAGAAUCAAA UUAUCAAC	Protein Argonaute 1A	RISC formation

Since it is difficult to extract microRNAs from the total RNA because of their small size (18-22nt), the amplification was carried out in a two-step process. A stem-loop RT primer was used in the first step to hybridize with the miRNAs in the total RNA and cDNA was synthesized in an RT reaction. In the second step, this cDNA was amplified using miRNA specific forward primer and universal reverse primer in a real-time PCR reaction using SYBR Green assay.

3.6.1 Primers for extracting miRNAs

In the present study, a stable stem-loop primer sequence of 44nt (5′- GTC GTA TCC AGT GCA GGG TCC GAG GTA TTC GCA CTG GAT ACG AC -3′) as reported by Chen *et al*. (2005) was used to lengthen the miRNA cDNA. The miRNA-specific stem-loop primers (Table 2) used in this work were designed (Subramanian, 2019) by combining the stem-loop sequence with the reverse complement of the six 3′ nt of the mature miRNA sequence.

Table 2 Sequence of stem-loop primers

Sl. No	miRNA name	miRNA sequence (5'..3')	miRNA specific stem loop sequence(5'..3')
1	miR-6928-5p	GGGGATTTTC AAGTACTGCA	GTCGTATCCAGTGCAGGGTCCGAGGTA TTCGCACTGGATACGACTGCAGT
2	miR-971-5p	TTTGATGATTT GAATTTA	GTCGTATCCAGTGCAGGGTCCGAGGTA TTCGCACTGGATACGACTAAATT

3.6.2 Primers for the amplification of miRNAs

Table 3 shows the primers used for amplification of miRNAs. The miRNA-specific primer designed by Subramanian (2019) was used as forward primer for each miRNA. It 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 ºC. The reverse primer was universal (Kramer, 2011) for all miRNAs that was specific to the 44nt stem-loop sequence (5'-CCAGTGCAGGGTCCGAGGTA-3').

Table 3 Sequence of primers used for the amplification of miRNAs

Sl.No	miRNA name	miRNA sequence (5'..3')	Forward primer (5'..3')	Universal reverse primer (5'..3')
1	miR-6928	GGGGATTTTC AAGTACTGCA	GAGGGCCGGGG ATTTTCAAGT	CCAGTGCAGGGT CCGAGGTA
2	miR- 971-5p	TTTGATGATTT GAATTTA	GGGGGGCGTTT GATGATTTG	CCAGTGCAGGGT CCGAGGTA

miRNA specific cDNA was synthesized from the RNA isolated from the leaf samples of control plants as well as BBrMV and BBTV infected (section 3.5.1) were

converted to cDNA. For the reverse transcription of miRNAs using stem-loop primers, a 10 µl reaction mix was prepared using the following components:

Components	**Volume (µl)**
cDNA synthesis buffer	2 µl
dNTP (500 µM)	1 µl
Stem-loop primers (10 µM)	0.5 µl
RNA samples (1 ng/µl)	2.5 µl
Reverse transcriptase enzyme	0.5 µl
Water	3.5 µl
Total volume	**10 µl**

The contents were mixed well and after giving a flash spin, the reaction was carried out in BIORAD's thermal cycler.

The following thermal profile was used for this reaction

16 ºC incubation: 30 min,

Step 1: 30 ºC for 30 sec

Step 2: 42 ºC for 30 sec

Step 3: 50 ºC for 1 sec

Steps 1 to 3 were repeated for 60 cycles.

Step 4: 75 ºC for 5 min

The cDNA samples were stored at -20 ºC

3.6.3 Assay for checking the amplification of miRNAs

The expression of miRNAs in control and infected plants was checked by PCR. miRNA specific, as well as universal primers specific to stem-loop sequence, were used to amplify miRNA specific cDNA. Components for PCR were added as follows

Components	**Volume (µl)**
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)	0.5 µl
cDNA (500 ng/µl)	1 µl
Nuclease free water	13.5 µl
Total volume	**20 µl**

The thermal profile followed was:

Step 1: 95 °C for 5 min

Step 2: 95 °C for 5 sec

Step 3: 60 °C for 10 sec

Step 4: 72 °C for 8 sec. Steps 2 to 4 were repeated for 30 cycles

Step 5: 72 °C for 5 min

After completion, the PCR products were separated on 1.5 per cent agarose gel. 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 Gel Doc™XR+ documentation system (BIO-RAD).

3.7AMPLIFICATION OF TARGET GENES

The primers specific to the target genes (Table 4) reported by Subramanian (2019) were used in this study. And the primers were designed using Primer3Plus software (Untergasser *et al.,* 2007). The specificity of primers was checked using NCBI Primer-BLAST software. The software designed by NCBI 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 amplification (Ye *et al*., 2012).

Table 4 Sequence of primers used for the amplification of target genes

Sl. No.	Target name	Forward primer(5'…3')	Reverse primer(5'…3')	Expected amplicon size (bp)
1	FAD dependent oxidoreductase (*FAD*)	TGTGCAAATCG TTTGCTTTC	GTATTGTTGCCC ATCCATGA	274
2	Argonaute protein (*ARG*)	TACTGGACAAA AGCCCCAAC	GACGTTTTTGGA CAACCACA	300

3.7.1 Assay for checking the amplification of target and reference gene

The cDNA of the infected and the healthy banana plants were checked for the amplification of target genes using the target gene-specific primers. The PCR components were added as follow

Components	Volume (µl)
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)	0.5 µl
cDNA (500 ng/µl)	1 µl
Nuclease free water	13.5 µl
Total volume	**20 µl**

The thermal profile followed was:

Step 1: 95 $^{\circ}$C for 3 min

Step 2: 95 $^{\circ}$C for 15 sec

Step 3: 55 $^{\circ}$C for 15 sec

Step 4: 72 °C for 45 sec. Steps 2 to 4 were repeated for 30 cycles

Step 5: 72 °C for 5 min

After completion, the PCR products were separated on 1.5 per cent agarose gel along with 50 kb 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 the gel documentation system (BIO-RAD).

3.8 RELATIVE EXPRESSION OF miRNAs AND THEIR TARGET GENES

RT-qPCR is one of the most widely used method for gene expression analysis. This technique was used to analyse the expression levels of miRNAs, target genes, and reference gene. The analysis was done in control and infected samples using CFX96 real-time machine (BIO-RAD). The 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 the expression of miRNAs and target genes in infected sample. RT-qPCR reactions for all the samples belonging to the same gene/ miRNA were run on the same plate. Two technical replicates were kept for each reaction. Non-template controls were included for each assay. A reaction mixture of 20 µl was prepared using the following components:

Components	**Volume (µl)**
cDNA (50 ng/µl)	5 µl
Forward primer (10 µM)	0.6 µl
Reverse primer (10 µM)	0.6 µl
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 sec, annealing at 60 °C for 10 sec, and extension at 72 °C for 8 sec.

Target and reference genes were amplified from the cDNA of both healthy and infected plants by initial denaturation at 95 $^{\circ}$C for 2 min, followed by 40 cycles of denaturation at 95 $^{\circ}$C for 15 sec, annealing at 55 $^{\circ}$C for is 15 sec, and extension at 72 $^{\circ}$C for 30 sec. The final extension was done at 72 $^{\circ}$C for 5 min. PCR was followed by melt curve analysis *i.e.* fluorescence signals at 530 nm wavelength were monitored from 65 $^{\circ}$C to 95 $^{\circ}$C at every 0.2 $^{\circ}$C to determine the product specificity.

Raw fluorescence data (RFU) was generated by CFX96 software. A threshold fluorescence passing through the exponential phase of the log RFU plot of all the reactions in an assay was set manually to generate threshold cycle (Cq) value. Cq value is the fractional number of cycles taken by each reaction to reach threshold fluorescence. Normalized relative quantities for each target gene and miRNA were generated by $\Delta\Delta$Cq method in excel using actin as the reference gene.

Results

4. RESULTS

The study entitled "Expression profiling of microRNAs associated with virus infection in banana cultivar Red Banana (*Musa* AAA)" was conducted at the Department of Plant Biotechnology, College of Agriculture, Vellayani, Thiruvananthapuram during 2018-2020. The results of the study are presented in this chapter.

4.1 COLLECTION OF SAMPLES

Leaf samples were collected from healthy uninfected and infected banana cultivar red banana plants showing symptoms of BBrMV and BBTV from the Instructional Farm, College of Agriculture, Vellayani (Section 3.1).

4.2 DNA ISOLATION FROM BBTV INFECTED SAMPLES

DNA was isolated from the leaf samples of control and infected plants of BBTV using Doyle and Doyle (1990) protocol.

4.2.1 Agarose gel electrophoresis

The isolated DNA on separation on agarose gel (0.8 per cent) showed the presence of intact genomic DNA bands (Plate 3).

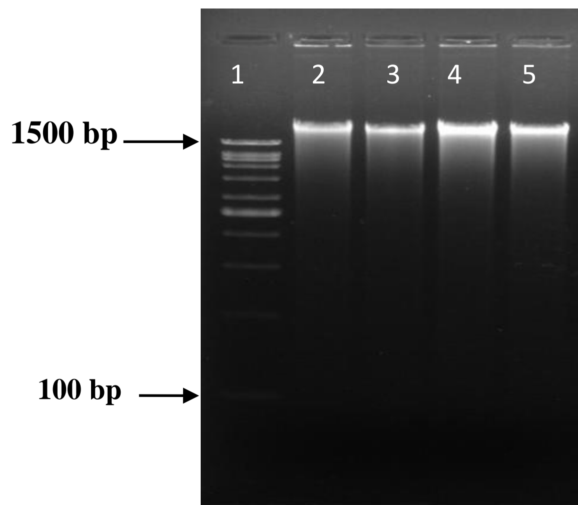


Plate 3. Gel profile of genomic DNA isolated from BBTV infected and uninfected samples

Lane 1: 100bp ladder, Lane 2: uninfected control, Lane 3-5: BBTV Infected

4.2.2 Quantification of DNA

DNA isolated was quantified using NanoDrop microvolume spectrophotometer showed concentrations ranging from 267.92 to 362.04 and A_{260}/A_{280} ratio ranging from 1.80 to 1.9 (Table 5).

Table 5 Quantity of DNA isolated from BBTV infected samples measured using NanoDrop spectrophotometer

	Sample	**A_{260}/A_{280}**	**A_{260}/A_{230}**	**Concentration (ng/µl)**
UNINFECTED PLANT/ CONTROL	C1	1.86	0.89	362.04
BBTV INFECTED	1	1.9	0.61	272.20
	2	1.80	0.79	268.22
	3	1.84	0.85	267.92

4.3 RNA ISOLATION FROM BBrMV AND BBTV INFECTED SAMPLES

RNA was isolated from the leaf samples of control and infected plants of BBrMV and BBTV was analysed using electrophoresis

4.3.1 Agarose gel electrophoresis

The RNA profile obtained on agarose gel (2 per cent) showed the presence of intact 28S rRNA and 18S rRNA bands in BBrMV (Plate 4) and BBTV (plate 5) infected samples.

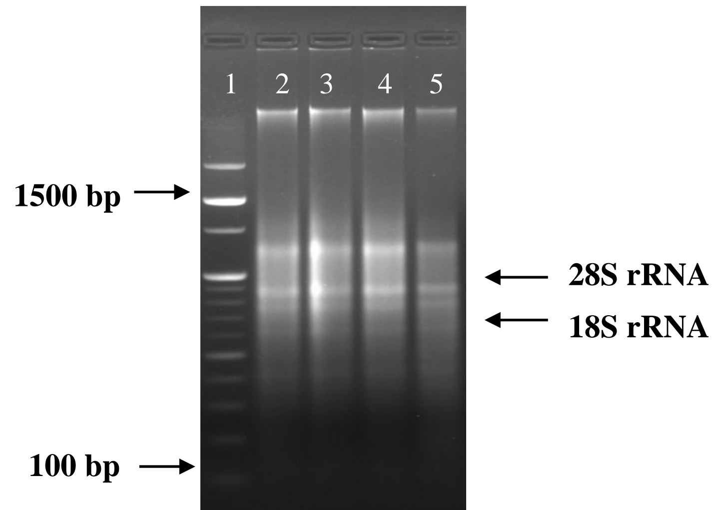


Plate 4. Gel profile of RNA isolated from BBrMV infected and uninfected samples

Lane 1: 100bp ladder, Lane 2: uninfected control, Lane 3-5: BBrMV infected

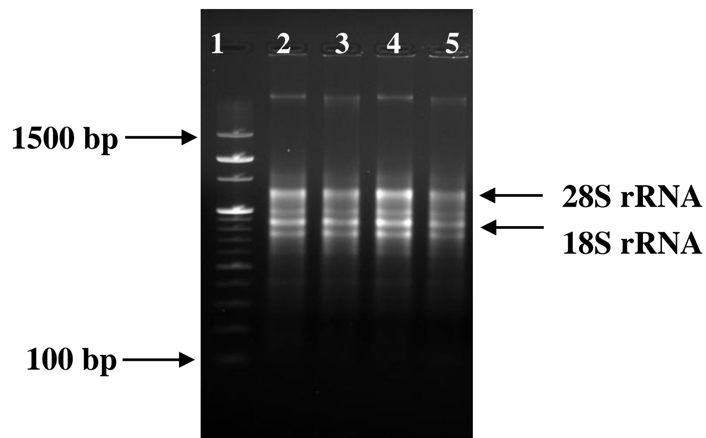


Plate 5. Gel profile of RNA isolated from BBTV infected and uninfected samples

Lane 1: 100 bp ladder, Lane 2: Uninfected control, Lane 3-5: BBTV infected

4.3.2 Quantification of RNA

The concentration of RNA quantified using NanoDrop microvolume spectrophotometer ranged from 304.67to 518.17and A_{260}/A_{280} ratio ranged from 2.02 to 2.07 in BBrMV infected samples (Table 6). In BBTV infected samples RNA concentrations ranged from 191.04 to 507.21 and A_{260}/A_{280} ratio ranged from 1.90 to 2.09 (Table 7)

Table 6 Quantity of RNA isolated from BBrMV infected samples measured using NanoDrop spectrophotometer

	Sample	A_{260}/A_{280}	A_{260}/A_{230}	Concentration (ng/µl)
UNINFECTED PLANT/ CONTROL	C1	2.07	2.11	507.21
BBrMV INFECTED	1	2.06	2.29	304.67
	2	2.02	1.84	488.07
	3	2.05	2.06	518.17

Table 7 Quantity of RNA isolated from BBTV infected samples measured using NanoDrop spectrophotometer

	Sample	A_{260}/A_{280}	A_{260}/A_{230}	Concentration (ng/µl)
UNINFECTED PLANT/ CONTROL	C1	2.07	2.11	507.21
BBTV INFECTED	1	2.09	2.25	447.30
	2	1.90	1.25	191.04
	3	2.02	1.99	345.32

4.3.3 cDNA synthesis and its confirmation

The RNA isolated from healthy control and infected BBrMV and BBTV was converted to complementary DNA using (Thermo Scientific) Verso cDNA synthesis kit. Synthesis was confirmed by doing PCR with β-actin gene primers spanning an intron. The PCR product on separation on 1.5 per cent gel showed the expected band corresponding to 102 bp in BBrMV (Plate 6) and BBTV (Plate 7) infected samples.

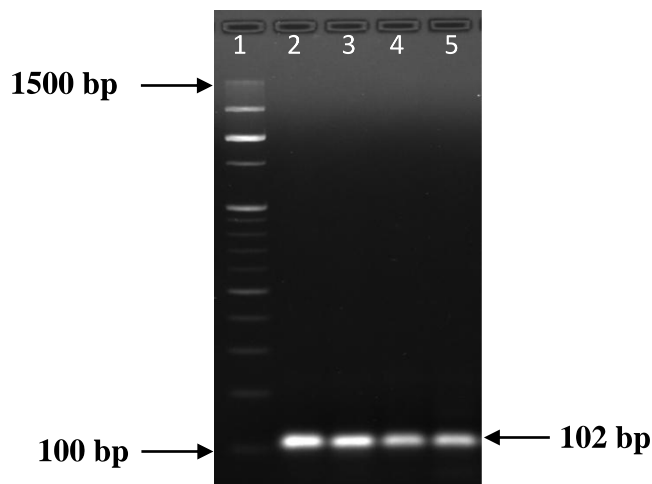


Plate 6. Gel profile of β-actin gene specific fragments amplified from cDNA of control and BBrMV infected samples

Lane 1: 100 bp ladder, Lane 2: Uninfected control, Lane 3-5: BBrMV infected

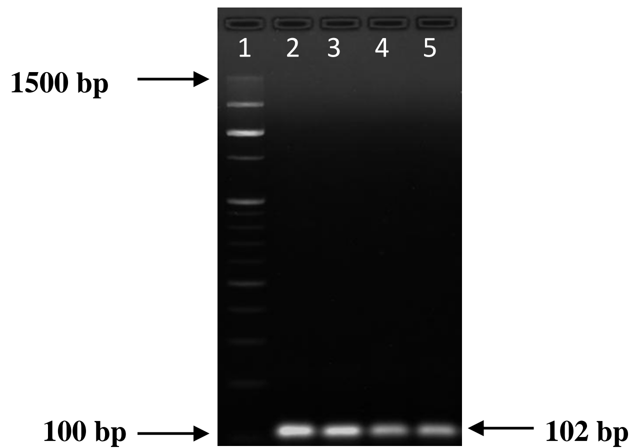


Plate 7. Gel profile of β-actin gene specific fragments amplified from cDNA of control and BBTV infected samples

Lane 1: 100 bp ladder, Lane 2: Uninfected control, Lane 3-5: BBTV infected

4.4 CONFIRMATION OF BBTV INFECTION IN SAMPLES USING COAT PROTEIN GENE-SPECIFIC PRIMER

The PCR product obtained using primer specific for coat protein gene of BBTV separated on 1.5 per cent agarose gel (Plate 8) showed bands corresponding to 516 bp.

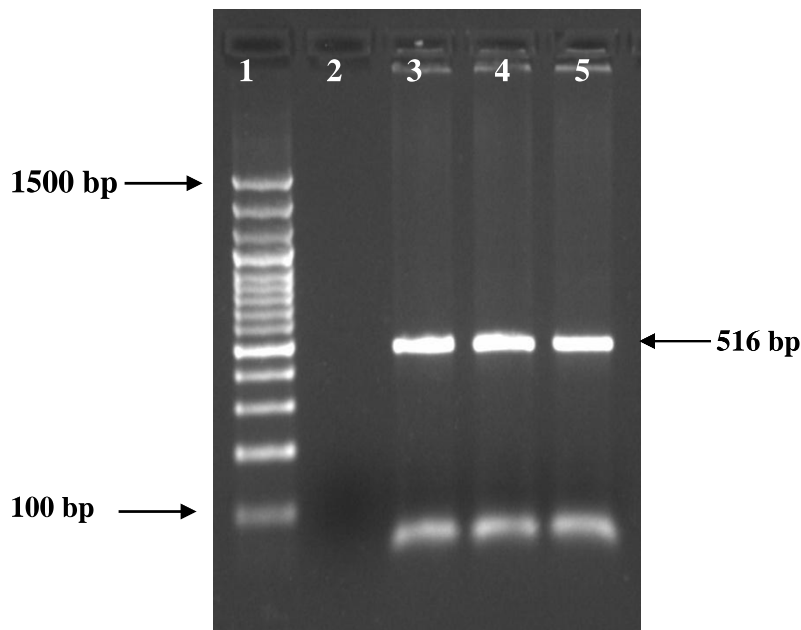


Plate 8. Confirmation of BBTV infection in samples using coat protein gene specific primer

Lane 1: 100 bp ladder, Lane 2: Uninfected control, Lane 3-5: BBTV infected

4.5 CONFIRMATION OF BBrMV INFECTION IN SAMPLES USING COAT PROTEIN GENE-SPECIFIC PRIMER

RT-PCR product of the infected and control samples of BBrMV using coat protein gene specific primer on1.5 percent agarose gel shown in Plate 9. All the infected samples showed an amplicon of expected size (745 bp).

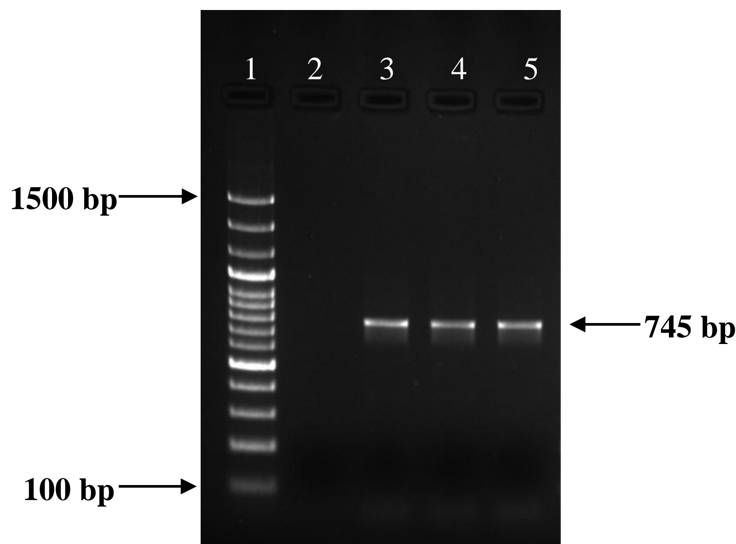


Plate 9. Confirmation of BBrMV infection in samples using coat protein gene specific primer

Lane 1: 100 bp ladder, Lane 2: Uninfected control, Lane 3-5: BBrMV infected

4.6 ISOLATION AND AMPLIFICATION OF miRNAs

Two miRNAs (miR-6928-5p and miR-971-5p) and their target genes (FAD dependent oxidoreductase and Protein Argonaute 1A) which were reported by Subramanian *et al*., 2019 were used for expression profiling of BBrMV and BBTV field infected samples of banana.

4.6.1 Assay for checking the amplification of miRNAs

miRNA was converted to complementary DNA using stem loop primers. The cDNA synthesized from miRNA was assessed by doing PCR with miRNA specific and

universal primers specific to stem loop. PCR produced amplicons of expected size (65 bp and 62 bp) for miR-6928-5p and miR-971-5p respectively (Plate 10, 12 and plate 11, 13).

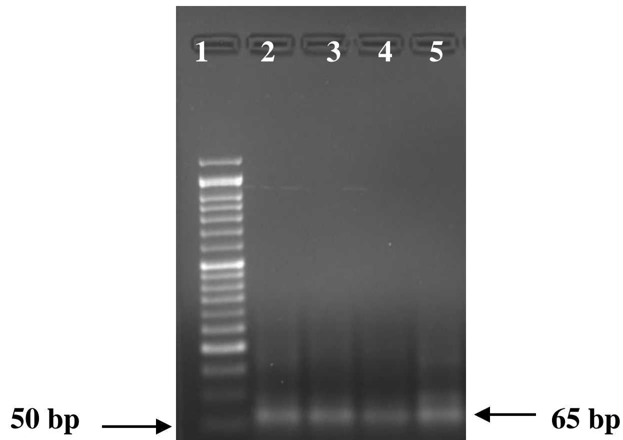


Plate 10. Amplicons of miR-6928-5p in BBrMV infected samples

Lane 1: 50 bp ladder, lane 2: uninfected control, lane 3 - 5: BBrMV infected.

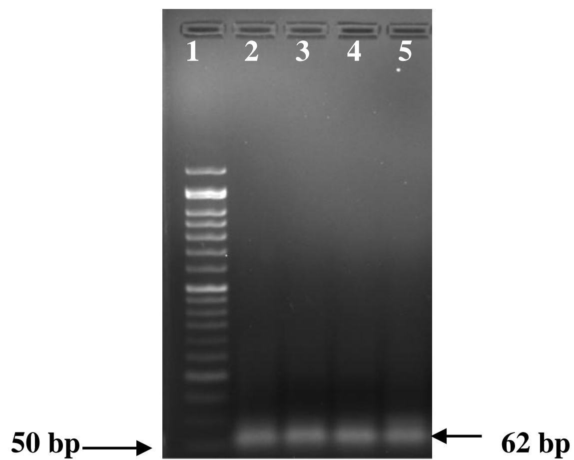


Plate 11. Amplicons of miR-971-5p in BBrMV infected samples

Lane 1: 50 bp ladder, lane 2: uninfected control, lane 3- 5: BBrMV infected

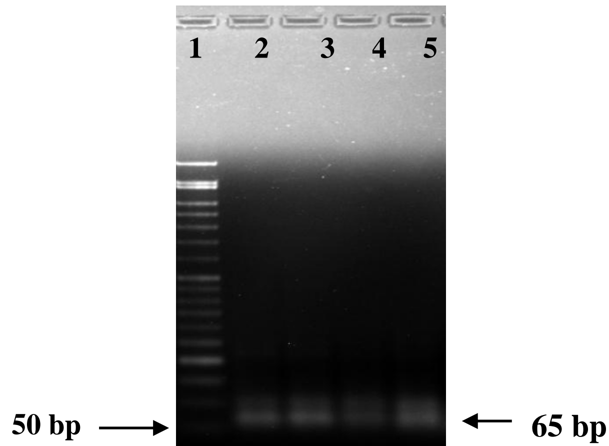


Plate 12. Amplicons of miR-6928-5p in BBTV infected samples

Lane 1: 50 bp ladder, lane 2: uninfected control, lane 3 -5: BBTV infected

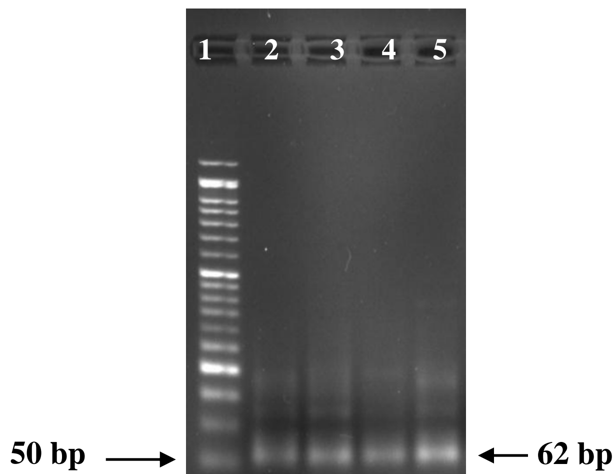


Plate 13. Amplicons of miR-971-5p in BBTV infected samples

Lane 1: 50 bp ladder, lane 2: uninfected control, lane 3 -5: BBTV infected

4.7 AMPLIFICATION OF TARGET GENES

4.7.1 Assay for checking the amplification of target and reference gene

Quality of target and reference gene amplification using primers designed in control and infected plants of BBrMV (Plate14, 15) and BBTV (Plate 16, 17) was checked by PCR and products were examined on agarose gel electrophoresis. Specific amplification was obtained for the assays in the samples analysed.

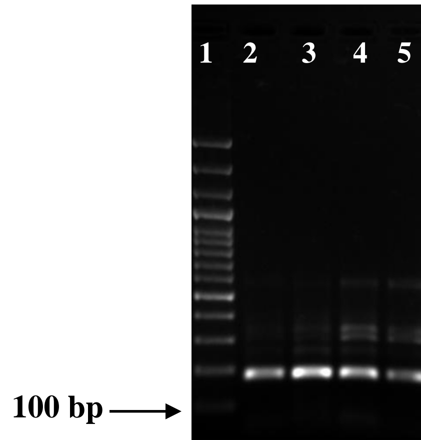


Plate 14. Amplicons of FAD dependent oxidoreductase gene (target of miR-6928-5p) in BBrMV infected samples

Lane 1: 100 bp ladder, lane 2: uninfected control, lane 3 -5: BBrMV infected.

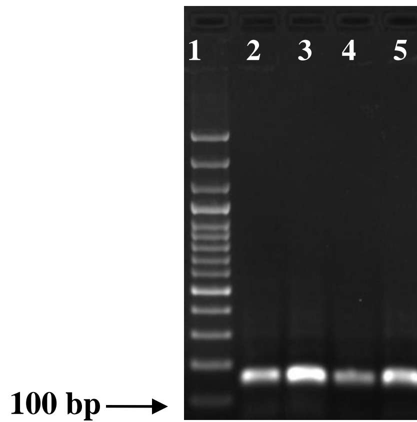


Plate 15. Amplicons of Argonaute protein gene (target of miR-971-5p) in BBrMV infected samples

Lane 1: 100 bp ladder, lane 2: uninfected control, lane 3- 5: BBrMV infected

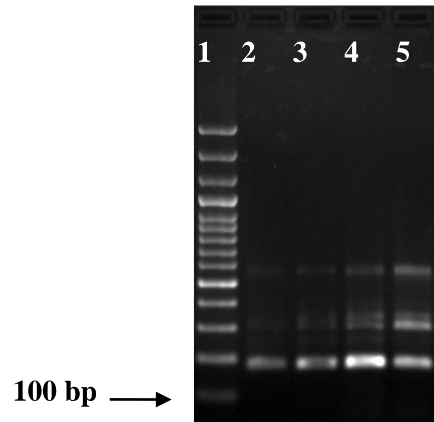


Plate 16. Amplicons of FAD dependent oxidoreductase gene (target of miR-6928-5p) in BBTV infected samples

Lane 1: 100 bp ladder, lane 2: uninfected control, lane 3-5: BBTV infected

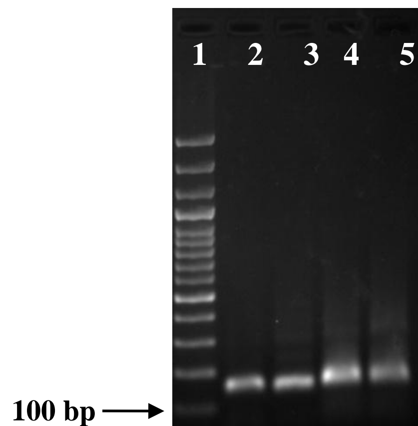


Plate 17. Amplicons of Argonaute protein gene (target of miR-971-5p) in BBTV infected samples

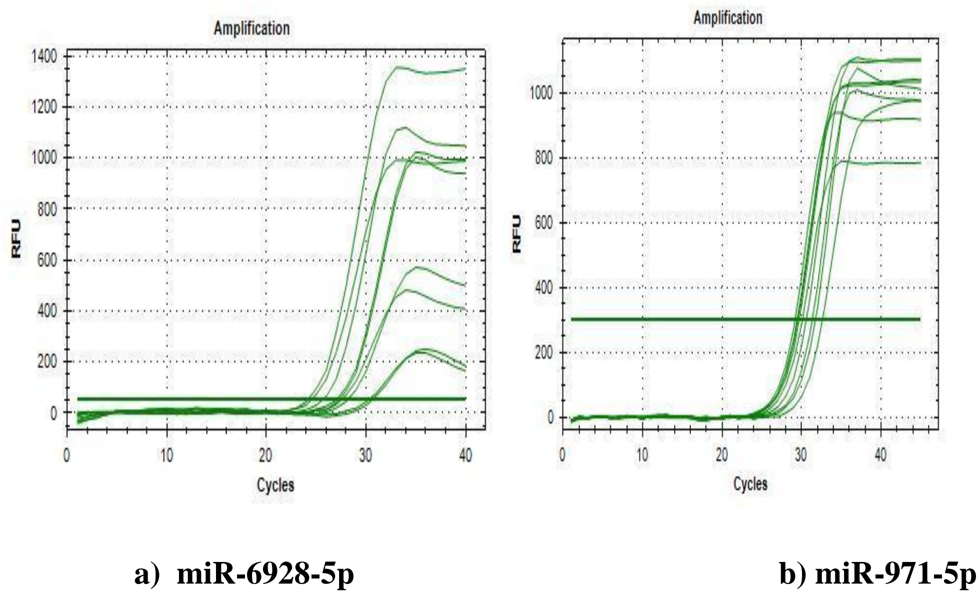
Lane 1: 100 bp ladder, lane 2: uninfected control, lane 3- 5: BBTV infected

4.8 RT-qPCR ANALYSIS

Expression of miRNAs and their corresponding target genes were assessed in the BBrMV and BBTV infected as well as healthy plants by RT-qPCR. For each set three biological replicates were used.

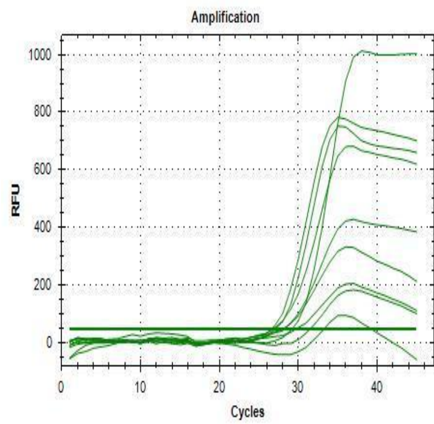
4.8.1 Raw expression data and Cq values of miRNAs and target genes

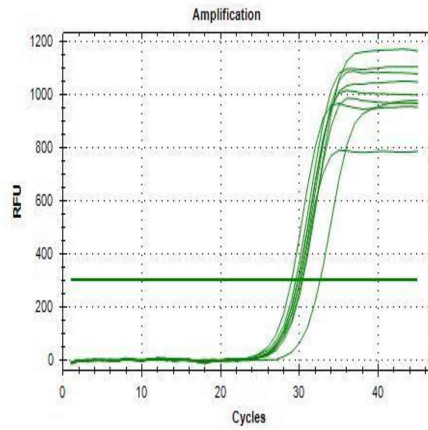
RT-qPCR was conducted with two technical replicates for each reaction. Amplification plot for each miRNA and their target genes were generated by RT-qPCR. Fig.1, 2, 3, and 4 shows the raw expression data of each miRNA and their target gene respectively. Cq values were generated for each assay by keeping a common threshold passing through the exponential phase of all samples. For miRNAs, the Cq value ranged from 24 to 32 (Fig. 1, 2). For target genes, it ranged from 21 to 27 (fig. 3, 4). Cq values for reference gene ranged from 21 to 23. The Cq values of miRNAs and their target genes as well as reference genes are presented in Tables 8-11.



a) miR-6928-5p **b) miR-971-5p**

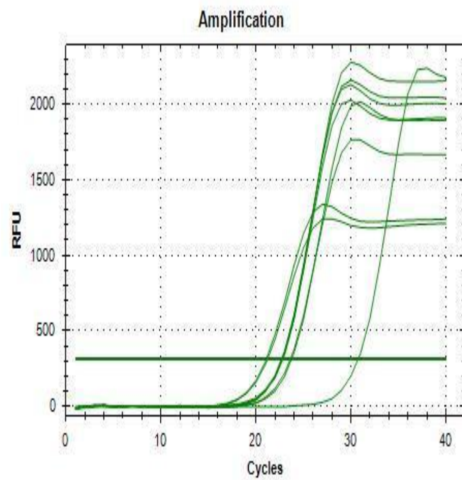
Fig. 1 Raw expression data of miRNAs in BBrMV infected

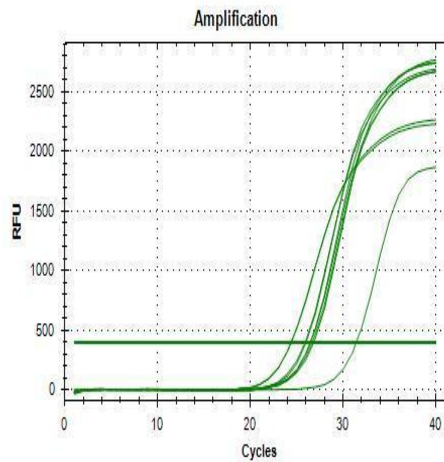




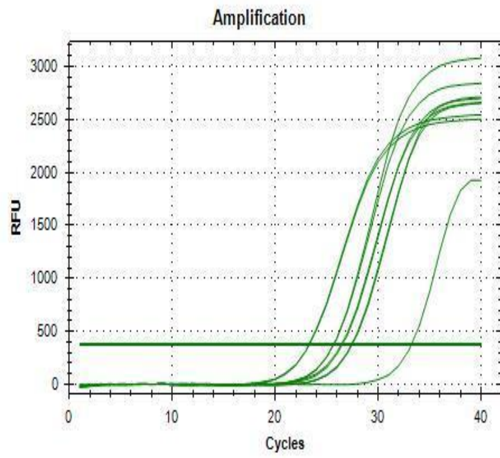
c) miR-6928-5p **d) miR-971-5p**

Fig. 2 Raw expression data of miRNAs in BBTV infected samples



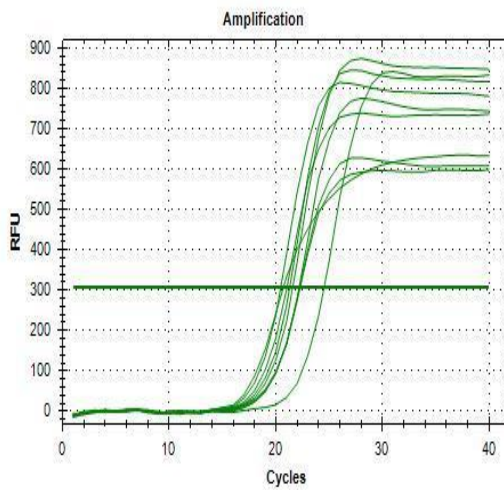


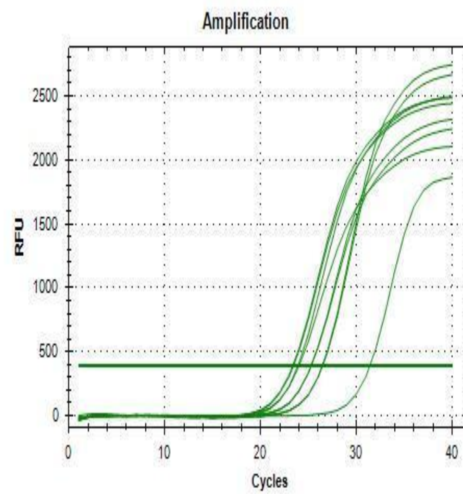
a) β- Actinb) FAD dependent oxidoreductase



c) Argonaute protein gene

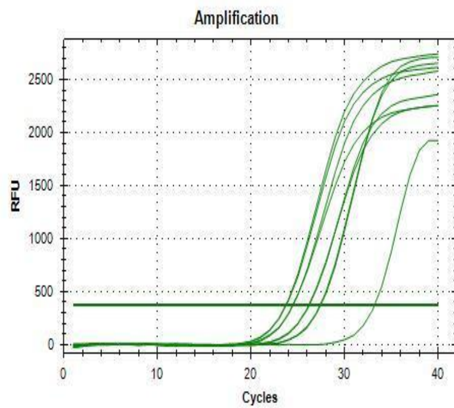
Fig. 3 Raw expression data of target genes in BBrMV infected samples





a) β- Actin

b) FAD dependent oxidoreductase



b) Argonaute protein gene

Fig. 4 Raw expression data of target genes in BBTV infected samples

Table 8 Cq values of miRNA in BBrMV infected samples

	miR-6928-5p	**miR-971-5p**
C1	24.31463	29.80996
1	27.8124	29.29771
2	27.13953	31.16122
3	30.41053	30.50487

Table 9 Cq values of miRNA in BBTV infected samples

	miR-6928-5p	**miR-971-5p**
C1	32.4054	29.80996
1	26.85429	29.40004
2	28.9771	30.23351
3	27.58273	30.05337

Table 10 Cq values of target genes in BBrMV infected samples

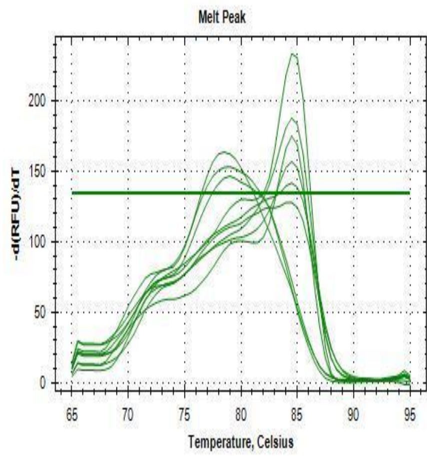
	β-Actin	***FAD***	***ARG***
C1	23.64766	26.45029	27.40229
1	21.13152	24.37654	23.25916
2	22.72381	26.72903	26.43621
3	22.72524	25.9405	25.63651

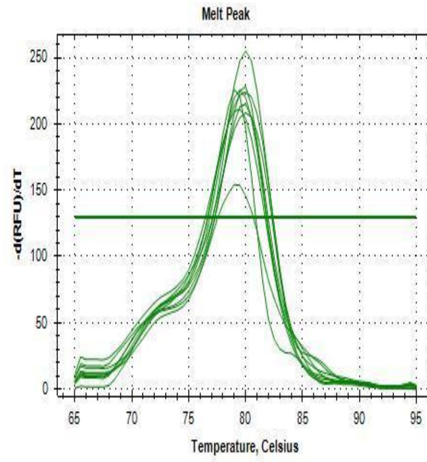
Table 11 Cq values of target genes in BBTV infected samples

	β-Actin	***FAD***	***ARG***
C1	23.64766	26.45029	27.40229
1	21.13152	23.39381	23.72644
2	22.72381	23.96659	24.46401
3	22.72524	25.2801	26.19456

4.8.2 Melt curve analysis

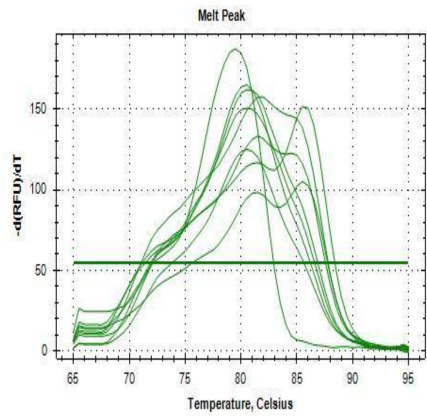
The specificity of RT-qPCR was determined by doing melt curve analysis. miRNAs except miR6928-5p showed single prominent peaks indicating good amplification (Fig. 5, 6). miR6928-5p showed nonspecific amplification which was confirmed by agarose gel electrophoresis. All the target genes studied showed specific amplification (Fig. 7, 8).

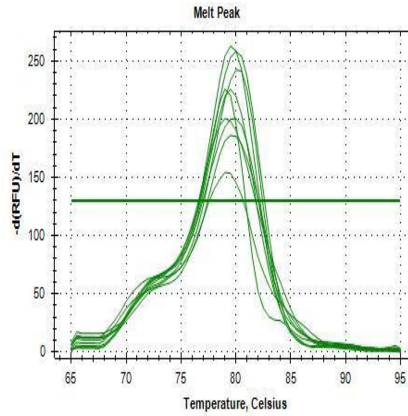




a) miR-6928-5p **b) miR-971-5p**

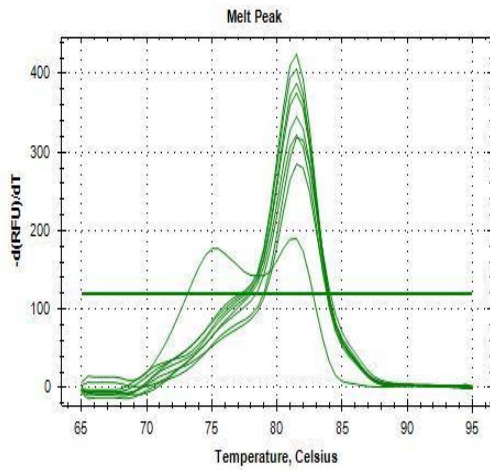
Fig. 5 Melt curve of miRNAs in BBrMV infected samples



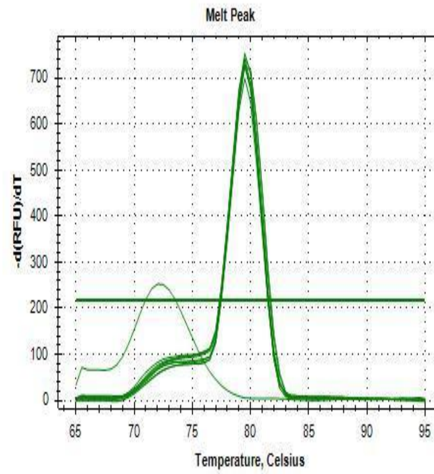


c) miR-6928-5p **d) miR-971-5p**

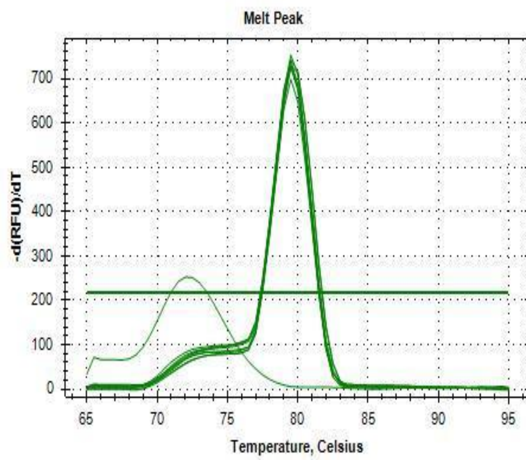
Fig. 6 Melt curve of miRNAs in BBTV infected samples



a) β- Actin

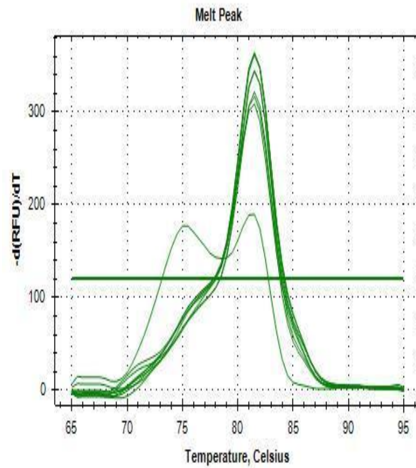


b) FAD dependent oxidoreductase

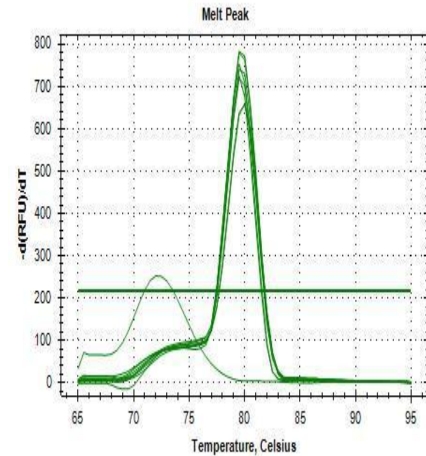


c) Argonaute protein gene

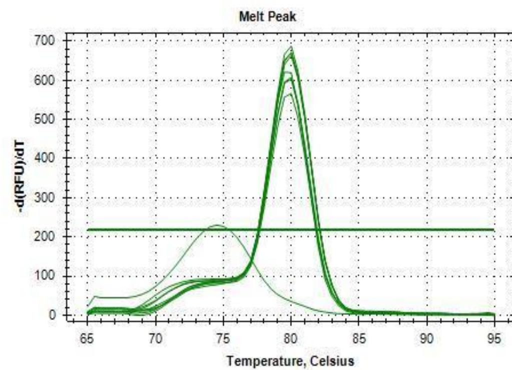
Fig. 7 Melt curve analysis of target genes in BBrMV infected samples



a) β- Actin



b) FAD dependent oxidoreductase



c) Argonaute protein gene

Fig. 8 Melt curve analysis of target genes in BBTV infected samples

4.9 RELATIVE EXPRESSION OF miRNAs AND THEIR TARGET USING REAL-TIME PCR

Cq values obtained for miRNA and target genes in different samples were normalised with the Cq values for β-actin in the corresponding samples. The relative expression values are shown in table 12-15 and are represented by bar diagrams (Fig 9, 10, 11 and 12).

miR-6928-5p was found to be down regulated in BBrMV infected samples compared to healthy samples. The least expression was observed in sample 3 with NRQ 0.01 (Table 12; Fig 9). On the other hand, in BBTV infected samples miR-6928-5p was expression increased compared to control. The relative expression value ranged from 2.44 fold to 11.05 fold (Table 13; Fig 11).

Target of miR-6928-5p, the FAD dependent oxidoreductase also showed lesser expression in BBrMV infected samples compared to healthy control. The decrease in expression was not as dramatic as the corresponding miRNA. NRQs ranged from 0.75 in sample 3 to 0.43 in sample 2. In all the BBrMV infected samples an inverse correlation was observed between miR-6928-5p and its target in their expression (Table 14; Fig 9).

In BBTV infected sample FAD dependent oxidoreductase expression was almost unchanged in infected samples. Expression of the gene showed a marginal increase in sample 1 (1.23 fold) and sample 2 (1.27 fold) and a marginal decrease in sample 3 (0.88 fold) (Table 15; Fig 11). Here also an inverse correlation was observed between the miRNA and its target in the infected samples.

miR-971-5p was found downregulated in both BBTV and BBrMV infected samples. In BBrMV infected sample the expression was lowered by 0.21 fold to a maximum of 0.33 fold (Table 12; Fig 10).In BBTV infected sample the least expression compared to control was noted in sample 2 (0.17 fold) followed by sample 1 (0.21 fold) and sample 3 (0.33 fold) (Table 13; Fig 12).

Target gene of miR-971-5p coding for Argonaute protein showed increased expression in BBrMV infected sample 1 (3.09 fold) and sample 3 (1.79 fold) while in sample 2 expression levels remained almost unchanged (1.03 fold) (Table 14; Fig. 10).

In BBTV infected sample arganaute protein had its expression increased in sample 1 (1.89 fold) and sample 2 (1.74 fold) and remained almost unchanged (0.9 fold) in sample 3 (Table 15; Fig 12). Here also in the virus infected samples, the miRNA and its target showed an inverse correlation.

Table 12 NRQ values of miRNAs in BBrMV infected samples

	miR-6928-5p	**miR-971-5p**
C1	1.00	1.00
1	0.08	0.25
2	0.06	0.21
3	0.01	0.33

Table 13 NRQ values of miRNAs in BBTV infected samples

	miR-6928-5p	**miR-971-5p**
C1	1.00	1.00
1	6.94	0.20
2	2.44	0.17
3	11.05	0.33

Table 14 NRQ values of target genes in BBrMV infected samples

	β-Actin	***FAD***	***ARG***
C1	1.00	1.00	1.00
1	1.00	0.74	3.09
2	1.00	0.43	1.03
3	1.00	0.75	1.79

Table 15 NRQ values of target genes in BBTV infected samples

	β-Actin	***FAD***	***ARG***
C1	1.00	1.00	1.00
1	1.00	1.23	1.89
2	1.00	1.27	1.74

3	1.00	0.88	0.90

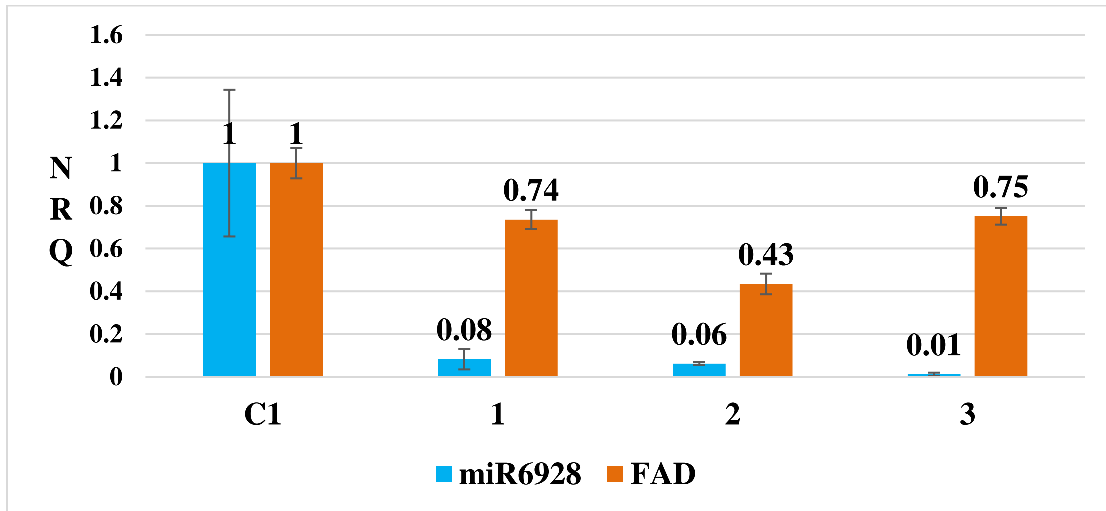


Fig. 9 Relative expression of miR-6928-5p and its target FAD dependent oxidoreductase in BBrMV infected samples

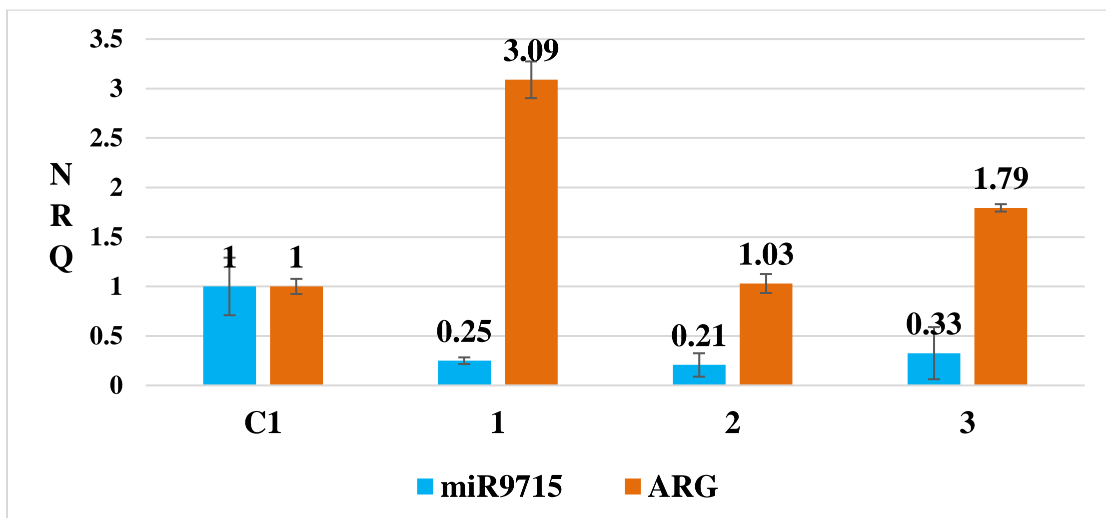


Fig. 10 Relative expression of miR-971-5p and its target Argonaute protein gene in BBrMV infected samples

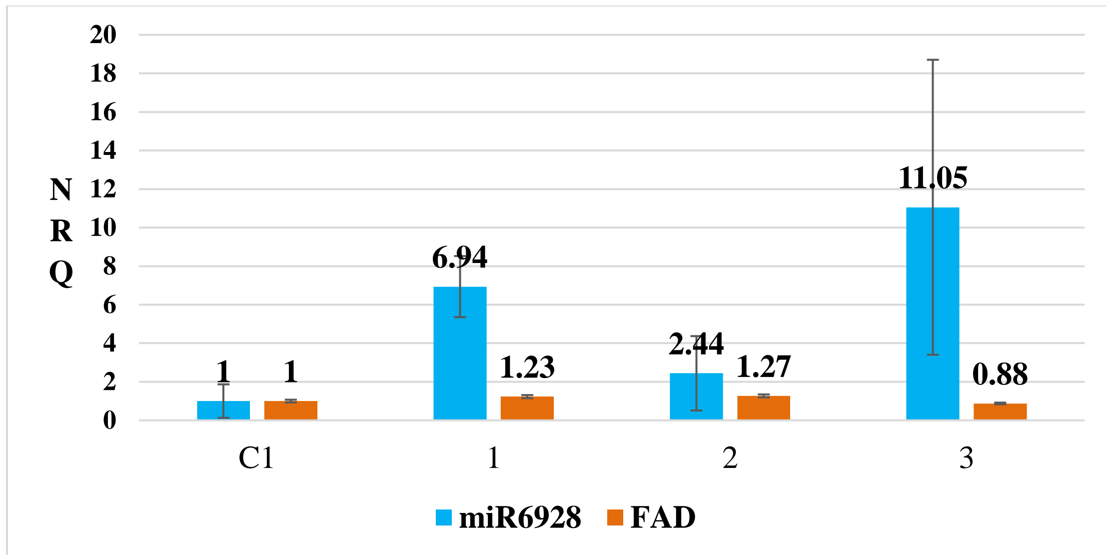


Fig. 11 Relative expression of miR-6928-5p and its target FAD dependent oxidoreductase in BBTV infected samples

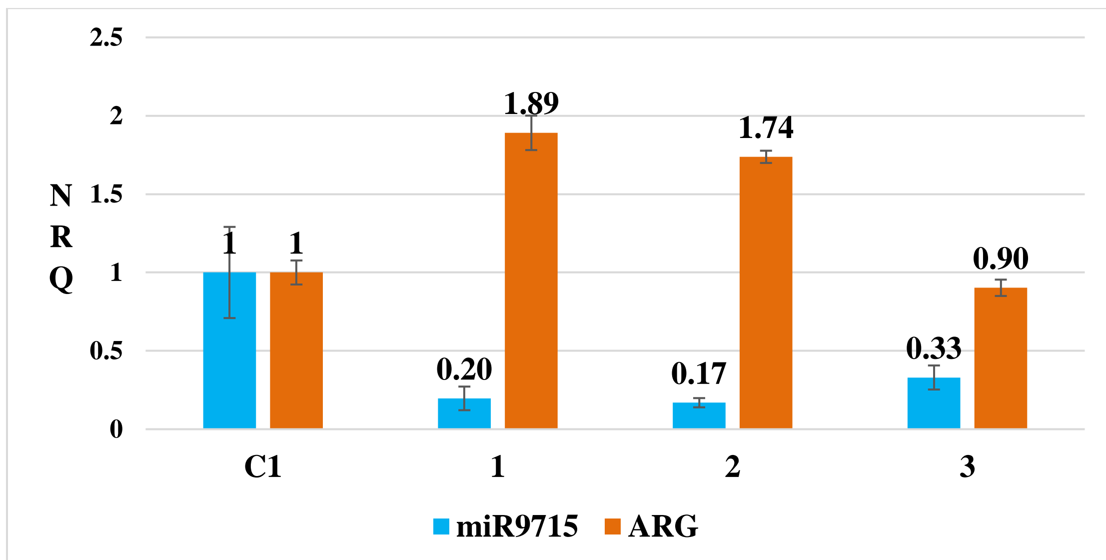


Fig. 12 Relative expression of miR-971-5p and its target Argonaute protein gene in BBTV infected samples

Discussion

5. DISCUSSION

Banana is the most important fruit crop and staple food source for millions of people in tropical and subtropical countries. It is the second extensively consumed fruit crop next to mango in India and due to its availability, low- cost of cultivation, taste and high nutritive value, the fruit is favored by people all over the world. The average production of banana is estimated to be 115.73 million tonnes in over 120 countries in which India ranks first with 30.8 million tonnes, which accounts for 13% of the area and 33% of the world's production (FAOSTAT, 2018).

Biotic and abiotic stresses are the major constraints in banana cultivation that affect production and productivity. Among them, the viral diseases such as *Banana bract mosaic virus* (BBrMV), *Banana bunchy top virus* (BBTV) are considered as the major concern for banana production because of their effects not only on yield and quality, as well as limitations to the international germplasm exchange of banana. BBrMV is a single-stranded RNA virus belonging to the potyviridae family and the disease was first noted in the year 1988 in Philippines (Magnaye and Espino, 1990). In India, the virus majorly affected the areas of Tamil Nadu, Kerala, Karnataka and Andhra Pradesh (Rodoni *et al*., 1997; Thomas *et al*., 1997). Banana bunchy top disease is caused by BBTV which is a multicomponent, circular, single-stranded DNA virus. BBTV is also the type of member of the genus Babuvirus in the family Nanoviridae (King *et al*., 2012). Banana aphid (*Pentalonia nigronervosa*) is recognized to be the main vector of BBrMV and BBTV (Magee, 1927).

Due to the lack of wild relatives resistant to the virus, it is not possible to do conventional breeding for developing disease resistant varieties. Several defense mechanisms have been shown by plants to resist the entry of pathogens. It has been proved that endogenous miRNAs show differential expression during biotic stress conditions. The involvement of miRNAs in biotic stresses has been reported in many crops. The levels of miR398 were found down regulated in *Arabidopsis* during biotic stress (Jagadeeshwaran *et al*., 2009). During *Begomoviruses* infection several

developmental processes were upregulated by the miRNA and it caused the suppression of viral targets (Amin *et al*., 2011). Sheeba *et al*. (2013) have predicted eighteen banana miRNAs from ESTs and BAC sequences and the expression profiling of three miRNAs (miR156, miR159, and miR166) after the infection of *Banana streak mysore virus* (BSMYV) in banana showed an increased expression of miR166. Ghag *et al*. (2015) observed several morphological changes in transgenic banana plants overexpressing *Musami*RNA156. In rice, miR444, a monocot-specific miRNA, has been reported to regulate resistance against *Rice stripe virus* (RSV) infection by up-regulation of the *OsRDR1* expression (Wang *et al*., 2016). During *Sugarcane mosaic virus* (SCMV) infection in maize plant, up regulation of miR168 and miR528, and down regulation of miR159 were observed (Xia *et al.,* 2018). Transgenic maize plants overexpressing miR408b exhibited reduced resistance to *Fusarium verticillioides* infection in a susceptible maize line (Zhou *et al.,* 2020).

As plants lack an immune system to act against viruses, miRNA-mediated gene regulation is a new strategy used to control plant viruses. microRNAs are an extensive class of endogenous, non-coding small regulatory RNAs that bind complementary to the mRNA molecules and negatively regulate gene expression at the post-transcription levels and they have a length of 20-24 nucleotides length in plants (Bartel, 2004; Zhang *et al*., 2006). Very few miRNAs have been identified associated with biotic stress in banana. So identification of differentially expressed miRNAs during viral infection can help in the development of targeted genetic transformation or gene editing approach for imparting virus resistance in banana.

In an earlier study conducted in the Department of Plant Biotechnology, 52 mature miRNAs were predicted using NovoMIR software, and over 142 targets were identified for these miRNAs using psRNATarget in the banana genome (Mathew, 2018). From a selected number of miRNAs that were validated, five were found to be associated with BBrMV infection in tissue culture raised banana var. Nendran (Subramanian, 2019). In the present study, two of these miRNAs and their targets were selected with an

objective to study the expression of selected miRNAs in BBrMV and BBTV infection in red banana (*Musa* AAA). The two miRNAs and their targets selected were miR-6928-5p (target: Flavin adenine dinucleotide dependent oxidoreductase gene) and miR-971-5p (target: Argonaute 1A protein).

The experiment was carried out in field-grown banana cultivar red banana (*Musa* AAA) plants to study their response towards BBrMV and BBTV infection. Leaf samples were collected from uninfected and infected red banana plants based on the visual symptoms of the BBrMV and BBTV infection. Later it was confirmed by PCR using primers specific to coat protein gene of the respective viruses. Amplicons of expected size produced (110bp) confirmed the presence of viruses in the samples. RNA isolated from the collected samples using the modified Rodrigues-Garcia protocol (Ekatpure *et al*., 2019) was found to be of good quality on gel electrophoresis and spectrophotometric measurements. The RNA isolated was converted to cDNA using gene-specific primers for target genes and stem-loop primers for miRNAs.

Expression analysis of miRNAs and their target gene was done by RT-qPCR. miRNAs could be successfully amplified using stem-loop primers and miRNA specific primers. The result of the experiment confirmed the successful conversion of cDNA in the case of miRNAs and target genes. Relative expression miRNAs and their target in the infected samples were done using RT-qPCR. Cq values were generated for each assay by keeping a common threshold passing through the exponential phase of all samples. For miRNAs, the Cq value ranged from 24 to 32. For target genes, it ranged from 21 to 27. Cq values for the reference gene ranged from 21 to 23. The specificity of the real-time PCR reaction was confirmed using melt curve analysis. miRNAs except miR-6928-5p showed single prominent peaks indicating good amplification. miR-6928-5p showed non-specific amplification which was confirmed by agarose gel electrophoresis. All the target genes studied showed specific amplification indicating the good quality of the primer used. The result of the RT-qPCR was normalized using the housekeeping gene, β-Actin with reference to the uninfected control in qBase plus software.

Two miRNAs selected in the study and their target genes showed expression in control plants as well as BBrMV and BBTV infected plants collected from the field. The expression of miR-6928-5p was found to upregulated in all the BBTV infected samples, with a maximum of 11.05 fold, whereas the expression of FAD dependent oxidoreductase was found to be downregulated indicating an inverse correlation between miR-6928-5p and its target during viral infection which emphasizes the possibility of the suppression of the target gene by the miRNA. FAD (flavin adenine dinucleotide) is a kind of redox-active flavoprotein coenzyme involved in a lot of enzymatic reactions. It also functions in the generation of ROS species in cells (Jaspers and Kangasjarvi, 2010). These are formed by the incomplete reduction (hydrogen peroxide – H_2O_2; superoxide radical – O·−2O2•−; hydroxyl radical – HO; etc.) or excitation (singlet oxygen – $^1O_2$) of molecular oxygen (Gechev *et al.*, 2006) and are strong oxidizers that can react with and damage a large variety of biological molecules (Petrov and Van Breusegem, 2012). They are regarded as byproducts of the aerobic way of life and are generated in different cellular compartments like chloroplasts, mitochondria and peroxisomes (Apel and Hirt, 2004). ROS actually has a dual role *in vivo* depending on their concentration, site and duration of action, previous exposures to stress, etc. (Miller *et al.*, 2010). In general, lower doses of ROS are employed as signals that mediate at least part of the responses towards stress while at higher concentrations they pose a significant threat that may eventually lead to programmed cell death (PCD) (Gechev and Hille, 2005). Prolonged drought stress is often associated with ROS accumulation, mainly due to decreased CO_2 fixation concomitant with increased electron leakage to triplet oxygen which may eventually lead to PCD (Gechev *et al.*, 2012). Fully functional ROS detoxifying systems are therefore of essential importance for the tolerance of plants towards drought and desiccation (Kranner *et al.*, 2002). Therefore one of the main strategies to restrict ROS propagation and avoid unnecessary PCD under drought is to reduce ROS formation by downregulating chlorophyll synthesis and other components of the photosynthetic machinery (Farrant *et al.*, 2007). Flavins, including FAD, have a tendency to auto oxidise oxygen molecules to superoxide during biotic and abiotic stress

(Massy, 1994). In a previous study conducted by Subramanian (2019), the *in vitro* raised plants infected with BBrMV showed upregulation of FAD dependent oxidoreductase during the different time intervals of study with a maximum increase at 24 h. In that study there was a marginal increase in the expression of miRNA upto 48 h and it came to normal level. In this study, the field grown plants infected with BBrMV showed low expression of both miR-6928-5p and its target gene compared to control. But here also, the infected plants were maintaining a higher level of target gene expression compared to the miRNA. It can be considered as a defense response of the infected plant.

The target mRNA transcripts coding for Protein Argonaute 1 A showed an inverse correlation with miR971-5p. It remained upregulated in both BBrMV and BBTV infected plants compared to control. The target gene showed 3.09 and 1.89 fold increase in its expression in BBrMV and BBTV respectively. The Argonaute protein is involved in the RISC formation (Garcia-Ruiz *et al.*, 2015) which is essential for the synthesis of miRNAs. The small RNAs are then sorted into specific AGO family proteins, which function as the core components of RNA silencing complexes (Tolia and Joshua-Tor, 2007; Hutvagner and Simard, 2008; Vaucheret, 2006). Small RNAs guide AGO proteins to their targets through complementary base pairing. AGO then silences these targets through RNA cleavage, translational interference, or chromatin modifications (Bartel, 2009; Brodersen and Voinnet, 2009; Fabian *et al.*, 2010). Regulation of gene expression through RNA silencing predominantly relies on the accurate functioning of DCL, AGO and RDR proteins which exist in multiple copies in the eukaryotic genomes. Plants encode a large number of AGOs that play key roles in RNA silencing mechanisms regulating plant development, response to biotic and abiotic stresses, DNA repair and antiviral defenses (Vaucheret, 2008). In tomato, the expression patterns of seven *DCL*, 15 *AGO* and six *RDR* genes in different tissues and organs detected using semi quantitative RT-PCR, revealed that some candidate genes were upregulated in response to *Tomato yellow leaf curl virus infection* and abiotic stress (Bai *et al.*, 2012). Eight *DCL*, 19 *AGO* and 11 *RDR* genes were identified in foxtail millet, and the expression of candidate genes representing each subfamily in response to dehydration, salinity and

abscisic acid (ABA) treatment was analysed using quantitative real-time PCR. The differential expression patterns of these genes at different time points in response to stress suggested that they play a role in the complex molecular network of the stress response (Yadav *et al*., 2015).

The present study indicates that both miR-6928-5p and miR971-5p are biotic stress-responsive in banana. The differential expression of their target genes in inverse correlation represents the initiation of defense response by the plants towards the virus infection.

Summary

6. SUMMARY

The study entitled "Expression profiling of microRNAs associated with virus infection in banana cultivar Red Banana (*Musa* AAA)" was conducted at the Department of Plant Biotechnology, College of Agriculture, Vellayani, Thiruvananthapuram during 2018-2020. The objective was to study the expression of selected miRNAs in *Banana bract mosaic virus* (BBrMV) and *Banana bunchy top virus* (BBTV) infection in banana cultivar Red Banana (*Musa* AAA).

Banana (*Musa spp*.) is the most important fruit crop and staple source for millions of people in tropical and subtropical countries. It is the second extensively consumed fruit crop next to mango in India and due to its availability, low- cost of cultivation, taste and high nutritive value it is favored by people all over the world. Biotic and abiotic stresses are the major constraints in banana cultivation. Among them, viral diseases are considered a major concern for banana production because of their effects on yield and quality, as well as limitations to the international germplasm exchange of banana. Due to the lack of wild virus-resistant relative of banana, it is not applicable to do conventional breeding methods for developing disease-resistant varieties. In order to combat this transgenic technology was developed against viral diseases (Sagi *et al.,* 1995) mainly targeting viral coat protein genes.

RNA interference technology or gene silencing brought new light to combat viral infections (Andrew Fire and Craig Mello, 1998).Gene silencing technology in plants is known as post transcriptional gene silencing (PTGS) mediated by miRNA and siRNA. The involvement of miRNAs has been reported in the stress responses in plants as well as important biological processes. The present study was to understand the response of selected miRNAs in banana genome towards BBrMV and BBTV infection using RT-qPCR, so as to understand their role during biotic stress. The salient findings of the study are summarized below.

In a study conducted earlier in the Department of Plant Biotechnology, 52 mature miRNAs were predicted in the banana genome using NovoMIR software, and over 142 targets were identified for these miRNAs using psRNATarget. On validation of a few miRNAs, five computationally predicted miRNAs were identified to be associated with BBrMV infection in tissue culture raised banana cv. Nendran and a differential expression of these miRNAs and their target genes were observed (Subramanian, 2019). In this study, two of them showing better responses were analysed in the field-grown banana plants of cv. Red banana infected with BBrMV and BBTV. The miRNAs selected were miR-6928-5p (target: Flavin adenine dinucleotide dependent oxidoreductase gene) and miR-971-5p (target: Argonaute protein).

For studying the expression of these miRNAs leaf samples were collected from healthy uninfected and infected Red banana plants showing symptoms of BBrMV and BBTV from the field. The plants were selected based on the visual symptoms of BBrMV and BBTV infection and later reconfirmed by conducting RT-PCR/normal PCR using the primers specific to coat protein genes of the viruses. All the infected samples showed the presence of specific amplicons of the expected size (745bp) compared to the uninfected samples.

RNA was isolated from the leaf samples of both control and infected plants using the modified method of Rodrigues-Garcia was reverse transcribed to cDNA. The PCR analysis confirmed the presence of both miRNAs in the banana cultivar. The expression of selected miRNAs and their corresponding target genes in the BBrMV and BBTV infected as well as healthy plants were analysed by RT-qPCR, by keeping three biological replicates for each. For miRNAs, the Cq value ranged from 24 to 32 and for the target genes, it ranged from 21 to 27. Cq values for the reference gene, β-actin ranged from 21 to 23. The melt curve analysis showed prominent peaks for the miRNAs and their target genes and the peaks obtained for were above the threshold line.

The Cq values of all the RT-qPCR assays for both control and infected samples were analysed using qBase plus software. The software generated comparative expression values of miRNAs and their target genes with respect to the control plants, normalized using the Cq values of β-actin (housekeeping gene).

The gene expression analysis conducted by RT-qPCR showed differential expression of miR-6928-5p and miR-971-5p in BBrMV and BBTV infected plants. The expression of miR-6928-5p was found to be down-regulated in BBrMV infected samples compared to healthy samples. The maximum NRQ obtained was 0.08 fold and the least was 0.01 fold. On the contrary, the BBTV infected samples showed an upregulation of miR-6928-5p compared to control. The relative expression value ranged from 2.44 fold to 11.05 fold.

The target of miR-6928-5p, FAD-dependent oxidoreductase also showed a lower expression in BBrMV infected samples compared to uninfected control. The NRQs ranged from 0.75 in sample 3 to 0.43 in sample 2. In all the infected samples the expression of miR-6928-5p and its target showed an inverse correlation.

In BBTV infected sample FAD-dependent oxidoreductase expression was almost unchanged in infected samples. Expression of the gene showed a marginal increase in sample 1 (1.23 fold) and sample 2 (1.27 fold) and a marginal decrease in sample 3 (0.88 fold). Here also the miR-6928-5p and its target maintained an inverse relationship in their expression.

The expression of miR-971-5p was found downregulated in both BBTV and BBrMV infected samples. In BBrMV infected sample the value ranged from 0.21 fold to a maximum of 0.33 fold. In BBTV infected samples expression decreased dramatically with the least value of 0.17 fold.

The target gene of miR-971-5p coding for Argonaute protein showed increased expression in BBrMV infected sample to a maximum of 3.09 fold. In BBTV infected

sample the Argonaute protein had its expression increased upto 1.89 fold. In all the infected samples the miRNA and its target showed an inverse correlation.

The study showed that both miR-6928-5p and miR-971-5p respond to BBrMV and BBTV infection if banana cv. Red banana.

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References

7. REFERENCES

Amin, I., Patil, B.L., Briddon, R.W., Mansoor, S., and Fauquet, C.M. 2011. A common set of developmental miRNAs are upregulated in *Nicotiana benthamiana* by diverse begomoviruses. *Virol. J.* 8(1): 143-151.

Andrew, F., Siqun, X.U., Mary, K.M., Steven, A.K., Samuel, E.D., and Craig, C. 1998. Potent and specific genetic interference by double stranded RNA in *Caenorhabditis elegans. Nature 391*: 806-811.

Anhalt, M.D. and Almeida, R.P.P. 2008. Effect of temperature, vector life stage and plant access period on transmission of *banana bunchy top virus* to banana. *J. Phytopathol.* 98: 743-748.

Apel, K. and Hirt, H. 2004. Reactive oxygen species: metabolism, oxidative stress, and signal transduction. *Annu. Rev. Plant Biol.* 55:373-399.

Bai, M., Yang, G.S., Chen, W.T., Mao, Z.C., Kang, H.X., Chen, G.H., Yang, Y.H., and Xie, B.Y. 2012. Genome-wide identification of Dicer-like, Argonaute and RNA-dependent RNA polymerase gene families and their expression analyses in response to viral infection and abiotic stresses in *Solanum lycopersicum*. *Gene* 501(1): 52-62.

Barah, P., Winge, P., Kusnierczyk, A., Tran, D.H., and Bones, A.M. 2013. Molecular signatures in *Arabidopsis thaliana* in response to insect attack and bacterial infection. *PLoS One* 8(3): 1-17.

Barozai, M.Y.K., Ye, Z., Sangireddy, S.R., and Zhou, S. 2018. Bioinformatics profiling and expressional studies of microRNAs in root stem and leaf of the bioenergy plant switchgrass (*Panicum virgatum* L.) under drought stress. *Agri Gene* 8: 1-8.

Bartel, D.P. 2004. MicroRNAs: genomics, biogenesis, mechanism and function. *Cell* 116: 281-297.

Bartel, D.P. 2009. MicroRNAs: target recognition and regulatory functions. *Cell* 136: 215-233.

Beetham, P.R., Harding, R.M., and Dale, J.L. 1999. *Banana bunchy top virus* DNA-2 to 6 are monocistronic. *Arch. Virol.* 144: 89-105.

Brodersen, P. and Voinnet, O. 2009. Revisiting the principles of microRNA target recognition and mode of action. *Nat. Rev. Mol. Cell Biol.* 10: 141-148.

Burns, T.M., Harding, R.M., and Dale J.L. 1995. The genome organization of *banana bunchy top virus*: analysis of six ssDNA components. *J. Gen. Virol.* 76: 1471-1482.

Chai, J., Feng, R.J., Shi, H.R., Ren, M.Y., Zhang, Y.D., and Wang, J.Y. 2015. Bioinformatic identification and expression analysis of banana microRNAs and their targets. *PLoS One* 10(4): 1-9.

Chen, C., Ridzon, D.A., Broomer, A.J., Zhou, Z., Lee, D.H., Ngyuyen, J.T., Barbisim, M., Xu, N.L., Mahuvaker, V.R., Anderson, M.R., Lao, K.Q., Lvak, K.J., and Guegler, K.J. 2005. Real time quantification of microRNAs by stem loop RT-PCR. *Nucleic Acids Res*. 33: 20-25.

Chen, C.M., Lu, Y.L., Sio, C.P., Wu, G.C., Tzou, W.S., and Pai, T.W. 2014. Gene ontology based housekeeping gene selection for RNA-seq normalization. *Elsevier* 67: 354-363.

Cherian, A.K., Menon, R., Suma, A., Nair, S., and Sudheesh, M.V. 2002. Impact of Banana bract mosaic diseases on the yield of commercial banana varieties of Kerala [abstract]. In: *Abstract, Global Conference on Banana and Plantain*; 28–31, October, 2002, Bangalore, p.155.

D'Hont, A., Denoeud, F., and Winter, P. 2012. The banana (*Musa acuminata*) genome and the evolution of monocotyledonous plants. *Nature* 488: 213-217.

Dale, J.L. 1987. Banana bunchy top: an economically important tropical plant virus disease. *Adv. Virus Res.* 33: 301–325.

Dehury, B., Panda, D., Sahu, J., Sahu, M., Sarma, K., Barooah, M., Sen, P., and Modi, M.K. 2013. In sillico identification and characterization of conserved miRNAs and their target genes in sweet potato (*Ipomoea batatas* L.) Expressed Sequence Tags (ESTs). *Plant Signal. Behav.* 8: 12-20.

Djami-Tchatchou, A.T., Sanan-Mishra, N., Ntushelo, K., and Dubery, I.A. 2017. Functional roles of microRNAs in agronomically important plants-potential as targets for crop improvement and protection. *Front. Plant Sci.* 8: 1- 24.

Doyle, J.J., and Doyle, J. 1990. Isolation of plant DNA from fresh tissue. *Focus* 12: 13-15.

Du, P., Wu, J., Zhang, J., Zhao, S., Zheng, H., Gao, G., Wei, L., and Li, Y. 2011. Viral infection induces expression of novel phased microRNAs from conserved cellular microRNA precursors. *PLoS Pathog*. 7(8): 1-13.

Ekatpure, S.C., Soni, K.B., Jadhav, P.R., Lekshmi, R.S., and Alex, S. 2019. Rapid and Efficient High Quality Viral RNA Isolation from *Banana Bract Mosaic Virus* Infected Banana cv. Grand Naine. *Int. J. Agric. Sci.* 11(6): 1-4.

Elangovan, R., Mohan, S., Arumugham, S., and Jeyarajan, R. 1990. A survey report in the incidence of major diseases of banana in Tamil Nadu. *S. Indian Hortic.* 38: 339-340.

Fabian, M.R., Sonenberg, N., and Filipowicz, W. 2010. Regulation of mRNA translation and stability by microRNAs. *Annu. Rev. Biochem.* 79: 351-379.

Fang, X. and Qi, Y. 2016. RNAi in plants: an argonaute-centered view. *Plant Cell* 28(2): 272-285.

FAOSTAT [Food and agriculture organization statistics]. 2018. FAOSTAT home page [online]. Available: www.fao.org › faostat

Farrant, J.M., Brandt, W.B., and Lindsey, G.G. 2007. An overview of mechanisms of desiccation tolerance in selected angiosperm resurrection plants. *Plant Stress* 1(1): 72-84.

Fauquet, C.M., Mayo, M.A., Maniloff, J., Desselberger, U., and Ball, L.A. eds., 2005. Virus taxonomy: *VIIIth report of the International Committee on Taxonomy of Viruses*. Amsterdam: *Academic Press*. 1259p.

Fire, A., Xu, S., Montgomery, M.K., Kostas, S.A., Driver, S.E., and Mello, C.C. 1998. Potent and specific genetic interference by double-stranded RNA in *Caenorhabditis elegans*. *Nature* 391(6669): 806-811.

Frazier, T.P., Xie, F., Friestaedter, A., and Zhang, B. 2010. Identification and characterization of microRNAs and their target genes in tobacco (*Nicotiana tabaccum*). *Planta* 10: 2-9.

Gao, P., Bai, X., Yang, L., Lv, D., Li, Y., Cai, H., Ji, W., Guo, D., and Zhu, Y. 2010. Over-expression of osa-MIR396c decreases salt and alkali stress tolerance. *Planta* 231(5): 991-1001.

Garcia-Ruiz, H., Takeda, A., Chapman, E.J., Sullivan, C.M., Fahlgren, N., Brempelis, K.J., and Carrington, J.C. 2015. *Arabidopsis* RNA-dependent RNA polymerases and dicer-like proteins in antiviral defense and small interfering RNA biogenesis during *Turnip mosaic virus* infection. *Plant Cell* 27: 944–945.

Gauhl, F. and Pasberg_Gauhl, C. 1994. Symptoms associated with *banana streak virus* (BSV). Plant Health Management Division, International Institute of Tropical Agriculture, Ibadan, Nigeria, pp. 20.

Gechev, T.S. and Hille, J. 2005. Hydrogen peroxide as a signal controlling plant programmed cell death. *J. Cell Biol.* 168: 17-20.

Gechev, T.S., Dinakar, C., Benina, M., Toneva, V., and Bartels, D. 2012. Molecular mechanisms of desiccation tolerance in resurrection plants. *Cell. Mol. Life Sci.* 69(19): 3175-3186.

Gechev, T.S., Van Breusegem, F., Stone, J.M., Denev, I., and Laloi, C. 2006. Reactive oxygen species as signals that modulate plant stress responses and programmed cell death. *BioEssays* 28(11): 1091-1101.

Ghag, S.B., Shekhawat, U.K., and Ganapathi, T.R. 2015. Small RNA profiling of two important cultivars of banana and overexpression of miRNA156 in transgenic banana plants. *PloS One* 10(5): 1-7.

Hammond, S.M., Bernstein, E., Beach, D., and Hannon, G.J. 2000. An RNA-directed nuclease mediates post-transcriptional gene silencing in Drosophila cells. *Nature* 404(6775): 293-296.

Harper, G., Hart, D., Moult, S., and Hull, R. 2002. Detection of *Banana streak virus* in field samples of bananas from Uganda. *Ann. Appl. Biol.* 141(3): 247-257.

Hewezi, T., Maier, T.R., Nettleton, D., and Baum, T.J. 2012. The *Arabidopsis* microRNA396-GRF1/GRF3 regulatory module acts as a developmental regulator in the reprogramming of root cells during cyst nematode infection. *Plant physiol.* 159(1): 321-335.

Hutvagner, G. and Simard, M.J. 2008. Argonaute proteins: key players in RNA silencing. *Nat. Rev. Mol. Cell Biol.* 9(1): 22-32.

Iskra-Caruana, M.L. 1990. The Banana Bunchy top disease (BBTD), a virus disease affecting banana and plantain. *Fruits*: 56-58.

Jadhav P.R. 2019. Development of resistance against *Banana bract mosaic virus* in *Musa* spp. Var. Grand Naine using small interfering RNA (siRNA). Ph. D. (Ag.) Thesis. Kerala Agricultural University, Thrissur, 68p.

Jagadeeswaran, G., Saini, A., and Sunkar, R. 2009. Biotic and abiotic stress down-regulate miR398 expression in *Arabidopsis*. *Planta* 229(4): 1009-1014.

Jaspers, P. and Kangasjarvi, J. 2010. Reactive oxygen species in abiotic stress signaling. *Physiol. Plant.*138 (4): 405-413.

Jin, W. and Wu, F. 2015. Identification and characterization of cucumber microRNAs in response to *Pseudoperonospora cubensis* infection. *Gene* 569(2): 225-232.

King, A., Adams, M., Carstens, E., and Lefkowitz, E. 2012. Virus taxonomy*: Ninth Report of the International Committee on Taxonomy of Viruses*. Amsterdam: *Elsevier Academic Press*. 1327p.

Kiranmani, G., Lavakumar, P., Hema, M., Venkatramana, M., Krishna Prasadji, J., Rao, M., and Sreenivasalu, P. 2005. Partial charecterization of a potyvirus causing gene regulation of banana in Andra Pradesh. *Indian J. Virol*. 16: 7-11.

Kramer, M.F. 2011. STEM-LOOP RT-qPCR for miRNAs. *Curr. Protoc. Mol. Biol*. 15: 10-20.

Kranner, I., Beckett, R.P., Wornik, S., Zorn, M., and Pfeifhofer, H.W. 2002. Revival of a resurrection plant correlates with its antioxidant status. *Plant J.* 31(1): 13-24.

Lee, R.C., Feinbaum, R.L., and Ambros, V. 1993. The *C. elegans* heterochronic gene lin-4 encodes small RNAs with antisense complementarity to lin-14. *Cell* 75(5): 843-854.

Li, W.X., Oono, Y., Zhu, J., He, X.J., Wu, J.M., Iida, K., Lu, X.Y., Cui, X., Jin, H., and Zhu, J.K. 2008. The Arabidopsis NFYA5 transcription factor is regulated transcriptionally and post transcriptionally to promote drought resistance. *Plant Cell* 20(8): 2238-2251.

Li, Y.F., Zheng, Y., Addo-Quaye, C., Zhang, L., Saini, A., Jagadeshwaran, G., Axtell, M.J., Zhang, W., and Sunkar, R. 2010. Transcription wide identification of microRNA targets in Rice. *Plant J.* 62: 742-759.

Liu, J., Zhang, X., Zhang, F., Hong, N., Wang, G., Wang, A., and Wang, L. 2015. Identification and characterization of microRNAs from in vitro-grown pear shoots infected with *Apple stem grooving virus* in response to high temperature using small RNA sequencing. *BMC Genomics* 16(1): 945.

Lockhart, B. and Jones, D. 2000. Banana streak. In: *Jones DR(ed) Diseases of banana, abaca, and enset.* CAB International, Wallingford, pp. 263-274

Lockhart, B.E.L. and Olszewski, N.E. 1993. Serological and genomic heterogeneity of banana streak badnavirus: implications for virus detection in Musa germplasm. In: *International Symposium on Genetic Improvement of Bananas for Resistance to Diseases and Pests;* 7-9, September, 1992, Montpellier (France), CIRAD-FLHOR.

Magee, C.J. P. 1930. A new virus disease of Bananas. *Agric. Gaz. N. S. W.* 41(12).

Magee, C.J.P. 1927. Investigation on the bunchy top disease of banana. *Comm. Austr. Countries Sci. Ind. Res. Bull.* 30: 64.

Magee, C.J.P. 1940. Transmission studies on the Banana bunchy top virus. *J. Austr. Inst. Agric. Sci*. 6: 109-110.

Magee, C.J.P. 1953. Some aspects of bunchy top disease of banana and other *Musa* spp. *J. Proc. R. Soc. N. S. W.* 87: 1-18.

Magnaye, L.V. and Espino, R.R.C. 1990. *Banana bract mosaic*, a new disease of banana, I. symptomatology. *Phillipp. Agri. Sci*. 73: 55-59.

Massey, V. 1994. Activation of molecular-oxygen by flavins and flavoproteins. *J. Biol. Chem*. 269: 22459-22462.

Mathew, K.S.A. 2018. Computational prediction of miRNAs in banana (*Musa* spp.) and evaluation of their role in virus infection. M.Sc. (Ag) Thesis, Kerala Agricultural University, Thrissur, 45p.

Miller, G.A.D., Suzuki, N., Ciftci-Yilmaz, S.U.L.T.A.N., and Mittler, R.O.N. 2010. Reactive oxygen species homeostasis and signaling during drought and salinity stresses. *Plant Cell Environ.* 33(4): 453-467.

Napoli, C., Lemieux, C., and Jorgensen, R. 1990. Introduction of a chimeric chalcone synthase gene into petunia results in reversible co suppression of homologous genes in trans. *Plant Cell* 2: 279-289.

Ni, Z., Hu, Z., Jiang, Q., and Zhang, H. 2012. Overexpression of gma-MIR394a confers tolerance to drought in transgenic *Arabidopsis thaliana*. *Biochem. Biophys. Res. Commun.* 427(2): 330-335.

Niblett, C.L., Pappu, S.S., Bird, J., and Lastra, R. 1994. Infectious chlorosis, mosaic and heart rot. *Compendium of tropical fruit disease. APS Press.* 88p.

OCEAH, Б. 1995. Identification of cucumber mosaic virus subgroup I isolates from banana plants affected by infectious chlorosis disease using RT-PCR. *Plant Dis.* 79(7): 713.

Ortiz, R. 2013. Conventional banana and plantain breeding. *Acta Hortic*. 986: 77-194

Palatnik, J.F., Allen, E., Wu, X., Schommer, C., Schwab, R., Carrington, J.C., and Weigel, D. 2003. Control of leaf morphogenesis by microRNAs. *Nature* 425: 257–263.

Pantaleo, V., Szittya, G., Moxon, S., Miozzi, L., Moulton, V., Dalmay, T., and Burgyan, J. 2010. Identification of grapevine microRNAs and their targets using high-throughput sequencing and degradome analysis. *Plant J.* 62(6): 960-976.

Park, W., Li, J., Song, R., Messing, J., and Chen, X. 2002. CARPEL FACTORY, a Dicer homolog, and HEN1, a novel protein, act in microRNA metabolism in *Arabidopsis thaliana*. *Curr. Biol.* 12(17): 1484-1495.

Petrov, V.D. and Van Breusegem, F. 2012. Hydrogen peroxide-a central hub for information flow in plant cells. *AoB Plants* 1-13.

Ponsubrya, R.K., Alex, S., Rajmohan, K., and Soni, K.B. 2014. Polymerase chain reaction based detection of *banana bunchy top virus* using coat protein based primers. *Int. J. Agric. Environ. Biotech.* 7(4): 765-767.

Rao, D.G., 1980. Studies on a new strain of banana mosaic virus in south India. In: *National Seminar on Banana Production Technology.* Tamil Nadu Agricultural University, Coimbatore, pp.155-159.

Reinhart, B.J., Weinstein, E.G., Rhoades, M.W., Bartel, B., and Bartel, D.P. 2002. MicroRNAs in plants. *Genes Dev*. 16(13): 1616-1626.

Rodoni, B.C., Ahlawat, Y.S., Varma, A., Dale, J.L., and Harding, R.M. 1997. Identification and characterization of *banana bract mosaic virus* in India. *Plant Dis.* 81(6): 669-672.

Rodoni, B.C., Dale, J.L. and Harding, R.M., 1999. Characterization and expression of the coat protein-coding region of banana bract mosaic potyvirus, development of diagnostic assays and detection of the virus in banana plants from five countries in Southeast Asia. *Arch. Virol.* 144(9): 1725-1737.

Sagi, L., Panis, B., Remy, S., Schoofs, H., De Smet, K., Swennen, R., and Bruno, P.A.C. 1995. Genetic transformation of banana and plantain (*Musa* spp.) via particle bombardment. *Bio/Technol.* 13(5): 481-485.

Selvarajan, R. and Jeyabaskaran, K.J. 2006. Effect of *Banana bract mosaic virus* (BBrMV) on growth and yield of cultivar Nendran (Plantain, AAB). *Indian Phytopathol*. 59: 496–500.

Selvarajan, R. and Singh, H.P. 1997. Occurrence, geographical distribution and electron microscopy of BBrMV in India [abstract]. In: *Abstracts*, *Proceedings of International Conference on Integrated Plant Disease Management for Sustainable Agriculture;* 10-15, November, 1997, IARI, New Delhi, p.223.

Selvarajan, R., Balasubramanian, V., and Sathiamoorthy, S. 2006. Vector transmission of *banana bract mosaic* and *banana streak viruses* in India. In: *Abstracts of XVI annual convention and international symposium on management of vector-borne viruses*; 7-10, February, 2006, ICRISAT, Hyderabad, p.110.

Sheeba, M.M., Selvarajan, R., and Mustaffa, M.M. 2013. Prediction and Identification of MicroRNA from Banana Infected with *Banana Streak Mysore Virus* (BSMYV). *Madras Agric. J.* 100(4-6): 513-518.

Song, S., Chen, X., Huang, D., Xu, Y., Zeng, H., Hu, X., Xu, B., Jin, Z., and Wang, W. 2016. Identification of miRNAs differentially expressed in *Fusarium* wilt-resistant and susceptible banana varieties. *S. Afr. J. Bot.* 106: 244-249.

Simon-Mateo, C. and Garcia, J.A. 2011. Antiviral strategies in plants based on RNA silencing. *Biochem. Biophys. Acta.* 1809: 722-731.

Singh, S.J. 2002. Viral disease on banana and their management, In: *souvenir Global conference on banana and plantain.* p.28-31.

Song, J.B., Gao, S., Sun, D., Li, H., Shu, X.X., and Yang, Z.M. 2013. miR394 and LCR are involved in Arabidopsis salt and drought stress responses in an abscisic acid-dependent manner. *BMC Plant Biol.* 13(1): 1-16.

Subramanian, A. 2019. Identification and charecterisation of virus responsive miRNAs in banana Musa (AAB) 'NENDRAN'. M.Sc. (Ag) thesis, Kerala Agricultural University, Thrissur, 74p.

Teune, J.H. and Steger, G. 2010. NovoMIR: De novo prediction of microRNA coding regions in a single plant genome. *J. Nucleic Acids* 25:55-65.

Thangavelu, R., Selvarajan, R., and Singh H.P. 2000. Status of banana streak virus and *banana bract mosaic virus* diseases in India. In: Singh, H.P. and Chadha K.L. (ed.), *Banana: improvement, production and utilization. Proceedings of the conference on challenges for banana production and utilization in 21st century*. AIPUB, NRCB, Trichy, pp.364-376.

Thomas, J.E., Geering, A.D.W., Gambley, C.F., Kessling, A.F., and White, M. 1997. Purification, properties, and diagnosis of banana bract mosaic potyvirus and its distinction from abaca mosaic potyvirus. *Phytopathol.* 87(7): 698-705.

Tolia, N.H. and Joshua-Tor, L. 2007. Slicer and the argonautes. *Nat. Chem. Biol. 3*(1): 36-43.

Untergasser, A., Nijveen, H., Rao, X., Bisseling, T., Gaurts, R., and Leunissen, J.A.M. 2007. Primer3Plus, an enhanced web interface to Primer3. *Nucleic Acids Res*. 35: 71-74.

Varghese, M.K. 1945. Bunchy top disease of plantain. *Indian Farming* 5: 239-240.

Vaucheret, H. 2008. Plant argonautes. *Trends Plant Sci.* 13(7): 350-358.

Vaucheret, H., Mallory, A.C., and Bartel, D.P. 2006. AGO1 homeostasis entails coexpression of miR168 and AGO1 and preferential stabilization of miR168 by AGO1. *Mol. Cell* 22: 129-154.

Vetten, H.J., Dale J.L., Grigoras, I., Gronenborn, B., Harding, R., and Randles, J.W. 2012. Family Nanoviridae. In: Virus taxonomy: ninth report of the international committee on taxonomy of viruses, pp. 395-404.

Wang, H., Jiao, X., Kong, X., Humaira, S., Wu, Y., Chen, X., Fang, R., and Yan, Y. 2016. A signalling cascade from miR444 to RDR1 in rice antiviral RNA silencing pathway. *Plant Physiol*. 170: 2365–2377.

Wang, J.W., Wang, L.J., Mao, Y.B., Cai, W.J., Xue, H.W., and Chen, X.Y. 2005. Control of root cap formation by microRNA-targeted auxin response factors in *Arabidopsis*. P*lant Cell* 17(8): 2204-2216.

Watanabe, S., Greenwell, A.M., and Bressan, A. 2013. Localization, concentration, and transmission efficiency of *Banana bunchy top virus* in four asexual lineages of Pentalonia aphids. *Viruses* 5(2): 758-776.

Watanabe, Y., Tomita, M., and Kansi, A. 2007. Computational methods for microRNA target prediction. *Method Enzymol*. 427: 65-89.

Watson, M.A. and Nixon, H.L. 1953. Studies on the feeding of *Myzus persicae* (sulz.) on radioactive plants. *Ann. Appl. Biol*. 40(3): 537-545.

Widyasari, K., Alazem, M., and Kim, K.H. 2020. Soybean Resistance to *Soybean Mosaic Virus*. *Plants* 9(2): 219.

Wightman, B., Ha, I., and Ruvkun, G. 1993. Post transcriptional regulation of the heterochronic gene lin-14 by lin-4 mediates temporal pattern formation in *C. elegans*. *Cell* 75(5): 855-862.

Williams, L., Grigg, S.P., Xie, M., Christensen, S., and Fletcher, J.C. 2005. Regulation of *Arabidopsis* shoot apical meristem and lateral organ formation by microRNA miR166g and its AtHD-ZIP target genes. *Dev.* 132(16): 3657-3668.

Xia, Z., Zhao, Z., Li, M., Chen, L., Jiao, Z., Wu, Y., Zhou, T., Yu, W., and Fan, Z. 2018. Identification of miRNAs and their targets in maize in response to *sugarcane mosaic virus* infection. *Plant Physiol. Biochem.* 125: 143-152.

Yadav, C. B., Muthamilarasan, M., Pandey, G., and Prasad, M. 2015. Identification, characterization and expression profiling of Dicer-Like, Argonaute and RNA-dependent RNA polymerase gene families in foxtail millet. *Plant Mol. Biol. Rep.* 33: 43-55.

Ye, J., Coulouris, G., Zaretskaya, I., Cutcutache, I., Rozen, S. and Madden, T.L. 2012. Primer-BLAST: a tool to design target-specific primers for polymerase chain reaction. *BMC Bioinform.* 13(1): 134.

Zhang, B. and Wang, Q. 2015. MicroRNA-based biotechnology for plant improvement. *J. Cell. Physiol.* 230(1): 1-15.

Zhang, B., Pan, X., Cobb, G.P., and Anderson, T.A. 2006. Plant microRNA: a small regulatory molecule with big impact. *Dev. Biol.* 289(1): 3-16.

Zhang, B.H., Pan, X.P., Wang, Q.L., George, P.C., and Anderson, T.A. 2005. Identification and characterization of new plant microRNAs using EST analysis. *Cell Res.* 15(5): 336-360.

Zhang, C., Ding, Z., Wu, K., Yang, L., Li, Y., Yang, Z., Shi, S., Liu, X., Zhao, S., Yang, Z.,Wang, Y., Zheng, L., Wei, J., Du, Z., Zhang, A., Miao, H., Li, Y., Wu, Z., and Wu, J. 2016. Suppression of jasmonic acid-mediated defense by viral-inducible microRNA319 facilitates virus infection in rice. *Mol. Plant* 9(9): 1302-1314.

Zhang, X., Zou, Z., Gong, P., Zhang, J., Ziaf, K., Li, H., Xiao, F., and Ye, Z. 2011. Over-expression of microRNA169 confers enhanced drought tolerance to tomato. *Biotechnol. Lett.* 33(2): 403-409.

Zhang, Y.C., Yu, Y., Wang, C.Y., Li, Z.Y., Liu, Q., and Xu, J. 2013. Overexpression of microRNA OsmiR397 improves rice yield by increasing grain size and promoting panicle branching. *Nat. Biotechnol*. 31: 848-852.

Zhou, M., Li, D., Li, Z., Hu, Q., Yang, C., Zhu, L., and Luo, H. 2013. Constitutive expression of a miR319 gene alters plant development and enhances salt and drought tolerance in transgenic creeping bentgrass. *Plant Physiol.* 161(3): 1375-1391.

Zhou, Z., Cao, Y., Li, T., Wang, X., Chen, J., He, H., Yao, W., Wu, J., and Zhang, H. 2020. MicroRNAs Are Involved in Maize Immunity against *Fusarium verticillioides* Ear Rot. *Genomics Proteomics Bioinformatics*: 1-32.

Appendix

APPENDIX I

DNA extraction buffer (Doyle and Doyle, 1990)

CTAB	2%
Tris-HCl, pH 8.0	100 Mm
NaCl	1.4M
EDTA	20mM
β mercaptoethanol	0.1% (w/v)

APPENDIX II

RNA extraction buffer (Ekatpure *et al.*, 2019)

Tris HCl	150mM
SDS	4% (w/v)
EDTA, pH 7.5	100 mM
β mercaptoethanol	2% (v/v)
PVP	3% (w/v)
RNase free water	Treated with diethyl pyrocarbonate (DEPC) 0.1%

Abstract

EXPRESSION PROFILING OF microRNAs ASSOCIATED WITH VIRUS INFECTION IN BANANA CULTIVAR RED BANANA (*Musa* AAA)

by

ASWATHY RAJAN
(2018-11-171)

ABSTRACT

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ABSTRACT

The study entitled "Expression profiling of microRNAs associated with virus infection in banana cultivar Red Banana (*Musa* AAA)" was conducted at the Department of Plant Biotechnology, College of Agriculture, Vellayani, Thiruvananthapuram during 2018-2020. The objective was to study the expression of selected miRNAs in *Banana bract mosaic virus* (BBrMV) and *Banana bunchy top virus* (BBTV) infection in banana cultivar Red Banana (*Musa* AAA).

In a study conducted in the Department of Plant Biotechnology, 52 mature miRNAs were predicted using NovoMIR software and over 142 targets were identified for these miRNAs using psRNATarget in the banana genome. On validation of these miRNAs, Subramanian (2019) found differential expression of a few of them in tissue culture grown banana var. Nendran infected with BBrMV. In this study, two of those miRNAs and their target genes i.e. miR-6928-5p (target: Flavin adenine dinucleotide dependent oxidoreductase gene) and miR-971-5p (target: Argonaute protein) were studied for their expression in field infected samples.

Banana plants of cultivar red banana infected with BBrMV and BBTV were collected from the Instructional Farm, College of Agriculture, Vellayani, based on the visual symptoms. The BBrMV and BBTV infections were reconfirmed by PCR using the primers specific to coat protein genes of the viruses. All the infected samples showed the presence of specific amplicons of the expected size (745bp) compared to the uninfected samples. RNA isolated from the leaf samples of both control and infected plants were reverse transcribed to cDNA. The PCR analysis confirmed the presence of both miRNAs and their target genes in the banana samples.

The expression analysis conducted by RT-qPCR showed differential expression of miR-6928-5p and miR-971-5p in BBrMV and BBTV infected plants. In BBrMV infected samples, miR-6928-5p was downregulated up to 0.01 fold and the BBTV infected samples showed an upregulation of this miRNA up to11.08 fold. The mRNA

transcript encoding FAD dependent oxidoreductase showed downregulation in BBrMV infected samples (upto 0.43 fold) and in BBTV infected samples the change was marginal. But within the infected plants both miRNA and its target showed an inverse correlation.

miR-971-5p was found to be down regulated in both BBrMV and BBTV infected samples (a maximum of 0.21 and 0.17 fold respectively). On the other hand, its target mRNA transcript encoding Argonaute protein showed a maximum of 3.09 and 1.89 fold increase in the BBrMV and BBTV infected samples. Here both miR-971-5p and its target gene showed an inverse correlation in their expression in both BBrMV and BBTV infection.

The study showed that both miR-6928-5p and miR-971-5p respond to BBTV and BBrMV infection in banana cultivar red banana (*Musa* AAA).